710011752

FORM 5
THE PATENT ACT, 1970
(39 OF 1970)
&
The Patents Rules, 2003
DECLARATION AS TO INVENTORSHIP
[See section 10(6) and rule 13(6)]

1. NAME OF APPLICANT (S)	Dr. Santosh M. Shinde, Prof. Sarita Goyal, Dr. Dashrath Gaikwad Dr. Pratap Vasantrao Pawar , DR.B.L.RAJU,Dr.T.Sunil

hereby declare that the true and first inventor(s) of the invention disclosed in the complete specification filed in pursuance of my / our application numbered dated / / is / are

2. INVENTOR (S)

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Dated this 1st day of Oct-2022
Signature:- Name of the signatory:- . Dr. Santosh M. Shinde

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Dated this 1st day of Oct-2022
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Name of the signatory:- Dr. Dashrath Gaikwad

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Dated this 1st day of Oct-2022
Signature:-
Name of the signatory:- Dr. Pratap Vasantrao Pawar

Name DR.B.L.RAJU
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Dated this 1st day of Oct-2022
Signature:-
Name of the signatory:- DR.B.L.RAJU

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Dated this 1st day of Oct-2022
Signature:-
Name of the signatory:- .Dr.T.Sunil

3. DECLARATION TO BE GIVEN WHEN THE APPLICATION IN INDIA IS FILED BY THE APPLICANT (S) IN THE CONVENTION COUNTRY :-

N. A.

Dated this ..... 1 ..... day of 10/22

Signature :-

Name of the signatory:- Dr T Somu

4. **STATEMENT**(to be signed by additional inventor(s) not mentioned in the application form)

I/We accept to the invention referred to in the above declaration, being included in the complete specification filed in pursuance of the stated application.

N.A

Dated this 1 day of 10 20 22

Signature of the additional inventor(s):-

Name :- Dr. T Somu

To,
The Controller of Patent
The Patent Office, at Chennai

Note

Repeat boxes in case of more than one entry

***To be signed by applicant(s)or by authorized registered patent agent otherwise where mentioned.**

***Name of the inventor and applicant should be given in full, family name in beginning**

***Complete address of the inventor and applicant should be given stating postal index no/code,state and country**

*** Strike the column which is/are not applicable**

2/2

710011754

FORM 2
THE PATENT ACT 1970
(39 of 1970)
&
The Patents Rules, 2003
PROVISIONAL/COMPLETE SPECIFICATION
(See section 10 and rule13)

1.TITLE OF THE INVENTION: Design of framework for Virtual and Augmented Reality in E-commerce for increase in footprints (Field: Management)

2.APPLICANT(S)

1. Dr. Santosh M. Shinde

2. Prof. Sarita Goyal

3. Dr. Dashrath Gaikwad

4.Dr. Pratap Vasantrao Pawar

5 DR.B.L.RAJU

6.Dr.T.Sunil

(b)NATIONALITY: INDIAN

ADDRESS: Annexure---2---

3. Field of Invention : Management

The field of Invention is related to management where in the focus is on the increase of footprints, which is very important for any business to grow. The framework will help in increase of footprints by making use of the latest technology which virtual reality.

Details of the Applicants Annexure- 2

1. Dr. Santosh M. Shinde Associate Professor S B Patil Institute of Management,pune , 9370113528 shinde.santosh1928@gmail.com College address - near akurdi railway station, Nigdi **Faddr:Hyderabad-500100**	
2. Prof. Sarita Goyal Incharge Principal, ASM'S CSIT COLLEGE PIMPRI saritagoyal2006@gmail.com 9637543834 Sr. No 29/1+2A, Near Sterling Honda, Pune-mumbai Highway, Pimpri, Pune 411018	
3. Dr. Dashrath Gaikwad Assistant Professor WPU RISE Digital learning Pvt Ltd 7507449143 dashrath88@gmail.com 11th floor ABZ baner balewadi phata Baner Pune	
4.Dr. Pratap Vasantrao Pawar Associate Professor, Deputy Director, Siddhant Institute of Business Management, Sudumbare, Pune. pvpawar.raj@gmail.com Mob. 9423270598. A/Po- Sudumbare, Chakan-Talegaon Road, Tal - Maval Dist - Pune Pune. Pincode 412109.	
5 DR.B.L.RAJU PROF & PRINCIPAL ACE ENGINEERING COLLEGE ANKUSHAPUR GHATKESAR **HYDERABAD-501301** MAIL: blraju2@gmail.com PHNO:9440925929	
6. Dr.T.Sunil Professor,CSE MRCE Sunil.tekale2010@gmail.com, 9885303678 **Hyderabad-100**	

Office of the Controller General of Patents, Designs & Trade Marks
Department of Industrial Policy & Promotion,
Ministry of Commerce & Industry,
Government of India

सत्यमेव जयते

INTELLECTUAL PROPERTY INDIA
PATENTS | DESIGNS | TRADE MARKS
GEOGRAPHICAL INDICATIONS

Application Details

APPLICATION NUMBER	202121031917
APPLICATION TYPE	ORDINARY APPLICATION
DATE OF FILING	15/07/2021
APPLICANT NAME	**Dr.Shrikant Balkrishna Jagtap**
TITLE OF INVENTION	NOVEL METHOD FOR SYNTHESIS OF C-3 METHYL AND HALO LAWSONEMONOXIMATES OF HO(III), ER(III) AND YB(III)
FIELD OF INVENTION	METALLURGY
E-MAIL (As Per Record)	patentpublication@gmail.com
ADDITIONAL-EMAIL (As Per Record)	patentpublication@gmail.com
E-MAIL (UPDATED Online)	
PRIORITY DATE	
REQUEST FOR EXAMINATION DATE	--
PUBLICATION DATE (U/S 11A)	29/07/2022

"FORM 1 THE PATENTS ACT 1970 (39 of 1970) and THE PATENTS RULES, 2003 **APPLICATION FOR GRANT OF PATENT** (See section 7, 54 and 135 and sub-rule (1) of rule 20)	(FOR OFFICE USE ONLY) 200174478

	Application No.	201721038426
	Filing date	30/10/2017
	Amount of Fee paid	17501/- रुपये के चालान/डी आर/पी ओ ऑर्डर द्वारा
	CBR No	CBR संख्या 20564 30/10/2017 के तहत प्राप्त हुए।
	Signature	

1. APPLICANT'S REFERENCE / IDENTIFICATION NO. (AS ALLOTTED BY OFFICE)	

2. TYPE OF APPLICATION [Please tick (√) at the appropriate category]

Ordinary (√)		Convention ( )		PCT-NP ( )	
Divisional ( )	Patent of Addition ( )	Divisional ( )	Patent of Addition ( )	Divisional ( )	Patent of Addition ( )

3A. APPLICANTS

Name in Full	Nationality	Country of Residence	Address of the Applicant	
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			City	Pune
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			Country	India
			Pin code	411007

3B. CATEGORY OF APPLICANT [Please tick (√) at the appropriate category]

Natural Person (√)	Other than Natural Person		
	Small Entity ( )	Startup ( )	Others ( )

4. INVENTORS [Please tick (√) at the appropriate category]				
Are all the inventors same as the applicants named above?			Yes ( )	No (√)
If "No", furnish the details of the inventor(s)				
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			City	Dist.- Pune
			State	Maharashtra
			Country	India
			Pin code	412 301
Dr. Jagtap Suresh D.			House No.	Herbal Medicine, Interactive Research School for Health Affairs (IRSHA), Bharati Vidyapeeth University
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			City	Pune
			State	Maharashtra
			Country	India
			Pin code	411 043

5. TITLE OF THE INVENTION **Novel process for extraction of Ephedrine from selected plants.**		
6. AUTHORISED REGISTERED PATENT AGENT(S)	IN/PA No.	-
	Name	-
	Mobile No.	-
7. ADDRESS FOR SERVICE OF APPLICANT IN INDIA	Name	Prof. Dr. Deokule Subhash S.
	Postal Address	Department of Botany, Savitribai Phule Pune University (SPPU), Ganeshkhind Road, Pune-411007. Maharashtra, India
	Telephone No.	020 2560 1217
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	Fax No.	020 2560 1217
	Email ID	deokule.ss@gmail.com

8. IN CASE OF APPLICATION CLAIMING PRIORITY OF APPLICATION FILED IN CONVENTION COUNTRY, PARTICULARS OF CONVENTION APPLICATION

- NOT APPLICABLE -

9. IN CASE OF PCT NATIONAL PHASE APPLICATION, PARTICULARS OF INTERNATIONAL APPLICATION FILED UNDER PATENT CO-OPERATION TREATY (PCT)

- NOT APPLICABLE -

10. IN CASE OF DIVISIONAL APPLICATION FILED UNDER SECTION 16, PARTICULARS OF ORIGINAL (FIRST) APPLICATION

- NOT APPLICABLE -

11. IN CASE OF PATENT OF ADDITION FILED UNDER SECTION 54, PARTICULARS OF MAIN APPLICATION OR PATENT

- NOT APPLICABLE -

12. DECLARATIONS

(i) Declaration by the inventor(s):

We, the above named inventors are the true & first inventors for this invention and declare that IRSHA, BVDU herein our assignee.

Signature:

Name: Prof. Dr. Deokule Subhash S. Date: 24.10.2017

Signature:

Name: Dr. Patale M. W. Date: 24.10.2017

Signature:

Name: Dr. Mungikar Rahul R. Date: 25/10/2017

Signature:

Name: Mr. Shilimkar Vaibhav C. Date: 25/10/2017

Signature:

Name: Dr. Jagtap Suresh D. Date: 25/10/2017

(ii) Declaration by the applicant(s) in the convention country

- NOT APPLICABLE -

Declaration by the applicants:

We the applicants hereby declares that:-

√	We are in possession of the above-mentioned invention.
√	The complete specification relating to the invention is filled with this application.
√	The invention as disclosed in the specification uses the biological material from India and the necessary permission from the competent authority shall be submitted by us before the grant of patent to us.
√	There is no lawful ground of objection to the grant of the patent to us.
√	We are the true & first inventors.
√	We are the assignee of true and first inventors.
√	The application or each of the applications, particulars of which are given in Paragraph-8, was the first application in convention countries in respect of our inventions.
√	We claim the priority from the above mentioned application(s) filed in convention countries and state that no application for protection in respect of the invention had been made in a convention country before that date by us or by any person from which we derive the title.
X	Our application in India is based on international application under Patent Cooperation Treaty (PCT) as mentioned in Paragraph-9.
X	The application is divided out of our application particulars of which is given in Paragraph-10 and pray that this application may be treated as deemed to have been filed on .....under section 16 of the Act.
X	The said invention is an improvement in or modification of the invention particulars of which are given in Paragraph-11.

13. FOLLOWING ARE THE ATTACHMENT WITH THE APPLICATION:

(a) Form 2

Item	Details	Fee	Remarks
Complete specification	No. of pages: 07	Rs 1750/-	
No. of Claims	No. of claims 07 No. of pages 01		

(b) Complete specification (2 copies). Drawing Sheet - 5 & figure 09

(c) Statement and undertaking on Form 3 Abstract - 1

(d) Declaration as to Inventorship on Form 5

(e) Total fee Rs. 1750 - in cash 2017 16:58

We hereby declare that to the best of my/our knowledge, information and belief the fact and matters stated herein are correct and I/we request that a patent may be granted to me/us for the said invention.

Dated this .....30.....day of ..October.....2017

Signature:

Prof. Dr. Deokule Subhash S.
Department of Botany,
Savitribai Phule Pune University (SPPU),
Ganeshkhind Road,
Pune- 411007. Maharashtra, India

To,
THE CONTROLLER OF PATENTS,
THE PATENT OFFICE, MUMBAI

E-2/2329/2017

1

FORM 2

THE PATENT ACT 1970

(39 OF 1970)

&

The patents rules, 2003

COMPLETE SPECIFICATION

(See section 10 and rule 13)

200174481

1. TITLE OF THE INVENTION:

Novel process for extraction of Ephedrine from selected plants

2. APPLICANT (S):

a.	**Name**	:	Prof. Dr. Deokule Subhash S.
b.	**Nationality**	:	Indian
c.	**Address**	:	Department of Botany, Savitribai Phule Pune University, Ganeshkhind Road, Pune-411007, Maharashtra, India.
a.	**Name**	:	Dr. Patale M. W.
b.	**Nationality**	:	Indian
c.	**Address**	:	Department of Botany, Savitribai Phule Pune University, Ganeshkhind Road, Pune-411007, Maharashtra, India.
a.	**Name**	:	Dr. Mungikar Rahul R.
b.	**Nationality**	:	Indian
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d.	**Name**	:	Mr. Shilimkar Vaibhav C.
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g.	**Name**	:	Dr. Suresh D. Jagtap
h.	**Nationality**	:	Indian
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3. PREAMBLE TO THE DESCRIPTION	
PROVISIONAL	**COMPLETE**
NOT APPLICABLE	The following specification describes the invention.

4. DESCRIPTION

Field of the Invention

This invention relates to a process of extraction of Ephedrine. The present invention particularly relates to an isolation of Ephedrine from selected plant sources which are the source of extraction of Ephedrine. This invention is based on new process to report and enhance the yield of Ephedrine. Aspect of the invention is through analytical method viz. Thin layer chromatography (TLC), infrared spectroscopy (IR), Nuclear magnetic resonance (NMR) techniques and confirmed presence of alkaloid ephedrine.

Background of the invention:

Ephedrine:

Ephedrine is a medication and stimulant. It is often used to prevent low blood pressure during spinal anesthesia. It has also been used for asthma, narcolepsy, and obesity but is not the preferred treatment. It is of unclear benefit in nasal congestion. It can be taken orally or through other routes.

Ephedrine is a sympathomimetic amine commonly used as an appetite suppressant, decongestant and to treat Hypotension associated with an aesthesia.

Plant based Source and uses:

Sida acuta Burm. f.

This is commonly known as 'Bala Phanijivika'. Roots are bitter, tonic, stomachic, diaphoretic and antipyretic. They are useful in form of decoction or infusion in febrile affections and some forms of dyspepsia and in mild cases debility due to various illnesses. Infusion of root with little ginger is given in intermittent fever and chronic bowel complaints. Expressed juice of the roots in the form of electuary is employed for the removal of intestinal worms.

Sida rhombifolia L. var. *rhomboidea* Linn.

This is known as 'Atibala' (Nadkarni, A.K.1927) Mahabala, and Sahadevi (Cooke, T.1958). The plant is useful in tuberculosis and rheumatism. In Canary Island the leaves are used as substitute for tea, stem abounds in mucilage and is employed as demulcent and emollient. It is also used internally in skin diseases and calculus troubles and as a diuretic and febrifuge with pepper.

Sida spinosa Linn.

It is known as 'Nagabala' (Nadkarni, A.K.1927), Jangali methi (Cooke, T.1967). The root, leaf and fruit destroy kapha and vata. It is used as tonic and cures ulcer, skin infections and kidney disorders. In Ayurveda, fruits are considered as an astringent and cooling. The leaves are demulcent, refrigerant and are useful in cases of gonorrhea, gleet, and scalding urine. The decoction of root bark and roots are used as demulcent in irritability of the bladder and in gonorrhea. The root acts as a gentle tonic, diaphoretic and as employed in mild cases of debility and fever (Kirtikar, K.R. and Basu, B.D. 1937).

Summary of the invention

This invention includes the source of four species from family Malvaceae. These plants will be finding their use for utilization as drug in different systems of medicines in different human aliments. The introduction part of this thesis explains the uses of plants and their medicinal importance. The plants are as follows:

1) *Sida acuta* Burm. f.
2) *Sida rhombifolia* L. var. *microphylla Cav Sida spinosa* Linn.
3) *Sida spinosa* Linn.

The plant *Sida acuta* Burm. f., *Sida rhombifolia* L. var. *microphylla* Cav., *Sida spinosa* Linn. showing presence of ephedrine. In materials and methods a method for extraction of ephedrine is present investigation is proposed.

In the present investigation alkaloids are present in the drugs and ephedrine is one of them. These were further detected quantitatively. Further these investigations are supported by thin layer chromatography (TLC), infrared spectroscopy (IR), Nuclear magnetic resonance (NMR), techniques and confirmed alkaloid ephedrine.

Detailed description of the figures

Figure 1: TLC of standard ephedrine hydrochloride, Sample A, Sample B & Sample C

Figure 2: IR of Standard ephedrine hydrochloride in Nujol

Figure 3: IR of Sample A, Seeds of *Sida spinosa* L. in Nujol

Figure 4: IR of Sample B, Seeds of *Sida acuta* Burm. f, in Nujol

Figure 5: IR of Sample C, Seeds of *Sida rhombifolia* var. *microphylla*, Cav. in Nujol

Figure 6: ^{1}H NMR spectrum of Standard ephedrine hydrochloride from Aldrich catalogue

Figure 7: ^{1}H NMR spectrum, Seeds of *Sida spinosa* L. in $CdCl_3$

Figure 8: ^{1}H NMR spectrum, Seeds of *Sida acuta* Burm. f. in $CdCl_3$

Figure 9: ^{1}H NMR spectrum, Seeds of *Sida rhombifolia* var. *microphylla* Cav. in $CdCl_3$

Detailed description of the invention

The present invention discloses a process for the extraction of Ephedrine from plant based sources. It has extracted from four different plants belonging to family Malvaceae. In this detailed description, the process of extraction of Ephedrine is described for a better understanding of the invention without any limitation.

Detailed experimental studies

Chromatography: After detection of the chemical constituents with the help of qualitative tests, they were further separated with chromatographic technique. Alkaloid and other chemical compounds were separated on silica gel.

Extraction and detection of alkaloid ephedrine:

Procedure: 2 g of finely powdered material was uniformly wet by slow addition and mixing of 10% aqueous sodium carbonate. The wet mass was shaken with 10 ml of benzene for one hour on shaker. The benzene extract was filtered and to this solution 2 ml of 0.1 N HCL added and shaken vigorously. The benzene residue was again washed 2-3 times by benzene and that residue filtered and filtrate was mixed in the previous extract of benzene. Air-drying separated the acids solution containing the alkaloid. Thus the benzene solution was concentrated and used further for TLC.

Loading of spot: After cooling activated TLC plates were used for loading the spots. Concentrated plant extract was used for loading. It was loaded 2-3 times and dried after successive loading. Methanol and liquid ammonia in proportion of 200:4 was used as solvent system. After saturation of chambers for half an hour, loaded TLC plates were kept for running in the above solvent system for half an hour. The TLC plates were then removed, air-dried and sprayed with ninhydrin reagent. (Ninhydrin reagent: 30 mg of ninhydrin dissolved in 10 ml of n-butanol and 0.3 ml glacial acetic acid.).

The plates were dried and then heated in oven at 110°C for 5-10 minutes. The TLC plate showed pinkish violet coloured spot. The range of R_f value varied from 0.5 to 0.7.

Chromatographic analysis of the sample of market drug and authentic ephedrine alkaloid was carried out by the same method. Crystals of ephedrine dissolved in benzene were used to load and compare the TLC.

Solvent system = Methanol: Ammonia in 200:4 respectively

Reagent – Fresh ninhydrin

After detection of ephedrine by TLC, preparatory TLC was performed. The silica gel of known R_f value was eluted and dissolved in chloroform. The extracted Ephedrine was dried and dissolved in $CdCl_3$ and used for NMR studies. Samples dissolved in Nujol were used for FTIR.

IR: Infrared spectroscopy is a reliable and sensitive method to identify the functional group present in the sample. The definite categorization is possible by comparing the peaks with the standards. IR spectra were taken on FTIR – 8201 PC of Shimadzu – Japan (Dyer, J.R.1969, Khandpue, R.S. and Ewing Galen W,1982). ^1H NMR Spectroscopy shows the proton signals by comparing these signals with standard chemical we can conclude the result (Ewing Galen W.1982).

Quantitative Estimation:

Quantitative estimation of alkaloid: Ephedrine

Materials: Chloroform, solvent ether, ammonia, sodium bicarbonate, cotton, charcoal, N/3 HCL, N/10 NaOH, NaCl,0.1 N H_2SO_4, Methyl red indicator.

Procedure: To the 20 g of plant powder, 200 ml of Chloroform and solvent ether (Diethyl ether) in 1:3 proportion added. 10 ml of 10% ammonia solution and 1 g of sodium carbonate was added to make it alkaline. It was kept for overnight shaking.

Wet cotton plugs with solvent ether were placed at the bottom of funnel. Activated charcoal was placed onto the cotton plug up to half of the funnel. Overnight shaked mixture was slowly poured in

funnel. A clear liquid was collected in 500 ml conical flask. This percolation or filtration was continued with 100 ml mixture of chloroform and solvent ether in 1:1.5 proportion (This 100 ml mixture was shaken with the residue remained in the conical flask). Again same percolation or filtration was continued with 100 ml solvent ether.

This combined percolates was shaken with successive portions of 40, 30, 20 and 20 ml of N/10 NaOH with drop wise addition till the solution was acidic to litmus. 10 g of anhydrous sodium carbonate was added and the solution was saturated with NaCl. The clear alkaline solution was extracted with 5 successive portions of 60, 50, 50, 30, and 25 ml of solvent ether. The combined ether extract was allowed to stand until clear and then filtered.

Ether solution and alkaline solution were separated in separating funnel. In ether solution, 10 ml 0.1 N sulfuric acid added to make it acidic and was kept for evaporation.

The beaker which contained the residue was washed with 20 ml of 0.1 N H_2SO_4, and transferred to conical flask. It was further washed with 20 ml of distilled water. Final volume became 50 ml 'A solution' and the solution was titrated with 0.1 N NaOH, using Methyl red as an indicator.

A blank titration was done with 30 ml 0.1 N H_2SO_4 and the difference in the reading indicates the amount of acid reacted with alkaloids. 1 ml of 0.1 N H_2SO_4 is equivalent to 0.01651 g of total alkaloid calculated as ephedrine. Pink colour indicated the end point.

Results and discussion

TLC, IR & NMR Studies:

Thin layer chromatography (TLC) was carried out by using specific solvent system for the development of silica gel plates. Spot and R_f value obtained by standard ephedrine hydrochloride, seeds of *Sida acuta* Burm. f. and *Sida rhombifolia* var. *microphylla* Cav. ARE approximately 0.7, while it is 0.5 in *Sida spinosa*, L (Figure 1. Sample A,B,C).

Infra- red spectroscopy (IR) peaks are obtained by standard ephedrine hydrochloride (Text. fig. 2), seeds of *Sida spinosa*, L (Text. fig. 3), seeds of *Sida acuta* Burm. f (Text. fig. 4) and *Sida rhombifolia* var. *microphylla* Cav. (Text. fig. 5) and are nearly of same wavelength and wave number / cm (Table 1).

^{1}H Nuclear magnetic resonance (NMR) was carried out for the identification of alkaloid. The five proton signals appeared as a multiplate in its ^{1}H NMR spectrum have clearly indicated the only one carbon atom is substituted. Further, the ^{1}H NMR shows the presence of 2-methyl group appeared as doublet at δ1.25 and singlet appeared at δ2.78.

The ^{1}H NMR spectrum shows signal at δ3.5, that indicates the presence of NH in the above compound. The signal appeared at δ5.4 (br) indicated, the presence of hydroxyl group. The above spectral data clearly indicated the presence of ephedrine in the samples. (Standard, Text fig 7), seed of *Sida spinosa* L. (Text. fig. 6), seeds of *Sida acuta* Burm. f, (Text. Fig 8) and *Sida rhombifolia* var. microphylla Cav. (Text. fig. 9).

References:

1) Nadkarni, A.K. (1927) Materia Medica, Popular Book Depot. Lamington Road, Bombay 3 rd edn, Vol. I,II

2) Cooke, T.(1967).The Flora of Presidency of Botany (2nd ed.) (Vol. I: 93-129). Botanical Survey of India

3) Dyer, J. (1969). Application of absorption spectroscopy of organic compounds. New delhi: Prentic Hall of India Pvt. Ltd.

4) Ewing Galen, W. (1982). Instrumental methods of chemical analysis (5th ed.) Singapore: McGraw Hill international editions.

5) Kirtikar, K.R. and Basu, B.D. (1937). Indian Medicinal Plants (2nd ed.) Parabasi Press Calcutta, India 1-2.

6) Khandpue, R.S. (1998). Handbook of analytical instruments. New Delhi: Tata McGraw Hill Publishing Company.

5. Claims

We claim:

1. A method for extraction of ephedrine using extraction, TLC, IR and NMR from four different plant species.
2. Extraction method as in claim 1 is applicable for studied plants as shown below.
   a) *Sida Acuta* Burm. f.
   b) *Sida rhombifolia* L. var. *microphylla* Cav.
   c) *Sida spinosa* Linn.
3. Extraction method present study as in claim 1, using plants from family Malvaceae as in claim 2 can be extracted using root powder in benzene as a solvent.
4. Method of extraction characterized in comprising the following process steps;
   a. Root powder of plant material (passed through 80 mesh size sieve) as in claim 2
   b. Methanol and liquid ammonia in proportion of 200:4 as a solvent system for TLC.
   c. Detected ephedrine by TLC further processed for preparatory TLC. The silica gel of known R_f value is eluted. It is dissolved in chloroform.
   d. Ephedrine extract then dried and dissolved in $CdCl_3$.
   e. Dissolution of samples in Nujol for FTIR and NMR.
5. Method of extraction according to claim 4 characterized in that the method is the production of a solution form (benzene extract).
6. Method used for extraction as in claim 1, for the plant as in claim claim 2, using extraction method as in claim 3 & 4, is capable for increase the yield of ephedrine.
7. According to any of the proceeding claims that exemplified, the said method shows increased enhanced yield and new sources for ephedrine.

Name : Prof. Dr. Deokule Subhash S Signature :

6. Date and Signature

Date: 24.10.2017

Place: Pune

Prof. Dr. Deokule Subhash S.
Department of Botany,
Savitribai Phule University of Pune (SPPU),
Ganeshkhind Road,
Pune-411007. Maharashtra, India

No. of sheet - 5

Figure 1: TLC of Standard ephedrine hydrochloride

A: seeds of *Sida spinosa* Linn.; B: Seeds of *Sida acuta* Burm. f.;
C: Seeds of *Sida rhombifolia* var. *microphylla* Cav

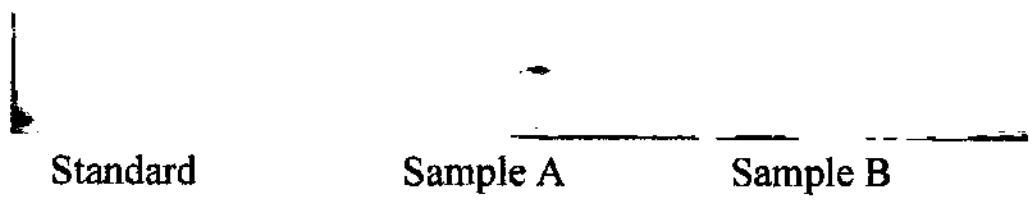
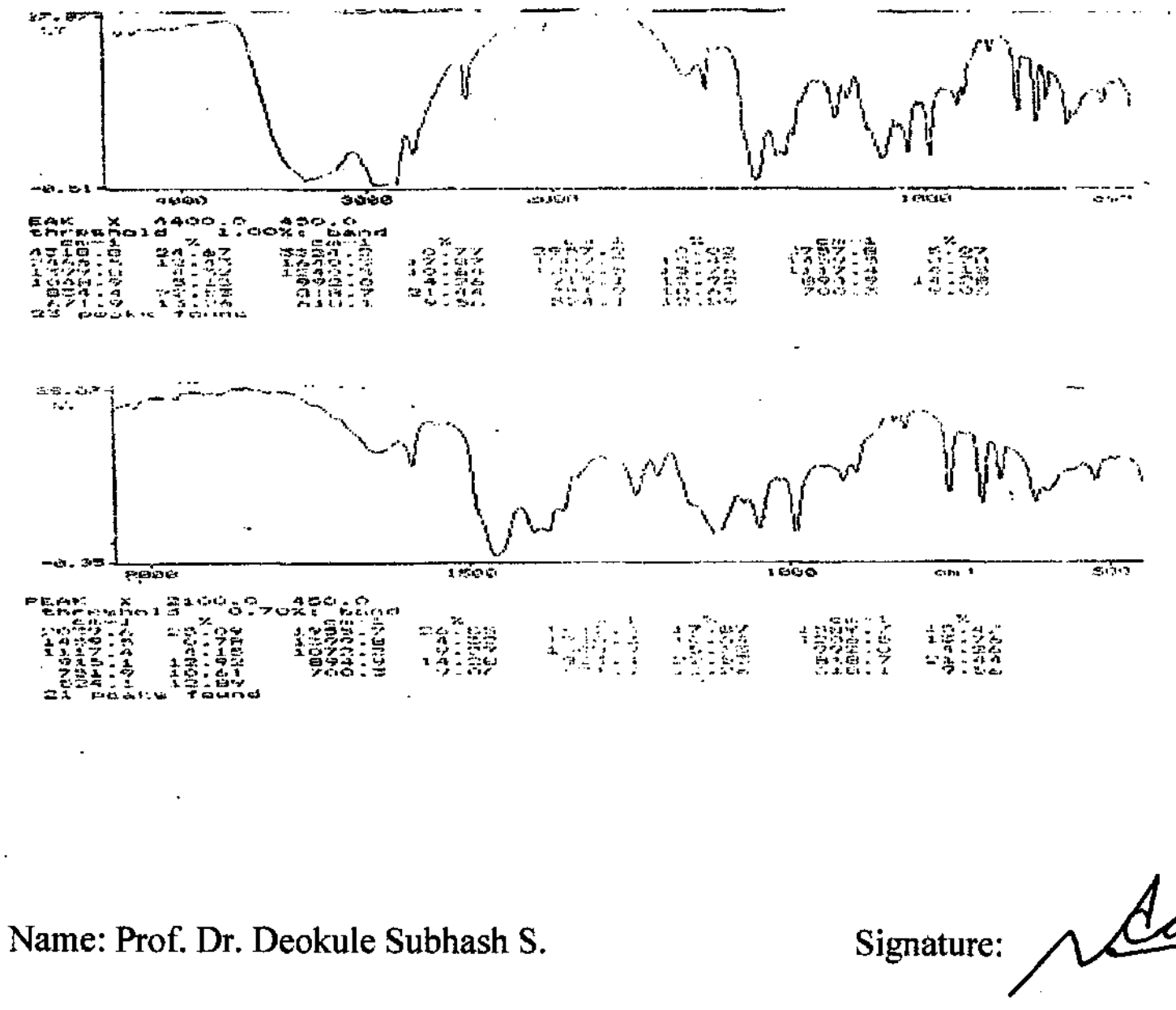


Figure 2: IR of Standard ephedrine hydrochloride in Nujol



Name: Prof. Dr. Deokule Subhash S.

Signature:

Figure 3: IR of Sample A, Sample B, Seeds of *Sida spinose*, L. in Nujol.

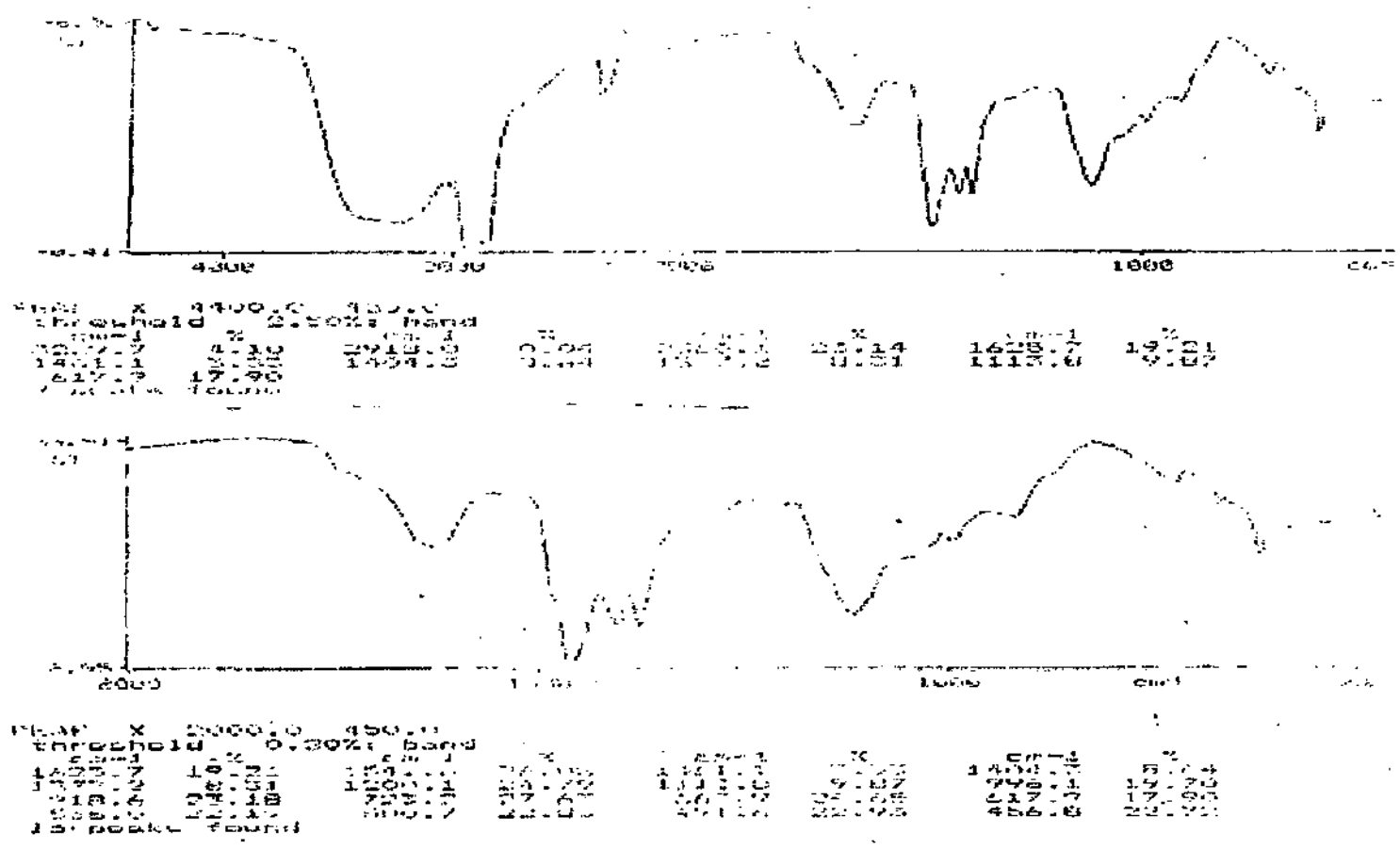
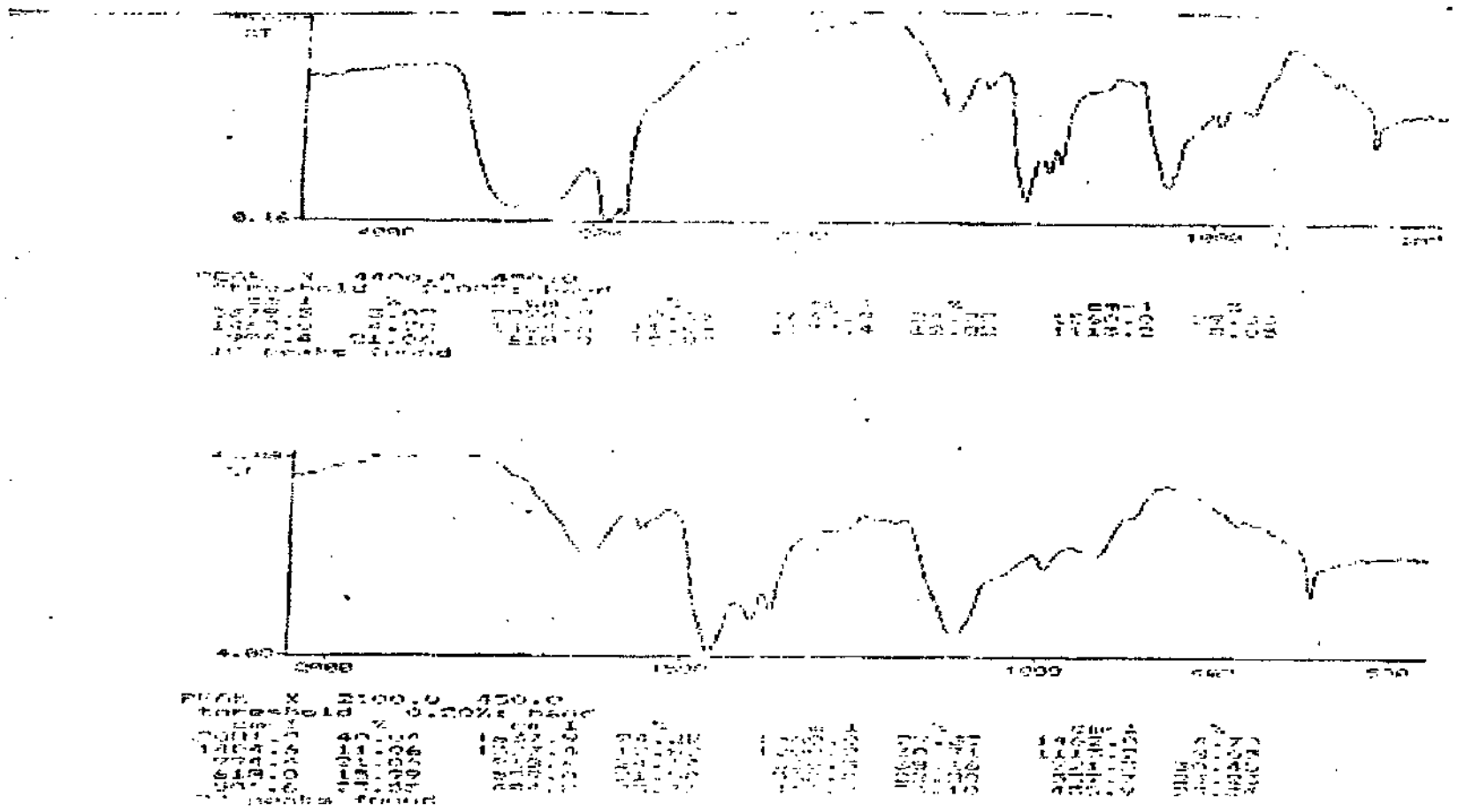


Figure 4: IR of Sample B, Seeds of *Sida acuta*, Burm. f, in Nujol.



Name: Prof. Dr. Deokule Subhash S.

Signature:

Figure 5: IR of Sample C, Seeds of Sida rhombifolia var. microphylla, Cav. in Nujol.

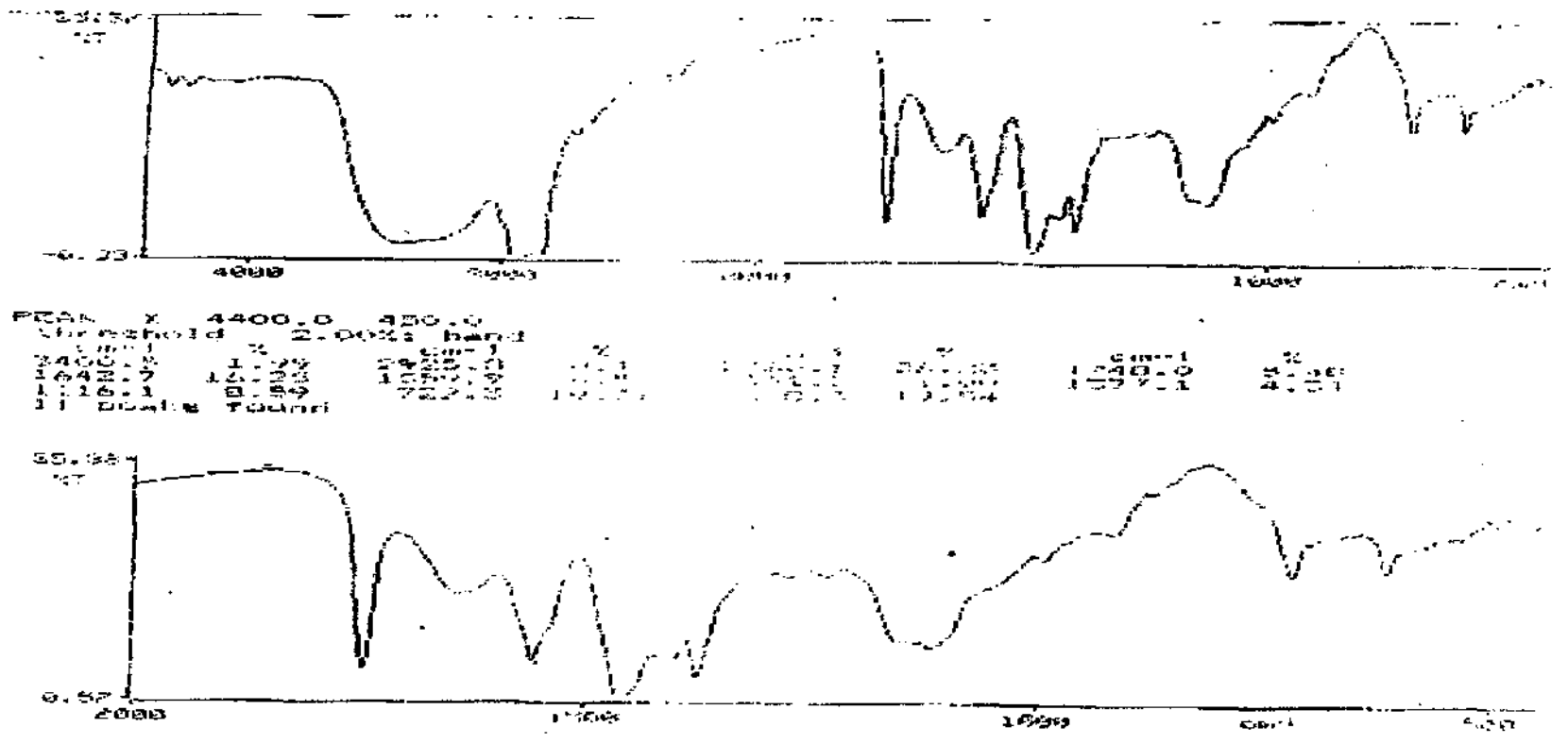
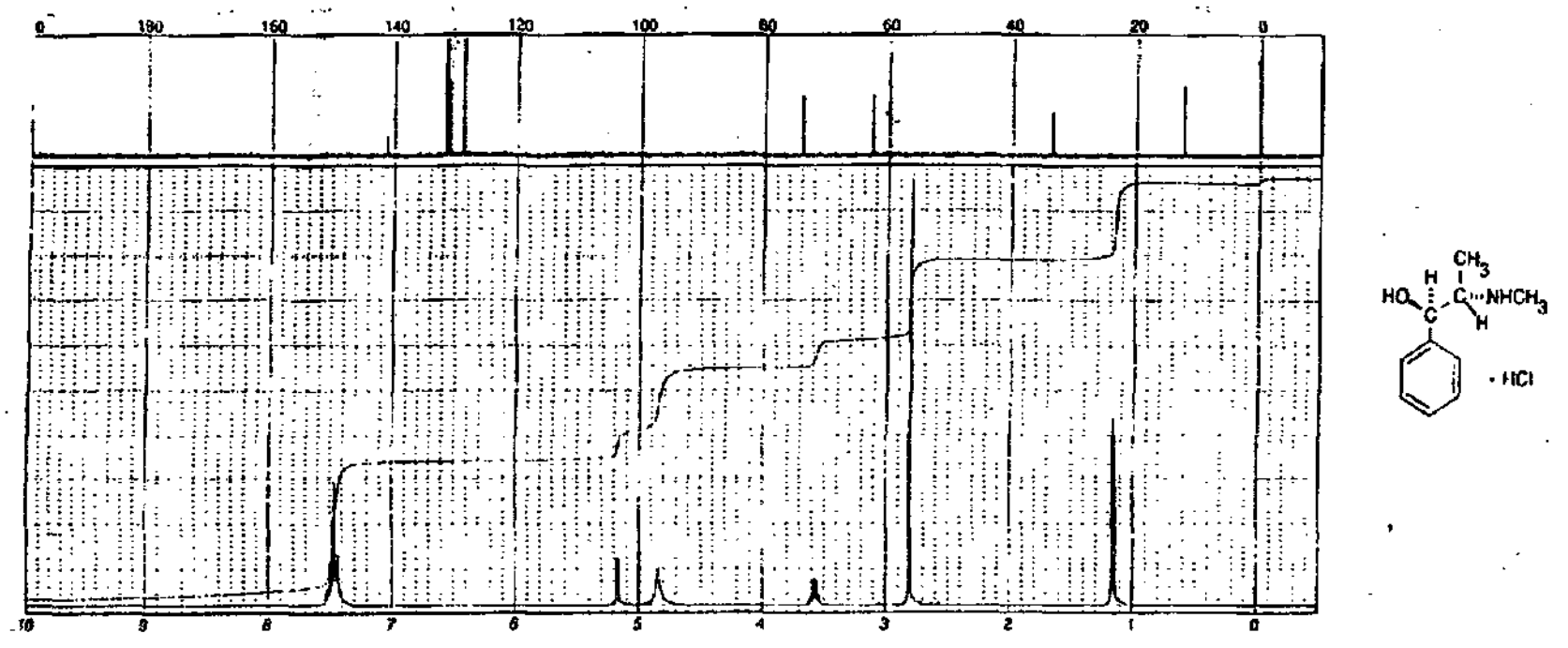


Figure 6: ^{1}H NMR spectrum of Standard ephedrine hydrochloride from Aldrich catalogue.



Name: Prof. Dr. Deokule Subhash S.

Signature:

Figure 7: ^{1}H NMR spectrum, Seeds of *Sida spinose*, L. in $CdCl_3$

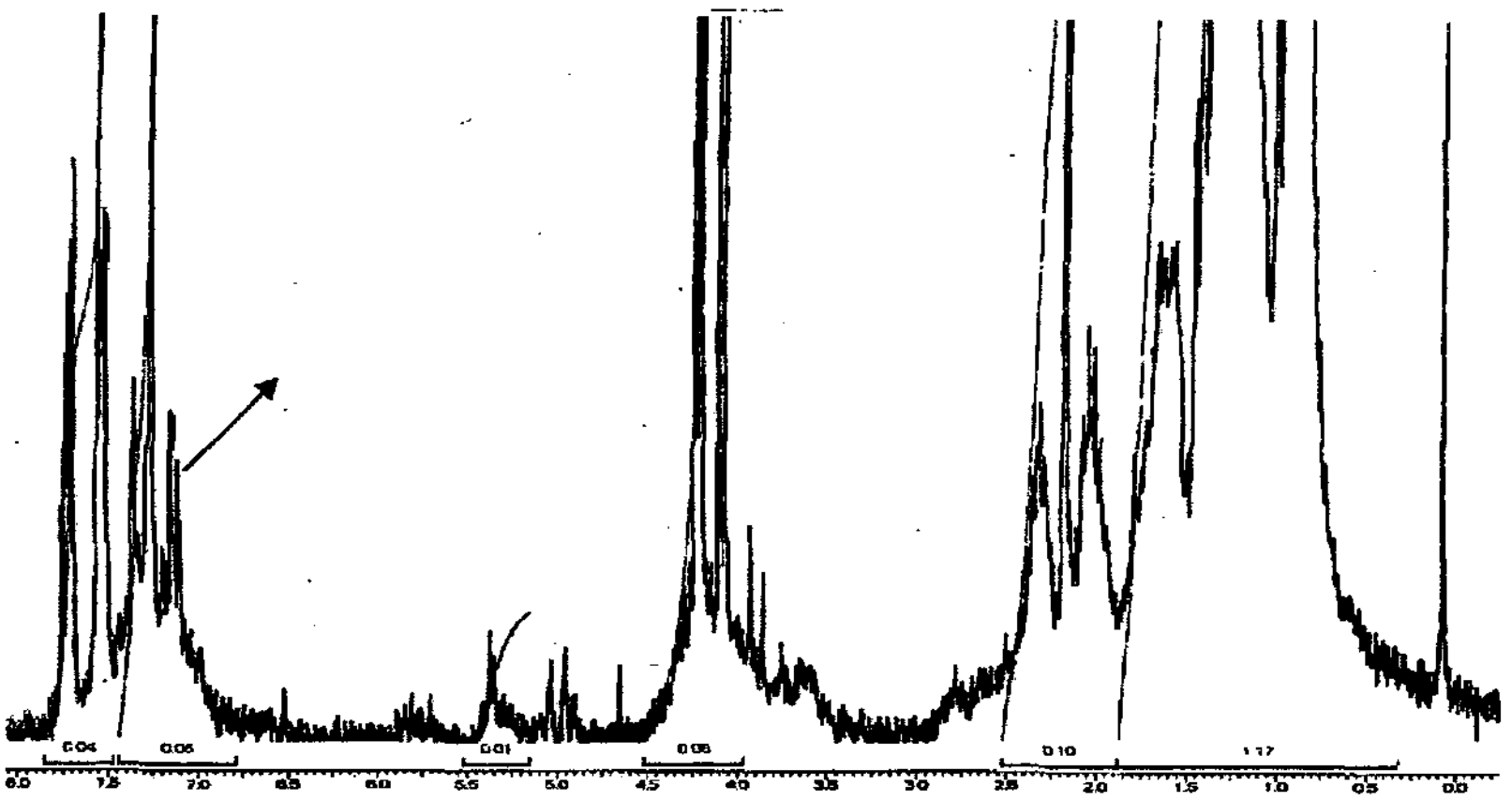
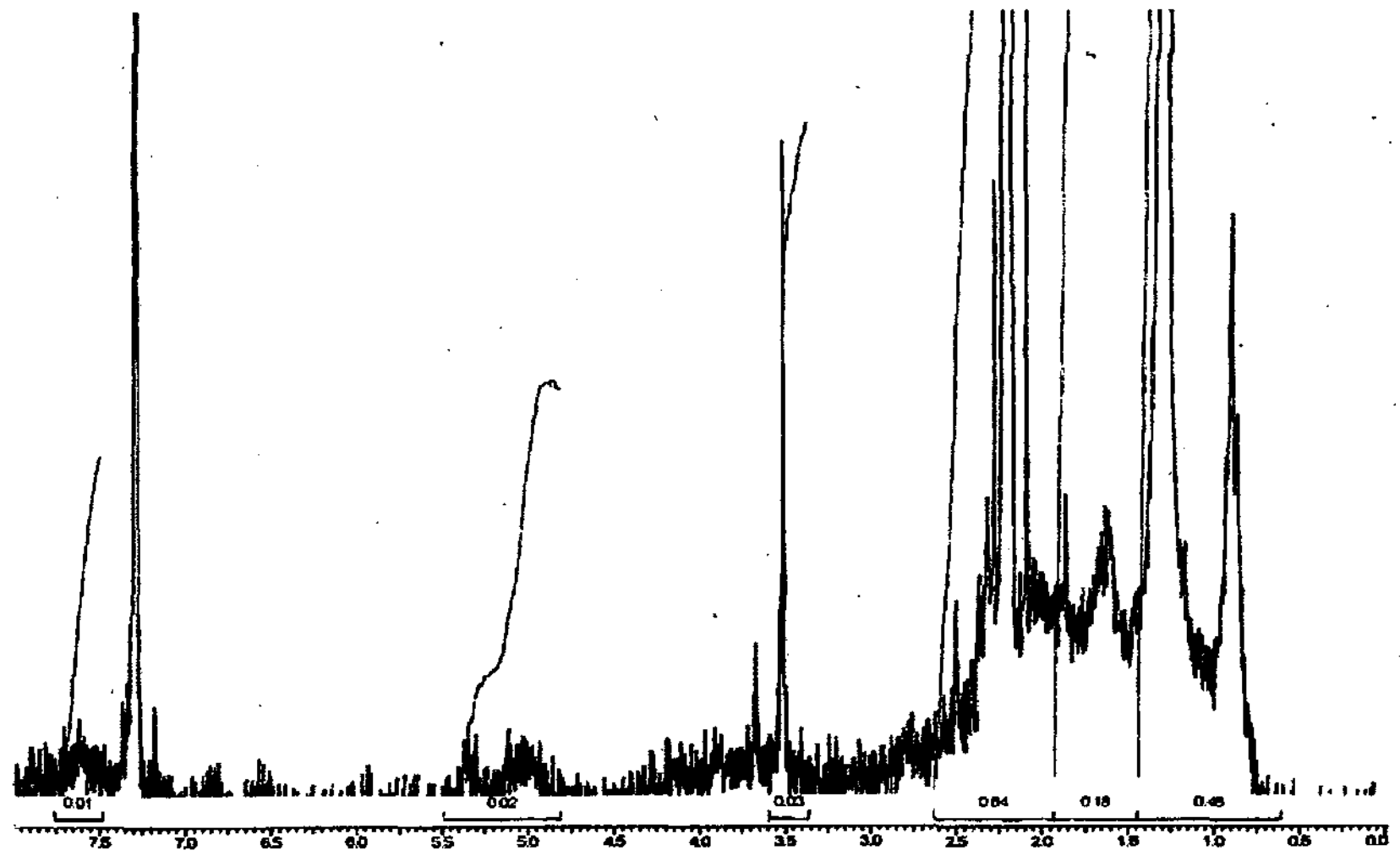


Figure 8: ^{1}H NMR spectrum, Seeds of *Sida acuta*, Burm. f. in $CdCl_3$



Name: Prof. Dr. Deokule Subhash S.

Signature:

Figure 9: ^{1}H NMR spectrum, Seeds of *Sida rhombifolia*, var. microphylla, Cav. in $CdCl_3$

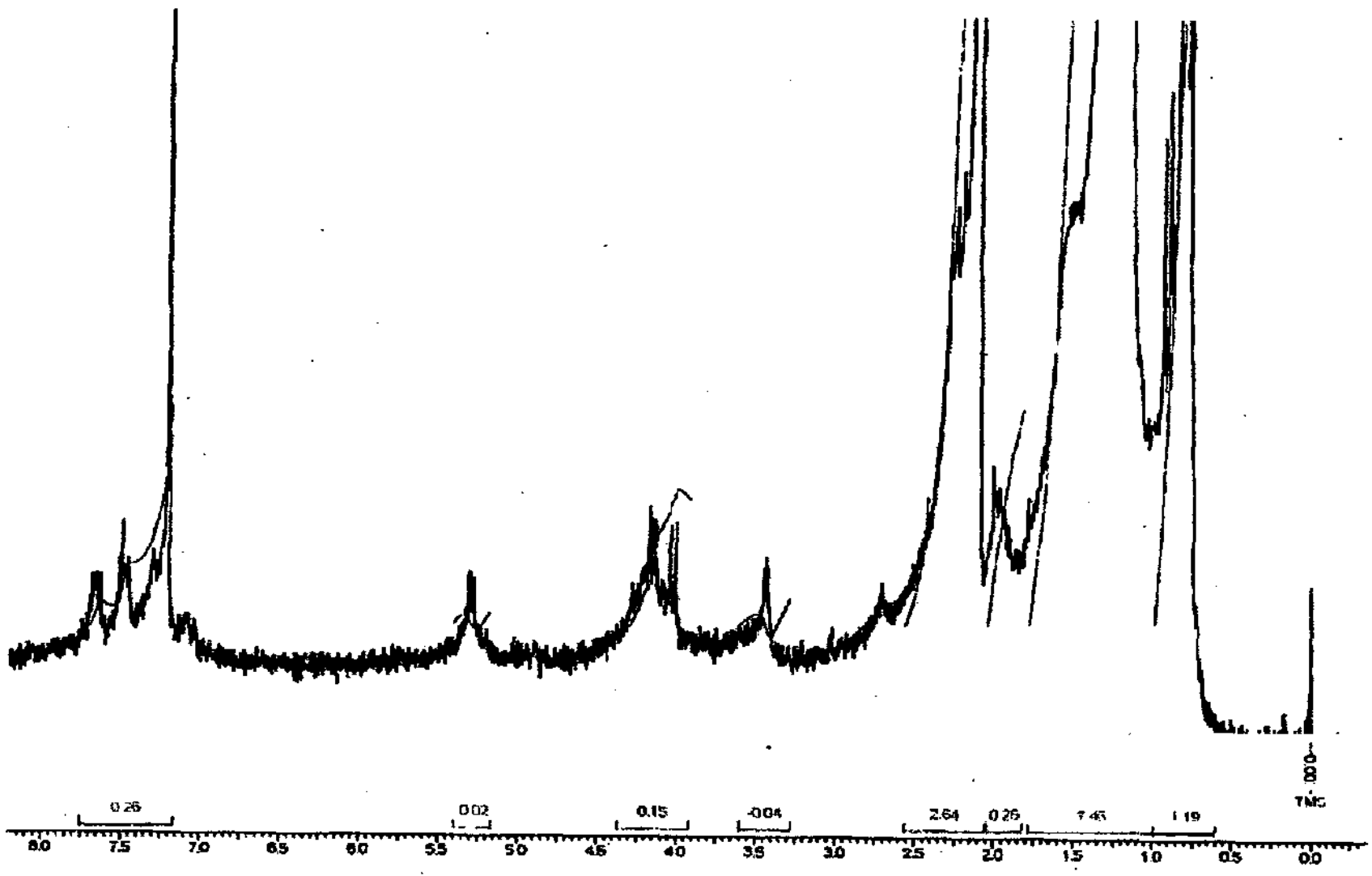


Table 1: Peak table of IR Scanning

FUNCTION AL GROUP	STANDARD		SAMPLE A		SAMPLE B		SAMPLE C	
	Position cm^{-1}	Intensity %T	Position cm^{-1}	Intensity %T	Position cm^{-1}	Intensity %T	Position cm^{-1}	Intensity %T
OH - Stretch, N- H Stretch	3324	0.7	3379	3.02	3229	4.1	3400	1.99
N - H bending	1640	17.8	1637	24.2	1625	119	1642	16
Aromatic	1399	4.3	1404	11	1404	8.6	1407	6.3

Name : Prof. Dr. S.S. Deokule

Deokule

Title :- "Novel Process for extraction of Ephedrine from plants"

7. ABSTRACT OF THE INVENTION

The present invention relates to a process is extraction process to increase the yield of ephedrine, particularly commercially useful biochemical with good demand by industry.

Sida cordifolia Linn. known as 'True Bala' consists alkaloid ephedrine. Hence it gives efficacy of the precious Ayurvedic drug Bala. But many times due to rare occurrence of *Sida cordifolia* Linn. It is substituted or adulterated with other genera (*Abutilon*) and species of *Sida* in Maharashtra's drug market samples.

Plants/seeds of *Sida acuta*, Burm. f.; *Sida spinosa* Linn. and *Sida rhombifolia* var. *microphylla* Cav. Shows presence of ephedrine. Therefore these species are recommended as a substitute for the true drug Bala i.e. *Sida cordifolia* Linn. On the basis of presence or absence of ephedrine, it is possible to identify *Sida* species for further use.

In the present investigation alkaloids present in the plants were detected quantitatively. Further these investigations were supported by thin layer chromatography (TLC), infrared spectroscopy (IR) and Nuclear magnetic resonance (NMR) techniques to confirm the presence and quantification of alkaloid ephedrine. The introduction part of this invention explains the uses of plants with increased yield of ephedrine content for its commercial use.

E-3/11423/2017

FORM 3

THE PATENTS ACT, 1970
[39 OF 1970]
and
THE PATENTS RULES, 2003

200174479

STATEMENT AND UNDERTAKING UNDER SECTION 8
(See Section 8; Rule 12)

1. Name of the applicants	I, Prof. Dr. Deokule Subhash S., Department of Botany, Savitribai Phule Pune University, Pune, hereby declare:
2. Name, address and nationality of the joint applicant	Mr. Shilimkar Vaibhav C. Department of Pharmacognosy, Seth Govind Raghunath Sable College of Pharmacy, Saswad, Taluka Purandhar, Dist. Pune; Dr. Mungikar Rahul R., Parisanstha, 24/4, Balaji Park 4, Talegaon Dabhade, Taluka- Maval, Dist.- Pune and Dr. Suresh D. Jagtap, Herbal Medicine, IRHSA, Bharati Vidyapeeth University, Pune. (i) that we have not made any application for the same/substantially the same invention outside India
3. Name and address of the assignee	(ii) that the rights in the applications have been assigned to Department of Botany, Savitribai Phule Pune University, Ganesh Khind road, Pune-7, Maharashtra, India. that we undertake that up to the date of grant of the patent by the Controller, we would keep him informed in writing the details regarding corresponding applications for the date of filling of such application Dated this 30 day of October 2017.
4. To be signed by the applicant or his authorized registered patent agent	
5. Name of the natural person who has signed	Prof. Dr. Deokule Subash S. Professor (Retd) Department of Botany, Savitribai Phule Pune University, Ganeshkhind road, Pune-7, Maharashtra, India.
IPO MUMBAI 30-10-2017	To, The Controller of Patents, The Patent Office, at Mumbai

E-5|1694|2017

FORM 5
THE PATENTS ACT, 1970
(39 of 1970)
&
The patents rules, 2003
DECLARATION AS TO INVENTORSHIP
[See section 10 (6) and rule 13(6)]

200174480

1. NAME OF APPLICANTS	**(i) Prof. Dr. Deokule Subhash S.**

Hereby declare that the true and first inventor(s) of the invention disclosed in the complete specification filed in pursuance of our application numbered..... Dated..30.-10.....are..2017.....

2. INVENTOR(S)

a.	**Name**	**:**	Prof. Dr. Deokule Subhash S.
b.	**Nationality**	**:**	Indian
c.	**Address**	**:**	Department of Botany, Savitribai Phule Pune University, Ganeshkhind Road, Pune-411007, Maharashtra, India
a.	**Name**	**:**	Dr. Patale M. W.
b.	**Nationality**	**:**	Indian
c.	**Address**	**:**	Department of Botany, Savitribai Phule Pune University, Ganeshkhind Road, Pune-411007, Maharashtra, India
a.	***Name***	**:**	*Dr. Mungikar Rahul R.*
b.	**Nationality**	**:**	Indian
c.	**Address**	**:**	Parisanstha, 24/4, Balaji Park 4, Rao Colony, Talegaon Dabhade, Taluka- Maval, Dist.- Pune - 410 506. Maharashtra, India.
a.	**Name**		Mr. Shilimkar Vaibhav C.
b.	**Nationality**		Indian
c.	**Address**		Department of Pharmacognosy, Seth Govind Raghunath Sable College of Pharmacy, Saswad, Taluka Purandhar, Dist. Pune – 412 301. Maharashtra, India.

a.	**Name**		Dr. Jagtap Suresh D.
b.	**Nationality**		Indian
c.	**Address**		Herbal Medicine Interactive Research School for Health Affairs (IRSHA), Bharati Vidyapeeth University, Pune Satara Road, Pune – 411 043. Maharashtra, India.

Dated this 30..... day of .October 2017

Signature:

Savitribai Phule Pune University *Dept. of Botany*

Prof. Dr. Deokule S. S.

PROFESSOR (Rtd.)
Department of Botany
Savitribai Phule Pune University
Pune - 411 007.

3. DECLARATION TO BE GIVEN WHEN THE APPLICATION IN INDIA IS FILED BY THE APPLICANTS IN THE CONVENTION COUNTRY:-

-NOT APPLICABLE-

4. STATEMENT (to be signed by the additional inventors not mentioned in the application form)

-NOT APPLICABLE-

To,

The Controller of Patent

The Patent Office, at Mumbai

200293026

FORM 18 THE PATENTS ACT 1970 (39 of 1970) & The Patents Rules, 2003 REQUEST/EXPRESS REQUEST FOR EXAMINATION OF APPLICATION FOR PATENT [See section 11B and rule 20(4)(ii), 24B(1)(i)]	(FOR OFFICE USE ONLY) RQ No: 2020 20 31 281 Filing Date: 08/10/2020 Amount of Fee Paid: रूपये नकद/चेक/मनी ऑर्डर द्वारा CBR No: 44001 CBR संख्या. 26226 ..दि.08/10/2020 Signature के नाम प्राप्त हुए।

1. APPLICANTS

(a)	Name:	Deokule Subhash Sadhu
(b)	Nationality:	Indian
(c)	Address:	Department of Botany, Savitribai Phule Pune University (SPPU), Pune- 411 007, Maharashtra, India.

रीकडिंग

2. Statement in case of request for examination made by the applicants

We hereby request that our application for patent no.: **201721038426** filed on **30/10/2017** for the invention titled **"Novel Process for extraction of Ephedrine from selected plants"** shall be examined under sections 12 and 13 of the Act.

3. Statement in case of request for examination made by any other interested person

I/We the interested person request for the examination of the application no._____dated _____filed by the applicant_____titled_____under sections 12 and 13 of the Act.

As an evidence of my/our interest in the application for patent following documents are submitted.

(a) _____

Not applicable

4. ADDRESS FOR SERVICE

Department of Botany, Savitribai Phule Pune University (SPPU), Pune- 411 007, Maharashtra, India. **Email:** deokule.ss@gmail.com. **Mobile:** 9371088210.

Dated this 8 day of October 2020

Deokule

Deokule Subhash Sadhu

To,
The Controller of Patents
The Patent Office,
Mumbai

SAVIRIBAI PHULE PUNE UNIVERSITY

(formerly University of Pune)

DEPARTMENT OF BOTANY
SAVITRIBAI PHULE PUNE UNIVERSITY
Ganeshkhind, Pune-411 007
Tele. No. : (020) 25601439, 25601438
Email : .....@unipune.ac.in

D.38044

Ref. No. : Bot/ E-101/13696/2012 **Date :** 30/10/2017

To,
The Controller of Patents,
The Patent Office,
Boudhik Sampada Bhavan,
S. M. Road, Antop Hill, Mumbai,
Maharashtra- 400037

200174477

Dear Sir,

Re: Submission of Complete Patent Specification

Please find herewith documents for a complete specification application entitled "**Novel process for extraction of Ephedrine from selected plants**" along with the prescribed fee of Rs. 1750/-.

The Learned Controller is requested to take the same on record and issue the official filing receipt for the same.

Yours faithfully,

Prof. Dr. Deokule Subhash S.
Department of Botany,
Savitribai Phule Pune University (SPPU),
Ganeshkhind Road,
Pune- 411007. Maharashtra, India

Enclosures:

1. Application for Grant of Patent [Form 1]
2. Complete specification [Form 2]
3. Statement and undertaking under section 8 [Form 3]
4. Declaration as to inventorship [Form 5]
5. Fees of Rs. 1750/- (by cash)

IPO MUMBAI 30-10-2017 16:58

INTELLECTUAL PROPERTY INDIA
एकस्व/PATENTS|अभिकल्प/DESIGNS|
व्यापार चिह्न/TRADE MARKS|भौगोलिक
उपदर्शन/GEOGRAPHICAL INDICATIONS

सत्यमेव जयते
भारत सरकार
GOVERNMENT OF INDIA

एकस्व कार्यालय /THE PATENT OFFICE
बौद्धिक सम्पदा भवन/ I.P.O. BUILDING
एंटोप हिल/Antop Hill,
एस.एम.रोड/ S.M.Road,
मुंबई/ Mumbai- 400037
दूरभाष /Tel. No.: (091)(022)24153651
फ़ैक्स/ Fax: 022-24130387
ई मेल/ Email: mumbai-patent@nic.in
वेबसाइट /Website:http://ipindia.nic.in

सं.संख्या/Ref.No /आवेदन संख्या/Application No/ 201721038424 दिनांक/Date of Dispatch/Email: 24/06/2021

सेवा मे,/To
PROF.DR. DEOKULE SUBHASH S.,
PROF.DR. DEOKULE SUBHASH S. DEPARTMENT OF BOTANY, SAVITRIBAI PHULE PUNE UNIVERSITY (SPPU), GANESHKHIND ROAD, PUNE-411 007, MAHARASHTRA, INDIA.
Email : deokule.ss@gmail.com

विषय: एकस्व अधिनियम, 1970 की धारा 12 व 13 तथा एकस्व नियम, 2003 के अधीन परीक्षण रिपोर्ट
Subject: Examination report under sections 12 & 13 of the Patents Act, 1970 and the Patents Rules, 2003.

1. उपर्युक्त आवेदन के संदर्भ मे परीक्षण रिपोर्ट (अर्थात, एकस्व नियम, 2003 (यथा संशोधित) के नियम 24-ख(3) में विनिर्दिष्ट आपत्तियों का प्रथम कथन) इसके साथ संलग्न है। यह रिपोर्ट परीक्षण हेतु अनुरोध दिनांक 08/10/2020 के उत्तर मे जारी की गयी है। परीक्षण रिपोर्ट का उत्तर दाखिल करने की अंतिम तिथि (अर्थात, इस रिपोर्ट में लगाई गयी सभी आवश्यकताओं के अनुपालन की अवधि) आवेदक को आपत्तियों का प्रथम कथन जारी होने की तिथि से छः माह है।
   Please find enclosed herewith an Examination Report (i.e. a first statement of objections as specified in Rule 24-B(3) of The Patents Rules, 2003 (as amended)) in respect of above-mentioned application. This report is issued with reference to a request for examination dated 08/10/2020.The last date for filing a response to the Examination Report (i.e. a period to comply with all the requirements raised in this examination report) is six months from the date on which the first statement of objections is issued to the Applicant.

2. यदि रिपोर्ट के अंतर्गत लगाई गयी आवश्यकताओं का अनुपालन एकस्व नियम, 2003 (यथा संशोधित) के नियम 24 ख(5) में विनिर्दिष्ट अवधि के भीतर अंदर अनुपालन नहीं किया गया तो एकस्व अधिनियम 1970 की धारा 21(1) के अधीन वर्तमान आवेदन को परित्यक्त माना जाएगा।
   The instant application shall be deemed to have been abandoned under Section 21(1) of The Patents Act, 1970, unless all the requirements raised in this report are complied with in the period as specified in Rule 24-B (5) of The Patents Rules, 2003 (as amended).

3. आपका ध्यान एकस्व नियम, 2003 के नियम 24 ख(6) के प्रावधानों की ओर भी आमंत्रित किया जाता है।
   Your attention is also invited to the provisions of Rule 24-B (6) of the Patents Rules 2003.

4. आपको सलाह दी जाती है कि शीघ्र निपटान हेतु अपना उत्तर शीघ्र प्रस्तुत करें।
   You are advised to file the reply at the earliest for early disposal.

Soumen Ghosh
नियंत्रक पेटेंट/ Controller of Patents

संलग्न/Enclosed: अपरोक्त अनुसार/As above

टिप्पणी: यह इलेक्ट्रोनिक रूप से उत्पन्न रिपोर्ट है।
NOTE: This is an electronically generated report.

सभी पत्राचार नियंत्रक एकस्व को उपरोल्लिखित पते पर भेजा जाये।
All communications should be sent to the Controller of Patents at the above mentioned address.

THE PATENT OFFICE

परीक्षण रिपोर्ट /Examination Report

आवेदन संख्या /Application Number	201721038424
दाखिल करने की तिथि /Date of Filing	30/10/2017
पूर्विक्ता दिनांक /Date of Priority	--
पीसीटी अंतर्राष्ट्रीय आवेदन की संख्या व दिनांक / PCT International Application No. & Date	--
आवेदक /Applicant	PROF.DR. DEOKULE SUBHASH S.
परीक्षण हेतु अनुरोध की संख्या व दिनांक /Request for Examination No. & Date	R20202031282 08/10/2020
प्रकाशन की तिथि /Date of Publication	03/05/2019

इस परीक्षण रिपोर्ट के चार भाग हैं, अर्थात रिपोर्ट का सारांश, विस्तृत तकनीकी रिपोर्ट, औपचारिक आवश्यकताएँ तथा रिकॉर्ड मे दस्तावेज़ /
This examination report consists of four parts, namely summary of the report, detailed technical report, formal requirements and documents on record.

भाग -1: रिपोर्ट का सारांश
PART-I: SUMMARY OF THE REPORT

क्र. सं. /Sl. No.	अधिनियम के तहत आवश्यकताओं पर विस्तृत टिप्पणियां /Requirements under the Act		दावों की संख्या /Claim Numbers	टिप्पणी /Remarks
1.	धारा 2(1)(ज) के तहत आविष्कार /Invention u/s 2(1)(j)	नवीनता /Novelty	दावे /Claims:	हाँ /Yes
			दावे /Claims: 1-6	नहीं /No
		आविष्कारी कदम / Inventive step	दावे /Claims:	हाँ /Yes
			दावे /Claims: 1-6	नहीं /No
		औद्योगिक उपयोगिता /Industrial Applicability	दावे /Claims: 1-6	हाँ /Yes
			दावे /Claims:	नहीं /No
2.	धारा 3 के अधीन पेटेंट-अयोग्यता (यदि हाँ, खंड 3(क-त) /Non-patentability u/s 3 (if yes, specify section3(a-p))		दावे /Claims: 1-6	हाँ /Yes 3(d)
			दावे /Claims:	नहीं /No
3.	[धारा 10(5) व 10(4) (ग)] के अधीन दावे /Claims [u/s 10(5) & 10(4) (c)]	स्पष्टता/ संक्षिप्तता /Clarity / Conciseness	दावे /Claims:	हाँ /Yes
			दावे /Claims: 1-6	नहीं /No

भाग –II विस्तृत तकनीकी रिपोर्ट
PART-II: DETAILED TECHNICAL REPORT

क. उद्धरित दस्तावेजों की सूची /A.List of documents cited:

(क) पेटेंट साहित्य / (a). Patent Literature :

कोई दस्तावेज़ उद्धृत नहीं है /No Document Cited

(ख) गैर-पेटेंट साहित्य /(b).Non-patent literature

क्र. सं.	दस्तावेजों का विवरण /Details of	प्रकाशन	उद्धरित दस्तावेज़ का प्रासंगिक विवरण (पृष्ठ व अनुच्छेद संख्या) /Relevant	अभिकथित आविष्कार के दावे /Relevant	अभिकथित आविष्कार

/ Sl.no	दस्तावेज़ों का विवरण /Details of documents	तिथि(दिन/माह/वर्ष) /Publication date	/Relevant description (page and paragraph no.) of cited document	के दावे /Relevant claims of cited document	के दावे /Claims of alleged invention
1	D1: Abyari, M. et.al. : Title: Enhanced Accumulation of Scopoletin in Cell Suspension Culture of Spilanthes acmella Murr. Using Precursor Feeding (Braz. Arch. Biol. Technol. v.59: e16150533, Jan/Dec 2016) (http://dx.doi.org/10.1590/1678-4324-2016150533)	15/02/2016	abstract and entire document		1-6
2	D2: Naikawadi, V.B. et.al. : Title: In vitro propagation and cell cultures of memory tonic herb Evolvulus alsinoides: a best source for elicited production of scopoletin (Appl Microbiol Biotechnol) (DOI 10.1007/s00253-015-7153-5)	01/12/2015	abstract and entire document		1-6
3	D3: Goy, P.A. et.al. : Title: Accumulation of scopoletin is associated with the high disease resistance of the hybrid Nicotiana glutinosa*Nicotiana debneyi (Planta (1993) 191: 200-206)	08/03/1993	abstract and entire document		1-6

ख. अधिनियम के तहत आवश्यकताओं पर विस्तृत टिप्पणियां /B. Detailed observations on the requirements under the Act:

(1).नवीनता / NOVELTY:

(I) ऊपर उद्धरित दस्तावेज़ के संदर्भ (1-6) मे दिये गए प्रकटन के पूर्वानुमान को ध्यान मे रखते हुए, निम्नलिखित कारणों से दावा(वों) (1-6) मे नवीनता की कमी है /
Claim(s) (1-6) lack(s) novelty, being anticipated in view of disclosure in the document cited above under reference D1 for the following reasons:

Claims 1-6 do not constitute an invention u/s 2(1)(j) of the Patents Act, 1970 as they do not involve novelty in view of the following documents separately:

D1: Abyari, M. et.al. : Title: Enhanced Accumulation of Scopoletin in Cell Suspension Culture of Spilanthes acmella Murr. Using Precursor Feeding (Braz. Arch. Biol. Technol. v.59: e16150533, Jan/Dec 2016) (http://dx.doi.org/10.1590/1678-4324-2016150533)

The present application relates to the various concentrations of casein hydrolysate (25, 50, 75, 100 mg/L) and L-phenylalanine (50, 100, 150, 200 µM/l) were incorporated in MS containing 15 µM BA plus 5 µM 2,4-D for enhancement of secondary metabolites in cell culture of Spilanthes acmella. The presence of casein hydrolysate in the nutrient medium improved the growth of cell biomass and the production of scopoletin. The addition of casein hydrolysate up to 75 mg/L stimulated the accumulation of scopoletin, but increasing excess 75 mg/L the level of casein hydrolysate reduced the production of scopoletin. The addition of L-phenylalanine in the nutrient medium was found to be more effective for production of secondary metabolite in S. acmella. The addition of 50 µM/L of L-phenylalanine in the medium increased scopoletin content to 27.12 ± 0.58 µg/g dry weight, compared to the scopoletin content of control at 7.89 ± 0.61 µg/g dry weight. The highest accumulation of scopoletin was observed in the 100 µM/L L-phenylalanine in cell suspension, which was 4.51 times more than the control. As a result, using moderate concentration of L-phenylalanine was ideal for the production of scopoletin. In general, casein hydrolysate was more effective than L-phenylalanine for production of scopoletin and growth of cell

biomass in the cell culture of S. acmella.

D1 discloses casein hydrolysate is a mixture of amino acids and peptides produced by enzymatic or acid hydrolysis of casein, that obtained by digesting casein with hydrochloric acid. It therefore acts as a good nutrient substrate and is especially suitable for the large-scale cultivation in micropropagation and secondary metabolites production. Casein hydrochloric acid hydrolysate was added to media primarily because of the organic nitrogen and growth-factor components. The increase of secondary metabolites content in cell culture cultivated on media with the addition of casein hydrolysate might be due to its sterols or amino acids content. Among the biotechnological methods, stimulation of metabolic pathways in the cultivated plant cells toward production of a desired compound most likely results in a dramatic increase in secondary metabolite yield. Scopoletin originates from phenylalanine, an upstream metabolic precursor through coumarins biosynthetic pathway. The addition of phenylalanine is expected to increase the level of targeted compounds. The results indicated that addition of high level of L-phenylalanine did not improve cell biomass in cell culture. The incorporation of L-phenylalanine in the nutrient medium was found to be more effective for the production of secondary metabolite in S. acmella. The addition of 50 µM/L of L-phenylalanine in the medium increased scopoletin content to 27.12 ± 0.58 µg/g D.W, while scopoletin content of control was 7.89 ± 0.61 µg/g D.W. The highest accumulation of scopoletin was detected at 100 µM/L L-phenylalanine concentration in cell suspension, which was 4.51 times more than the control. As results using moderate concentrations of L-phenylalanine was ideal for the production of scopoletin (abstract and entire document).

Since all the necessary technical features of claims 1-6 can easily be anticipated by considering the prior art document D1, hence novelty of claims 1-6 cannot be acknowledged.

(2).आविष्कारी कदम / INVENTIVE STEP:

(I) ऊपर उद्धरित दस्तावेज़(जों) के संदर्भ D1, D2 and D3 मे स्पष्ट अध्यापन(नों) को ध्यान मे रखते हुए, निम्नलिखित कारणों से दावा(वों) (1-6) मे आविष्कारी कदम की कमी है
Claim(s) (1-6) lack(s) inventive step, being obvious in view of teaching (s) of cited document(s) above under reference D1, D2 and D3 for the following reasons:

Claims 1-6 do not constitute an invention u/s 2(1)(ja) of the Patents Act, 1970 as they do not involve inventive step in view of the following documents:

D1: Abyari, M. et.al. : Title: Enhanced Accumulation of Scopoletin in Cell Suspension Culture of Spilanthes acmella Murr. Using Precursor Feeding (Braz. Arch. Biol. Technol. v.59: e16150533, Jan/Dec 2016) (http://dx.doi.org/10.1590/1678-4324-2016150533)

D2: Naikawadi, V.B. et.al. : Title: In vitro propagation and cell cultures of memory tonic herb Evolvulus alsinoides: a best source for elicited production of scopoletin (Appl Microbiol Biotechnol) (DOI 10.1007/s00253-015-7153-5)

D3: Goy, P.A. et.al. : Title: Accumulation of scopoletin is associated with the high disease resistance of the hybrid Nicotiana glutinosa*Nicotiana debneyi (Planta (1993) 191: 200-206)

In addition to D1, D2 discloses Evolvulus alsinoides L. is used for preparation of ‘Shankhapushpi’, an important popular ayurvedic drug that contributes considerably to the improvement of memory power. The improvement is attributed to the presence of furanocoumarin scopoletin, a metabolite with a wide range of biological activities. This report describes, for the first time, an in vitro culture system for propagation and enhanced production of scopoletin. Different concentrations of auxins and cytokinins individually and in combination were used in Murashige and Skoog (MS) medium to induce shoot regeneration in cotyledonary nodal explants and callus formation in leaf explants. The best response was achieved in MS medium fortified with 5.0 µM 6-benzyladenine (BA) in which 96 % of cultures produced 7.6 ± 0.6 shoots per explant. Regenerated shoots were rooted on MS medium with 5.0 µM indole-3-acetic acid (IAA). Plantlets were successfully acclimatized and established in soil. MS medium fortified with 10 µM BA+5.0 µM IAA showed maximum growth and accumulation of scopoletin in cell cultures. Cell cultures could be maintained over 24 months. The influences of auxins, cytokinins, organic acids, amino acids, and fungal-derived elicitors on production of scopoletin were studied. Presence of either L-arginine, sodium pyruvate, or yeast extract highly promoted scopoletin production as compared with control and achieved 75.02-, 72.13-, and 57.98-fold higher accumulation, respectively. The results presented herein have laid solid

foundation for large-scale production of scopoletin and further investigation of its purification and utilization as a novel pharmaceutical drug (abstract and entire document).

D3 discloses the high disease resistance of the amphidiploid hybrid of Nicotiana glutinosa x Nicotiana debneyi is associated with high constitutive levels of two phenolic compounds as analysed by high-performance liquid chromatography. The structures of these two compounds were elucidated by means of gas chromatography-tandem mass spectrometry, fluorescence- and light-spectrophotometry to be those of scopolin and scopoletin. They reached levels of 4 nmol·(g FW)−1 and 35 nmol·(g FW)−1, respectively, in leaf tissues of the hybrid, about 10–50 times the amount found in the parental species. Scopoletin showed a direct antimicrobial activity against Cercospora nicotianae, Phytophthora parasitica var. Nicotianae, Pseudomonas syringae pvs. Tabaci and syringae and tobacco mosaic virus when added to synthetic growth media, mixed with the inoculum or sprayed onto tobacco plants prior to inoculation. We postulate that the high amount of toxic phenolics in the leaves of the hybrid N. Glutinosa x N. Debneyi contributes to its high disease resistance (abstract and whole document).

Therefore, from the combined teaching of documents D1-D3, any person skilled in the art can arrive at the method for extraction of scopoletin using in-vitro culture as disclosed in the current application without any technical advancement. To prove an inventive step, the applicant should relate the distinguished features of the present application over the cited prior art documents to a surprising technical effect or make plausible that this distinguishing feature is not obvious in light of the prior art documents. Therefore the subject matter of claims 1-6 lacks an inventive step in view of the cited documents D1-D3.

(3).पेटेंट अयोग्यता /NON PATENTABILITY:

(I) निम्नलिखित कारणों से धारा 3 के खंड (3(d))के प्रावधान के तहत दावा(वे) (1-6) सांविधिक रूप से पेटेंट योग्य नहीं हैं /
Claim(s) (1-6) are statutorily non-patentable under the provision of clause (3(d)) of Section 3 for the following reasons:

The subject matter of claims 1-6 falls under the purview of section 3(d) of the Patents (Amendment) Act, 2005 as the method for extraction of scopoletin using in-vitro culture is a mere use of a known method as disclosed by the citations. This is not allowable as it lacks technical advancement.

(4).प्रकटन की दक्षता /SUFFICIENCY OF DISCLOSURE:

(I) आविष्कार मे उपयोग की गयी जैविक सामग्री के स्रोत व भौगोलिक उद्गम की सूचना.
Information of source and geographical origin of biological material used in the invention:

As per the requirement u/s.10(4)(ii)(D) you have to disclose the source and geographical origin of the biological material, i.e., Spilanthes acmella used in the invention.

(5).स्पष्टता एवं संक्षिप्तता /CLARITY AND CONCISENESS:

(I) दावा(वे) 1-6 के संबंध मे स्पष्ट रूप से परीभाषित नहीं हैं.
Claim(s) 1-6 are not clearly worded in respect of:

Claims 1-6 are drafted in such a way that they do not sufficiently define the invention as they do not contain any technical features. Therefore, the claims are unclear. Again claims do not define the technical aspect of the invention for which protection is sought leading to ambiguity and clarity in the nature of scope for protection.

(6).राष्ट्रीय जैव विविधता अधिनियम (एनबीए) का अनुमोदन आवश्यक है /National Biodiversity Act(NBA) Approval Required :

(I) Attention of the applicant is invited towards Section 6 of the Biodiversity Act 2002 which mandates that if biological material obtained from India is used in the application for Patent, then permission and other information for making application for patent should be obtained from the National Biodiversity Authority and details should be furnished in the application Form 1 column 9 (iii). Relevant application form (Form 3,Rule 18) for such permission is available in the website of National Biodiversity Authority

(7).अन्य आवश्यकताएँ /OTHERS REQUIREMENTS:

(I)

The term "as in claim" used in the successive dependent claims shall be replaced by the term "as claimed in".

भाग – III: औपचारिक आवश्यकताएँ /PART-III: FORMAL REQUIREMENTS

आपत्तियां /Objections	टिप्पणी /Remarks
Statement & Under Taking (Form 3 Details)	Details regarding the search and/or examination report including claims of the application allowed, as referred to in rule 12(3) of the Patents Rules, 2003, in respect of same or substantially the same invention filed in all the major patent offices along with appropriate translation wherever applicable, should be submitted within a period of six months from the date of receipt of this communication as provided under section 8(2) of the Patents Act, 1970. Details regarding application for patents which may be filed outside India from time to time for the same or substantially the same invention should be furnished within six months from the date of filing of the said application under clause (b) of sub section (1) of section 8 of the Patents Act, 1970 and rule 12(1) of the Patents Rules, 2003.
Permission from NBA	As per the requirement u/s.10(4)(ii)(D) you have to disclose the source and geographical origin of the biological material, i.e., Spilanthes acmella used in the invention. Your attention is hereby invited to section 6 of the Biological Diversity Act 2002, which mandates that if a biological material procured from India is used in an application for patent, permission for making an application for the patent obtained from the National Biodiversity Authority should accompany the application or if not already submitted with the application, should be submitted before the grant of the patent. Also other actions/information as required by the NBA is to be fulfilled under intimation to this office. The relevant application form for such permission is available in the website of National Biodiversity Authority. In the present case it appears that no such approval has been filed with the Patent Office. Therefore, you are advised to act as per the provisions of the Biological Diversity Act, 2002.
Format of Specification (rule 13)	Complete specification should contain the numbering to every 5th line of each page of specification and each page of claims at right half of the left margin u/r 9(1)(d) of the Patents (Amendment) Rules, 2013.
Other Deficiencies	Applicant is asked to file a fresh Form-1 (i.e. Patent Application Form) in the prescribed format, wherein all declarations in column 12(iii), especially with respect to the use of biological material from India and NBA approval, should be clearly attended.

भाग-IV: रिकॉर्ड मे दस्तावेज़ /PART-IV: DOCUMENTS ON RECORD

निम्नलिखित दस्तावेज़ों के आधार पर यह परीक्षण रिपोर्ट तैयार की गयी है
The examination report has been prepared based on the following documents:

कार्यसूची तिथि / Docket Date	कार्यसूची संख्या /Docket Number	पूर्विष्टि संख्या विवरण /Entry Number Description
30 Oct 2017	38031	1-New Application For Patent With Provisional /Complete Specification
30 Oct 2017	38031	OTHERS(NON CASH)
30 Oct 2017	38031	2-Complete After Provisional Specification - Form 2 Check For No. OF Pages & Claims
30 Oct 2017	38031	5-Declaration As To Inventorship - Form 5
30 Oct 2017	38031	3-Statement & Undertaking - Form 3
08 Oct 2020	57750	28(i)-Request For Examination After 18 months Publication - Form 18

नियंत्रक का नाम /Name of the Controller: Soumen Ghosh

नियंत्रक स्थान /Controller Location: Kolkata

टिप्पणी: परीक्षण रिपोर्ट का उत्तर दाखिल करने की अंतिम तिथि / Note: Last date for filing response to the Examination Report: 24/12/2021

"FORM 1 THE PATENTS ACT 1970 (39 of 1970) and THE PATENTS RULES, 2003 **APPLICATION FOR GRANT OF PATENT** (See section 7, 54 and 135 and sub-rule (1) of rule 20)	(FOR OFFICE USE ONLY) 200174470

1750 फीस नकद/चेक/मनी आर्डर द्वारा	Application No.	201721038424
CBR संख्या 20560 दि. 30/10/2017	Filing date	30-10-2017
के तहत प्राप्त हुए।	Amount of Fee paid	₹. 1750/-
	CBR No	20560
रोकड़िया	Signature	
1. APPLICANT'S REFERENCE / IDENTIFICATION NO. (AS ALLOTTED BY OFFICE)		

2. TYPE OF APPLICATION [Please tick (√) at the appropriate category]

Ordinary (√)		Convention ( )		PCT-NP ( )	
Divisional ( )	Patent of Addition ( )	Divisional ( )	Patent of Addition ( )	Divisional ( )	Patent of Addition ( )

3A. APPLICANTS

Name in Full	Nationality	Country of Residence	Address of the Applicant	
Prof. Dr. Deokule Subhash S.	Indian	India	House No.	Department of Botany, Savitribai Phule Pune University (SPPU).
			Street	Ganeshkhind Road,
			City	Pune
			State	Maharashtra
			Country	India
			Pin code	411007

3B. CATEGORY OF APPLICANT [Please tick (√) at the appropriate category]

Natural Person (√)	Other than Natural Person		
	Small Entity ( )	Startup ( )	Others ( )

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4. INVENTORS [Please tick (√) at the appropriate category]				
Are all the inventors same as the applicants named above?			Yes ( )	No (√)
If "No", furnish the details of the inventor(s)				
Name in Full	Nationality	Country of Residence	Address of the Inventor	
Prof. Dr. Deokule Subhash S.	Indian	India	House No.	Department of Botany, Savitribai Phule Pune University (SPPU)
			Street	Ganeshkhind Road,
			City	Pune
			State	Maharashtra
			Country	India
			Pin code	411007
Dr. Mohammad Abyari	Iranian	Iran	House No.	Department of Botany, Savitribai Phule Pune University (SPPU)
			Street	Ganeshkhind Road,
			City	Pune
			State	Maharashtra
			Country	India
			Pin code	411007
Dr. Gaikwad Sanjay K.	Indian	India	House No.	Cell and Molecular Biology, Rajiv Gandhi Institute of Biotechnology (RGIBT), Bharati Vidyapeeth University
			Street	Pune Satara Road
			City	Pune
			State	Maharashtra
			Country	India
			Pin code	411 043
Dr. Shirsath Mahendra S.	Indian	India	House No.	Flat no. 1/103, Govardhan enclave housing society, Tawri Pada, Near RTO, Kalyan (West).
			Street	Tawri Pada
			City	Mumbai
			State	Maharashtra
			Country	India
			Pin code	431 301
Dr. Jagtap Suresh D.	Indian	India	House No.	Herbal Medicine, Interactive Research School for Health Affairs (IRSHA), Bharati Vidyapeeth University
			Street	Pune Satara Road
			City	Pune
			State	Maharashtra
			Country	India
			Pin code	411 043

5. TITLE OF THE INVENTION **Enhanced accumulation of Scopoletin in cell suspension culture of *Spilanthes acmella* Murr. using precursor feeding**		
6. AUTHORISED REGISTERED PATENT AGENT(S)	IN/PA No.	-
	Name	-
	Mobile No.	-
7. ADDRESS FOR SERVICE OF APPLICANT IN INDIA	Name	**Prof. Dr. Deokule Subhash S.**
	Postal Address	**Department of Botany, Savitribai Phule Pune University (SPPU), Ganeshkhind Road, Pune-411007. Maharashtra, India.**
	Telephone No.	**020 2560 1439**
	Mobile No.	**09371088210**
	Fax No.	**020 25690498**
	Email ID	**deokule.ss@gmail.com**
8. IN CASE OF APPLICATION CLAIMING PRIORITY OF APPLICATION FILED IN CONVENTION COUNTRY, PARTICULARS OF CONVENTION APPLICATION - NOT APPLICABLE -		
9. IN CASE OF PCT NATIONAL PHASE APPLICATION, PARTICULARS OF INTERNATIONAL APPLICATION FILED UNDER PATENT CO-OPERATION TREATY (PCT) - NOT APPLICABLE -		
10. IN CASE OF DIVISIONAL APPLICATION FILED UNDER SECTION 16, PARTICULARS OF ORIGINAL (FIRST) APPLICATION **- NOT APPLICABLE -**		
11. IN CASE OF PATENT OF ADDITION FILED UNDER SECTION 54, PARTICULARS OF MAIN APPLICATION OR PATENT **- NOT APPLICABLE -**		

12. DECLARATIONS

(i) Declaration by the inventor(s):

We, the above named inventors are the true & first inventors for this invention and declare that IRSHA, BVDU herein our assignee.

Signature:

Name: Prof. Dr. Deokule Subhash S. Date: 24.10.2017

Signature:

Name: Dr. Mohammad Abyari Date: 24.10.2017

Signature:

Name: Dr. Gaikwad Sanjay K. Date: 29-10-2017

Signature:

Name: Dr. Shirsath Mahendra S. Date: 3|10|2917

Signature:

Name: Dr. Jagtap Suresh D. Date: 3|10|2017

(ii) Declaration by the applicant(s) in the convention country

- NOT APPLICABLE -

Declaration by the applicants:

We the applicants hereby declares that:-

√	We are in possession of the above-mentioned invention.
√	The complete specification relating to the invention is filled with this application.
√	The invention as disclosed in the specification uses the biological material from India and the necessary permission from the competent authority shall be submitted by us before the grant of patent to us.
√	There is no lawful ground of objection to the grant of the patent to us.
√	We are the true & first inventors.
√	We are the assignee of true and first inventors.
√	The application or each of the applications, particulars of which are given in Paragraph-8, was the first application in convention countries in respect of our inventions.
√	We claim the priority from the above mentioned application(s) filed in convention countries and state that no application for protection in respect of the invention had been made in a convention country before that date by us or by any person from which we derive the title.
X	Our application in India is based on international application under Patent Cooperation Treaty (PCT) as mentioned in Paragraph-9.
X	The application is divided out of our application particulars of which is given in Paragraph-10 and pray that this application may be treated as deemed to have been filed on .....under section 16 of the Act.
X	The said invention is an improvement in or modification of the invention particulars of which are given in Paragraph-11.

13. FOLLOWING ARE THE ATTACHMENT WITH THE APPLICATION:

(a) Form 2

Item	Details	Fee	Remarks
Complete specification	No. of pages: 09	Rs - 1750/-	
No. of Claims	No. of claims 06 No. of pages 01		

(b) Complete specification (2 copies). Drawing sheet - 4 & Figure - 7

(c) ~~X~~ Statement and undertaking on Form 3

(d) ~~X~~ Declaration as to Inventorship on Form 5 Abstract - 1

(e) Total fee Rs. 1750 in cash.

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We hereby declare that to the best of my/our knowledge, information and belief the fact and matters stated herein are correct and I/we request that a patent may be granted to me/us for the said invention.

Dated this ....30.....day of ...October.....2017.

Signature:

Prof. Dr. Deokule Subhash S.
Department of Botany,
Savitribai Phule Pune University (SPPU),
Ganeshkhind Road,
Pune- 411007. Maharashtra, India

To,

THE CONTROLLER OF PATENTS,

THE PATENT OFFICE, MUMBAI

FORM 2

THE PATENT ACT 1970

(39 OF 1970)

&

The patents rules, 2003

COMPLETE SPECIFICATION

(See section 10 and rule 13)

1. TITLE OF THE INVENTION:

Enhanced accumulation of Scopoletin in cell suspension culture of *Spilanthes acmella* Murr. using precursor feeding

2. APPLICANT (S):

a.	**Name**	:	Prof. Dr. Deokule Subhash S.
b.	**Nationality**	:	Indian
c.	**Address**	:	Department of Botany, Savitribai Phule Pune University, Ganeshkhind Road, Pune-411007, Maharashtra, India.
a.	**Name**	:	Dr. Mohammad Abyari
b.	**Nationality**	:	Iranian
c.	***Address***	:	*Department of Botany, Savitribai Phule Pune University,* Ganeshkhind Road, Pune-411007, Maharashtra, India.
a.	**Name**	:	Mr. Gaikwad Sanjay K.
b.	**Nationality**	:	Indian
c.	**Address**	:	Cell and Molecular Biology, Rajiv Gandhi Institute of Biotechnology (RGIBT), Bharati Vidyapeeth University, Pune Satara Road, Pune -411 043. Maharashtra, India.
d.	**Name**	:	Dr. Shirsath Mahendra S.
e.	**Nationality**	:	Indian
f.	**Address**	:	Flat no. 1/103, Govardhan enclave housing society, Tawri Pada, Near RTO, Kalyan (West). Mumbai – 431 301. Maharashtra, India.
g.	**Name**	:	Dr. Jagtap Suresh D.
h.	**Nationality**	:	Indian
i.	**Address**	:	Herbal Medicine Interactive Research School for Health Affairs (IRSHA), Bharati Vidyapeeth University, Pune Satara Road, Pune – 411 043. Maharashtra, India. .

3. PREAMBLE TO THE DESCRIPTION	
PROVISIONAL	**COMPLETE**
NOT APPLICABLE	The following specification describes the invention.

4. DESCRIPTION

Field of the Invention

This invention relates to a process of using organic supplements for enhancement of production of Scopoletin in the selected plant species. The present study reported the effect of casein hydrolysate and L-phenylalanine on the growth of cell biomass and production of Scopoletin in cell culture of *Spilanthes acmella*.

Background of the invention:

Spilanthes acmella Murr. (family Asteraceae) is commonly known as toothache plant and pellitary. About 60 species of *Spilanthes* have been reported from various parts of world, and they are mostly perennials of warmer climate (DAS, 2014).

The plant has various applications in pharmaceuticals such as an anti-toothache pain relief (Singh and Chaturvedi, 2012), swelling and gum infections (Pandey et al., 2007), periodontosis (Adler, 2006), and in mouthwashes (Shimada and Gomi, 1995). In addition, its extract is an active component added to the body as beauty-care cosmetics for fast-acting muscle relaxant to accelerate repair of functional wrinkles (Belfer, 2007). The plant extract was also used for stimulating, reorganizing, and strengthening the collagen network in anti-age applications such as anti-wrinkle cream formulations (Schubnel, 2007; Demarne and Passaro 2008). *S. acmella* contains a wide diversity of biologically active secondary metabolites such as scopoletin (6-methoxy-7-hydroxycoumarin), which is one of its powerful ingredients and belongs to Coumarins, a class of phenolic secondary metabolites. Coumarins are derived from the phenylpropanoid pathway in plants, and based on their anti-microbial properties, and production in response various stress events (Chong et al., 2002, Gachon et al., 2004), they are thought to have defense role in plants.

Scopoletin has attracted the more attention because of its use in the treatment of cardiovascular diseases and as antitumor and antithyroid agent (DAS, 2014). In addition to this, Scopoletin also possesses antioxidant, antimicrobial, anti-inflammatory, antipyretic, and hepatoprotective properties (Taguchi et al., 2001). Scopoletin also has antifungal (against Candida fungi), antihistamine and antiviral properties, as well as in reducing high blood pressure by dilating the blood vessels (Tanton 2008). Scopoletin has been historically used for menstrual cramps with severe lower back and pelvic pains (Hudson, 2007). Plants are valuable sources of a wide range of secondary metabolites used as pharmaceuticals, agrochemicals, flavors, fragrances, colors, biopesticides and food additives. The production of these compounds is often low (less than 1% dry weight) and depends greatly on the physiological and developmental stages of the plant (Dixon, 2001; Oksman-Caldentey and Inzé 2004). Several strategies, such as manipulating the nutrient, optimizing the culture conditions, feeding of precursor and elicitation have been used to substantially improve the yields of secondary metabolites in plant cell cultures (Ramachandra Rao and Ravishankar, 2002). Other techniques such as hairy root culture, biotransformation, immobilization, and elicitations are also applied for the increased production of secondary metabolites. On the basis of the knowledge of the biosynthetic pathways, several organic compounds have been added to the culture medium in order to increase the synthesis of secondary metabolites. The exogenous supply of a biosynthetic precursor to culture medium may enhance the yield of the desired product. This approach is more useful when the precursors are inexpensive.

In the present study, we report the effect of casein hydrolysate and L-phenylalanine on the growth of cell biomass and production of scopoletin in cell culture of *Spilanthes acmella*. To the best of our knowledge, this is the first report on using of organic supplements for enhancement of production of Scopoletin in the species.

Summary of the invention

Spilanthes acmella Murr. is an annual herb belonging to family Asteraceae, commonly known as toothache plant. *S. acmella* Murr. contains a wide array of compounds with a diverse range of bioactivity. One such compound, Scopoletin (6-methoxy-7-hydroxycoumarin), has attracted the most attention because of its use in cardiovascular disease, and antitumor and antithyroid treatment. In addition to this, Scopoletin also possesses antioxidant, antimicrobial, anti-inflammatory, antipyretic and hepatoprotective properties. Scopoletin also acts as an antifungal (which assists in fighting Candida), an antihistamine and an anti-viral, as well as reducing high blood pressure by dilating blood vessels. *Scopoletin haa* also been historically used as a specific medicine for menstrual cramps with severe low back and bearing-down pelvic pain.

The present study demonstrates an optimized and reliable *in vitro* propagation protocol. *S. acmella* Murr. is an important endangered plant using different explants, shoot regeneration, rooting of shoots and leaves and establishment of plantlets which could be used for clonal propagation and mass multiplication. Among two additives precursors and organic supplements, L-phenylalanine and casein hydrolysate, the latter was found to be the most efficient enhancer for the stimulation of bioaccumulation of Scopoletin in *S. acmella*. Therefore, it could be used industrially to produce higher yields of Coumarins compounds in this species. In brief, our results demonstrate that the addition of organic supplements to cell cultures is quite effective for large-scale biosynthesis and constant production of Scopoletin as well as for the conservation of germplasm.

Detailed description of the figures

Figure 1: a: Seedling plantlet after 40 days **b:** callus on MS plus 15 µM BA with 5 µM 2,4-D **c:** Cell suspension culture **d:** Cell biomass.

Figure 2: Effect of different concentrations of casein hydrolysate on dry weigh (DW) and accumulation of scopoletin in cell culture of *Spilanthes acmella*.

Figure 3: Effect of different concentrations of Phenylalanine on dry weight (DW) and accumulation of scopoletin in cell culture of *Spilanthes acmella*.

Figure 4: HPTLC chromatogram of scopoletin standard

Figure 5: HPTLC fingerprint profile of scopoletin in leaf derived regenerated shoot biomass of *Spilanthes acmella* Murr.

Figure 6: HPTLC Spectrum of scopoletin at 366 nm in leaf derived regenerated shoot biomass of *Spilanthes acmella* Murr.

Figure 7: HPTLC chromatogram of scopoletin in leaf derived regenerated shoot biomass grown on MS + 10 µM BA + 1 µM IAA.

Detailed description of the invention

The present invention discloses a process for the enhanced accumulation and extraction of Scopoletin in cell suspension culture of *Spilanthes acmella* Murr using precursor feeding. In this detailed description, the process including culture condition, maintenance of cell culture and extraction of Scopoletin is described for a better understanding of the invention without any limitation.

Detailed experimental studies

Plant materials and culture conditions

Plants were obtained from nursery of University of Pune. The authentic plant material *Spilanthes acmella* Murr. was collected from Pune area. Callus culture was developed from leaf explant of the aseptically germinated *S. acmella* seedlings (Fig. 1a) and maintained on MS medium supplemented with 15 µm 6-benzyladenine plus 5 µm 2,4-dichlorophenoxyacetic acid as growth regulators. The cell culture (Fig. 1c) were exposed to various concentrations of casein hydrolysate and L-phenylalanine. For cell culture experiments, 250 ml flasks containing 40 ml medium were inoculated with cells. Cell suspensions were incubated on a rotary shaker at 100 ± 10 rpm at 25 ± 2°C under 16 h- 8 h light-dark regime, using fluorescent lamps at a light intensity of 35 µmol m^2s^{-1}. The cultures were harvested after 28 days and dried in oven to be used for secondary metabolites estimation.

Establishment of growth and maintenance of cell cultures

Suspension cultures of *S. acmella* were developed from leaf-derived callus (Fig. 1b and d) at stationary phase of growth (10 –12 days post inoculation). A callus piece (1 g) was tenderly broken up in a sterile petri dish to generate 20–30 smaller pieces. The small pieces were transferred aseptically into 250 ml flasks containing 40 ml of MS media with 3% (w/v) sucrose and 15 µm BA plus 5 µm 2,4-D. Suspension cultures were routinely sub cultured every 2 weeks by transferring into 10 ml of the previous culture, to 40 ml (Murashige and Skoog 1962) medium supplemented with 3% (w/v) sucrose, 15 µm BA plus 5 µm 2,4-D at pH 5.6. To establish growth and production kinetics, the cultures were harvested and analyzed for biomass and scopoletin content. Dry weight was estimated by centrifuging the harvested suspension cells at 3,000 rpm for 15 min, collecting the cells and drying to constant weight at 60°C.

L-phenylalanine experiment

The different concentrations of L-phenylalanine (Sigma) (50, 100, 150, 200 µM) were incorporated in cell culture media (MS +15 µM BA plus 5 µM 2,4-D). L-phenylalanine was dissolved in distilled water (0.1-1%) and then was filter sterilized in the laminar airflow by passing through a Millipore membrane (0.22 Dmpore size) (Millipore Corporation, USA) and added to sterilized (autoclaved) medium when the temperature of cell suspension cultures medium reached 50°C.

Casein hydrolysate experiment

The various concentrations of casein hydrolysate (Sigma) (25, 50, 75, 100 mg/L) were incorporated in MS containing 15 µM BA + 5 µM 2, 4-D. Appropriate concentrations of casein hydrolysate were first dissolved in distilled water and added to the medium before setting pH and sterilization. Its effect on the dry weight and secondary metabolites were investigated. It is noteworthy that the *S. acmella* cultures were developed from leaf-derived callus. Scopoletin standard was procured from Sigma Aldrich. The standard was prepared by dissolving 5 mg of the compound in 5 ml of HPLC grade methanol. The solution was then stored at –20°C. The standard solution was filtered through a 0.20

μm membrane filter before HPTLC analysis and run at least thrice to check the repeatability and precision of results. TLC plates containing fluorescent indicator after derivatization with anisaldehyde/sulphuric acid were examined for scopoletin content in UV-366 nm. To prepare samples, 500 mg of dried powdered samples were soaked in methanol for 12 h. The methanolic samples were then centrifuged in a high-speed refrigerated centrifuge (R-4C REMI make, India) at 5,000 rpm for 10 min. The supernatant was transferred into a new tube and the residue was re-extracted thrice with 10 ml methanol. Thereafter, the residue was discarded and the supernatant was pooled, filtered, and evaporated to dryness in a rotatory evaporator (Buchi Rotavapor R-200, Japan) at 40°C. CAMAG analytical HPTLC system was used for estimation of scopoletin. The methanolic fraction thus obtained was redissolved in HPLC grade methanol, filtered through a 0.20 μm membrane filter prior to analysis, and aliquots of 10 μL of clean solution were injected into HPTLC system with CAMAG TLC scanner3. The developing solvent was toluene: ethyl acetate: formic acid (7:3:1). TLC plates, containing fluorescent indicator after derivatization with anisaldehyde sulphuric acid, were examined for scopoletin content in UV-366 nm. Finally, scopoletin content was calculated by area of standard and area of sample.

Statistical analysis

The experiment was performed in triplicate. Observations on the dry weight (DW) and scopoletin content were recorded at 8 day intervals. Standard error of the mean was calculated and represented as bars in the graph. The data was analyzed statistically using SPSS software version 16, and differences among treatment means were determined using the least significant difference (LSD) at the 0.05 level of probability.

Results

Effect of casein hydrolysate on the cell culture

The influence of casein hydrolysate on growth and accumulation of scopoletin in cell cultures of *Spilanthes acmella* are depicted in Figure 2. Significant increase in accumulation of scopoletin was achieved in the cell culture medium supplemented with different concentration of casein hydrolysate (25, 50, 75 and 100 mg/L). Also, casein hydrolysate in 50 and 75 mg/L concentrations caused a significant increase in cell-biomass.

Figure 2 - Effect of different concentrations of casein hydrolysate on dry weigh (DW) and accumulation of scopoletin in cell culture of *Spilanthes acmella*.

Dry weight of callus that cultured on MS medium with 25 mg/L was 0.43 ± 0.03 g, while the biomass of callus produced on control medium was 0.41 ± 0.03 g. The dry weight of cell biomass gradually increased with the increase in concentration of casein hydrolysate. However, the biomass growth declined with the incorporation of higher concentrations of casein hydrolysate (>75 mg/L). The incorporation of 50, 75 and 100 mg/L casein hydrolysate in cell culture medium increase statistically significant in scopoletin content, while fortification of 75 mg/L of casein hydrolysate produced the maximum dry weight of the cell culture. It was 0.57 ± 0.03 g, which was about 1.39 fold more than the control (0.41 ± 0.01 g). Results of this part of the experiment revealed that the addition of casein hydrolysate in cell culture was effective for the production of cell biomass and accumulation of scopoletin. Among the different concentrations, addition of 75 mg/L casein hydrolysate was ideal for scopoletin production.

Effect of l-phenylalanine on cell culture The results presented in Figure 3 shows the influence of various concentrations of Phenylalanine (0, 50, 100, 150, and 200 μM) on the growth (DW) and scopoletin production in cell culture of *S. acmella*. Supplementation of moderate concentrations of L-

phenylalanine (100 - 150 µM) in the nutrient medium of the cell culture was statistically effective for growth of cell biomass (Fig. 3). The inclusion of high concentrations of L-phenylalanine was ineffective for stimulation growth of cell biomass. However, the inclusion of 100 µM L-phenylalanine in cell culture showed highest (with statistically significant differences) growth of cell biomass (0.451 ± 0.01 g) and accumulation of scopoletin. The dry weight of cell culture at 150 mg/L L-phenylalanine concentration was 0.42 ± 0.01g. These results showed that the addition of 100 or 150 µM/L L-phenylalanine in cell cultures media was effective for biomass production in *S. acmella*. The inclusion of low concentrations of L-phenylalanine (50 µM) resulted in increased scopoletin content by about 3.43 times over the control. Among the different concentrations of L phenylalanine tested, optimum increase in scopoletin in cell culture (35.63 ± 0.56 µg/g DW) was obtained at 100 µM L-phenylalanine. These results suggested that the addition of moderate concentration of L-phenylalanine, was beneficial for accumulation of secondary metabolite in cell culture. This enhanced content was estimated and confirmed using HPTLC (Figure 4-7).

Discussion

Casein hydrolysate is a mixture of amino acids and peptides produced by enzymatic or acid hydrolysis of casein, that obtained by digesting casein with hydrochloric acid. It therefore acts as a good nutrient substrate and is especially suitable for the large-scale cultivation in micropropagation and secondary metabolites production (Kayser and Quax, 2007). Casein hydrochloric acid hydrolysate was added to media primarily because of the organic nitrogen and growth-factor components. The increase of secondary metabolites content in cell culture cultivated on media with the addition of casein hydrolysate might be due to its sterols or amino acids content (Heble 1985). Comparable results of increased secondary metabolite contents were reported in cell culture of Panax ginseng with increased in the concentration of casein hydrolysate (Wu et al., 2005). They reported 0.5 g/L of casein hydrolysate with combined 0.2 mM sorbitol enhanced saponin yield 3.5-fold over the control. The enhancement of *artemisinin production on MS supplemented with 0.5 g/L casein hydrolysate in cell cultures of Artemisia annua* was reported by Woerdenbag et al. (1993). Addition of casein hydrolysate in genetically transformed cultures of *Coleus forskohlii* for enhancement of forskolin production was reported by Mukherjee et al. (2000). Precursor feeding has been a successful approach for enhancement of secondary metabolites in different medicinal plants. In particular feeding of loganin, secologanin and tryptamine has been studied extensively. After feeding geraniol, 10- hydroxygeraniol or loganin resulted in a significant increase of tabersonine accumulation (Morgan and Shanks, 2000). Similarly, Namdeo (2004) reported that the effect of tryptophan addition on ajmalicine in *Coleus roseus* cells cultured in Zenk's production medium, maximum ajmalicine (310 µg/g dry weight) was recorded in medium with 100 mg/L tryptophan, followed by 292 and 140 µg/g dry weight ajmalicine in medium with 250 and 50 mg/L tryptophan. Moreno et al. (1993) studied the effect of feeding different terpenoid precursors on alkaloid production. They observed that the addition of secologanin, its precursor loganin and loganic acid increased the accumulation of ajmalicine and strictosidine. Among the biotechnological methods, stimulation of metabolic pathways in the cultivated plant cells toward production of a desired compound most likely results in a dramatic increase in secondary metabolite yield (Wang et al., 2001). Scopoletin originates from phenylalanine, an upstream metabolic precursor through coumarins biosynthetic pathway. The addition of phenylalanine is expected to increase the level of targeted compounds (Shinde et al. 2009). The results indicated that addition of high level of L-phenylalanine did not improve cell biomass in cell culture. The incorporation of L-phenylalanine in the nutrient medium was found to be more effective for the production of secondary metabolite in *S. acmella*. The addition of 50 µM/L of L-phenylalanine in the medium increased

scopoletin content to 27.12 ± 0.58 µg/g D.W, while scopoletin content of control was 7.89 ± 0.61 µg/g D.W. The highest accumulation of scopoletin was detected at 100 µM/L L phenylalanine concentration in cell suspension, which was 4.51 times more than the control. As results using moderate concentrations of L phenylalanine was ideal for the production of scopoletin. By contrast, higher levels of L- phenylalanine did not improve the accumulation of scopoletin. Similar results on the inclusion of L-phenylalanine in the medium on cell culture of different plants were reported previously (Cusidó et al., 1999; Brincat et al. 2002). The addition of 0.2 Mm/L of L-phenylalanine to cell culture of Cistanche deserticola resulted in 75% higher production of phenylethanoid glycosides, compared with the cell culture without precursors (Ouyang et al. 2005). Paclitaxel yields in the cell culture of *Taxus cuspidata* were improved up to six times by the addition L-phenylalanine and other potential paclitaxel side-chain precursors (e.g., benzoic acid, N-benzoylglycine and serine) (Fett-Neto and DiCosmo 1996). The present study and previous reports suggest that the L-phenylalanine amino acid has effective role for production of secondary metabolites in cell culture of different plant species. Singh and Chaturvedi (2010) presented the first report on quantification of scopoletin from the leaves of in vitro and field-grown plants of *S. acmella*. Their study revealed that even the uninfected leaves of Spilanthes could accumulate the scopoletin. They compared the scopoletin content in the tissue-cultured plants of Spilanthes produced from plants with known scopoletin content. These findings open up the possibility of producing Spilanthes plants with the desired metabolite content which will help the pharmaceutical industry to achieve better output by using superior quality raw materials. Dandin et al. (2014) reported that in vitroregenerated plants of *Spilanthes oleracea* L. had higher amounts (mean = 3.42 µg g–1 DW) of scopoletin compared to in vivo plants (range 0.01 – 3.04 µg g–1 DW).

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5. Claims

We claim:

1. A method for extraction of scopoletin using in-vitro culture, TLC and HPTLC from selected plant.
2. In-vitro method as in claim 1 is applicable for studied plan viz. *Spilanthes acmella.*
3. Extraction method present study as in claim 1, using plants as in claim 2 Scopoletin in cell suspension culture can be extracted by elicitation using precursor feeding.
4. Method of extraction characterized in comprising the following process steps;
   a. Callus culture was developed from leaf explants of the aseptically germinated *S. acmella* seedlings and maintained on MS medium supplemented with 15 µm 6-benzyladenine plus 5 µm 2,4-dichlorophenoxyacetic acid as growth regulators.
   b. The cell cultures were exposed to various concentrations of casein hydrolysate and L-phenylalanine.
   c. Further inoculated cell suspensions were incubated on a rotary shaker, using fluorescent lamps at a light intensity of 35 µmol m^2s^{-1}.
   d. To establish growth and production kinetics, the cultures were harvested and analyzed for biomass and scopoletin content. Dry weight was estimated by centrifuging the harvested suspension cells at 3,000 rpm for 15 min, collecting the cells and drying to constant weight at 60°C.
   e. The different concentrations of L-phenylalanine (Sigma) (50, 100, 150, 200 µM) were incorporated in cell culture media (MS +15 µM BA plus 5 µM 2,4-D).
   f. The various concentrations of casein hydrolysate (Sigma) (25, 50, 75, 100 mg/L) were incorporated in MS containing 15 µM BA + 5 µM 2, 4-D.
   g. TLC and HPTLC was carried out for estimation.
5. Method used for extraction as in claim 1, for the plant as in claim claim 2, using extraction method as in claim 3 & 4, is capable for increase the yield of Scopoletin.
6. According to any of the proceeding claims that exemplified, the said method shows increased enhanced yield of scopoletin.

Name : Prof. Dr. Deokule Subhash S Signature :

6. Date and Signature

Date: 24.10.2017

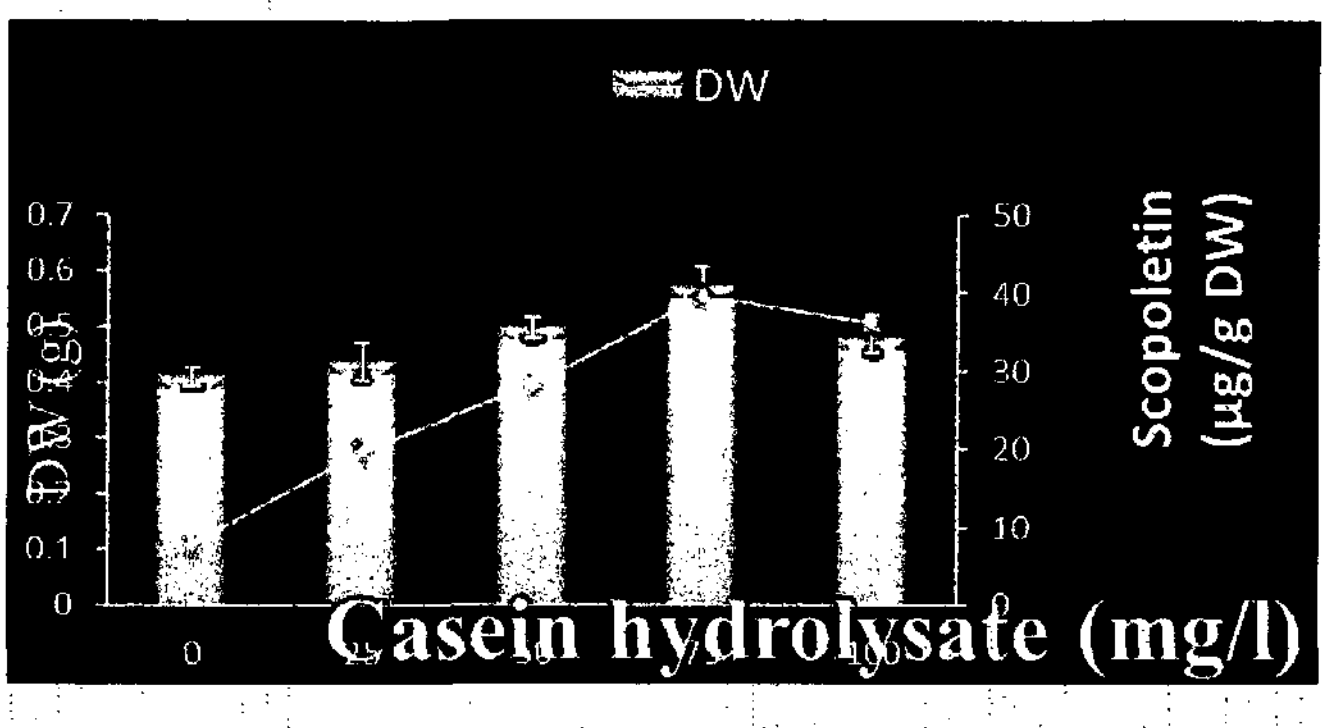
Place: Pune

Prof. Dr. Deokule Subhash S.
Department of Botany,
Savitribai Phule University of Pune (SPPU),
Ganeshkhind Road,
Pune- 411007. Maharashtra, India

Figure 1: Different stages of Scopoletin production through tissue culture

a: Seedling plantlet after 40 days.
b: Callus on MS plus 15 µM BA with 5 µM 2,4-D.
c: Cell suspension culture.
d: Cell biomass.

Figure 2: Effect of different concentrations of casein hydrolysate on dry weigh (DW) and accumulation of scopoletin in cell culture of *Spilanthes acmella*.



Name: Prof. Dr. Deokule Subhash S. Signature:

Figure 3: Effect of different concentrations of L phenylalanine on dry weight (DW) and accumulation of scopoletin in cell culture of *Spilanthes acmella*

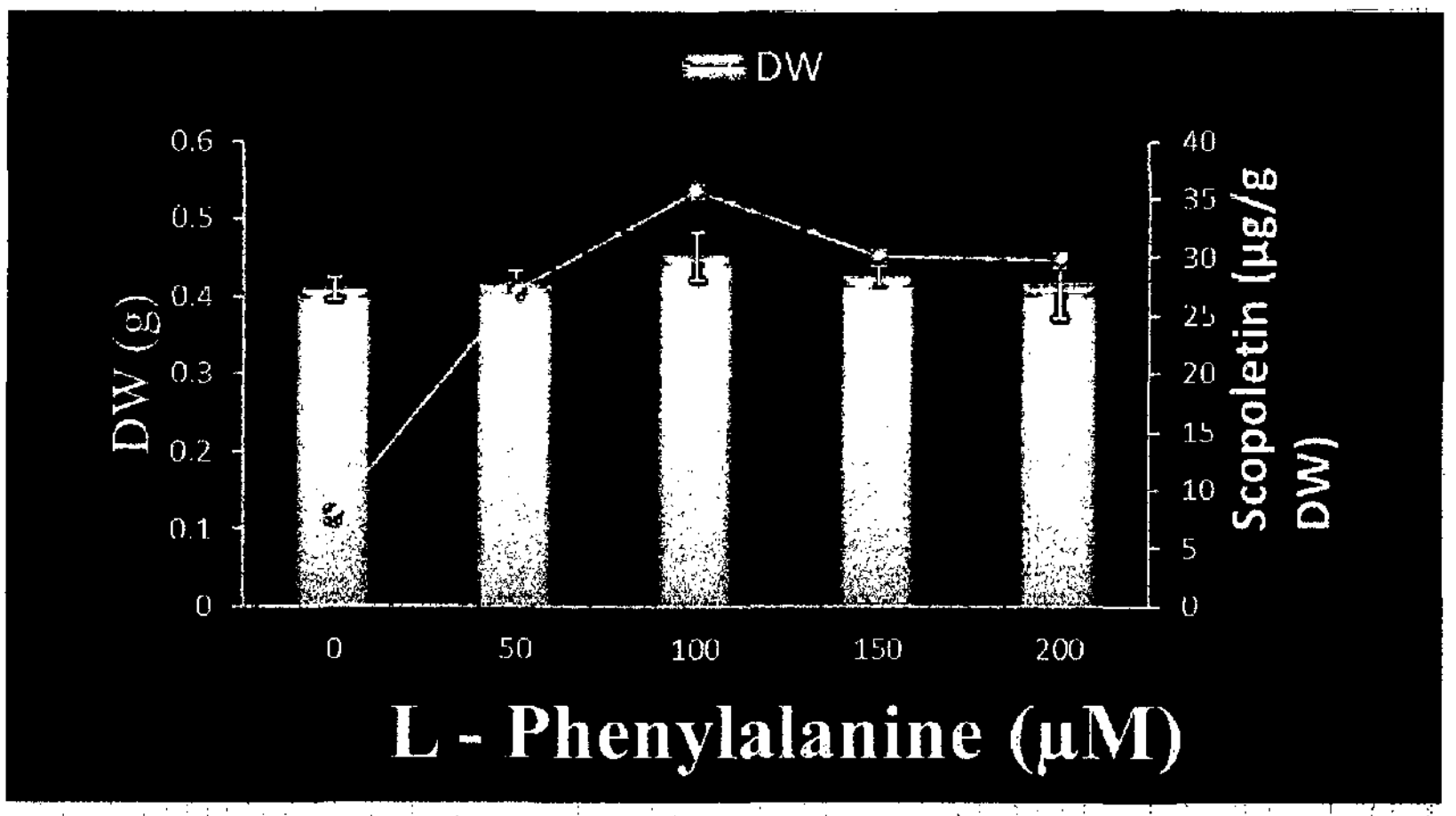
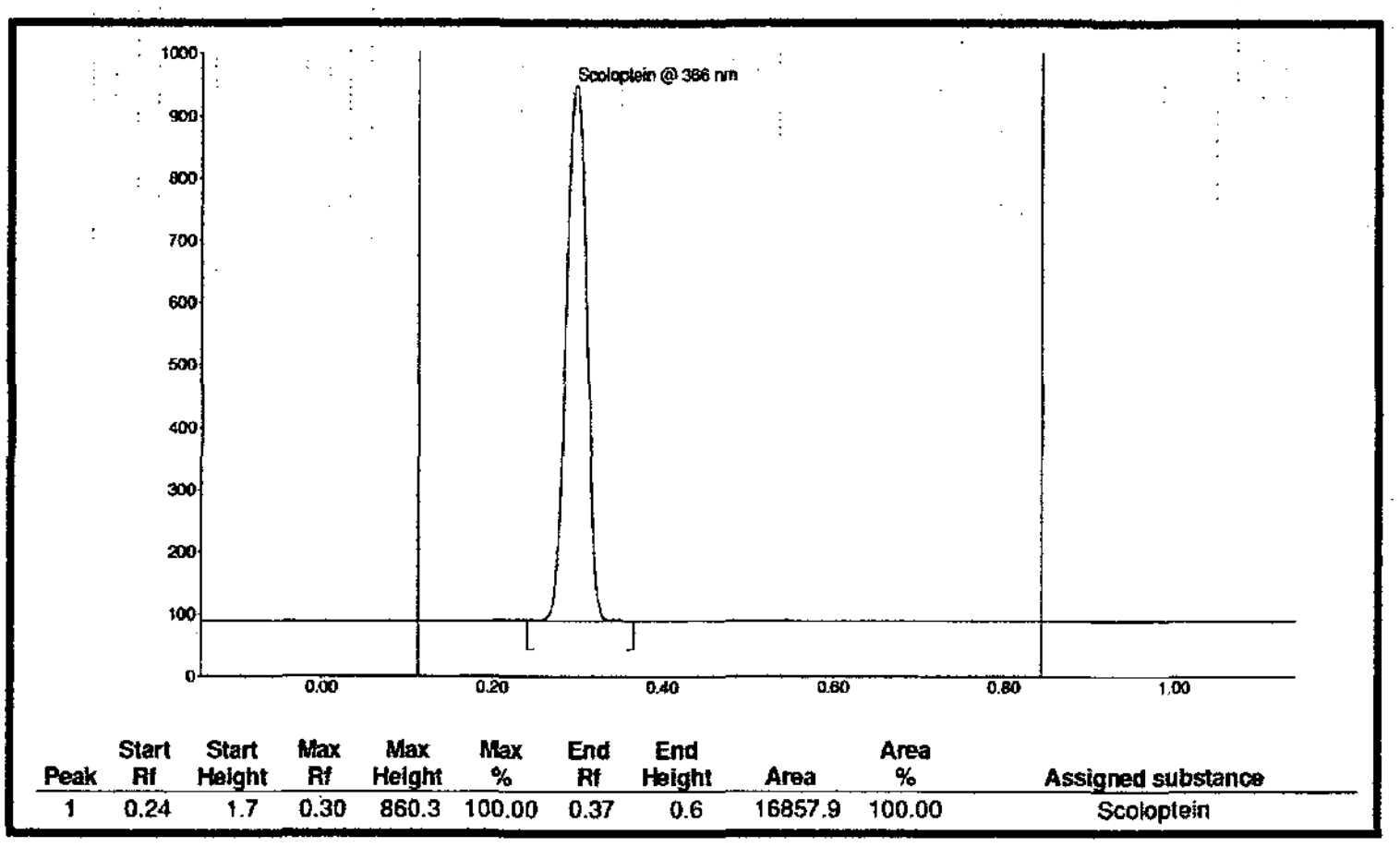


Figure 4: HPTLC chromatogram of scopoletin standard



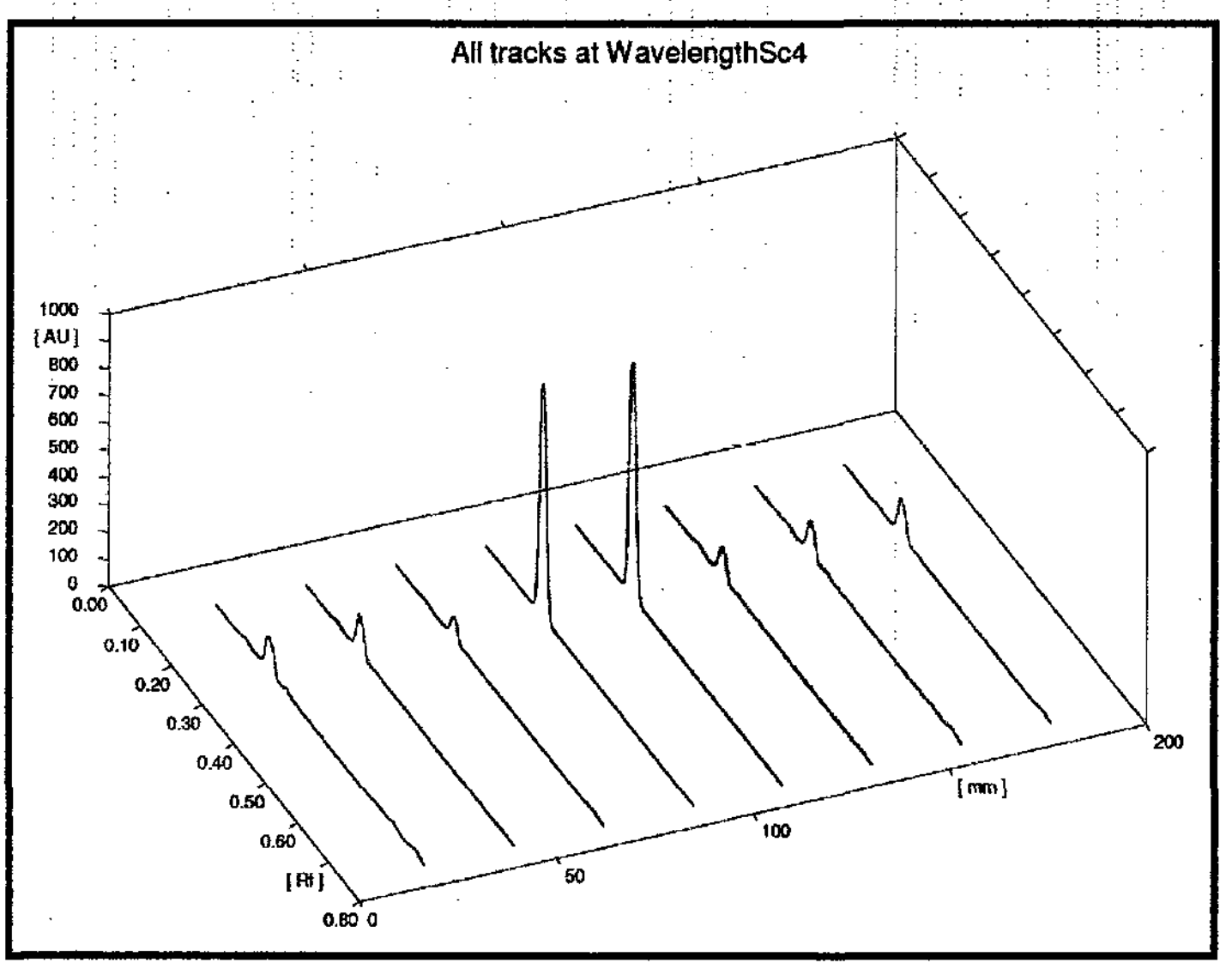
Peak	Start Rf	Start Height	Max Rf	Max Height	Max %	End Rf	End Height	Area	Area %	Assigned substance
1	0.24	1.7	0.30	860.3	100.00	0.37	0.6	16857.9	100.00	Scoloptein

Name: Prof. Dr. Deokule Subhash S.

Signature:

Figure 5: HPTLC fingerprint profile of scopoletin in leaf derived regenerated shoot biomass of *Spilanthes acmella* Murr.

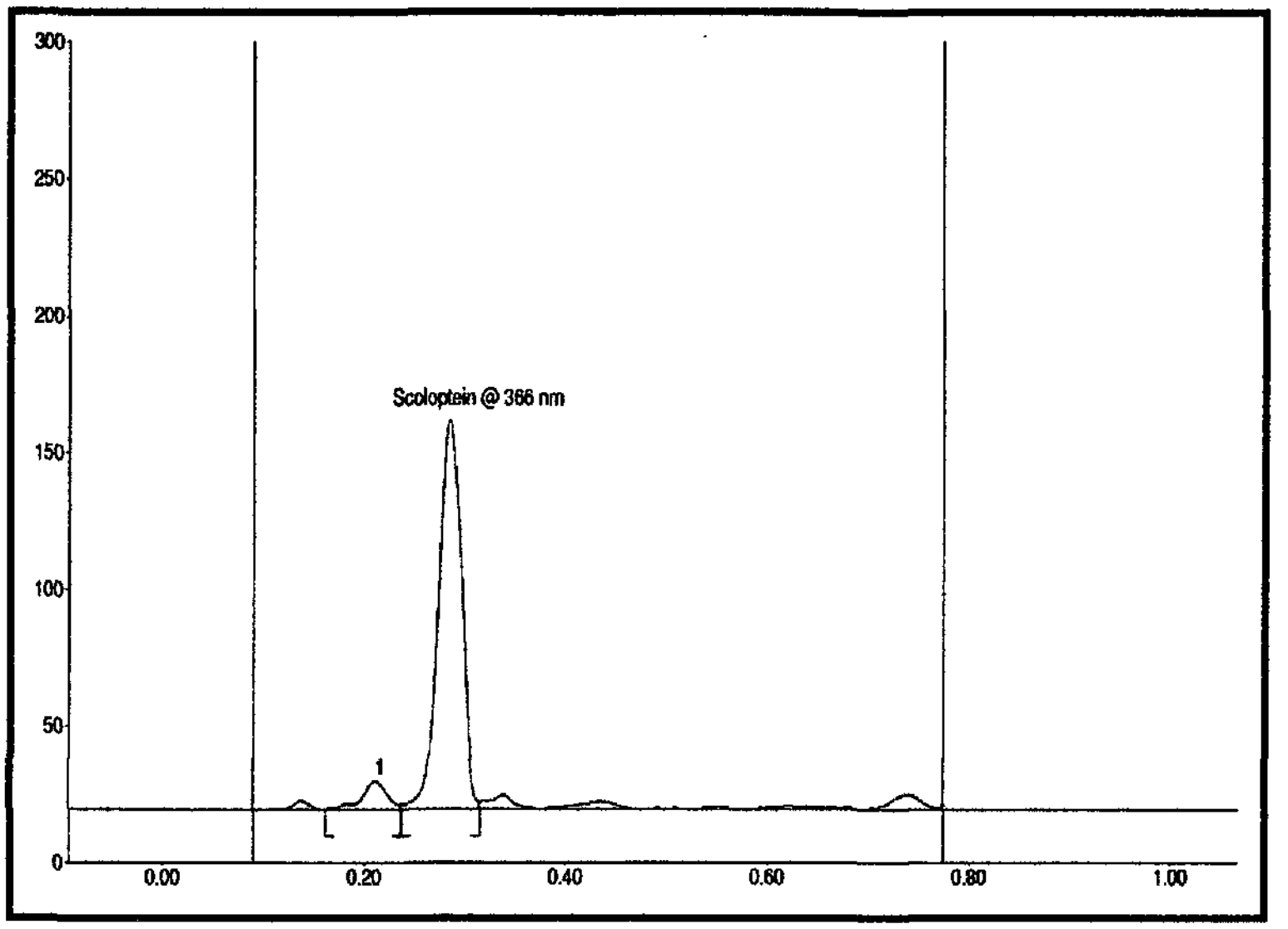
Figure 6: HPTLC Spectrum of scopoletin at 366 nm in leaf derived regenerated shoot biomass of *Spilanthes acmella* Murr.



Name: Prof. Dr. Deokule Subhash S.

Signature:

Figure 7: HPTLC chromatogram of scopoletin in leaf derived regenerated shoot biomass grown on MS + 10 µM BA + 1 µM IAA.



Name: Prof. Dr. Deokule Subhash S.

Signature:

Title :- "Enhanced accumulation of Scopoletin in cell suspension culture of Spilanthes acmella Murr. using Precursor feeding"

7. ABSTRACT OF THE INVENTION

In this study, the various concentrations of casein hydrolysate (25, 50, 75, 100 mg/L) and L-phenylalanine (50, 100, 150, 200 µM/l) were incorporated in MS containing 15 µM BA plus 5 µM 2,4-D for enhancement of secondary metabolites in cell culture of *Spilanthes acmella*. The presence of casein hydrolysate in the nutrient medium improved the growth of cell biomass and the production of scopoletin. The addition of casein hydrolysate up to 75 mg/L stimulated the accumulation of scopoletin, but increasing excess 75 mg/L the level of casein hydrolysate reduced the production of scopoletin. The addition of L-phenylalanine in the nutrient medium was found to be more effective for production of secondary metabolite in *S. acmella*. The addition of 50 µM/L of L-phenylalanine in the medium increased scopoletin content to 27.12 ± 0.58 µg/g dry weight, compared to the scopoletin content of control at 7.89 ± 0.61 µg/g dry weight. The highest accumulation of scopoletin was observed in the 100 µM/L L phenylalanine in cell suspension, which was 4.51 times more than the control. As a result, using moderate concentration of L-phenylalanine was ideal for the production of scopoletin. In general, casein hydrolysate was more effective than L-phenylalanine for production of scopoletin and growth of cell biomass in the cell culture of *S. acmella* which was confirmed using HPTLC.

& D/11419/2017

FORM 3
THE PATENTS ACT, 1970
[39 OF 1970]
and
THE PATENTS RULES, 2003
STATEMENT AND UNDERTAKING UNDER SECTION 8
(See Section 8; Rule 12)

200174471

1. Name of the applicants	I, Deokule Subhash S., Department of Botany, Savitribai Phule Pune University, Pune-7, hereby declare:
2. Name, address and nationality of the joint applicant	Dr. Gaikwad Sanjay K., Cell and Molecular Biology, Rajiv Gadhi Institute of Biotechnology, Bharati Vidyapeeth University, Pune Satara Road, Pune – 411043; Dr. Shirsath Mahendra S. Flat no. 1/103, Govardhan enclave housing society, Tawri Pada, Near RTO, Kalyan (West); Dr. Suresh D. Jagtap, Herbal Medicine, IRHSA, Bharati Vidyapeeth University, Pune – 411 043. (i) that we have not made any application for the same/substantially the same invention outside India
3. Name and address of the assignee	(ii) that the rights in the applications have been assigned to Department of Botany, Savitribai Phule Pune University, Ganeshkhind road, Pune-7, Maharashtra, India. that we undertake that up to the date of grant of the patent by the Controller, we would keep him informed in writing the details regarding corresponding applications for the date of filling of such application Dated this 30 day of October 2017
4. To be signed by the applicant or his authorized registered patent agent	Deokule
5. Name of the natural person who has signed	Prof. Dr. Deokule Subhash S. Professor (Retd) Department of Botany, Savitribai Phule Pune University, Ganeshkhind road, Pune-7, Maharashtra, India.
	To, The Controller of Patents, The Patent Office, at Mumbai

1693/2017

FORM 5
THE PATENTS ACT, 1970
(39 of 1970)
&
The patents rules, 2003
DECLARATION AS TO INVENTORSHIP
[See section 10 (6) and rule 13(6)]

200174472

1. NAME OF APPLICANTS	**(i) Prof. Dr. Deokule Subhash S.**

Hereby declare that the true and first inventor(s) of the invention disclosed in the complete specification filed in pursuance of our application numbered..... Dated 30-10 .....are 2017.....

2. INVENTOR(S)

a. **Name** : Prof. Dr. Deokule Subhash S.

b. **Nationality** : Indian

c. **Address** : Department of Botany, Savitribai Phule Pune University, Ganeshkhind Road, Pune-411007, Maharashtra, India

a. **Name** : Dr. Mohammad Abyari

b. **Nationality** : Iranian

c. **Address** : Department of Botany, Savitribai Phule Pune University, Ganeshkhind Road, Pune-411007, Maharashtra, India

a. **Name** : Dr. Gaikwad Sanjay K.

b. **Nationality** : Indian

c. **Address** : Cell and Molecular Biology, Rajiv Gadhi Institute of Biotechnology (RGBT), Bharati Vidyapeeth University, Pune Satara Road, Pune – 411043. Maharashtra, India

a. **Name** : Dr. Shirsath Mahendra S.

b. **Nationality** : Indian

c. **Address** : Flat no. 1/103, Govardhan enclave housing society, Tawri Pada, Near RTO, Kalyan (West). Mumbai- 431 301. Maharashtra, India.

a.	**Name**	**:**	Dr. Suresh D. Jagtap
b.	**Nationality**	**:**	Indian
c.	**Address**	**:**	Herbal Medicine, Interactive Research School for Health Affairs (IRSHA), Bharati Vidyapeeth University, Pune Satara Road, Pune – 411 043. Maharashtra, India.

Dated this ...30..... day of October 2017

Signature:

Prof. Dr. Deokule S. S.

Rtd. PROFESSOR
Department of Botany
Savitribai Phule Pune University
Pune - 411 007.

3. *DECLARATION TO BE GIVEN WHEN THE APPLICATION IN INDIA IS FILED BY THE APPLICANTS IN THE CONVENTION COUNTRY:-*

-NOT APPLICABLE-

4. STATEMENT (to be signed by the additional inventors not mentioned in the application form)

-NOT APPLICABLE-

To,

The Controller of Patent

The Patent Office, at Mumbai

200293025

FORM 18 THE PATENTS ACT 1970 (39 of 1970) & The Patents Rules, 2003 REQUEST/EXPRESS REQUEST FOR EXAMINATION OF APPLICATION FOR PATENT [See section 11B and rule 20(4)(ii), 24B(1)(i)]	(FOR OFFICE USE ONLY) RQ No: R202020231282 Filing Date: 08/10/2020 Amount of Fee Paid: रूपये चेक/मनी ऑर्डर द्वारा CBR No: 4400/ 26226 दि.08/10/2020 Signature CBR संख्या के जरिए प्राप्त हुए।

1. APPLICANTS

(a)	Name:	Deokule Subhash Sadhu
(b)	Nationality:	Indian
(c)	Address:	Department of Botany, Savitribai Phule Pune University (SPPU), Pune- 411 007, Maharashtra, India.

2. Statement in case of request for examination made by the applicants

We hereby request that our application for patent no.: **201721038424** filed on **30/10/2017** for the invention titled **"Enhanced accumulation of Scopoletin in cell suspension culture of *Spilanthes acmella* Murr,"** shall be examined under sections 12 and 13 of the Act.

3. Statement in case of request for examination made by any other interested person

I/We the interested person request for the examination of the application no._____dated _____filed by the applicant_____titled_____under sections 12 and 13 of the Act.

As an evidence of my/our interest in the application for patent following documents are submitted.

(a) _____

Not applicable

4. ADDRESS FOR SERVICE

Department of Botany, Savitribai Phule Pune University (SPPU), Pune- 411 007, Maharashtra, India. **Email:** deokule.ss@gmail.com. **Mobile:** 9371088210.

Dated this 8 day of October 2020

Deokule

Deokule Subhash Sadhu

To,
The Controller of Patents
The Patent Office,
Mumbai

SAVIRIBAI PHULE PUNE UNIVERSITY

(formerly University of Pune)

DEPARTMENT OF BOTANY
SAVITRIBAI PHULE PUNE UNIVERSITY
Ganeshkhind, Pune-411 007
Tele. No. : (020) 25601439, 25601438
Email : .....@unipune.ac.in

Ref. No. : Bot/

Date : 30/10/2017

D-38031

Bot/13693/2017

To,
The Controller of Patents,
The Patent Office,
Boudhik Sampada Bhavan,
S. M. Road, Antop Hill, Mumbai,
Maharashtra- 400037

200174469

Dear Sir,

Re: Submission of Complete Patent Specification

Please find herewith documents for a complete specification application entitled "**Enhanced accumulation of Scopoletin in cell suspension culture of *Spilanthes acmella* Murr. using precursor feeding**" along with the prescribed fee of Rs. 1750/-.

The Learned Controller is requested to take the same on record and issue the official filing receipt for the same.

Yours faithfully,

Prof. Dr. Deokule Subhash S.
Department of Botany,
Savitribai Phule Pune University (SPPU),
Ganeshkhind Road,
Pune- 411007. Maharashtra, India

Enclosures:

1. Application for Grant of Patent [Form 1]
2. Complete specification [Form 2]
3. Statement and undertaking under section 8 [Form 3]
4. Declaration as to inventorship [Form 5]
5. **Fees of Rs. 1750/- (by cash)**

"FORM 1 THE PATENTS ACT 1970 (39 of 1970) and THE PATENTS RULES, 2003 **APPLICATION FOR GRANT OF PATENT** (See section 7, 54 and 135 and sub-rule (1) of rule 20)	(FOR OFFICE USE ONLY) 200174917

1750 कार्यालय में प्राप्त की तारीख 03/11/2017	Application No.	201721039233
CBR संख्या 20878 दि. 03/11/2017	Filing date	03-11-2017
के सहित प्राप्त हुए।	Amount of Fee paid	₹. 1750/-
रोकड़िया	CBR No	20878
	Signature	

1. APPLICANT'S REFERENCE / IDENTIFICATION NO. (AS ALLOTTED BY OFFICE)	

2. TYPE OF APPLICATION [Please tick (√) at the appropriate category]

Ordinary (√)		Convention ( )		PCT-NP ( )	
Divisional ( )	Patent of Addition ( )	Divisional ( )	Patent of Addition ( )	Divisional ( )	Patent of Addition ( )

3A. APPLICANTS

Name in Full	Nationality	Country of Residence	Address of the Applicant	
Prof. Dr. Deokule Subhash S.	Indian	India	House No.	Department of Botany, Savitribai Phule University of Pune (SPPU)
			Street	Ganeshkhind Road,
			City	Pune
			State	Maharashtra
			Country	India
			Pin code	411007

3B. CATEGORY OF APPLICANT [Please tick (√) at the appropriate category]

Natural Person (√)	Other than Natural Person		
	Small Entity ( )	Startup ( )	Others ( )

4. INVENTORS [Please tick (√) at the appropriate category]				
Are all the inventors same as the applicants named above?			Yes ( )	No (√)
If "No", furnish the details of the inventor(s)				
Name in Full	**Nationality**	**Country of Residence**	**Address of the Inventor**	
Prof. Dr. Deokule Subhash S.	Indian	India	House No.	Department of Botany, Savitribai Phule University of Pune (SPPU)
			Street	Ganeshkhind Road
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			State	Maharashtra
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			City	Pune
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			Street	Jambulwadi Road,
			City	Pune
			State	Maharashtra
			Country	India
			Pin code	411 046.
Dr. Mungikar Rahul R.	Indian	India	House No.	Parisanstha, 24/4, Balaji Park 4, Rao Colony, Talegaon Dabhade, Taluka- Maval, Dist.- Pune
			Street	Rao Colony Road,
			City	Pune
			State	Maharashtra
			Country	India
			Pin code	410 506
Mr. Shilimkar Vaibhav C.	Indian	Indian	House No.	Department of Pharmacognosy, Seth Govind Raghunath Sable College of Pharmacy, Saswad
			Street	Taluka -Purandhar
			City	Dist.- Pune
			State	Maharashtra
			Country	India
			House No.	Department of Pharmacognosy, Seth Govind Raghunath Sable College of Pharmacy, Saswad

Dr. Jagtap Suresh D.	Indian	India	House No.	Herbal Medicine, Interactive Research School for Health Affairs (IRSHA), Bharati Vidyapeeth University
			Street	Pune Satara Road
			City	Pune
			State	Maharashtra
			Country	India
			Pin code	411 043

5. TITLE OF THE INVENTION

New source and extraction method for colchicines in seed and tubers of *Arisaema murrayi*

6. AUTHORISED REGISTERED PATENT AGENT(S)	IN/PA No.	-
	Name	-
	Mobile No.	-
7. ADDRESS FOR SERVICE OF APPLICANT IN INDIA	Name	Prof. Dr. Deokule Subhash S.
	Postal Address	Department of Botany, Savitribai Phule University of Pune (SPPU), Ganeshkhind Road, Pune- 411007. Maharashtra, India
	Telephone No.	020 2560 1217
	Mobile No.	09371088210
	Fax No.	020 2560 1217
	Email ID	deokule.ss@gmail.com

8. IN CASE OF APPLICATION CLAIMING PRIORITY OF APPLICATION FILED IN CONVENTION COUNTRY, PARTICULARS OF CONVENTION APPLICATION

- NOT APPLICABLE -

9. IN CASE OF PCT NATIONAL PHASE APPLICATION, PARTICULARS OF INTERNATIONAL APPLICATION FILED UNDER PATENT CO-OPERATION TREATY (PCT)

- NOT APPLICABLE -

10. IN CASE OF DIVISIONAL APPLICATION FILED UNDER SECTION 16, PARTICULARS OF ORIGINAL (FIRST) APPLICATION

- NOT APPLICABLE -

11. IN CASE OF PATENT OF ADDITION FILED UNDER SECTION 54, PARTICULARS OF MAIN APPLICATION OR PATENT

- NOT APPLICABLE -

12. DECLARATIONS

(i) Declaration by the inventor(s):

We, the above named inventors are the true & first inventors for this invention and declare that IRSHA, BVDU herein our assignee.

Signature:

Name: Prof. Dr. Deokule Subhash S. Date: 02.11.2017

Signature:

Name: Dr. Pawar R. M. Date: 2/11/2017

Signature:

Name: Dr. Nagarkar Bhagyashri E. Date: 24/10/2017

Signature:

Name: Dr. Mungikar Rahul R. Date: 25/10/2017

Signature:

Name: Mr. Shilimkar Vaibhav C. Date: 02/11/2017

Signature:

Name: Dr. Jagtap Suresh D. Date: 2/11/2017

(ii) Declaration by the applicant(s) in the convention country

- NOT APPLICABLE -

Declaration by the applicants:

We the applicants hereby declares that:-

√	We are in possession of the above-mentioned invention.
√	The complete specification relating to the invention is filled with this application.
√	The invention as disclosed in the specification uses the biological material from India and the necessary permission from the competent authority shall be submitted by us before the grant of patent to us.
√	There is no lawful ground of objection to the grant of the patent to us.
√	We are the true & first inventors.
√	We are the assignee of true and first inventors.
√	The application or each of the applications, particulars of which are given in Paragraph-8, was the first application in convention countries in respect of our inventions.
√	We claim the priority from the above mentioned application(s) filed in convention countries and state that no application for protection in respect of the invention had been made in a convention country before that date by us or by any person from which we derive the title.
X	Our application in India is based on international application under Patent Cooperation Treaty (PCT) as mentioned in Paragraph-9.
X	The application is divided out of our application particulars of which is given in Paragraph-10 and pray that this application may bè treated as deemed to have been filed on .....under section 16 of the Act.
X	The said invention is an improvement in or modification of the invention particulars of which are given in Paragraph-11.

13. FOLLOWING ARE THE ATTACHMENT WITH THE APPLICATION:

(a) Form 2

Item	Details	Fee	Remarks
Complete specification	No. of pages: 05	Rs. 1750/-	
No. of Claims	No. of claims: 07 No. of pages: 01		
No. of drawing sheets	: 03		
No. of figures	: 05		
No. of tables	: 00		
No. of Abstract pages	: 01		

(b) Complete specification (1 copy)
(c) Statement and undertaking on Form 3
(d) Declaration as to Inventorship on Form 5
(e) Total fee Rs. 1750/- in cash.

We hereby declare that to the best of my/our knowledge, information and belief the fact and matters stated herein are correct and I/we request that a patent may be granted to me/us for the said invention.

Dated this ....03.....day of .November.....2017.

Signature:

Prof. Dr. Deokule Subhash S.
Department of Botany,
Savitribai Phule University of Pune (SPPU),
Ganeshkhind Road,
Pune- 411007. Maharashtra, India

To,

THE CONTROLLER OF PATENTS,

THE PATENT OFFICE, MUMBAI

FORM 2
THE PATENT ACT 1970
(39 OF 1970)
&
The patents rules, 2003
COMPLETE SPECIFICATION
(See section 10 and rule 13)

200174920

1. TITLE OF THE INVENTION:

New source and extraction method for colchicines in seeds and tubers of *Arisaema murrayi*

2. APPLICANT (S):

a.	**Name**	:	Prof. Dr. Deokule Subhash S.
b.	**Nationality**	:	Indian
c.	**Address**	:	Department of Botany, Savitribai Phule Pune University, Ganeshkhind Road, Pune-411007, Maharashtra, India.
a.	**Name**	:	Dr. Pawar R. M.
b.	**Nationality**	:	Indian
c.	**Address**	:	Department of Botany, Savitribai Phule Pune University, Ganeshkhind Road, Pune-411007, Maharashtra, India.
a.	**Name**	:	Dr. Nagarkar Bhagyashri E.
b.	**Nationality**	:	Indian
c.	**Address**	:	Herbs Foundation, A1-604, Dream City, Behind Telco Coloney, Dattanagar, Ambegaon, Pune – 411 046. Maharashtra, India.
a.	**Name**	:	Dr. Mungikar Rahul R.
b.	**Nationality**	:	Indian
c.	**Address**	:	Parisanstha, 24/4, Balaji Park 4, Rao Colony, Talegaon Dabhade, Taluka- Maval, Dist.- Pune - 410 506. Maharashtra, India.
a.	**Name**	:	Mr. Shilimkar Vaibhav C.
b.	**Nationality**	:	Indian
c.	**Address**	:	Department of Pharmacognosy, Seth Govind Raghunath Sable College of Pharmacy, Saswad, Taluka Purandhar, Dist. Pune – 412 301. Maharashtra, India.
a.	**Name**	:	Dr. Jagtap Suresh D.
b.	**Nationality**	:	Indian
c.	**Address**	:	Herbal Medicine, Interactive Research School for Health Affairs (IRSHA), Bharati Vidyapeeth University, Pune Satara Road, Pune – 411 043. Maharashtra, India.

3. PREAMBLE TO THE DESCRIPTION

PROVISIONAL	COMPLETE
NOT APPLICABLE	The following specification describes the invention.

4. DESCRIPTION

Field of the Invention

This invention relates to a process of extraction of Colchicine. This invention is based on new process to report and enhance the yield of Colchicine. Aspect of the invention is through analytical method viz. layer chromatography (TLC), infrared spectroscopy (IR) and Nuclear magnetic resonance (NMR) techniques.

Background of the invention:

Colchicine is a medication most commonly used to treat gout. It is a toxic natural product and secondary metabolite, originally extracted from plants of the genus *Colchicum*.

Adverse effects are primarily gastrointestinal upset at high doses. In addition to gout, colchicine is used to treat familial Mediterranean fever, pericarditis and Behçet's disease.

Colchicine is an alternative for those unable to tolerate NSAIDs in gout. At high doses, side effects (primarily gastrointestinal upset) limit its use. At lower doses, which are still effective, it is well tolerated. It is also used as an anti-inflammatory agent for long-term treatment of Behçet's disease. It appears to have limited effect in relapsing polychondritis, as it may only be useful for the treatment of chondritis and mild skin symptoms. Colchicine is also used in addition to other therapy in the treatment of pericarditis. Colchicine is also used widely in the treatment of familial Mediterranean fever, in which it reduces attacks and the long-term risk of amyloidosis.

It has been studied for the prevention of postoperative complications after heart surgery. Occurrence of atrial fibrillation can be reduced by a third. It has also been investigated in the prevention and treatment of postpericardiotomy syndrome.

***Arisaema murrayi* Hook. (Aracaea):**

Arisaema is a large and diverse genus of the flowering plant family Araceae. It is known as Sapkanda or Rankanda. Local Vaidoos are being used corn in the treatment of cancer. The chances of recovery depend upon doses prescribed by the Vaidoos. Perhaps it may be consumed by human and may cause death. The fruits of this plant fascinate children, being crimson red in color and eaten by them. Sometimes, cattle's are being fed with dried spike and the fruits along with fodder and causes death.

A. murrayi is medicinal and poisonous plant which has been used as homicide, suicide or as an accidental poisoning in forensic studies. It is commonly called as Cobra lily or Snake lily (Cooke 1958). The local tribal people are not touching this plant as they believe that this plant is highly poisonous (Chopra et al., 1965; Nadkarni, 1977; Modi, 1977; Parikh, 1985, Thothathriet et al., 1985).

Summary of the invention

This invention includes the new source and extraction method for colchicines in seed and tubers of *Arisaema murrayi*. These plants are known as Sapkanda or Rankanda. Local Vaidoos are being used corn in the treatment of cancer. *Arisaema murrayi* Hook. (Aracaea) are medicinal and poisonous plants which have been used as homicide, suicide or as an accidental poisoning in Forensic studies.

Colchicine is a medication most commonly used to treat gout. It is a toxic natural product and secondary metabolite, originally extracted from plants of the genus *Colchicum*.

Adverse effects are primarily gastrointestinal upset at high doses. In addition to gout, colchicine is used to treat familial Mediterranean fever, pericarditis and Behçet's disease.
Some investigation uses has been studied for the prevention of postoperative complications after heart surgery. Occurrence of atrial fibrillation can be reduced by a third. It has also been investigated in the prevention and treatment of post pericardiotomy syndrome.
After detection of Colchicine with the help of TLC from *A. murrayi* seeds and tubers, Rf values were calculated. Infra-red spectroscopy (IR) peaks were obtained by standard colchicine are nearly of same wavelength, and wave number ^{1}H Nuclear magnetic resonance (NMR) was carried out for the identification of colchicine.

Detailed description of the figures

Figure 1: a) *Arisaema murrayi* Hook. ¼ N.S. along with tubers, leaves and inflorescence.
b) *Arisaema murrayi* Hook. Fruit.

Figure 2: *Arisaema murrayi* Hook. T.S. of Tuber showing anatomical details and cell contents like raphides and potassium oxalate crystals 10*40

Figure 3: TLC of *Arisaem amurrayi* seed extract, Authentic Colchicine and *Arisaema murrayi* tuber extract.

Figure 4:NMR spectra of colchicine

Figure 5: IR spectra of colchicine

Detailed description of the invention

The present invention discloses a process for the extraction of Colchicine from plant *Arisaema murrayi*. In this detailed description, the process of extraction of Colchicineis described for a better understanding of the invention.

Detailed experimental studies

Collection of plants:

Plants were collected from different Western Ghats region of Maharashtra. Efforts were made to collect these plants in flowering and fruiting conditions for the correct botanical identification. The plant material was finely ground, dried and extracted with boiling methanol.

Chromatography:

Since the chemical constituents were detected with the help of histochemical and phytochemical tests, these were further studied with chromatography separation. These include alkaloids and other chemical compounds, separated on silica gel and also on paper.

Thin layer chromatography (TLC):

TLC involves the principle of adsorption. The adsorbent is spread as thin layers on an inner support viz. glass plate. Different substance shows different adsorption's which are depending on the solvent system used. Here the stationary phase is silica gel, which mobile phase is any solvent/s. Silica gel, while most active (silica gel is an amorphous porous substance often used with impregnating agent like formamide in 25% acetone).

Paper chromatography involves the principle of partition coefficient. Thus, the solute showing different rates of solubility in two solvent can be separated.

Apparatus-

Glass plate of the thickness 1.8 mm to 5.5 mm was used for preparing uniform layers. In the present investigation microscopic slide of 26 mm to 76 mm thickness were used.

Spraying procedure-

55 g of silica gel was homogeneously mixed with 130 ml of distilled water as well as in chloroform. The water slurry was evenly spread on the glass plates with a rod to a desired thickness. The plates were dried for 24 hours and before use activated in oven at 110° C, cooled and used for loading samples.

Application of the substance mixture for separation-

After testing the uniformity of the silica gel layer 2.4 mm margin from the layer was stripped off with thumb. A layer or point was marked with sharp pencil. The sample was applied to the starting point with the help of very small capillaries.

Solvent system-

Since the work on the chemical condition constituents and their chromatography separation has not been reported so far, different types of solvents of different polarity were tried to get better separation of chemical constituents of unknown identity in the selected plants.

Chromatographic Chambers-

Rectangular glass chamber were used in ascending TLC studies. The chamber filled with solvents to a depth about 0.5 cm. Then the plates were placed in chambers for development. The separation was carried out at room temperature. To avoid 'Edge effect' the chromatographic chambers were saturated at room temperature for 30-60 minutes before use. For the development of TLC plates ascending method was used. For visualization of colorless substances, U.V. camps, sprayers and heaters were used (Stahl 1969).

RF values of the active constituents in various solvent systems were determined with TLC as well as paper chromatography.

After detection of Colchicine with the help of TLC from *A. murrayi* seeds and tubers, Rf values were calculated. Infra-red spectroscopy (IR) peaks were obtained by standard colchicine are nearly of same wavelength, and wave number ^1H Nuclear magnetic resonance (NMR) was carried out for the identification of colchicine.

Estimation of Colchicine:

100 mL of 70% ethanol + 1 gm material placed in percolator, ethanol was evaporated and added with 20 ml Distilled water, filter through filter paper and collected in separating funnel. The remnant of resin and oil on filter paper with 20 ml Distilled water, Filtered through filter paper and mix with previous solution in the separating funnel then combined aqueous extract saturated with Chloroform 20, 15, 15 and 10 ml which allowed to evaporate till dryness. Residues dissolved in 30 ml. Distilled water (or hot water) and filter in beaker and allowed cool then added 3 ml. Phosphotungstate acid (10 g Sodium tungstate + 6 g Sodium phosphate + 50 ml water acidified with HNO_3) and 7 ml of dil. H_2SO_4. Entire mixture shakes vigorously till precipitation and allowed to settle and centrifuged, discarded the supernatant. Precipitate suspend in H_2O, transfer it in separating funnel, which contains 30 ml. Chloroform. Repeated it and add 10 ml of $CHCl_3$, combined solution evaporated to dryness and treat with 5 ml ethanol. Evaporated it again and finally dried in 100°C temp and calculated the weight of Colchicine and used for NMR and IR.

Results:

In Colchicine test, it was reported in *Arisaema murrayi* tuber and fruit. Using chloroform methanol and water solvent systems for the development of silica gel plates followed TLC studies extract. The color spots of chemical constituents obtained by *A. murrayi* are crimson red. Colchicine and potassium oxalate crystals, Raphides and Sphaerophides are found in the *Arisaema murrayi* (Fig, 2). Rf values of

these chemical compounds are as given bellow (Fig 3).

Arisaema murrayi tuber – 0.4791 mg/g

Colchicine – 0.4791 mg/g

Arisaema murrayi fruit – 0.4791 mg/g

Further the presence of colchicines was confirmed using NMR and IR (Fig 4 & 5).

References:

Chopra *et al.* (1965). Poisonous Plnats of India (2nd edition ICAR). (Vol.1 & 11) New Delhi:123-124, 764 and 804

Cooke (1958). The Flora of the Presidency of Bombay, Botanical Survey of India, Calcutta, I, 194 & 239, III, 36, 121, 821, 827 & 828.

Nadkarni (1927). KM Naderkarni's Indian Materia Medica, Popular Book Depot Lamington Road Bombay, (3rd edition), I: 137, 339, 725, 1054 and 1104.

Modi (1977). Modi's Text Book of Medical Jurisprudence and Toxicology, N.M. Tripathi Pvt. Ltd., (20th edition), Bombay

Parikh (1985). Parikh's Text Book of Medical Jurisprudence and Toxicology, CBS Publishers and Distributors, (4th edition), Medico Legal Center, Bombay

Thothathri*et al.* (1985). Selected Poisonous Plants From The Tribal Areas of India. B.S.I. Culcutta.

5. Claims

We claim:

1. A method for new source and extraction of colchicines using TLC, IR and NMR from selected plant.
2. Extraction method as in claim 1 is applicable for studied plants namely *Arisaema murrayi* Hook.
3. Extraction method in present study as in claim 1, using plants from family Araceae in claim 2 can be extracted using fruit and tuber powder in methanol as a solvent.
4. Method of extraction characterized in comprising the following process steps;
   a. Fruit and root powder of plant material as in claim 2 and extracted in methanol as in claim 3.
   b. Methanol and liquid ammonia in proportion of 200:3 as a solvent system for TLC.
   c. Detected colchicine by TLC further processed for preparatory TLC. The silica gel of known R_f value is eluted. It is dissolved in chloroform.
   d. Colchicine extract is then dried and dissolved in $CHCl_3$.
   e. Dissolution of samples in nujol for IR and NMR
5. Method of extraction according to claim 4 characterized in that the method is the production of a solution form (methanol extract)
6. Method used for extraction as in claim 1, for the plant as in claim claim 2, using extraction method as in claim 3 & 4, is capable for increase the yield of Colchicine.
7. According to any of the proceeding claims that exemplified, the said method shows increased enhanced yield and new sources for Colchicine.

Name : Prof. Dr. Deokule Subhash S Signature :

6. Date and Signature

Date: 02.11.2017

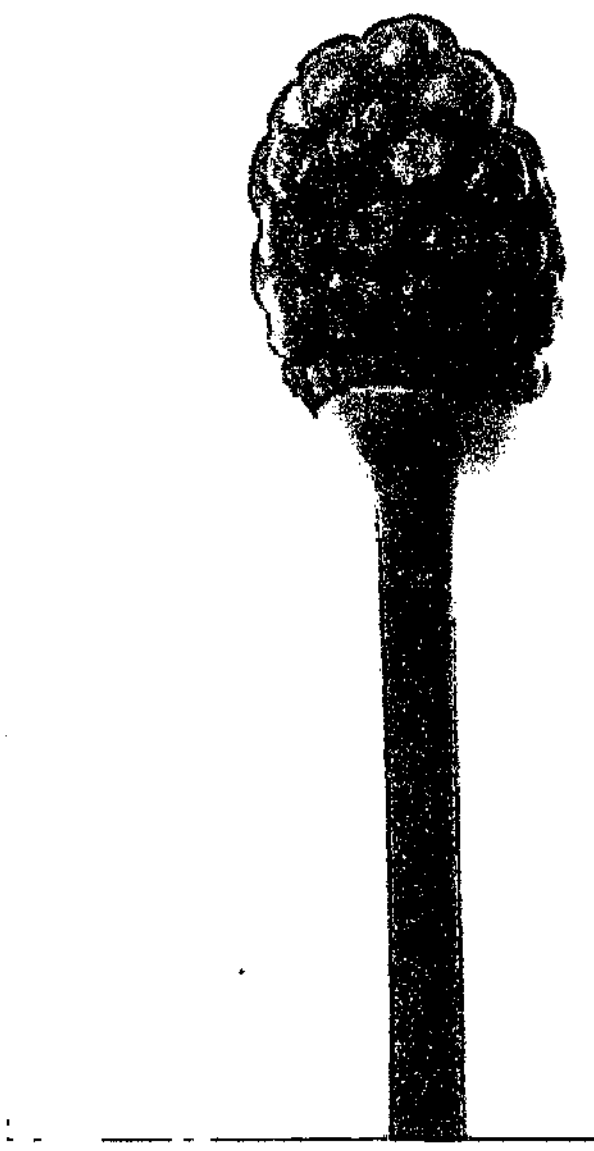
Place: Pune

Prof. Dr. Deokule Subhash S.
Department of Botany,
Savitribai Phule University of Pune (SPPU),
Ganeshkhind Road,
Pune- 411007. Maharashtra, India

Figure 1: *Arisaema murrayi* Hook.

a) ¼ N.S. along with tubers, leaves, inflorescence.

b) Fruit



Name: Prof. Dr. Deokule Subhash S.

Signature:

Figure 2: *Arisaemamurrayi* Hook. T.S. of Tuber showing anatomical details and cell contents like raphides and potassium oxalate crystals 10*40

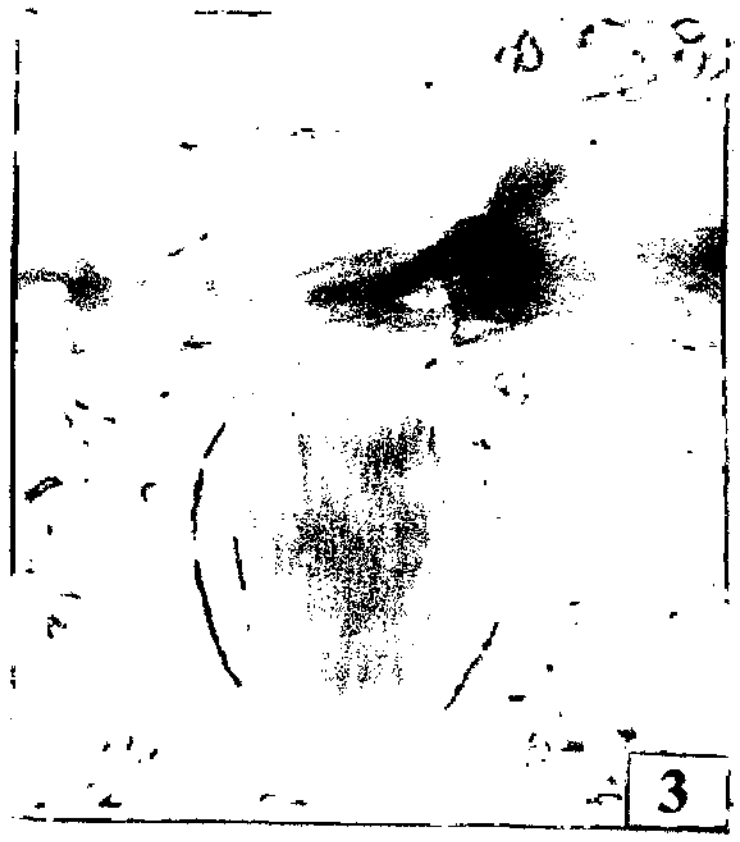


Figure 3: TLC of *Arisaemamurrayi* seed extract, Authentic Colchicine and *Arisaemamurrayi* tuber extract.

ASE CH ATE

ASE : *Arisaema murrayi* seed extract
CH : Authentic Colchicine
ATE : *Arisaema murrayi* tuber extract

Name: Prof. Dr. Deokule Subhash S. Signature:

Figure 4) NMR spectra of colchicine

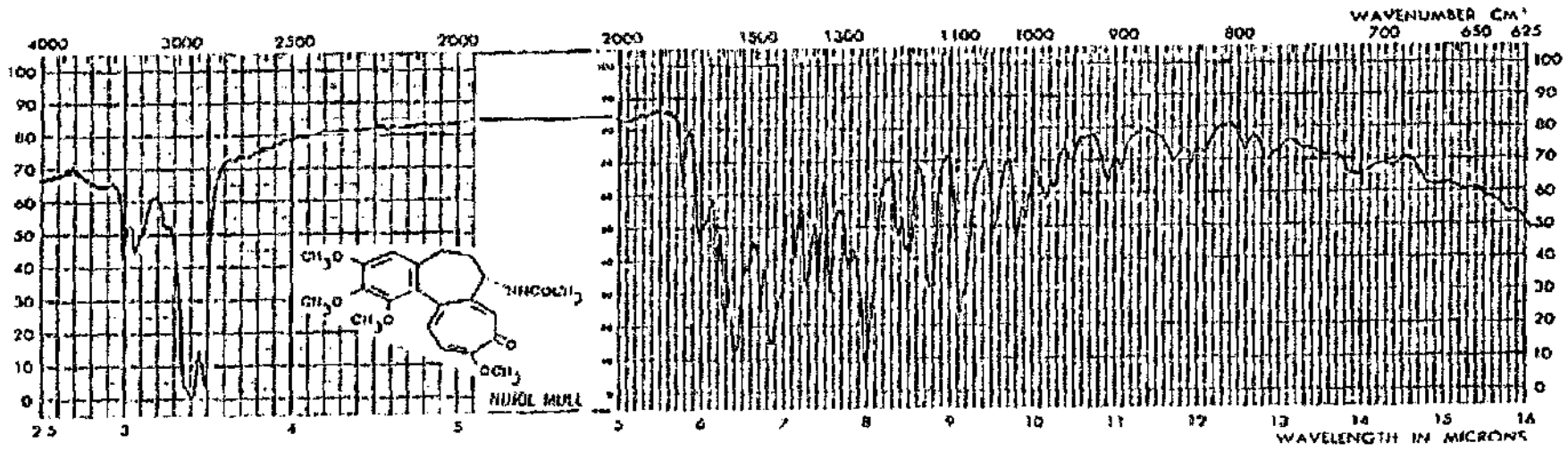


Figure 5: IR spectra of colchicine

Name: Prof. Dr. Deokule Subhash S.

Signature:

TITLE: New source and extraction method for colchicines in seed and tubers of *Arisaema murrayi*

7. ABSTRACT OF THE INVENTION

The present invention relates to a process is extraction process to increase the yield of Colchicine, particularly commercially useful biochemical with good demand by industry.

Arisaema murrayi Hook. consists Colchicine and potassium oxalate crystals, Raphides and Sphaerophides in fruit and tuber. Therefore this species is recommended as substitute source of Colchicine.

Present investigation includes the species viz. *Arisaema murrayi* Hook. of family Araceae. In the present investigation Colchicine and potassium oxalate crystals, Raphides and Sphaerophides are found to be present in the drugs. Colchicine was further detected quantitatively. Further these investigations were supported by thin layer chromatography (TLC), infrared spectroscopy (IR), Nuclear magnetic resonance (NMR), techniques and confirmed the presence as well as quantified the Colchicine. The introduction part of this invention explains the uses of plants with increased yield of ephedrine content for its commercial use.

E-3/11626/2017

FORM 3

THE PATENTS ACT, 1970

[39 OF 1970]

and

THE PATENTS RULES, 2003

STATEMENT AND UNDERTAKING UNDER SECTION 8

(See Section 8; Rule 12)

200174918

1. Name of the applicants	I, Prof. Dr. Deokule Subhash S., Department of Botany, Savitribai Phule Pune University, Pune-7,hereby declare:
2. Name, address and nationality of the joint applicant	Dr. Nagarkar Bhagyashri E., Herbs Foundation, A1-604, Dream City, Behind Telco Coloney, Dattanagar, Ambegaon, Pune; Dr. Mungikar Rahul R., Parisanstha, 24/4, Balaji Park 4, Talegaon Dabhade, Taluka- Maval, Dist.- Pune; Mr. Shilimkar Vaibhav C. Department of Pharmacognosy, Seth Govind Raghunath Sable College of Pharmacy, Saswa, Pune; Dr. Jagtap Suresh D., Herbal Medicine, Interactive Research School for Health Affairs (IRSHA), Bharati Vidyapeeth University, Pune Satara Road, Pune – 411 043. Maharashtra, India. (i) that we have not made any application for the same/substantially the same invention outside India
3. Name and address of the assignee	(ii) that the rights in the applications have been assigned to Department of Botany, Savitribai Phule Pune University, Ganeshkhind road, Pune-7, Maharashtra, India. that we undertake that up to the date of grant of the patent by the Controller, we would keep him informed in writing the details regarding corresponding applications for the date of filling of such application Dated this 03 day of November2017
4. To be signed by the applicant or his authorized registered patent agent	Deokule
5. Name of the natural person who has signed	Prof. Dr. Deokule Subash S. Professor (Retd) Department of Botany, Savitribai Phule Pune University, Ganeshkhind road, Pune-7, Maharashtra, India.
	To, The Controller of Patents, The Patent Office, at Mumbai

E-5/1724/2017.

FORM 5
THE PATENTS ACT, 1970
(39 of 1970)
&
The patents rules, 2003
DECLARATION AS TO INVENTORSHIP
[See section 10 (6) and rule 13(6)]

200174919

1. NAME OF APPLICANTS	(i) Prof. Dr. Deokule Subhash S.

Hereby declare that the true and first inventor(s) of the invention disclosed in the complete specification filed in pursuance of our application numbered.....
Dated 03-11-2017 .....are

2. INVENTOR(S)

a.	**Name**	:	Prof. Dr. Deokule Subhash S.
b.	**Nationality**	:	Indian
c.	**Address**	:	Department of Botany, SavitribaiPhule Pune University, Ganeshkhind Road, Pune-411007, Maharashtra, India
a.	**Name**	:	Patale M. W.
b.	**Nationality**	:	Indian
c.	**Address**	:	Department of Botany, Savitribai Phule Pune University, Ganeshkhind Road, Pune-411007, Maharashtra, India
a.	**Name**	:	Dr. Nagarkar Bhagyashri E.
b.	**Nationality**	:	Indian
c.	**Address**	:	Herbs Foundation, A1-604, Dream City, Behind Telco Coloney, Dattanagar, Ambegaon, Pune – 411 046. Maharashtra, India.
a.	**Name**	:	Dr. Mungikar Rahul R.
b.	**Nationality**	:	Indian
c.	**Address**	:	Parisanstha, 24/4, Balaji Park 4, Rao Colony, Talegaon Dabhade, Taluka- Maval, Dist.- Pune - 410 506. Maharashtra, India.

a.	**Name**	:	Mr. Shilimkar Vaibhav C.
b.	**Nationality**	:	Indian
c.	**Address**	:	Department of Pharmacognosy, Seth Govind Raghunath Sable College of Pharmacy, Saswad, Taluka Purandhar, Dist. Pune – 412 301. Maharashtra, India.
d.	**Name**	:	Dr. Jagtap Suresh D.
e.	**Nationality**	:	Indian
f.	**Address**	:	Herbal Medicine, Interactive Research School for Health Affairs (IRSHA), Bharati Vidyapeeth University, Pune Satara Road, Pune – 411 043. Maharashtra, India.

Dated this 03 **day of** November **2017.**

Signature:

Savitribai Phule Pune University ★Dept. of Botany★

Prof. Dr. Deokule Subhash. S.

Rtd. PROFESSOR
Department of Botany
Savitribai Phule Pune University
Pune - 411 007.

3. DECLARATION TO BE GIVEN WHEN THE APPLICATION IN INDIA IS FILED BY THE APPLICANTS IN THE CONVENTION COUNTRY:-

-NOT APPLICABLE-

4. STATEMENT (to be signed by the additional inventors not mentioned in the application form)

-NOT APPLICABLE-

To,

The Controller of Patent

The Patent Office, at Mumbai

200293028

FORM 18

THE PATENTS ACT 1970
(39 of 1970)
&
The Patents Rules, 2003
REQUEST/EXPRESS REQUEST FOR EXAMINATION OF APPLICATION FOR PATENT
[See section 11B and rule 20(4)(ii), 24B(1)(i)]

(FOR OFFICE USE ONLY)

RQ No: R20202023/283
Filing Date: 08/10/2020
Amount of Fee Paid: रूपए नकद/चैक/मनी ऑर्डर द्वारा
CBR No: 44001 संख्या. 26226 दि.08/10/2020
Signature के सहस प्राप्त हुए।

1. APPLICANTS

(a)	Name:	Deokule Subhash Sadhu
(b)	Nationality:	Indian
(c)	Address:	Department of Botany, Savitribai Phule Pune University (SPPU), Pune- 411 007, Maharashtra, India.

रोकड़िया

2. Statement in case of request for examination made by the applicants

We hereby request that our application for patent no.: **201721039233** filed on **03/11/2017** for the invention titled **"Novel source and extraction method for colchicine in seed and tubers of *Arisaema murrayi* Hook."** shall be examined under sections 12 and 13 of the Act.

3. Statement in case of request for examination made by any other interested person

I/We the interested person request for the examination of the application no._____dated _____filed by the applicant_____titled_____under sections 12 and 13 of the Act.

As an evidence of my/our interest in the application for patent following documents are submitted.

(a) _____

Not applicable

4. ADDRESS FOR SERVICE

Department of Botany, Savitribai Phule Pune University (SPPU), Pune- 411 007, Maharashtra, India. **Email:** deokule.ss@gmail.com. **Mobile:** 9371088210.

Dated this 8 day of October 2020

Deokule

Deokule Subhash Sadhu

To,
The Controller of Patents
The Patent Office,
Mumbai

SAVIRIBAI PHULE PUNE UNIVERSITY

(formerly University of Pune)

DEPARTMENT OF BOTANY
SAVITRIBAI PHULE PUNE UNIVERSITY
Ganeshkhind, Pune-411 007
Tele. No. : (020) 25601439, 25601438
Email : .....@unipune.ac.in

Ref. No. : Bot/

Date : 03-11-2017

D-38759

To,

The Controller of Patents,
The Patent Office,
Boudhik Sampada Bhavan,
S. M. Road, Antop Hill, Mumbai,
Maharashtra- 400037

R 01/13977/2017

200174916

Dear Sir,

Re: Submission of Complete Patent Specification

Please find herewith documents for a complete specification application entitled "**New source and extraction method for colchicines in seed and tubers of *Arisaema murrayi***" along with the prescribed fee of Rs. 1750/-.

The Learned Controller is requested to take the same on record and issue the official filing receipt for the same.

Yours faithfully,

Prof. Dr. Deokule Subhash S.
Department of Botany,
Savitribai Phule Pune University (SPPU),
Ganeshkhind Road,
Pune- 411007. Maharashtra, India

Enclosures:

1. Application for Grant of Patent [Form 1]
2. Complete specification [Form 2]
3. Statement and undertaking under section 8 [Form 3]
4. Declaration as to inventorship [Form 5]
5. Fees of Rs. 1750/- (by cash)

"FORM 1 THE PATENTS ACT 1970 (39 of 1970) and THE PATENTS RULES, 2003 **APPLICATION FOR GRANT OF PATENT** (See section 7, 54 and 135 and sub-rule (1) of rule 20)		**(FOR OFFICE USE ONLY)** 200174923

	Application No.	201721039237
	Filing date	03/11/2017
	Amount of Fee paid	1750/- रूपय चेक/चेक/मनी ऑर्डर द्वारा
	CBR No	CBR संख्या 20880 दि. 03/11/2017 के तहत प्राप्त हुए।
	Signature	

1. APPLICANT'S REFERENCE / IDENTIFICATION NO. (AS ALLOTTED BY OFFICE)	

2. TYPE OF APPLICATION [Please tick (√) at the appropriate category]

Ordinary (√)		Convention ( )		PCT-NP ( )	
Divisional ( )	Patent of Addition ( )	**Divisional** ( )	**Patent of** Addition ( )	**Divisional** ( )	Patent of Addition ( )

3A. APPLICANTS

Name in Full	Nationality	Country of Residence	Address of the Applicant	
Prof. Dr. **Deokule Subhash S.**	**Indian**	**India**	**House No.**	**Department** of Botany, Savitribai Phule **University** of Pune **(SPPU).**
			Street	**Ganeshkhind Road,**
			City	**Pune**
			State	**Maharashtra**
			Country	**India**
			Pin code	411007

3B. CATEGORY OF APPLICANT [Please tick (√) at the appropriate category]

Natural Person (√)	Other than Natural Person		
	Small Entity ( )	**Startup ( )**	**Others ( )**

4. INVENTORS [Please tick (√) at the appropriate category]				
Are all the inventors same as the applicants named above?		Yes ( )	No (√)	
If "No", furnish the details of the inventor(s)				
Name in Full	Nationality	Country of Residence	Address of the Inventor	
Prof. Dr. Deokule Subhash S.	Indian	India	House No.	Department of Botany, Savitribai Phule University of Pune (SPPU)
			Street	Ganeshkhind Road,
			City	Pune
			State	Maharashtra
			Country	India
			Pin code	411007
Dr. Joseph Tessy	Indian	India	House No.	Department of Botany, Savitribai Phule University of Pune (SPPU)
			Street	Ganeshkhind Road,
			City	Pune
			State	Maharashtra
			Country	India
			Pin code	411007
Dr. Mokat Digambar N.	Indian	India	House No.	Department of Botany, Savitribai Phule University of Pune (SPPU)
			Street	Ganeshkhind Road,
			City	Pune
			State	Maharashtra
			Country	India
			Pin code	411007
Ms. Raut Sharayu G.	Indian	India	House No.	701, Heramb Residency apartment
			Street	Behind Ekalavya College, Bhujbal township, Kothrud
			City	Pune
			State	Maharashtra
			Country	India
			Pin code	411 038
Dr. Jagtap Suresh D.	Indian	India	House No.	Herbal Medicine, Interactive Research School for Health Affairs (IRSHA), Bharati Vidyapeeth University
			Street	Pune Satara Road
			City	Pune
			State	Maharashtra
			Country	India
			Pin code	411043

5. TITLE OF THE INVENTION **Novel process to extract Lupeol and Saponin from selected plants**		
6. AUTHORISED REGISTERED PATENT AGENT(S)	IN/PA No.	-
	Name	-
	Mobile No.	-
7. ADDRESS FOR SERVICE OF APPLICANT IN INDIA	Name	**Prof. Dr. Deokule Subhash S.**
	Postal Address	**Department of Botany, Savitribai Phule University of Pune (SPPU), Ganeshkhind Road, Pune- 411007. Maharashtra, India.**
	Telephone No.	020 2560 1217
	Mobile No.	09371088210
	Fax No.	020 2560 1217
	Email ID	deokule.ss@gmail.com
8. IN CASE OF APPLICATION CLAIMING PRIORITY OF APPLICATION FILED IN CONVENTION COUNTRY, PARTICULARS OF CONVENTION APPLICATION **- NOT APPLICABLE -**		
9. IN CASE OF PCT NATIONAL PHASE APPLICATION, PARTICULARS OF INTERNATIONAL APPLICATION FILED UNDER PATENT CO-OPERATION TREATY (PCT) **- NOT APPLICABLE -**		
10. IN CASE OF DIVISIONAL APPLICATION FILED UNDER SECTION 16, PARTICULARS OF ORIGINAL (FIRST) APPLICATION **- NOT APPLICABLE -**		
11. IN CASE OF PATENT OF ADDITION FILED UNDER SECTION 54, PARTICULARS OF MAIN APPLICATION OR PATENT **- NOT APPLICABLE -**		

12. DECLARATIONS

(i) Declaration by the inventor(s):

We, the above named inventors are the true & first inventors for this invention and declare that IRSHA, BVDU herein our assignee.

Signature:

Name: Prof. Dr. Deokule Subhash S. Date: 02.11.2017

Signature:

Name: Dr. Joseph Tessy Date: 3.11.2017

Signature:

Name: Dr. Mokat Digambar N. Date 2.11.2017

Signature:

Name: Ms. Raut Sharayu G. Date: 2.11.2017.

Signature:

Name: Dr. Jagtap Suresh D. Date: 3/11/2017

(ii) Declaration by the applicant(s) in the convention country

- NOT APPLICABLE -

Declaration by the applicants:

We the applicants hereby declares that:-

√	We are in possession of the above-mentioned invention.
√	The complete specification relating to the invention is filled with this application.
√	The invention as disclosed in the specification uses the biological material from India and the necessary permission from the competent authority shall be submitted by us before the grant of patent to us.
√	There is no lawful ground of objection to the grant of the patent to us.
√	We are the true & first inventors.
√	We are the assignee of true and first inventors.
√	The application or each of the applications, particulars of which are given in Paragraph-8, was the first application in convention countries in respect of our inventions.
√	We claim the priority from the above mentioned application(s) filed in convention countries and state that no application for protection in respect of the invention had been made in a convention country before that date by us or by any person from which we derive the title.
X	Our application in India is based on international application under Patent Cooperation Treaty (PCT) as mentioned in Paragraph-9.
X	The application is divided out of our application particulars of which is given in Paragraph-10 and pray that this application may be treated as deemed to have been filed on .....under section 16 of the Act.
X	The said invention is an improvement in or modification of the invention particulars of which are given in Paragraph-11.

13. FOLLOWING ARE THE ATTACHMENT WITH THE APPLICATION:

(a) Form 2

Item	Details	Fee	Remarks
Complete specification	No. of pages: 08	Rs. 1750/-	
No. of Claims	No. of claims: 07 No. of pages: 01		
No. of drawing sheets	: 07		
No. of figures	: 08		
No. of tables	: 00		
No. of abstract pages	: 01		

(b) Complete specification (1 copy).

(c) Statement and undertaking on Form 3

(d) Declaration as to Inventor ship on Form 5

(e) Total fee Rs. 1750/- in cash.

We hereby declare that to the best of my/our knowledge, information and belief the fact and matters stated herein are correct and I/we request that a patent may be granted to me/us for the said invention.

Dated this ....03.....day of ..November.....2017.

Signature:

Prof. Dr. Deokule Subhash S.

Department of Botany,

Savitribai Phule University of Pune (SPPU),

Ganeshkhind Road, Pune- 411007. Maharashtra.

To,

THE CONTROLLER OF PATENTS,

THE PATENT OFFICE, MUMBAI

E-2/2420/2017

FORM 2

THE PATENT ACT 1970

(39 OF 1970)

&

The patents rules, 2003

COMPLETE SPECIFICATION

(See section 10 and rule 13)

200174926

1. TITLE OF THE INVENTION:

Novel process to extract Lupeol and Saponin from selected plants

2. APPLICANT (S):

a. **Name** : Prof. Dr. Deokule Subhash S.

b. **Nationality** : **Indian**

c. **Address** : Department of Botany, Savitribai Phule Pune University, **Ganeshkhind Road, Pune-411007, Maharashtra, India**

a. **Name** : **Dr. Joseph Tessy**

b. **Nationaiity** : Indian

c. **Address** : **Department of Botany, Savitribai Phule Pune University, Ganeshkhind Road, Pune-411007, Maharashtra, India**

a. **Name** : Dr. Mokat Digambar N.

b. **Nationality** : Indian

c. **Address** : Department of Botany, Savitribai Phule Pune University, **Ganeshkhind Road, Pune-411007, Maharashtra, India**

a. **Name** : Ms. Raut Sharayu G.

b. **Nationality** : Indian

c. **Address** : **Flat no. 701, Heramb Residency apartment, Behind Ekalavya College, Bhujbal township, Kothrud, Pune – 411 038, Maharashtra, India.**

a. **Name** : **Dr. Jagtap Suresh D.**

b. **Nationality** : **Indian**

c. **Address** : **Herbal Medicine**

Interactive Research School for Health Affairs (IRSHA),

Bharati Vidyapeeth University, Pune Satara Road, Pune – 411 043.

Maharashtra, India.

3. PREAMBLE TO THE DESCRIPTION	
PROVISIONAL NOT APPLICABLE	**COMPLETE** The following specification describes the invention.

4. Description

Field of the Invention

This invention relates to a process to enhance the yield of Lupol and Saponin having anti-dysenteric activity from *Barringtonia acutangula* (L.) Gaertn. and *Naregamia alata* W. & Arn. Aspect of the invention is through test for Saponin, High Performance Thin Layer Chromatography (HPTLC), and High Performance Liquid Chromatography (HPLC).

Background of the invention:

***Barringtonia acutangula* (L.) Gaertn.:**

It is known as Indian oak. This comes under the family Barringtoniaceae. It is seen in deciduous and evergreen forest, of India mostly along the banks of rivers, streams. This plant is seen in Kasargode, Kottayam, Thrissur, Kollam, Alappuzha, Palakkad and Malapuram Districts of Kerala (Sasidharan, 2004). This is a medium sized glabrous tree 10-15m in height with pale grey slender young branches with rough dark simple and alternate leaves and bears beautiful flowers (Pandey & Chadha, 1996).

The juice of the leaves is a remedy for diarrhoea. The bark has medicinal properties and it is used as an astringent. It is administered during diarrhoea and blennorrhoea and as a febrifuge in malaria (Jain, 1991).

Pal et al. (1991 & 1994) reported the presence of triterpenoid glucoside and saponins in *Barringtonia acutangula*. Other constituent reported from the plants are, barringtonic acid, Acutangulosides A-F, Monodesmosidic Saponins, Nine triterpene saponins, acutanguloside A-F, acutanguloside D-F methyl esters (5a-7a), single triterpene aglycone (1), three monodesmosidic glucuronide saponins of barringtogenol C namely barringtosides A, B and C, barringtoside A, 3-o-beta-D-xylopyranosyl (1->3)-[beta-D-galactopyranosyl (1->2)]-beta-D-glucuronopyranosyl barringtogenol C; barringtoside B, 3-o-beta-D-xylopranosyl (1-3)-] beta-D-galactopyranosyl (1->2)]-beta-D- glucuronopyranosyl barringtogenol C; barringtoside B, 3-o-beta-D-xylopyranosyl (1->3)-] beta-D-galactopyranosyl (1->2)]-beta-D-glucuronopyranosyl-21-o-tigloyl-28-o-isobutyryl barringtogenol C; barringtoside C, 3-o-alpha-L-arabinopyranosyl(1->3)-[beta- D-galactopyranosyl (1->2)]-beta-D-glucuronopyranosyl barringtogenol C. Saponin present in plant is responsible for anti-diarrhoeal activity (Anantaraman and Pillai, 1956; Barua and Chakraborti, 1964, 1965; Barua and Dutta, 1968; Barua et al., 1972, 1963; Braun and Mouchacea, 2000; Chakraborti and Barua, 1963, Mills et al., 1994).

***Naregamia alata* W. & Arn.:**

It belongs to family Meliaceae. The plant is a small, under shrub upto 30 cm in height. This is the monotypic genus native of India. It is found to be growing in Western Ghats, from north to south ascending up to 900m. It is commonly found throughout South India in all districts of Kerala. It is endemic to peninsular India (Hooker, 1872).

The whole plant is medicinally important. It is acrid, sweet, cooling, aromatic, alexeteric, vulnerary, emetic, cholagogue, expectorant, depurative and antipyretic. The root contains an alkaloid called naregamin, wax, gum, asparagines, starch etc. It is pungent and aromatic but not having any characteristic taste. The plant is being used in indigenous system of medicine in the treatment of

various ailments such as in acute dysentery, wounds, ulcers, vitiate conditions of pitta and vata, halitosis, cough, asthma, bronchitis, splenomegaly, scabies, purities, dyspepsia, catarrh, anaemia, malarial fevers, rheumatism and skin itching (Nadkarni, 1927, Jain, 1991). Decoction of the stem and leaves has been used in treatment of dysentery. *Naregamia* has recently been tried in Madras to treat acute dysentery and also as an emetic and expectorant. Roots are used as emetic, cholagogue, expectorant, and in the treatment of acute dysentery and chronic bronchitis as reported by Dymock et al. (1890), Ambasta (1986) and Agarwal (1997). *Naregamia* is a wonderful drug in the case of dysentery, treated by a decoction of the bark in rice water. Lupol and Saponin present in plants are having anti-dysentric activity from *Barringtonia acutangula* and *Naregamia alata*.

Saponin:

Saponins are a class of chemical compounds found in particular abundance in various plant species. More specifically, they are amphipathic glycosides grouped phenomenologically by the soap-like foaming they produce when shaken in aqueous solutions, and structurally by having one or more hydrophilic glycoside moieties combined with a lipophilic triterpene derivative.
Saponins have historically been understood to be plant-derived, but they have also been isolated from marine organisms such as sea cucumber. Saponins are indeed found in many plants. Most saponins, which readily dissolve in water, are poisonous to fish. Therefore, in ethnobotany, they are primarily known for their use by indigenous people in obtaining aquatic food sources.

Lupeol:

Lupeol is a pharmacologically active triterpenoid. It has several potential medicinal properties. Lupeol is found in a variety of plants, including mango, *Acacia visco* and *Abronia villosa*. It is also found in dandelion coffee. Lupeol is produced by several organisms from squalene epoxide. Dammarane and baccharane skeletons are formed as intermediates. The reactions are catalyzed by the enzyme lupeol synthase. Lupeol has a complex pharmacology, displaying antiprotozoal, antimicrobial, anti-inflammatory, antitumor and chemopreventive properties.

Summary of the invention:

This invention relates to a new process to enhance the yield of Lupol and Saponin from *Barringtonia acutangula* and *Naregamia alata*. Aspect of the invention is through test for Saponin, High Performance Thin Layer Chromatography (HPTLC).

In phytochemistry, plants were analyzed by qualitative tests for the presence of saponin. The phytochemical investigations were further supported by HPTLC analysis using solvent system consisted of toluene: acetone : acetic acid (GAA) in ratio of 8.9 : 0.9 : 0.2. The active principle lupeol appeared as blue band on visual observation after derivatization in the HPTLC analysis at an Rf value of 0.42. Quantitative analysis was achieved using standard lupeol. The amount of lupeol present in *Barrintonia acutangula* 0.05% and *Naregamia alata* lupeol is absent.

In case of saponin the solvent system used was ethylacetate : ethanol (96%) : water : ammonia (25%) in ratio (6.5 : 2.5 : 9 : 1) respectively. The standard saponin was spotted 0.1 µg/µl. The derivatization was carried out by using anisaldehyde -sulfuric acid reagent and later heating it at 100°C for 10 minutes. Active principle saponin appeared in bluish band on visual observation after derivatization in the HPTLC analysis at an Rf value 0.06. Quantitative analysis was achieved using standard saponin.

Detailed description of the figures:

Figure 1: *Barrintonia acutangula*
Figure 2: *Naregamia alata*
Figure 3: HPTLC of Saponin
Figure 4: Saponin – Standard
Figure 5: Saponin 3D Image
Figure 6: HPTLC of Lupeol
Figure 7: Lupeol – Standard
Figure 8: Lupeol 3D Image

Detailed description of the invention:

Detailed experimental studies

The leaves of *Barringtonia acutangula* (Figure 1) and *Naregamia alata* (Figure 2) collected from different parts of Kerala, India and identified by BSI of India western circle. Voucher specimens have been deposited in the Botanical Survey of India (BSI) Western Circle Pune, India, and also in the department of Botany University of Pune. The leaves were shade dried and pulverized. The pulverized plant materials were extracted with ethanol. These extracts were initially screened for antidysenteric.

Test for saponins:

Sections were placed directly in 1 drop of concentrated H_2SO_4 on a slide, which gives a characteristic sequence of colour reactions, beginning immediately with yellow, changing to red within 30 minutes and finally becoming violet or blue green in a short time.

To determine localization of the saponin, sections were put in saturated Barium hydroxide solution for about 24 hrs. Sections were washed with calcium chloride then placed in Potassium dichromate. Yellow colour indicated the presence of saponins.

High performance thin layer chromatography (HPTLC):

HPTLC technique was followed for the qualitative analysis and the confirmation of chemicals present in the studied plants.

HPTLC is a versatile separation technique included various steps as given below:

1) Selection of HPTLC plates and adsorbent
2) Sample preparation
3) Application of sample
4) Development (separation)
5) Detection including post-chromatographic derivatization
6) Quantification
7) Documentation

1) Selection of HPTLC plates and adsorbent:

For preparative TLC work, plates with adsorbent thickness of 1.0 - 2.0 mm are available in addition to chemically modified layers. Aluminum sheet (0.1 mm thick) sheets as support offer the same advantage as polyester support but with increased temperature resistance. However, with eluent containing high concentration of mineral acids or concentrated ammonia one may find problem, as they will chemically attack aluminum. Aluminum sheets are otherwise compatible with organic solvents and organic acids such as formic acid and acetic acid.

Plate size: The pre-coated TLC/ HPTLC plates in size of 20 x 20 cm with aluminum or polyester support are procured mainly for economic reasons. Before handling the pre-coated plates for any experimental work, it is important to note direction of the application of adsorbent as chromatographic developments have to be performed in that direction only. Pre-washing of pre-coated plates with large surface area absorb not only water vapours and the impurities from atmosphere but other volatile substances often condense particularly after the packing has been opened and exposed to laboratory. To avoid any possible interference, due to impurities with the chromatographic separation particularly in case of quantitative work, pre-washing of plates were done.

Activation of pre-coated plates: Plates exposed to high humidity or kept on hand for long time may have to be activated by placing in oven at 110- 120° C for 30 minutes prior to sample spotting.

2) Sample Preparation:

Dissolved dosage form with complete recovery of intact compound(s) of interest and minimum of matrix with a suitable concentration of analyte(s) for direct application on the HPTLC plate. Besides, maximizing the yield of analyte(s) in the selected solvent stability of analytes during extraction and analysis must be considered and ensured. Therefore, the choice of a suitable solvent for a given analysis is very important. For normal phase chromatography using silica gel pre-coated plates (more than 80-90% HPTLC analysis is done using silica gel as adsorbent) solvent for dissolving the sample should be non-polar and volatile as far as possible. It is preferable to keep the solvent as simple as possible and quantity employed is limited to ensure complete extraction of analytes and minimum of extraneous components. Sample and reference substances should be dissolved in the same solvent to ensure comparable distribution at starting zones.

3) Application of sample:

Applied 1-10 µl volume for TLC and 0.5 – 5 for HPTLC keeping the size of starting zone(s) down to minimum; 2 - 4 mm (TLC) and 0.5-1 mm (HPTLC) in the concentration range of 0.1-1 µg/µl for TLC/ HPTLC. However, volume and concentration primarily depend on the component under analysis and their sensitivity to various detection techniques.

4) Development (Mobile phase):

Mobile phase was selected by taking into consideration chemical properties of analytes and the adsorbent layer.

Pre-conditioning (Chamber saturation):

Drying cupboard or hot plates are employed. Hot plates with regulated range of temperature i.e. 50-190° C ± 2° C are extensively being employed for heating the chromatogram.

5) Detection and visualization:

As soon as the development process is complete the plate is removed from the chamber and dried to remove the mobile phase completely. The zones can be located by various physical chemical biological-physiological methods. There is apparently no difficulty in detecting colored substances or colorless substances in short wave ultra-violet (UV) region (254 nm) or with intrinsic fluorescence such as riboflavin quinine sulphate.

6) Quantitative:

Spraying and dipping techniques are used for applying detection reagents. However, in addition to other reasons as enumerated below dipping followed by evaporation is essential both for precision and repeatability in ultimate quantitative analysis.

7) Documentation:

The use of application scheme and labeling every single chromatogram can avoid mistake in respect of order of application. It is preferable to apply each sample and reference solution twice by following data - pair method. A lead pencil can be used to write on the chromatoplate. The plate should never be marked below the starting point, as layer is likely to get damaged affecting chromatographic distribution of the substances under analysis which may ultimately lead to error in scanning. The best way to label the chromatoplate is to mark above the level of solvent point, immediately after development is completed, the solvent point should be marked both on left and right hand edges of the plate, this facilitated calculation of Rf values. The practice of cutting a scratch across the whole layer is no longer in use. The type of plate, chamber system, composition of mobile phase, running time and detection method should all be recorded. HPTLC protocol format given in the text may be adopted for recording all the relevant data.

Results:

Phytochemical test:

In phytochemistry, plants were analyzed by qualitative tests for the presence of various chemical constituents saponin. The phytochemical investigations were further supported by HPTLC analysis.

High Performance Thin Layer Chromatography (HPTLC):

In the case of lupeol analysis along with the mehanolic extract, standard lupeol was also spotted 0.1 µg/µl (0..4µg/µl on the plate conc). The solvent system consisted of toluene: acetone : acetic acid (GAA) in ratio of 8.9 : 0.8 : 0.3. Derivatization was carried out by dipping the plate in anisaldehyde -sulfuric acid reagent and later heating it at 100°C for 10 minutes. The developed plate scanned at 580 nm using TLC scanner 3 and associate integration software (wincats 1.4.2 version).

In case of saponin the solvent system used was ethylacetate : ethanol (96%) : water : ammonia (25%) in ratio (6.8 : 2.7 : 9 : 1) respectively. The standard saponin was spotted 0.1 µg/µl. The derivatization was carried out by using anisaldehyde -sulfuric acid reagent and later heating it at 100°C for 10 minutes (Wagner and Bladt, 1996). The developed plates were scanned at 540nm using TLC scanner 3 and associate integration software (wincats 1.4.2 version).

Analytical studies (Lupeol):

(Figure: 3, 4 and 5)

The active principle lupeol appeared as blue band on visual observation after derivatization in the HPTLC analysis at an Rf value of 0.42. Quantitative analysis was achieved using standard lupeol. The amount of lupeol present in *Barrintonia acutangula* 0.05% and *Naregamia alata* lupeol is absent.

Analytical studies (Saponin):

(Figure: 6,7 and 8)

The active principle saponin appeared in bluish band on visual observation after derivatization in the HPTLC analysis at an Rf value 0.06. Quantitative analysis was achieved using standard saponin. The amount of Saponin present in *Barrintonia acutangula* 0.4 % and amount of saponin present in *Naregamia alata* 1.20 % on dry wait basis respectively.

References:

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Pal et al. (1994), Sapomins from *Barringtonia acutangula* , Phytochemistry, 35(5) :1315-1318.

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5. Claims

We claim:

1. A method for extraction of Lupeol and Saponin using extraction, HPTLC from two different plant species.
2. Extraction method as in claim 1 is applicable for studied plants as shown below.
   a) *Barrintonia acutangula* and
   b) *Naregamia alata*
3. Extraction method present study as in claim 1, using plants as in claim 2 can be extracted using leaf powder in methanol as a solvent.
4. Method of extraction characterized in comprising the following process steps;
   a. Leaf powder of plant material (passed through 80 mesh size sieve) as in claim 2
   b. Leaf powder was extracted in methanol as solvent.
   c. In case of Lupeol, the solvent system consisted of toluene: acetone : acetic acid (GAA) in ratio of 8.9 : 0.8 : 0.3.
   d. Derivatization was carried out by dipping the plate in anisaldehyde -sulfuric acid reagent and later heating it at 100°C for 10 minutes.
   e. In case of saponin the solvent system used was ethylacetate : ethanol (96%) : water : ammonia (25%) in ratio (6.8 : 2.7 : 9 : 1) respectively.
   f. The derivatization was carried out by using anisaldehyde -sulfuric acid reagent and later heating it at 100°C for 10 minutes.
   g. Yields were compared with those standard values of lupeol and saponin.
5. Method of extraction according to claim 4 characterized in that the method is the production of a solution form (methanol extract)
6. Method used for extraction as in claim 1, for the plant as in claim claim 2, using extraction method as in claim 3 & 4, is capable for increase the yield of lupeol and saponin.
7. According to any of the proceeding claims that exemplified, the said method shows increased enhanced yield and new sources for lupeol and saponin.

Name : Prof. Dr. Deokule Subhash S Signature : Deokule

6. Date and Signature

Date: 02.11.2017

Place: Pune

Deokule

Prof. Dr. Deokule Subhash S.
Department of Botany,
Savitribai Phule University of Pune (SPPU),
Ganeshkhind Road,
Pune- 411007. Maharashtra, India

Figure 1: Flowering twig of *Barrintonia acutangula*

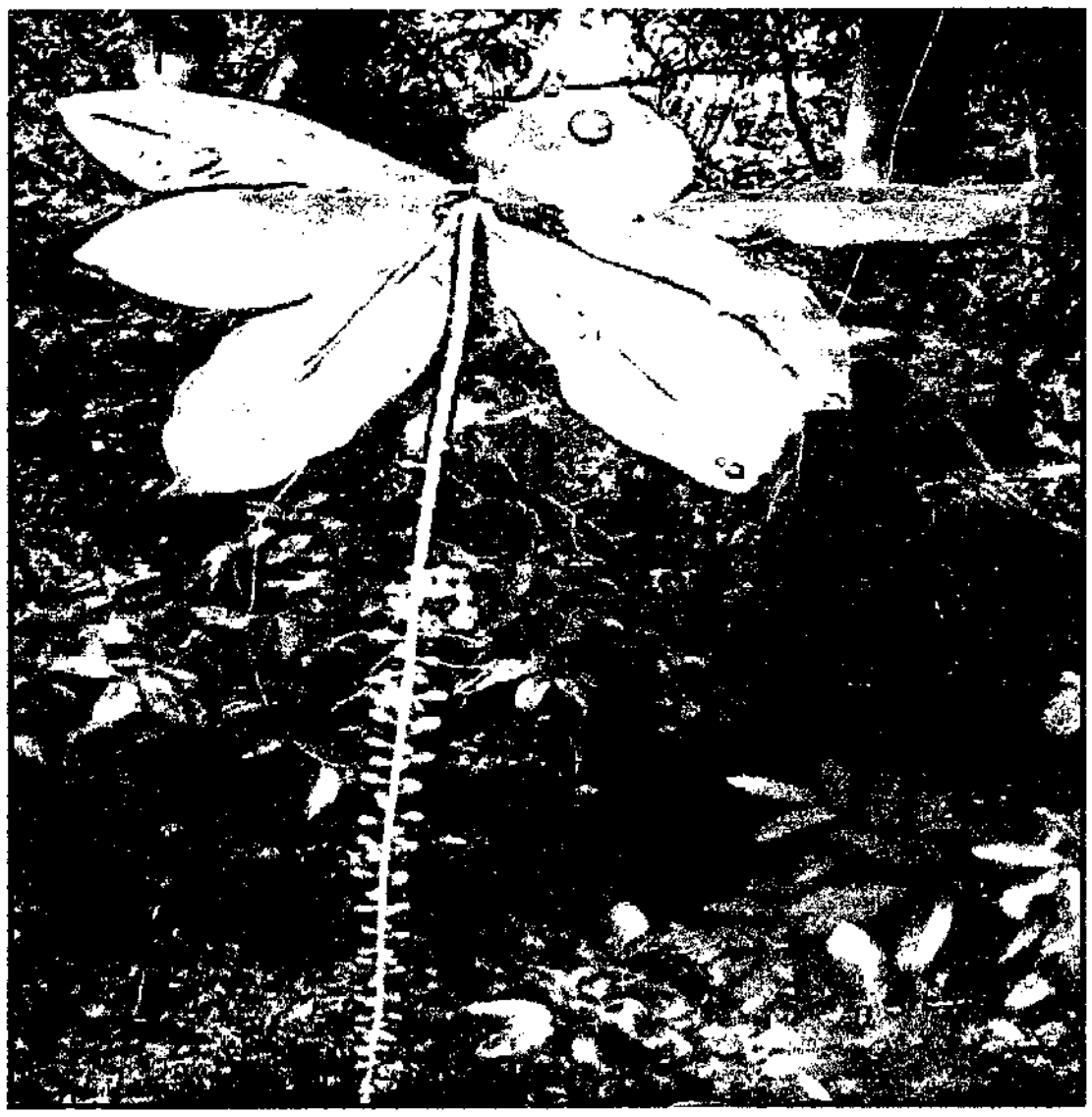

Figure 2: Habit of *Naregamia alata*

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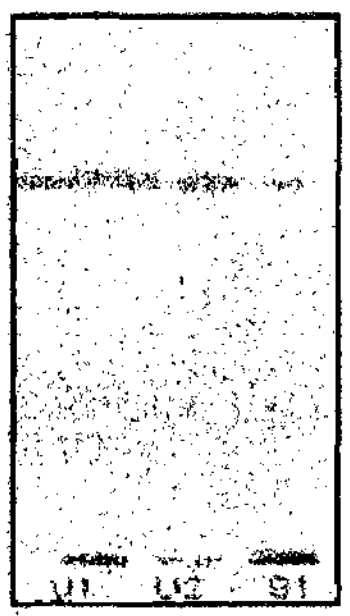
Signature:

Figure 3: HPTLC of Saponin

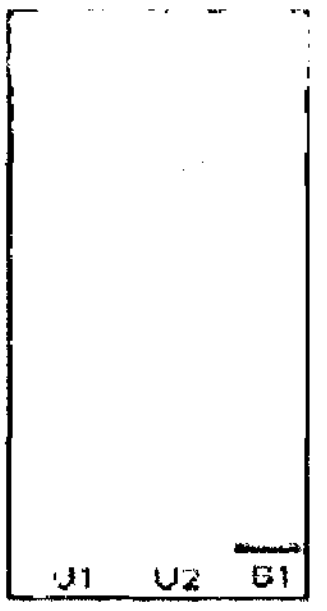
HPTLC analysis - Saponin



Saponin at 254 nm
(before derivatization)

Saponin at 366 nm
(after derivatization)

Saponin at 366 nm
(before derivatization)



Saponin at 540nm
(after derivatization)

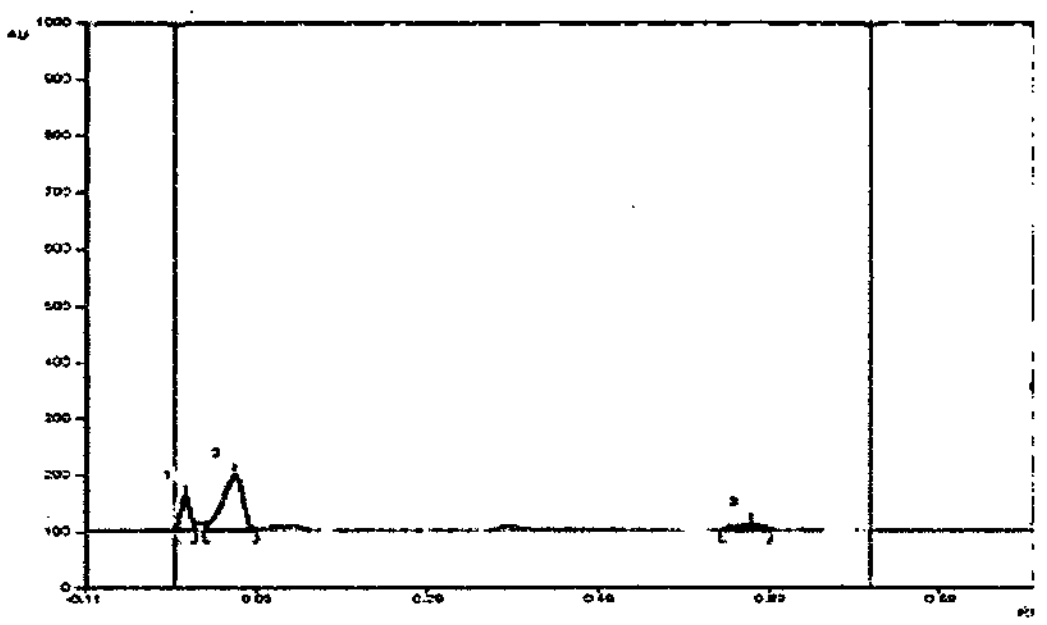
U1 - *B. acutangula*, U2 - *N. alata*, S1 - *Standard*

Name: Prof. Dr. Deokule Subhash S.

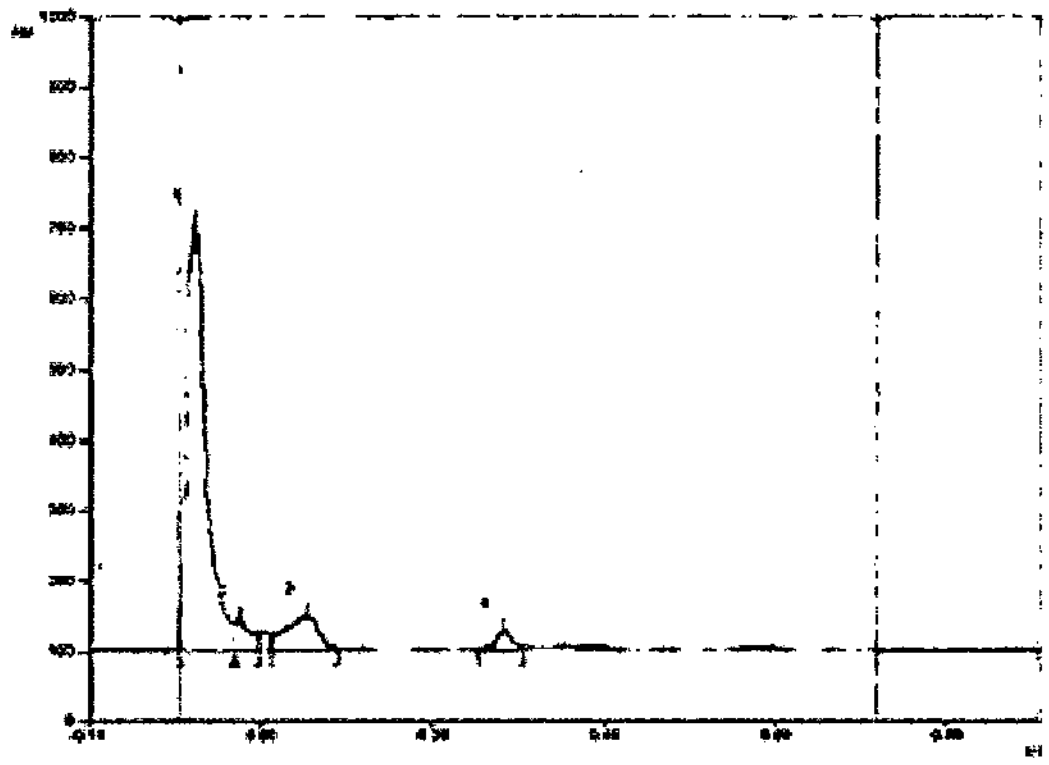
Signature:

Figure 4: Saponin

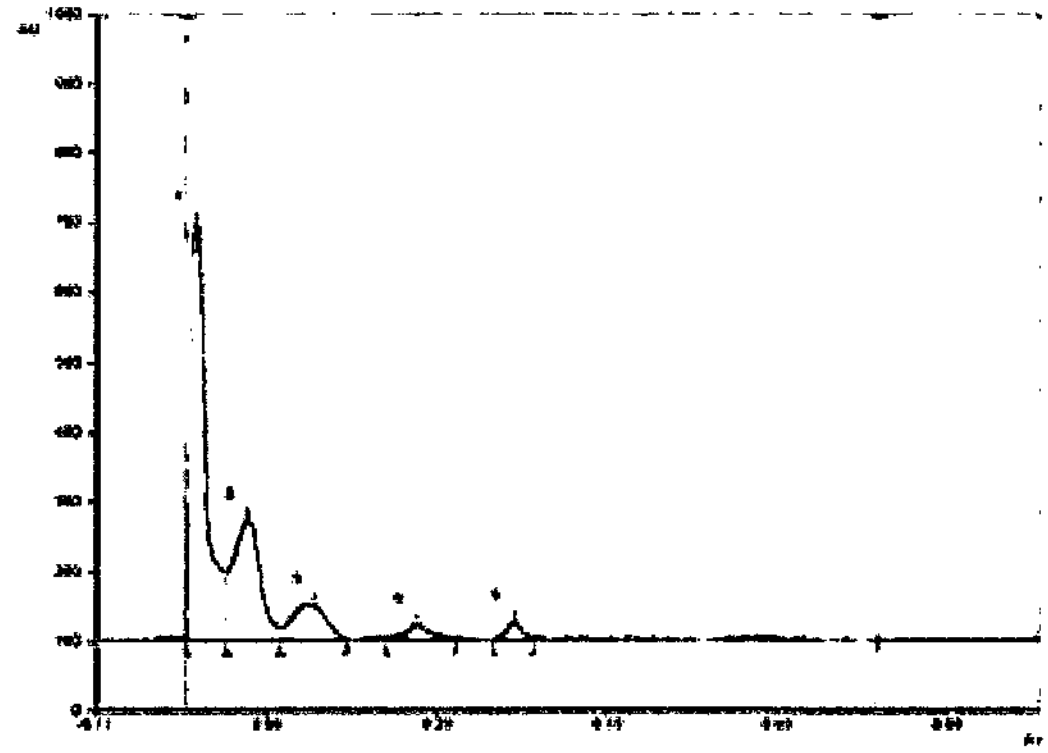
a) Standard



b) *Barrintonia acutangula*



c) *Naregamia alata*



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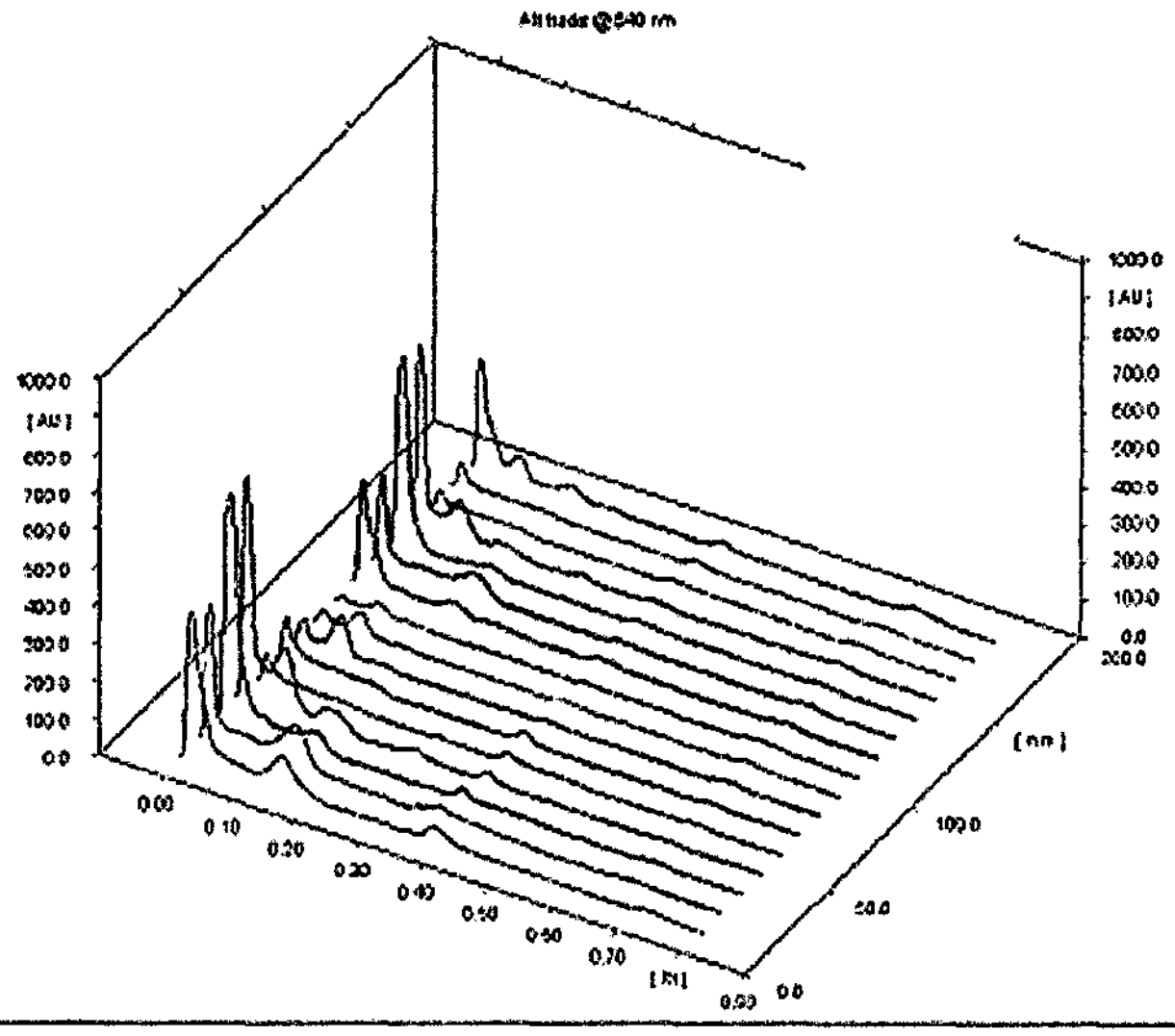
Signature:

Figure 5: Saponin 3D Image



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Signature:

Figure 6: HPTLC of Lupeol

HPTLC analysis - Lupeol

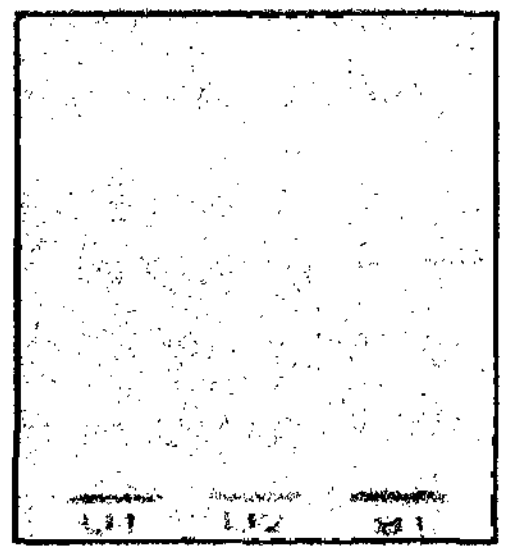


Image of lupeol 254 nm before derivatisation

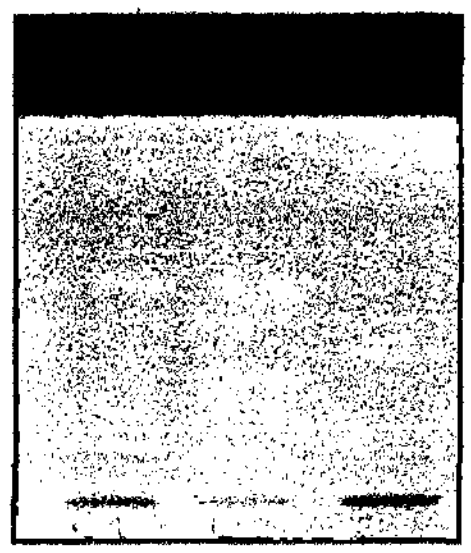


Image of lupeol 365 nm before derivatisation

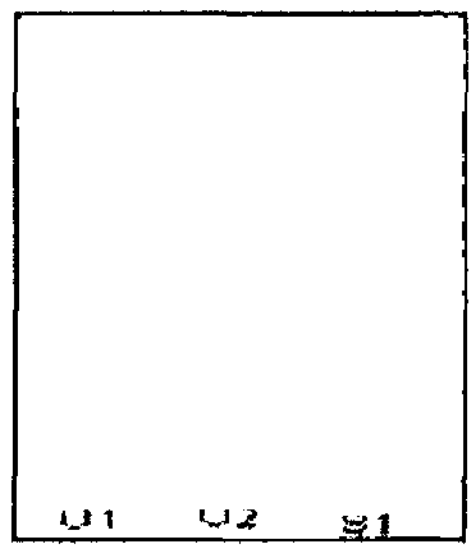


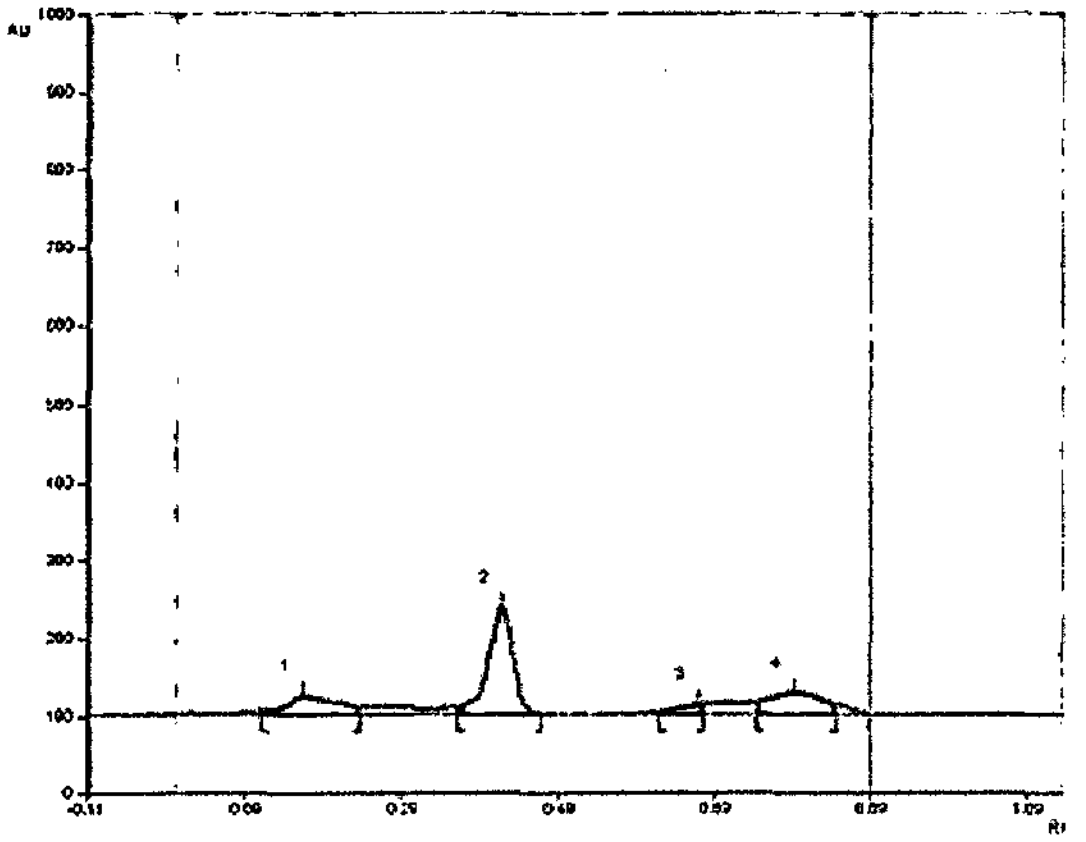
Image of lupeol 540 nm after derivatisation

U1 - *B. acutangula*, U2 - *N. alata*, S1 - *Standard*

Name: Prof. Dr. Deokule Subhash S. Signature:

Figure 7: Lupeol

a) Standard



b) *Barrintonia acutangula*

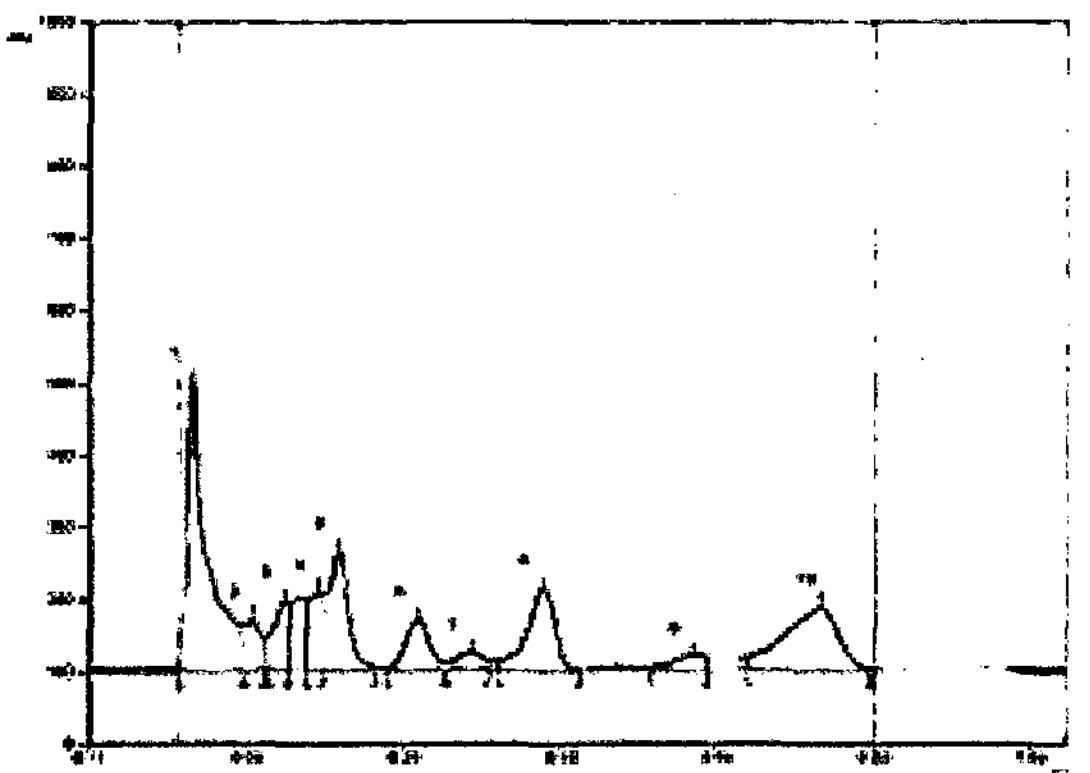


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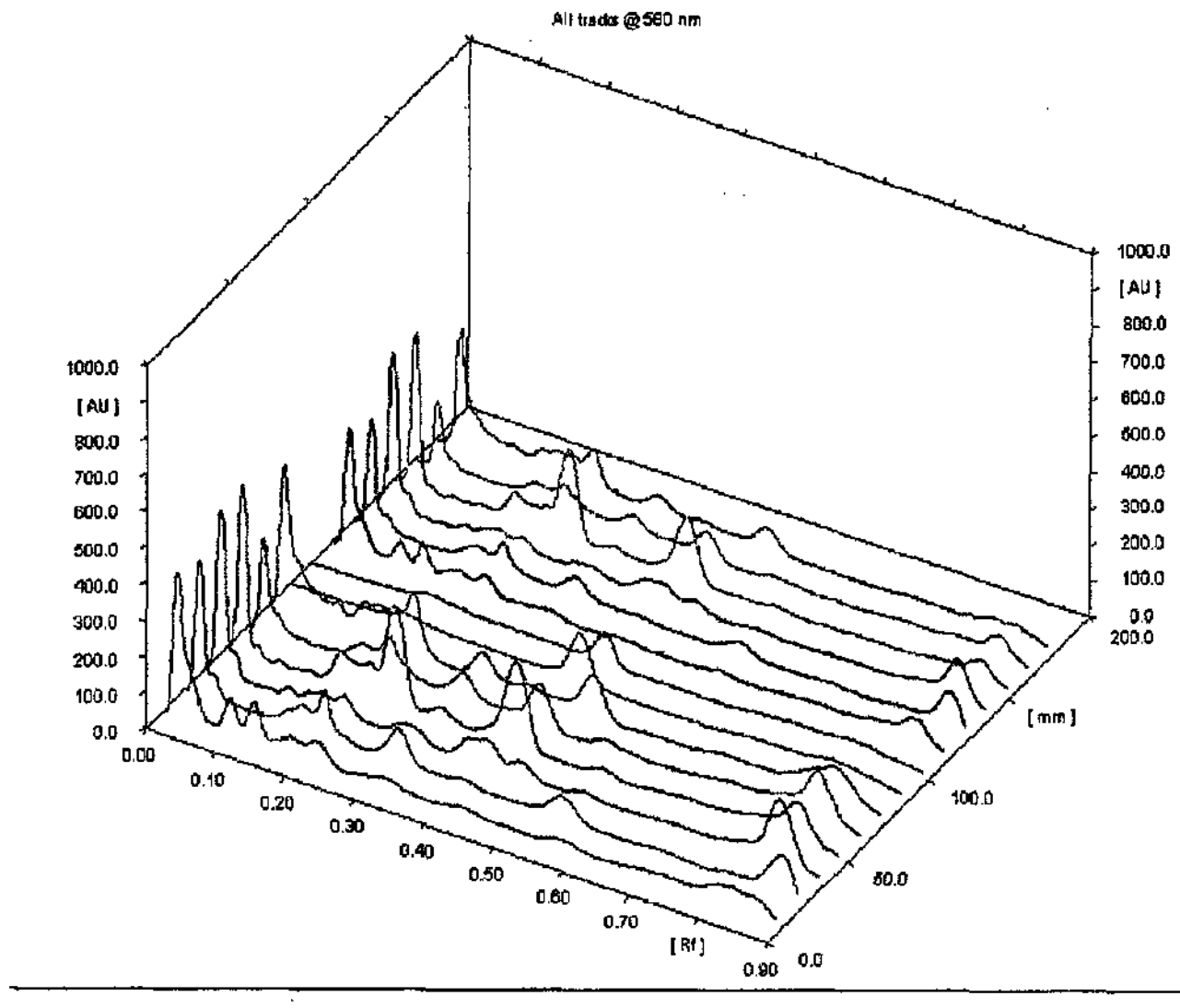
Figure 8: Lupeol 3D Image

LUPEOL

3 D Image Lupeol



Name: Prof. Dr. Deokule Subhash S.

Signature:

Title: Novel process to extract Lupeol and Saponin from selected plants

7. ABSTRACT OF THE INVENTION

The present study focuses on the potentiality of medicinal plants viz. *Barringtonia acutangula* (L.) Gaertn. and *Naregamia alata* W. & Arn. For the presence of secondary metabolites viz. lupeol and saponins. Both plants were collected and extacted in methanol. In phytochemistry, plants were analyzed by qualitative tests for the presence of saponin. The phytochemical investigations were further supported by HPTLC analysis using solvent system consisted of toluene: acetone : acetic acid (GAA) in ratio of 8.9 : 0.9 : 0.2. The active principle lupeol appeared as blue band on visual observation after derivatization in the HPTLC analysis at an Rf value of 0.42. Quantitative analysis was achieved using standard lupeol. The amount of lupeol present in *Barrintonia acutangula* is 0.05%, whereas it was found to be absent in *Naregamia alata.*

In case of saponin the solvent system used was ethylacetate : ethanol (96%) : water : ammonia (25%) in ratio 6.5 : 2.5 : 9 : 1 respectively. The standard saponin was spotted 0.1 µg/µl. The derivatization was carried out by using anisaldehyde -sulfuric acid reagent and later heating it at 100°C for 10 minutes. Active principle saponin appeared in bluish band on visual observation after derivatization in the HPTLC analysis at an Rf value 0.06. Quantitative analysis was achieved using standard saponin.

E-3/11627/2017

FORM 3

THE PATENTS ACT, 1970

[39 OF 1970]

and

THE PATENTS RULES, 2003

200174924

STATEMENT AND UNDERTAKING UNDER SECTION 8

(See Section 8; Rule 12)

1. Name of the applicants	I, Prof. Dr. Deokule Subhash S., Department of Botany, Savitribai Phule Pune University, Pune-411 007; hereby declare:
2. Name, address and nationality of the joint applicant	Dr. Suresh D. Jagtap, Herbal Medicine, IRHSA, Bharati Vidyapeeth University, Pune – 411 043; Ms. Sharayu Gajanan Raut, Flat no. 701, Heramb Residency apartment, Behind Ekalavya College, Bhujbal township, Kothrud, Pune – 411 038. (i) that we have not made any application for the same/substantially the same invention outside India.
3. Name and address of the assignee	(ii) that the rights in the applications have been assigned to Department of Botany, Savitribai Phule Pune University, Ganeshkhind road, Pune-7, Maharashtra, India. that we undertake that up to the date of grant of the patent by the Controller, we would keep him informed in writing the details regarding corresponding applications for the date of filling of such application Dated this 03 day of November 2017.
4. To be signed by the applicant or his authorized registered patent agent	Deokule
5. Name of the natural person who has signed	Prof. Dr. Deokule Subash S. Professor (Retd) Department of Botany, Savitribai Phule Pune University, Ganeshkhind road, Pune-7, Maharashtra, India.
	To, The Controller of Patents, The Patent Office, at Mumbai

E-5|1725|2017

FORM 5

THE PATENTS ACT, 1970

(39 of 1970)

&

The patents rules, 2003

DECLARATION AS TO INVENTORSHIP

[See section 10 (6) and rule 13(6)]

200174925

1. NAME OF APPLICANTS	(i) Prof. Dr. Deokule Subhash S.

Hereby declare that the true and first inventor(s) of the invention disclosed in the complete specification filed in pursuance of our application numbered..... Dated.....03-11-2017.....are.....

2. INVENTOR(S)

a.	**Name**	:	Prof. Dr. Deokule Subhash S.
b.	**Nationality**	:	Indian
c.	**Address**	:	Department of Botany, Savitribai Phule Pune University, Ganeshkhind Road, Pune-411007, Maharashtra, India
a.	**Name**	:	Dr. Tessy Joseph
b.	**Nationality**	:	Indian
c.	**Address**	:	Department of Botany, Savitribai Phule Pune University, Ganeshkhind Road, Pune-411007, Maharashtra, India
a.	**Name**	:	Dr. Digambar N. Mokat
b.	**Nationality**	:	Indian
c.	**Address**	:	Department of Botany, Savitribai Phule Pune University, Ganeshkhind Road, Pune-411007, Maharashtra, India
a.	**Name**	:	Ms. Sharayu Gajanan Raut
b.	**Nationality**	:	Indian
c.	**Address**	:	Flat no. 701, Heramb Residency apartment, Behind Ekalavya College, Bhujbal township, Kothrud, Pune – 411 038.

a.	**Name**	**:**	**Dr. Suresh D. Jagtap**
b.	**Nationality**	**:**	**Indian**
c.	**Address**	**:**	**Herbal Medicine** **Interactive Research School for Health Affairs (IRSHA), Bharati Vidyapeeth University, Pune** **Satara Road, Pune – 411 043.**

Dated this 03 **day of** November **2017**

Signature:

Savitribai Phule Pune University ★ Dept. of Botany ★

Prof. Dr. Deokule S. S.

Rtd. PROFESSOR
Department of Botany
Savitribai Phule Pune University
Pune - 411 007.

3. DECLARATION TO BE GIVEN WHEN THE APPLICATION IN INDIA IS FILED BY THE APPLICANTS IN THE CONVENTION COUNTRY:-

-NOT APPLICABLE-

4. STATEMENT (to be signed by the additional inventors not mentioned in the application form)

-NOT APPLICABLE-

To,
The Controller of Patent
The Patent Office, at Mumbai

200293027

FORM 18 THE PATENTS ACT 1970 (39 of 1970) & The Patents Rules, 2003 REQUEST/EXPRESS REQUEST FOR EXAMINATION OF APPLICATION FOR PATENT [See section 11B and rule 20(4)(ii), 24B(1)(i)]	(FOR OFFICE USE ONLY) RQ No: R202020031284 Filing Date: 08/10/2020 Amount of Fee Paid: CBR No: 44001 रूपये नकद/चेक/मनी ऑर्डर द्वारा Signature CBR संख्या. 26226 ..दि.08/10/2020 के तहत प्राप्त हुए।

1. APPLICANTS

(a)	Name:	Deokule Subhash Sadhu
(b)	Nationality:	Indian
(c)	Address:	Department of Botany, Savitribai Phule Pune University (SPPU), Pune- 411 007, Maharashtra, India.

रोक दिए

2. Statement in case of request for examination made by the applicants

We hereby request that our application for patent no.: **201721039237** filed on **03/11/2017** for the invention titled **"Novel Process to extract Lupeol and Saponin from selected plants"** shall be examined under sections 12 and 13 of the Act.

3. Statement in case of request for examination made by any other interested person

I/We the interested person request for the examination of the application no._____dated _____filed by the applicant_____titled_____under sections 12 and 13 of the Act.

As an evidence of my/our interest in the application for patent following documents are submitted.

(a) _____

Not applicable

4. ADDRESS FOR SERVICE

Department of Botany, Savitribai Phule Pune University (SPPU), Pune- 411 007, Maharashtra, India. **Email:** deokule.ss@gmail.com. **Mobile:** 9371088210.

Dated this 8 day of October 2020

Deokule

Deokule Subhash Sadhu

To,
The Controller of Patents
The Patent Office,
Mumbai

SAVIRIBAI PHULE PUNE UNIVERSITY

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Ref. No. : Bot/

Date : 03-11-2017

D.38766

E-101/13929/2017

To,
The Controller of Patents,
The Patent Office,
Boudhik Sampada Bhavan,
S. M. Road, Antop Hill, Mumbai,
Maharashtra- 400037

200174922

Dear Sir,

Re: Submission of Complete Patent Specification

Please find herewith documents for a complete specification application entitled "**Novel process to extract Lupeol and Saponin from selected plants**" along with the prescribed fee of Rs. 1750/-.

The Learned Controller is requested to take the same on record and issue the official filing receipt for the same.

Yours faithfully,

Deokule

Prof. Dr. Deokule Subhash S.
Department of Botany,
Savitribai Phule Pune University (SPPU),
Ganeshkhind Road,
Pune- 411007. Maharashtra, India

Enclosures:

1. Application for Grant of Patent [Form 1]
2. Complete specification [Form 2]
3. Statement and undertaking under section 8 [Form 3]
4. Declaration as to inventorship [Form 5]
5. Fees of Rs. 1750/- (by cash)

IPO MUMBAI 03 11 2017 18:51

"FORM 1 THE PATENTS ACT 1970 (39 of 1970) and THE PATENTS RULES, 2003 **APPLICATION FOR GRANT OF PATENT** (See section 7, 54 and 135 and sub-rule (1) of rule 20)	(FOR OFFICE USE ONLY) 200174908

	Application No.	201721039231
1750/- रुपये चेक/डी.डी. आदेश द्वारा CBR संख्या 2087 दि. 03/11/2017 के तहत प्राप्त हुए।	Filing date	03-11-2017
	Amount of Fee paid	₹. 1750/-
	CBR No	2087
	Signature	
1. APPLICANT'S REFERENCE / IDENTIFICATION NO. (AS ALLOTTED BY OFFICE)		

2. TYPE OF APPLICATION [Please tick (√) at the appropriate category]

Ordinary (√)		Convention ( )		PCT-NP ( )	
Divisional ( )	Patent of Addition ( )	Divisional ( )	Patent of Addition ( )	Divisional ( )	Patent of Addition ( )

3A. APPLICANTS

Name in Full	Nationality	Country of Residence	Address of the Applicant	
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			State	Maharashtra
			Country	India
			Pin code	411 007.

3B. CATEGORY OF APPLICANT [Please tick (√) at the appropriate category]

Natural Person (√)	Other than Natural Person		
	Small Entity ( )	Startup ( )	Others ( )

4. INVENTORS [Please tick (√) at the appropriate category]				
Are all the inventors same as the applicants named above?			Yes ( )	No (√)
If "No", furnish the details of the inventor(s)				
Name in Full	Nationality	Country of Residence	Address of the Inventor	
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5. TITLE OF THE INVENTION

New formulation for the management of glycosidase and oxidative stress.

6. AUTHORISED REGISTERED PATENT AGENT(S)	IN/PA No.	
	Name	
	Mobile No.	
7. ADDRESS FOR SERVICE OF APPLICANT IN INDIA	Name	Prof. Dr. Deokule Subhash S.
	Postal Address	Department of Botany, Savitribai Phule Pune University (SPPU), Ganeshkhind Road, Pune-411007. Maharashtra, India
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8. IN CASE OF APPLICATION CLAIMING PRIORITY OF APPLICATION FILED IN CONVENTION COUNTRY, PARTICULARS OF CONVENTION APPLICATION

- NOT APPLICABLE -

9. IN CASE OF PCT NATIONAL PHASE APPLICATION, PARTICULARS OF INTERNATIONAL APPLICATION FILED UNDER PATENT CO-OPERATION TREATY (PCT)

- NOT APPLICABLE -

10. IN CASE OF DIVISIONAL APPLICATION FILED UNDER SECTION 16, PARTICULARS OF ORIGINAL (FIRST) APPLICATION

- NOT APPLICABLE -

11. IN CASE OF PATENT OF ADDITION FILED UNDER SECTION 54, PARTICULARS OF MAIN APPLICATION OR PATENT

- NOT APPLICABLE -

12. DECLARATIONS

(i) Declaration by the inventor(s):

We, the above named inventors are the true & first inventors for this invention and declare that IRSHA, BVDU herein our assignee.

Signature:

Name: Prof. Dr. Deokule Subhash S. Date: 2/11/2017

Signature:

Name: Dr. Adhav Rahul Mohaniraj Date: 2/11/2017

Signature:

Name: Mr. Jagtap Kartikey Tanaji Date: 1/11/17

Signature:

Name: Dr. Singh Elangbam Athoiba Date: 1/11/17

Signature:

Name: Dr. Mahendra S. Shirsath Date: 1/11/2017

Signature:

Name: Dr. Jagtap Suresh D. Date: 2/11/2017

(ii) Declaration by the applicant(s) in the convention country

- NOT APPLICABLE -

Declaration by the applicants:

We the applicants hereby declares that:-

√	We are in possession of the above-mentioned invention.
√	The complete specification relating to the invention is filled with this application.
√	The invention as disclosed in the specification uses the biological material from India and the necessary permission from the competent authority shall be submitted by us before the grant of patent to us.
√	There is no lawful ground of objection to the grant of the patent to us.
√	We are the true & first inventors.
√	We are the assignee of true and first inventors.
√	The application or each of the applications, particulars of which are given in Paragraph-8, was the first application in convention countries in respect of our inventions.
√	We claim the priority from the above mentioned application(s) filed in convention countries and state that no application for protection in respect of the invention had been made in a convention country before that date by us or by any person from which we derive the title.
X	Our application in India is based on international application under Patent Cooperation Treaty (PCT) as mentioned in Paragraph-9.
X	The application is divided out of our application particulars of which is given in Paragraph-10 and pray that this application may be treated as deemed to have been filed on .....under section 16 of the Act.
X	The said invention is an improvement in or modification of the invention particulars of which are given in Paragraph-11.

13. FOLLOWING ARE THE ATTACHMENT WITH THE APPLICATION:

(a) Form 2

Item	Details	Fee	Remarks
Complete specification	No. of pages: 12	1750/-	
No. of Claims	No. of claims - 5 No. of pages - 1		
~~No of drawing sheets~~	—		
No. of figures	04 &02 pages		
No. of tables	07		
No of abstract pages	01		

(b) Complete specification (1 copies)

(c) Statement and undertaking on Form 3

(d) Declaration as to Inventorship on Form 5

(e) Total fee Rs. 1750/- in cash.

We hereby declare that to the best of my/our knowledge, information and belief the fact and matters stated herein are correct and I/we request that a patent may be granted to me/us for the said invention.
Dated this 03.....day of ..November.....2017.

Signature:

Prof. Dr. Deokule Subhash S.
Department of Botany,
Savitribai Phule Pune University (SPPU),
Ganeshkhind Road,
Pune- 411007. Maharashtra, India

To,

THE CONTROLLER OF PATENTS,

THE PATENT OFFICE, MUMBAI

FORM 2

THE PATENT ACT 1970

(39 OF 1970)

&

The patents rules, 2003

COMPLETE SPECIFICATION

(See section 10 and rule 13)

1. TITLE OF THE INVENTION:

New formulation for the management of glycosidase and oxidative stress.

2. APPLICANT (S):

a. Name : Prof. Dr. Deokule Subhash S.
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a. Name : Mr. Jagtap Kartikey Tanaji
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d. Name : Dr. Singh Elangbam Athoiba
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a. Name : Dr. Suresh D. Jagtap
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c. Address : Herbal Medicine
Interactive Research School for Health Affairs (IRSHA), Bharati Vidyapeeth University, Pune Satara Road, Pune – 411 043. Maharashtra, India.

3. PREAMBLE TO THE DESCRIPTION	
PROVISIONAL	**COMPLETE**
NOT APPLICABLE	The following specification describes the invention.

4. DESCRIPTION

Field of the Invention

Developed a new combination formulation for α-amylase, α-Glucosidase inhibitory activity and antioxidant activity of selected medicinal plants. Quantification of chemicals viz. phenols and flavonoids which are responsible for above mentioned activities.

Background of the invention:

Diabetes mellitus (DM) has turned out to be one of the major and emerging public health problems of the world. There has been perpetuating increase in the number of diabetic patients almost in all countries, especially in India which disreputably got nicknamed as the "Diabetes Capital of the World" (Mohan *et al.*, 2007). DM is not a disease but a heterogeneous group of symptoms endorsing a endocrine disorder being driven by a defective and deficient process of insulin secretion or it is a group of metabolic alterations characterized by hyperglycemia caused by insulin secretion defects, action or both and characterized by chronic hyperglycemia or increased blood glucose level with disturbances in carbohydrates, fat and protein metabolism resulting from absolute or relative lack of insulin secretion (WHO, 1999). Type 2 diabetes or non-insulin dependent diabetes mellitus (NIDDM) is a major metabolic disorder increasing worldwide. Diabetes, recently been described as perfect epidemic (Lau, 2001), is rapidly emerging as a global health care problem that threatens to reach pandemic levels by 2030. It is estimated approximately 285 million people worldwide or 6.6% adult have diabetes, 70% of whom live in low and middle income countries. This number is expected to increase by more than 50% in the next 20 years if prevention programmes are not put in place (Bagchi and Preuss, 2012).

Rate of the type 2 diabetes have increased markedly over the last 50 years in parallel with obesity. As of 2010, there are approximately 285 million people with the disease compared to around 30 million in 1985. Long term complications from high blood sugar can include heart disease, strokes, diabetic retinopathy where eyesight is affected, kidney failure that may require dialysis, and poor circulation in the limbs leading to amputations. The acute complication of ketoacidosis, a feature of type 1 diabetes, is uncommon. Complications are the one of the major rationalization of morbidity and mortality in DM, literature survey reveal that as early as 700-200 BC, DM was a dominant disease in India and on the basis genetically based disorder and other one resulting from dietary indiscretion it is classified under two categories. (Oubre *et al.*, 1997).

Classification of Diabetes Mellitus:

Diabetes Mellitus can be classified into two major categories i.e. Type 1 (T1DM) and Type 2 (T2DM). T1DM is often genetically-associated and immune-mediated and often referred to as juvenile diabetes, is insulin dependent. Individuals with T1DM have an absolute deficiency in insulin secretion and can be identified by serological evidence of autoimmune-mediated destruction of pancreatic islets or by genetic markers. However, this form of diabetes only accounts for 5-10% of those with diabetes (American Diabetes Association, 2009). The rate of β-cells destruction in this form of diabetes is usually rapid in infants and children. However, it can occur at any age, even as late as eighties or nineties in life. Markers responsible for this destruction include islet cell autoantibodies (ICAs), insulin autoantibodies

(IAAs), glutamic acid decarboxylase autoantibodies (GAD65), and autoantibodies to tyrosine phosphatase IA-2 and IA-2α. One and more of these autoantibodies are present in 85-90% of individuals when fasting hyperglycemia is initially detected. There is another form of T1DM where the pathogenicity is less well understood and hence known as idiopathic diabetes. Individuals in this category usually have permanent insulinopenia but lack signs of autoimmunity. This form of diabetes is strongly inherited. Hormone replacement therapy is not absolutely necessary for survival in this case as the degree of β-cell dysfunction varies among individuals (Van Genugten *et al.*, 2006).

The most common type of diabetes, T2DM, accounts for 90-95% of those with diabetes. Individuals in this category can either have predominant insulin resistance with relative insulin deficiency or predominant insulin secretory defect with insulin 3 resistance (Moller, 2001). The etiology of this form of diabetes is wide and complicated, ranging from abnormalities in lipoprotein metabolism, central or visceral obesity, to cardiovascular risk factors such as hypertension. However, pancreatic islets destruction does not occur in T2DM. On the contrary, insulin resistance may cause patient to have normal or even higher level of insulin. This form of diabetes is always associated with obesity. It's becoming more common in developed and developing countries, afflicting younger generations victimized by a global epidemic of overweight and obesity (American Diabetes Association, 2009). There is another type of diabetes diagnosed during pregnancy named gestational diabetes. Most of the cases resolve with delivery, but the condition may persist in some cases as unrecognized glucose intolerance may have begun before the pregnancy. Evaluation of gestational diabetes should be done early in the pregnancy except for those in low risk group, who (Kim, 2010).

- Are less than 25 years old
- Have a normal BMI
- Have no family history of diabetes
- Have no history of abnormal glucose metabolism
- Have no history of poor obstetric outcome

Glycosidase enzymes:

α- Amylase-

α- Amylase is an enzyme that catalyses the hydrolysis of starch into sugars α-amylase breaks down long-chain carbohydrates, ultimately yielding in to maltose and maltotriose from maltose, or amylose, from amylopectin glucose and "limit dextrin". The reason behind it that it can be act anywhere on the substrate, α-amylase is comparatively faster-acting than β-amylase. In animals, it is a major digestive enzyme, and its optimum pH is 6.7–7.0.

α- Glucosidase-

Alpha-glucosidase hydrolyzes terminal non-reducing (1→4)-linked alpha-glucose residues to release a single alpha-glucose molecule. Alpha-glucosidase is a carbohydrate-hydrolase that releases alpha-glucose as opposed to beta-glucose. Beta-glucose residues can be released by glucoamylase, a functionally similar enzyme. The substrate selectivity of alpha-glucosidase is due to subsite affinities of the enzyme's active site (Chiba, 1997).

In Human Physiology-

Although amylase found in various tissues but the most prominently observed in pancreatic juice and saliva, out of them each has its own isoforms of human α-amylase. On the basis of isoelectric focusing they behave differently and can be also separated with the help of specific monoclonal antibodies.

Salivary amylase-

Salivary amylase is an enzyme produced in the salivary glands that helps for breakdown of starch in to dextrin and maltose and this form of amylase known as ptyalin. It will break the large insoluble starch molecules like amylodextrin, erythrodextrin, and achrodextrin, into soluble starches. Salivary amylase is inactivated in the stomach by gastric acid (Fried *et al.*, 1987). After that further breakdown of starch done by pancreatic α-amylase.

Pancreatic amylase-

Pancreatic α-amylase cleaves randomly the α(1-4) glycosidic linkages of amylose to yield maltotriose, maltose, or dextrin. When food passes to the small intestine, the remainder of the starch molecules are catalyzed mainly to maltose by pancreatic amylase. This step in starch digestion occurs in the first section of the small intestine (the duodenum), the region into which the pancreatic juices empty. The by-products of amylase hydrolysis are ultimately broken down by other enzymes into molecules of glucose, which are rapidly absorbed through the intestinal wall.

Domain architecture-

α-Amylases contained number of different protein domains. The *Bacillus stearothermophilus* BSTA is comprised of a single polypeptide chain which is folded into 3 domains: A, B and C. Catalytic domain of α-Amylases consisting structure of a $(\beta/\alpha)_8$-barrel containing the active site, interrupted by a ~70-amino acid calcium-binding domain protruding between beta strand 3 and alpha helix 3, and a carboxyl-terminal Greek key beta-barrel domain. Several other α-Amylases contain a β-sheet domain, generally at the C terminus. This domain is organised as a 5-stranded anti-parallel beta-sheet (Kadziola *et al.*, 1994). Usually at the C terminus of the several alpha-amylases contain an all- β domain, (Machius *et al.*, 1995).

Disease relevance:

Inhibition of α -Glucosidase may cause several diseases

1) Pompe Disease
2) Diabetes
3) Azoospermia
4) Anti-viral agents

Antioxidants:

Antioxidants are the compounds that can delay or inhibit the oxidation of lipids or other molecules by inhibiting the initiation or propagation of oxidative chain reactions. (Choe and Min 2009), redox active compounds which help to prevent radical formation or remove them before damage can occur or repair oxidative damage. They eliminate damaged molecules by directly scavenging or neutralizing free radicals or other reactive oxygen species (ROS) and reactive nitrogen species (RNS). Oxidative damage to cells and macromolecule is considered to be the cause of several diseases such as coronary heart disease, a cataractogenesis, various neurodegenerative diseases including Alzheimer's disease, cancer and aging oxidative injury involves free radical induced damage from both endogenous and exogenous sources (Lobo *et al.*, 2010)

Several studies have shown that dietary antioxidants play an important role in preventing degenerative diseases associated with ageing such as cancer, cardio-vascular diseases, cataract, neurodegenerative diseases and immunological decline (Rahman, 2007).

Many spices, fruits, vegetables and medicinal plants contain potential antioxidant compounds, such as vitamins A, C and E, β-carotene, α-tocopherol, carotenoids, flavonoids, isoflavones, anthocyanins, polyphenols, tannins and other phenolics constituents etc. (Ghasemi *et al.*, 2009).

Natural antioxidants, especially phenolics and flavonoids are safe and also bioactive. Therefore in recent years considerable attention has been directed towards the identification of plants with antioxidant ability that may be used for human consumption (Jain *et al.*, 2009). Vegetables and their products are the major source of antioxidants in the diet and many reports are available regarding their analysis (Moure *et al.*, 2001; Bunea *et al.*, 2008; Murcia *et al.*, 2009). Phenolics are widespread constituents of plant foods (fruits, vegetables, cereals, olive, legumes, chocolate, etc.) and beverages (tea, coffee, beer, wine, etc.), and partially responsible for the overall organoleptic properties of plant foods.

The anti-oxidant properties of plants reduces oxidative stress which ultimately leads to prevention of many of conditions including diabetes.

Plants and plant constituents are potential sources of anti diabetic and antioxidant drugs:

Plants are a valuable source of new natural products. Despite the availability of different approaches for the discovery of therapeuticals, natural products still remain as one of the best reservoirs of new structural types (Hostettmann, 1998). In the past decade, the essential oils and various extracts of plants have provoked interest as sources of natural products. They have been screened for their potential uses as alternative remedies for the treatment of many infectious diseases and the preservation of foods (Jang *et al.*, 2007). Since ancient times, plants have been an exemplary source of medicine and many of the currently available drugs have been derived directly or indirectly from them.

***Artiplex hortensis* L.**

Atriplex hortensis L. is a hardy, annual plant, belongs to family Amaranthaceae, growing up to 1.8 m (6ft) by 0.3 m (1ft in) at a fast rate according to the variety and soil. It bears flower from Jul to August, and the seeds ripen in September. The leaves are used as vegetable or in salads. Leaves favoured raw or cooked (Hedrick, 1972) or used like a spinach. They have a bland flavour and are traditionally mixed with sorrel leaves in order to modify the acidity of the latter (Facciola, 1990). The leaves are diuretic, emetic and purgative (Polunin, 1969 & Duke, 1983). They are also said to be a stimulant to the metabolism and an infusion is used as a spring tonic and a remedy for tiredness and nervous exhaustion. They have been suggested as a folk remedy for treating plethora and lung ailments. The seeds, mixed with wine and are said to cure yellow jaundice. The fruits are purgative and emetic (Duke, 1983).

***Chenopodium album* L.**

Chenopodium album L. belongs to family Amaranthaceae. It is an erect, branched, annual herb. Leaves are alternate, simple ovate to rhomboid-oval, uppermost leaves mostly lanceolate, sometimes linear and sessile, glabrous, usually white with a mealy-covering, particularly on young leaves. Inflorescence is irregular spikes clustered in panicles at the ends of the branches. The leaves and tender branches may be used as a vegetable, and also mixed in curd as 'Raita' (Maheshwari, 1963). The leaves may be taken in the form of an infusion or decoction as a laxative and anthelminthic. It has also been recommended by Hindu physicians as a treatment for hepatic disorders and splenic enlargement (Chopra *et al.*, 1958).

***Senna glauca* Roxb.**

Senna glauca Lam. belongs to family Caesalpiniaceae. This is a medium to large tree reaching up to height: 18-20'. Leaves ovate, pointed, alternate, dark green, leaflets. Bright golden flowers borne in clusters and appear on almost every branch. Young leaves are boiled and consumed for cough and sore throat. A decoction of the roots is used against gonorrhoea. The leaves are used in the treatment of dysentery. The flowers are purgative (Gritsanapan and Nualkaew, 2009).

Summary of the invention

Present innovation deals with the development of new formulation having potential anti-diabetic and anti-oxidant potential. In the present investigation, following three plants were used.

1) *Artiplex hortensis* L.
2) *Chenopodium album* L.
3) *Senna glauca* Roxb.

These investigations are supported by determination of total flavonoid content, determination of total phenol content, DPPH free radical scavenging activity, ABTS radical scavenging activity, determination of α-Amylase inhibitory activity, determination of α-glucosidase inhibitory activity, determination of IC_{50} values. As results, the quantitative estimation of total phenols and flavonoids suggests that the *S. glauca* leaves are found to be rich source of phenolics and flavonoids and its chloroform and ethanol extracts showed excellent DPPH and ABTS reduction. Based on IC_{50} values, it was revealed that the chloroform and ethanol extracts of both *C. album* and *A. hortesnsis* showed maximum radical scavenging activity. Overall, *C. album* and *A. hortesnsis* would be a good source of antioxidants providing health-promoting effects in human.

The study indicated that, the ethanolic extract of *A. hortesnsis* showed the maximum alpha amylase and alpha glucosidase inhibitory activity. Furthermore, combination of ethanolic extract as new formulation (CAAG11) of *A. hortensis* and *C. album* showed enhanced alpha amylase and alpha glucosidase inhibitory activity.

Detailed description of the figures

Figure 1: *Atriplex hortensis* L.
Figure 2: *Chenopodium album* L.
Figure 3: *Senna glauca* Roxb.

Detailed description of the tables:

Table 1: Information about the plants selected for the study
Table 2: Total Phenol content mg gallic acid equivalent (GAE)/g
Table 3: Total Flavonoid content mg of quercetin equivalent/ g
Table 4: DPPH free radical scavenging activity
Table 5: ABTS free radical scavenging activity
Table 6: α-Amylase inhibitory activity
Table 7: α-Glucosidase inhibitory activity

Detailed description of the Graphs:

Graph 1: Total phenol content mg. gallic acid equivalent (GAE)/g
Graph 2: Total flavonoid content mg of quercetin equivalent/ g

Detailed description of the invention

Detailed experimental studies

Plant materials were collected from nearby areas of Pune district, Maharashtra, India. Efforts were made to collect plant material during flowering and fruiting conditions for the correct botanical identification (Table 1). The plants selected were *Artiplex hortensis*, *Chenopodium album*, *Senna glauca* (Figure 1-3).

Preparation of extract:

Leaves of freshly collected plant samples of each 50 gm. were chopped and placed in the filter Paper (No. 89) in a classical soxhlet apparatus and successively extracted with 170 ml of chloroform, ethanol, methanol and ethyl acetate solvents for 3 hrs. Extracts were cooled to room temperature. The extracts were filtered through whatman filter paper No. 1, and the filtrate was concentrated under reduced pressure by rotary evaporator. These extracts were used in the present study.

Determination of total phenol content:

Total phenol content was determined by using standard protocol of (Madaan *et al.*, 2011) with some modifications. For the preparation of Standard gallic acid graph, 10 mg of gallic acid was dissolved in 100 ml of 50% methanol (100 µg/ml) and then further diluted to 3.1, 6.25, 12.5, 25, 50 and 100 µg/ml. One ml aliquot of each dilution was taken in a test tube and diluted with 10 ml of distilled water. Then 1.5 ml Folin Ciocalteu's reagent was added and allowed to incubate at room temperature for 5 min. Four ml of 20% (w/w) Na_2CO_3 was added in each test tube, the final 25 ml total reaction volume was adjusted with distilled water and left to stand for 30 min at room temperature. Absorbance of the standard was measured at 765 nm using UV/VIS spectrophotometer (Sican 2301, inkarp) against blank, i.e. distilled water.

One ml aliquot of the sample was taken in a test tube and diluted with 10 ml of distilled water. Then 1.5 ml Folin Ciocalteu's reagents was added and allowed to incubate at room temperature for 5 min. Four ml of 20% (w/w) Na_2CO_3 was added, final volume adjusted with distilled water up to the mark of 25 ml, agitated and left to stand for 30 min at room temperature. Absorbance of the sample was measured at 765 nm against blank, i.e. distilled water. The total phenol content was calculated from the regression equation prepared from a range of concentrations of gallic acid and optical densities for the concentrations.

Determination of total flavonoid content:

The total flavonoid content was determined by following the protocol of (Kamtekar *et al.*, 2014) and was calculated from the regression equation prepared from a range of concentrations of quercetin and optical densities for the concentrations. Total flavonoid content was determined by using Aluminium chloride ($AlCl_3$) colorimetric assay. One ml of sample aliquots and one ml standard quercetin solution (100, 200, 400, 600, 800, 1000 µg/ml) was positioned into test tubes separately and 4ml of distilled water and 0.3 ml of 5% sodium nitrite solution was added into each test tube. After 5^{th} minutes, 0.3 ml of 10 % aluminum chloride was added. At 6^{th} minute, 2 ml of 1 M sodium hydroxide was added. Finally, volume was making up to 10 ml with distilled water and mix well. At the end of reaction yellowish orange colour was appeared. The absorbance was measured spectrophotometrically at 510 nm. The blank was performed using distilled water. Quercetin was used as standard. The samples were performed in triplicates.

DPPH free radical scavenging activity:

DPPH (2, 2-diphenyl,1-picrylhydrazyl) radical scavenging activity was determined according to the method of (Tekao *et al.*, 1994) adopted with suitable modifications (Kumarasamy *et al.*, 2007). The DPPH (Hi-Media) stock solution of were prepared in methanol to achieve the concentration of 1mg/ml.

Dilutions of plant extracts in various solvents were made to obtain concentrations of 100 to 400 µg/ml. Diluted plant extracts (1ml each) were allowed to react with 1 ml of methanolic solution of DPPH in concentration of 1mg/ml at room temperature. After 30 min incubation in darkness at room temperature (23° C), the absorbance values were measured spectrophotometrically at 517 nm against the blank. Control sample contained all the reagents except the extract. Percentage inhibition was calculated using equation = [(A control – A sample) /A control] × 100, where A control is the absorbance of negative control and A sample is the absorbance of the reaction mixture, while IC_{50} values were estimated from the % inhibition versus concentration plot, using a non-linear regression algorithm. Ascorbic acid was used as positive control. The lower the IC_{50} value indicates high antioxidant capacity. All tests were performed in triplicate, and expressed in Mean±SE.

ABTS radical scavenging activity:

ABTS radical scavenging activity was performed by following the method of (Re *et al.*, 1999) with some modifications. ABTS (Hi-Media) radical cation was produced by reacting ABTS stock solution 7 mM with 2.45 mM potassium persulfate (final concentration) by dissolving in distilled water (1:1) ratio and allowing the mixture to incubate 16-20 hrs. for the formation of ABTS radical cation at room temperature. Before use, this solution was diluted with ethanol to get an absorbance of 0.700 at 734 nm.

Dilutions of plant extracts in various solvents were made to obtain concentrations of 100 to 400 µg/ml. The plant extract at various concentrations with 1ml of ABTS solution was homogenized and its absorbance was recorded spectrophotometrically (sican 2301, inkarp). at 734 nm. Against blank i.e., Ethanol. Trolox was used as positive control. As for the antiradical activity, ABTS scavenging activity was expressed as IC_{50} (µg/ml). The percent inhibition of ABTS radical scavenging activity was calculated using the following formula: ABTS scavenging activity (%) = (A0 –A1) /A0 ×100 Where A0 is the absorbance of the negative control, and A1 is the absorbance of the sample. All tests were performed in triplicate, and expressed in Mean±SE.

Determination of α-Amylase inhibitory activity:

The α-Amylase inhibition activity was measured by following the method of with some modification. Porcine pancreatic α-amylase (4 units/ml) MP Biomedicals (Cat. No: 191239) was dissolved in 0.1 M phosphate buffer saline, pH 6.9. Plant extracts Stock solutions for inhibition assay in various solvents were made to obtain concentrations of 100 to 400 µg/ml and were were added to a solution containing starch (1 g/l) and phosphate buffer (165 µl). The reaction was initiated by adding enzyme solution (75 µl) to the incubation medium. After 10 min of incubation, the reaction was terminated by adding 250 ml dinitrosalicylic (DNS) reagent (1% 3, 5- dinitrosalicylic acid, 0.2% phenol, 0.05% Na_2SO_3 and 1% NaOH in aqueous solution). The mixtures were heated at 100°C for 10 min in order to stop the reaction. Thereafter, 250 µl of 40% potassium sodium tartarate solution was added to the mixtures to stabilize the color. After cooling to room temperature, the absorbance was recorded at 540 nm using a microplate reader. Acrabose was used as positive control.

Inhibitory activity was expressed as inhibition % and was calculated as follows:

$$\%\ \boldsymbol{Inhibition\ of\ \alpha - Amylase} = \frac{(\boldsymbol{Absorbance\ of Control}) - (\boldsymbol{Absorbance\ of Sample})}{(\boldsymbol{Absorbance\ of Sample})} \times 100$$

Determination of α-glucosidase inhibitory activity:

α -Glucosidase inhibitory activity was measured using p-nitrophenyl a-D-glucopyranoside as the substrate (Kwon *et al.*, 2008), α -Glucosidase (Sigma Chemical Co St. Louis M.O. USA), solution (0.006%) was prepared in 0.02 M phosphate buffer (pH 6.3). The enzyme solution (0.13 ml) was incubated with extract (0.13 ml) and 0.02 M phosphate buffer (0.45 ml for 1 hr. at 250 C. After pre incubation, 2 M p-nitrophenyl o-D-glucopyranoside (0.67 ml) was added to the reaction mixture. The mixture was then incubated for another 30 minutes at 30^0 C. The reaction was terminated by adding 1 M Na_2CO_3 solution (2 ml). Determination of the amount of p-nitro phenol formed was read by using a microplate reader at 405 nm.

Inhibitory activity was expressed as inhibition % and was calculated as follows:

$$\%\ Inhibition\ of\ \alpha-\ Glucosidase = \frac{(Absorbance\ of\ Control) - (Absorbance\ of\ Sample)}{(Absorbance\ of\ Sample)} \times 100$$

New combination formulation:

Ethanolic extract of leaves of these three plants were combined together in 1:1 proportion and named this formulation as CAAH11 and studied for α-Amylase inhibitory activity and α-glucosidase inhibitory activity as mentioned above.

Determination of IC_{50} values:

Regression equations were prepared from the concentrations of the extracts and percentage inhibition in different systems of assay. IC_{50} values (Concentration of sample required to inhibit) were calculated from these regression equations. A lower IC_{50} value indicates higher inhibitory activity (Gupta *et al.*, 2003).

Results and discussion

The selection of solvents and its extraction efficiency are significant to address antioxidant and antidiabetic activity. In present investigation, solvents were selected based on the relative polarity from moderate to excellent in the order Chloroform (0.25) < Ethanol (0.65)< Methanol (0.76).

The results showed that, the total phenolic contents in the selected plants species for the study varied considerably and ranged from 3.71 to 35.66 mg GAE/g. The highest phenolic content was observed in *S. glauca* leaf ethyl acetate extract i.e. 35.66 mg GAE/g. and lowest phenolic content was observed in *C. album* ethyl acetate extract i.e. 3.71 mg GAE/g.

The highest Phenols and content was observed in *S. glauca* leaf ethyl acetate extract i.e. 2.59 quercetin equivalent/ g. and lowest flavonoid content was observed in *C. album* ethanol extract i.e. 0.06 quercetin equivalent/g. (Table 3, Graph 1, 2).

The DPPH radical scavenging assay was done for all the three solvent extracts of Ethanol, Methanol and Chloroform. Among the plants studied the highest DPPH free radical scavenging activity was observed in *C. album* chloroform extract with IC_{50} value 0.6 µg/ml. The lowest inhibition percentage was observed in *A. hortesnsis* ethanol extract with IC_{50} value 3.4 µg/ml (Table no. 4)

Among the plants studied the highest ABTS free radical scavenging activity was observed in *A. hortesnsis* ethanol extract with IC_{50} value 2.0 µg/ml. The present study reveals the good source for the antioxidant studies by using the DPPH radical scavenging assay is an easy, rapid and sensitive method for screening of antioxidant in present studied plants (Table no. 5)

Among the plants studied in determination of α-Amylase inhibitory activity, it was observed that ethanolic extract of *A. hortesnsis* showed highest inhibitory activity against the enzyme with IC_{50} value

0.3 µg/ml (Table no. 6).

Among the plants studied in determination of α- Glucosidase inhibitory activity, it was observed that ethanolic extract of *A. hortesnsis* showed highest inhibitory activity against the enzyme with IC_{50} value 1.4 µg/ml. The lowest inhibitory activity was observed in *S. glauca* leaf methanolic extract with IC_{50} value 2.2 µg/ml (Table no. 7).

Finally, it was observed that, combination of extract of selected above three plants in the form of new combination formulation viz. CAAH11 showed highest α-Amylase inhibitory activity and α-Glucosidase inhibitory activity with IC_{50} value 0.3 µg/ml and 1.1 µg/ml respectively (Table no. 6,7). This enhanced activity is may be due to synergestic effect of combined secondary metabolites of selected extracts of three plants.

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5. Claims

We claim:

1. A new herbal combination formulation (CAAH11), the said formulation comprising 1:1 proportion of combination:
    a. Leaves extract of *A. hortensis* L. in ethanol,
    b. Leaves extract of *C. album* L. in ethanol and
    c. Leaves extract of *Senna glauca* Roxb. in ethnol.
2. A formulation as in claim 1 can be administered orally in the form of suspension, sachets, tablets or capsules.
3. Herbal formulation as in claim 1 and administered as in claim 2, wherein the said formulation Herbal formulation as in claim 1 and administered as in claim 2, wherein the formulation shown potent anti-oxidant activity.
4. A formulation is capable of substantially attenuate the α-Amylase inhibitory activity and α-Glucosidse inhibitory activity. Thus it has anti-diabetic activity.
5. Herbal formulation as in claim 1 and administered as in claim 2, wherein the said formulation is capable of inhibiting biochemical markers of diabetes.

Name : Prof. Dr. Deokule Subhash S Signature :

6. Date and Signature

Date: 02/11/2017

Place: Pune

Prof. Dr. Deokule Subhash S.
Department of Botany,
Savitribai Phule University of Pune (SPPU),
Ganeshkhind Road,
Pune- 411007. Maharashtra, India

Figure 1: *Atriplex hortensis* L.

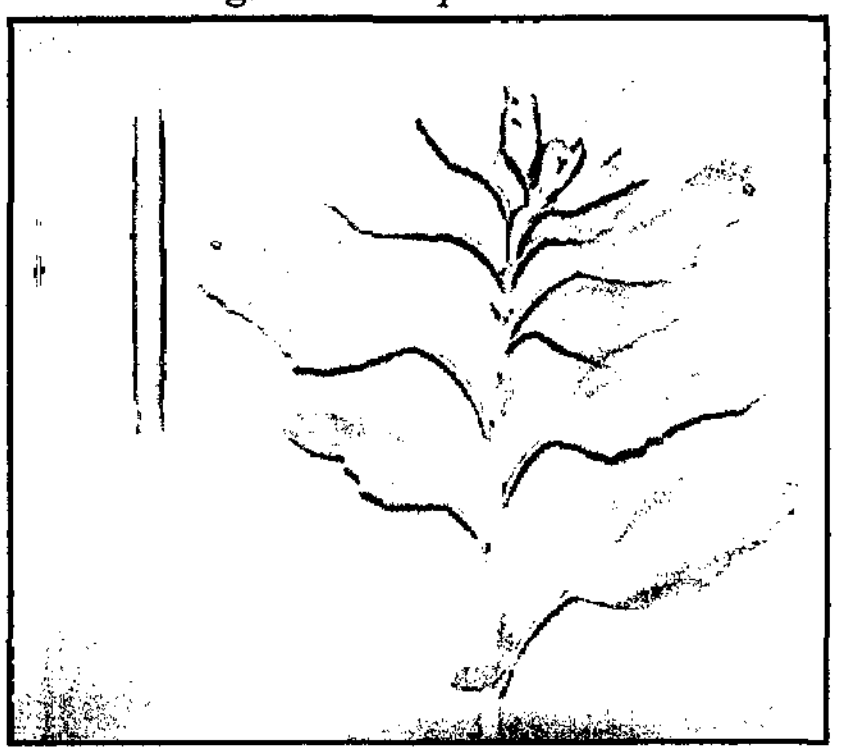


Figure 2: *Chenopodium album* L

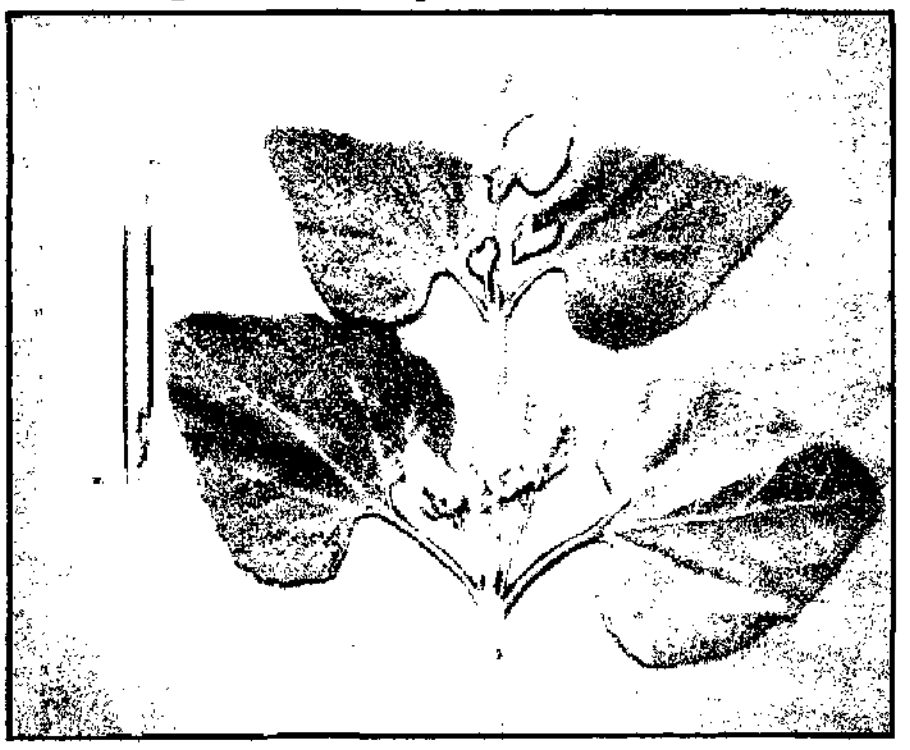
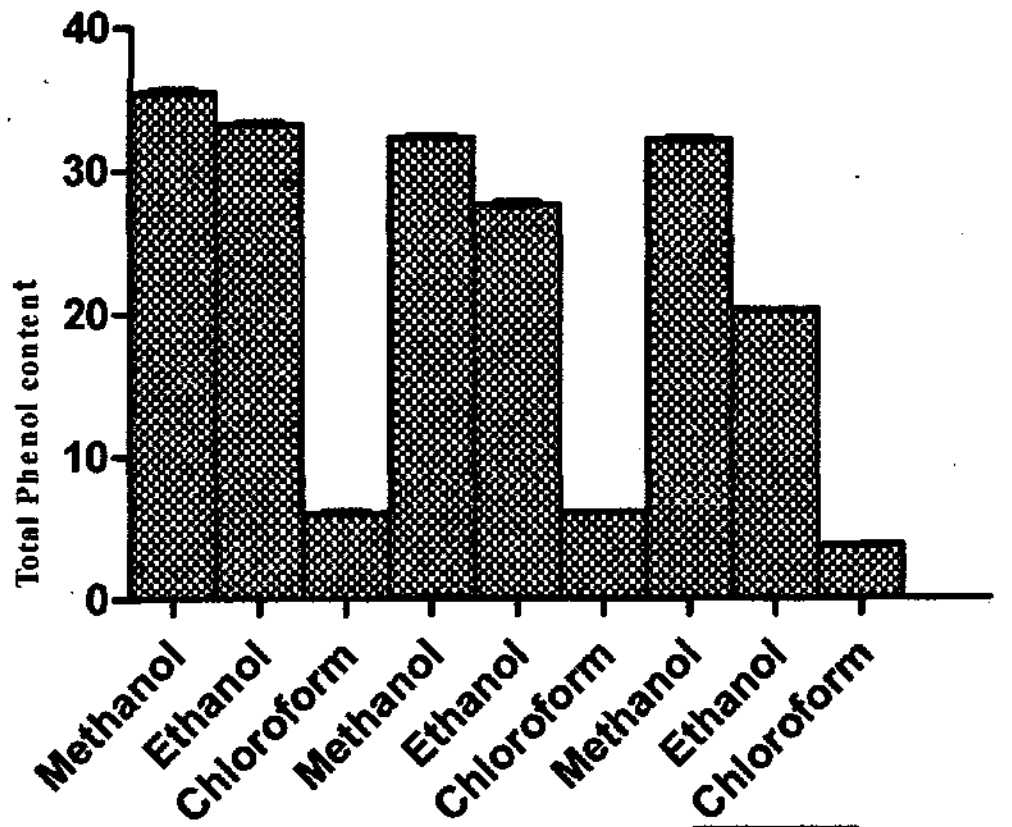


Figure 3: *Senna glauca* Roxb.

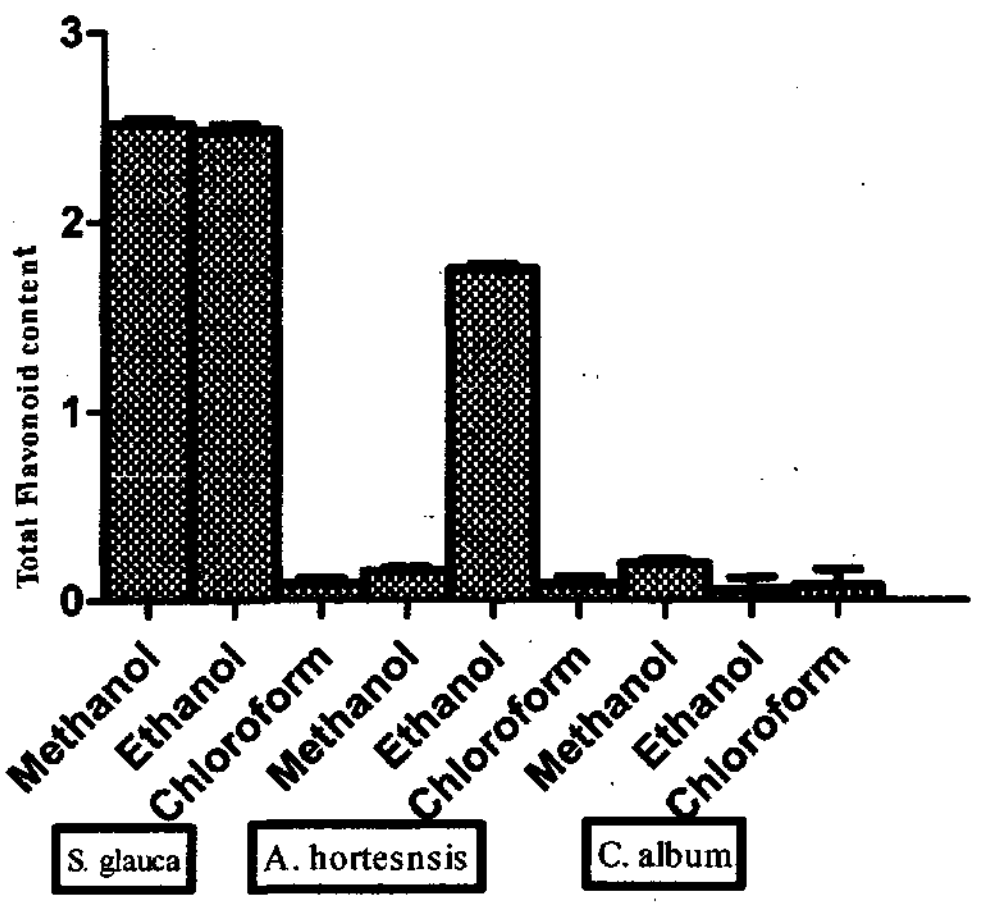
Name: Prof. Dr. Deokule Subhash S.

Signature:

Graph 1: Total phenol content mg. gallic acid equivalent (GAE)/g



Graph 2: Total flavonoid content mg of quercetin equivalent/ g



Name: Prof. Dr. Deokule Subhash S.

Signature:

Table 1: Information about the plants selected for the study

Sr. No.	Plant Materials	Family	Plant part Used	Collected from	Accession Number
1	*Atriplex hortensis* L.	Amaranthaceae	Leaves	Sangvi, District- Pune	67833
2	*Chenopodium album* L.	Amaranthaceae	Leaves	Sangvi, District- Pune	68518
3	*Senna glauca* Roxb.	Caesalpiniaceae	Leaves	Pune University Campus, District- Pune	30618

Table 2: Total Phenol content mg gallic acid equivalent (GAE)/g

Sr. No.	Plant Name	Extract	Total phenol content
1	*S. glauca* leaf	Methanol	35.52±0.026
		Ethanol	33.29±0.015
		Chloroform	06.10±0.026
2	*A. hortesnsis*	Methanol	32.33±0.021
		Ethanol	27.63±0.017
		Chloroform	06.18±0.010
3	*C. album*	Methanol	32.14±0.009
		Ethanol	20.32±0.015
		Chloroform	03.85±0.009

Table 3: Total Flavonoid content mg of quercetin equivalent/g

Sr. No.	Plant Name	Extract	Total Flavonoid content
1	*S. glauca* leaf	Methanol	2.52±0.023
		Ethanol	2.48±0.035
		Chloroform	0.09±0.032
2	*A. hortesnsis*	Methanol	0.16±0.018
		Ethanol	1.75±0.026
		Chloroform	0.09±0.035
3	*C. album*	Methanol	0.20±0.019
		Ethanol	0.06±0.062
		Chloroform	0.08±0.081

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Table 4: DPPH free radical scavenging activity

Sr. No.	Plant Name	Extract	IC_{50} Values (µg/ml)
1	*S. glauca*	**Methanol**	5.3±0.021
		Ethanol	5.0±0.026
		Chloroform	1.1±0.015
2	*A. hortesnsis*	**Methanol**	4.7±0.025
		Ethanol	3.4±0.017
		Chloroform	2.9±0.014
3	*C. album*	**Methanol**	7.7±0.023
		Ethanol	2.0±0.012
		Chloroform	0.6±0.010

Table 5: ABTS free radical scavenging activity

Sr. No.	Plant Name	Extract	IC_{50} Values (µg/ml)
1	*S. glauca*	**Methanol**	7.7±0.023
		Ethanol	2.3±0.014
		Chloroform	3.0±0.024
2	*A. hortesnsis*	**Methanol**	4.8±0.032
		Ethanol	2.0±0.014
		Chloroform	4.4±0.022
3	*C. album*	**Methanol**	2.4±0.016
		Ethanol	4.1±0.012
		Chloroform	7.3±0.025

Name: Prof. Dr. Deokule Subhash S. Signature:

Table 6: α-Amylase inhibitory activity

Sr. No.	Plant Name	Extract	IC_{50} Values (µg/ml)
1	*S. glauca* leaf	Methanol	0.7±0.023
		Ethanol	0.5±0.014
		Chloroform	0.7±0.011
2	*A. hortesnsis*	Methanol	0.7±0.015
		Ethanol	0.3±0.018
		Chloroform	0.6±0.017
3	*C. album*	Methanol	0.7±0.017
		Ethanol	0.8±0.020
		Chloroform	0.8±0.012
4	CAAH11	Ethanol	0.3±0.008

Table 7: α-Glucosidase inhibitory activity

Sr. No.	Plant Name	Extract	IC_{50} Value (µg/ml)
1	*S. glauca* leaf	Methanol	2.2±0.08
		Ethanol	1.5±0.06
		Chloroform	1.8±0.07
2	*A. hortesnsis*	Methanol	1.6±0.09
		Ethanol	1.4±0.08
		Chloroform	2.5±0.011
3	*C. album*	Methanol	2.4±0.018
		Ethanol	3.1±0.012
		Chloroform	3.3±0.015
4	CAAH11	Ethanol	1.1±0.07

Name: Prof. Dr. Deokule Subhash S. Signature:

TITLE: New formulation for the management of glycosidase and oxidative stress.

7. ABSTRACT OF THE INVENTION

Diabetes mellitus (DM) has turned out to be one of the major and emerging public health problems of the world. There has been perpetuating increase in the number of diabetic patients almost in all countries, especially in India which disreputably got nicknamed asthe "Diabetes Capital of the World" (Mohan et al., 2007). Diabetes mellitus is a complex and a diverse group of disorders that disturbs the metabolism of carbohydrate, fat and protein.

Antioxidants are the compounds that can delay or inhibit the oxidation of lipids or the molecules by inhibiting the initiation or propagation of oxidative chain reactions. (Choe and Min, 2009), redox active compounds which help to prevent radical formation or remove them before damage can occur or repair oxidative damage. They eliminate damaged molecules by directly scavenging or neutralizing free radicals or other reactive oxygen species (ROS) and reactive nitrogen species (RNS).

Human use of plants as medicinal purpose predates recorded history. Medicinal plant use data in many forms has been heavily utilized in the development of pharmacopoeias and formularies, providing a major focus in global healthcare, as well as contributing substantially to the drug development process for alternate of synthetic drug. Since ancient times, plants have been an exemplary source of medicine and many of the currently available drugs have been derived directly or indirectly from them. Ayurveda and other Indian literature mention the use of plants in treatment of various human ailments. The ethnobotanical information reports about 800 plants that may possess anti- diabetic potential.

In present innovation, deals with the development of new formulation having potential anti-diabetic and anti-oxidant potential. In overall study following plants were investigated.

1) *Artiplex hortensis* L.
2) *Chenopodium album* L.
3) *Senna glauca* Roxb.

These investigations are supported by determination of total flavonoid content, determination of total phenol content, DPPH free radical scavenging activity, ABTS radical scavenging activity, determination of α-Amylase inhibitory activity, determination of α-glucosidase inhibitory activity, determination of IC_{50} values. As results, the quantitative estimation of total phenols and flavonoids suggests that the *S. glauca* leaves are found to be rich source of phenolics and flavonoids and its chloroform and ethanol extracts showed excellent DPPH and ABTS reduction. Based on IC_{50} values, it was revealed that the chloroform and ethanol extracts of both *C. album* and *A. hortesnsis* showed maximum radical scavenging activity. Overall, *C. album* and *A. hortesnsis* would be a good source of antioxidants providing health-promoting effects in human.

The study indicated that, the ethanolic extract of *A. hortesnsis* showed the maximum alpha amylase and alpha glucosidase inhibitory activity. Furthermore, combination of ethanolic extract of leaves of selected three plants in the formof new combination formulation viz. CAAH11, showed enhanced alpha amylase and alpha glucosidase inhibitory activity.

E- 3/11625/2017.

FORM 3
THE PATENTS ACT, 1970
[39 OF 1970]
and
THE PATENTS RULES, 2003
STATEMENT AND UNDERTAKING UNDER SECTION 8
(See Section 8; Rule 12)

200174909

1. Name of the applicants	I, Deokule Subhash S., Department of Botany, Savitribai Phule Pune University, Pune-7, hereby declare:
2. Name, address and nationality of the joint applicant	Mr. Jagtap Kartikey Tanaji, Herbal Medicine, IRHSA, Bharati Vidyapeeth University, Pune – 411 043; Dr. Singh Elangbam Athoiba, Rajiv Gadhi Institute of Biotechnology, Bharati Vidyapeeth University, Pune Satara Road, Pune – 411043; Dr. Shirsath Mahendra S. Flat no. 1/103, Govardhan enclave housing society, Tawri Pada, Near RTO, Kalyan (West); Dr. Jagtap Suresh D., Herbal Medicine, IRHSA, Bharati Vidyapeeth University, Pune – 411 043. (i) that we have not made any application for the same/substantially the same invention outside India
3. Name and address of the assignee	(ii) that the rights in the applications have been assigned to Department of Botany, Savitribai Phule Pune University, Ganesh Khind road, Pune-7, Maharashtra, India. that we undertake that up to the date of grant of the patent by the Controller, we would keep him informed in writing the details regarding corresponding applications for the date of filling of such application Dated this 03 day of November 2017
4. To be signed by the applicant or his authorized registered patent agent	Deokule
5. Name of the natural person who has signed	Prof. Dr. Deokule Subash S. Professor (Rtd.) Department of Botany, Savitribai Phule Pune University, Ganeshkhind road, Pune-7, Maharashtra, India.
	To, The Controller of Patents, The Patent Office, at Mumbai

E-57/1723/2017

FORM 5
THE PATENTS ACT, 1970
(39 of 1970)
&
The patents rules, 2003
DECLARATION AS TO INVENTORSHIP
[See section 10 (6) and rule 13(6)]

200174910

1. NAME OF APPLICANTS	(i) Prof. Dr. Deokule Subhash S.

Hereby declare that the true and first inventor(s) of the invention disclosed in the complete specification filed in pursuance of our application numbered..... Dated..03-11-2017.....are

2. INVENTOR(S)

a.	**Name**	:	Prof. Dr. Deokule Subhash S.
b.	**Nationality**	:	Indian
c.	**Address**	:	Department of Botany, Savitribai Phule Pune University, Ganeshkhind Road, Pune-411007, Maharashtra, India
a.	**Name**	:	Rahul Mohaniraj Adhav
b.	**Nationality**	:	Indian
c.	**Address**	:	Department of Botany, Savitribai Phule Pune University, Ganeshkhind Road, Pune-411007, Maharashtra, India
a.	**Name**	:	Mr. Jagtap Kartikey Tanaji
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a.	**Name**	:	Singh Elangbam Athoiba Mr. Jagtap Kartikey Tanaji
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c.	**Address**	:	Cell and Molecular Biology, Rajiv Gadhi Institute of Biotechnology (RGBT), Bharati Vidyapeeth University, Pune Satara Road, Pune – 411043. Maharashtra, India

a.	**Name**	:	Dr. Shirsath Mahendra S.
b.	**Nationality**	:	Indian
c.	**Address**	:	Flat no. 1/103, Govardhan enclave housing society, Tawri Pada, Near RTO, Kalyan (West). Mumbai- 431 301. Maharashtra, India.
a.	**Name**	:	Dr. Suresh D. Jagtap
b.	**Nationality**	:	Indian
c.	**Address**	:	Herbal Medicine, Interactive Research School for Health Affairs (IRSHA), Bharati Vidyapeeth University, Pune Satara Road, Pune – 411 043. Maharashtra, India.

Dated this 03.... day of ..November....2017

Signature:

Savitribai Phule Pune University ★Dept. of Botany★

Prof. Dr. Deokule S. S.
Rtd. PROFESSOR
Department of Botany
Savitribai Phule Pune University
Pune - 411 007.

3. DECLARATION TO BE GIVEN WHEN THE APPLICATION IN INDIA IS FILED BY THE APPLICANTS IN THE CONVENTION COUNTRY:-

-NOT APPLICABLE-

4. STATEMENT (to be signed by the additional inventors not mentioned in the application form)

-NOT APPLICABLE-

To,

The Controller of Patent

The Patent Office, at Mumbai

SAVIRIBAI PHULE PUNE UNIVERSITY

(formerly University of Pune)

DEPARTMENT OF BOTANY
SAVITRIBAI PHULE PUNE UNIVERSITY
Ganeshkhind, Pune-411 007
Tele. No. : (020) 25601439, 25601438
Email : .....@unipune.ac.in

Ref. No. : Bot/ D-38753 **Date :** 3/11/2017

&01/13973/2017

To,
The Controller of Patents,
The Patent Office,
Boudhik Sampada Bhavan,
S. M. Road, Antop Hill, Mumbai,
Maharashtra- 400037

200174907

Dear Sir,

Re: Submission of Complete Patent Specification

Please find herewith documents for a complete specification application entitled **"New formulation for the management of glycosidase and oxidative stress"** along with the prescribed fee of Rs. 1750/-.

The Learned Controller is requested to take the same on record and issue the official filing receipt for the same.

Yours faithfully,

Prof. Dr. Deokule Subhash S.
Department of Botany,
Savitribai Phule Pune University (SPPU),
Ganeshkhind Road,
Pune- 411007. Maharashtra, India

Enclosures:

1. Application for Grant of Patent [Form 1]
2. Complete specification [Form 2]
3. Statement and undertaking under section 8 [Form 3]
4. Declaration as to inventorship [Form 5]
5. Fees of Rs. 1750/- (by cash)

IPO 03-Nov-2017 16:48

200293027

FORM 18 THE PATENTS ACT 1970 (39 of 1970) & The Patents Rules, 2003 REQUEST/EXPRESS REQUEST FOR EXAMINATION OF APPLICATION FOR PATENT [See section 11B and rule 20(4)(ii), 24B(1)(i)]	(FOR OFFICE USE ONLY) RQ No: R202020031284 Filing Date: 08/10/2020 Amount of Fee Paid: CBR No: 44001 रूपये नकद/चेक/मनी ऑर्डर द्वारा Signature CBR संख्या. 26226 ..दि.08/10/2020 के तहत प्राप्त हुए।

1. APPLICANTS

(a)	Name:	Deokule Subhash Sadhu
(b)	Nationality:	Indian
(c)	Address:	Department of Botany, Savitribai Phule Pune University (SPPU), Pune- 411 007, Maharashtra, India.

रोक दिए

2. Statement in case of request for examination made by the applicants

We hereby request that our application for patent no.: **201721039237** filed on **03/11/2017** for the invention titled **"Novel Process to extract Lupeol and Saponin from selected plants"** shall be examined under sections 12 and 13 of the Act.

3. Statement in case of request for examination made by any other interested person

I/We the interested person request for the examination of the application no._____dated _____filed by the applicant_____titled_____under sections 12 and 13 of the Act.

As an evidence of my/our interest in the application for patent following documents are submitted.

(a) _____

Not applicable

4. ADDRESS FOR SERVICE

Department of Botany, Savitribai Phule Pune University (SPPU), Pune- 411 007, Maharashtra, India. **Email:** deokule.ss@gmail.com. **Mobile:** 9371088210.

Dated this 8 day of October 2020

Deokule

Deokule Subhash Sadhu

To,
The Controller of Patents
The Patent Office,
Mumbai

"FORM 1 THE PATENTS ACT 1970 (39 of 1970) and THE PATENTS RULES, 2003 **APPLICATION FOR GRANT OF PATENT** (See section 7, 54 and 135 and sub-rule (1) of rule 20)	(FOR OFFICE USE ONLY) 200188618

	Application No.	201821000396
	Filing date	04/01/2018
	Amount of Fee paid	21101 रूपये नकद/चेक/मनी ऑर्डर द्वारा CBR संख्या 161 दि. 04/01/2018 के जरिए प्राप्त हुए।
	CBR No	
	Signature	

1. APPLICANT'S REFERENCE / IDENTIFICATION NO. (AS ALLOTTED BY OFFICE)	

2. TYPE OF APPLICATION [Please tick (√) at the appropriate category]

Ordinary (√)		Convention ( )		PCT-NP ( )	
Divisional ( )	Patent of Addition ( )	Divisional ( )	Patent of Addition ( )	Divisional ( )	Patent of Addition ( )

3A. APPLICANTS

Name in Full	Nationality	Country of Residence	Address of the Applicant	
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			Street	Ganeshkhind Road,
			City	Pune
			State	Maharashtra
			Country	India
			Pin code	411 007.

3B. CATEGORY OF APPLICANT [Please tick (√) at the appropriate category]

Natural Person (√)	Other than Natural Person		
	Small Entity ( )	Startup ( )	Others ( )

4. INVENTORS [Please tick (√) at the appropriate category]				
Are all the inventors same as the applicants named above?		Yes ( )	No (√)	
If "No", furnish the details of the inventor(s)				
Name in Full	Nationality	Country of Residence	Address of the Inventor	
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			Pin code	411007.
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			City	Pune
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			Pin code	411007.
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Dr. Nagarkar Bhagyashri E.	Indian	India	House No.	Herbs Foundation, A1-604, Dream City, Behind Telco Coloney, Dattanagar, Ambegaon, Pune.
			Street	Jambulwadi Road,
			City	Mumbai
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			Country	India
			Pin code	411 046.
Dr. Jagtap Suresh D.			House No	Herbal Medicine, Bharati Vidyapeeth (Deemed to be University), Interactive Research School for Health Affairs (IRSHA)
			Street	Pune Satara Road
			City	Pune
			State	Maharashtra
			Country	India
			Pin code	411 043.

5. TITLE OF THE INVENTION

Development of new herbal lotion for the management of dermatophytic infections

6. AUTHORISED REGISTERED PATENT AGENT(S)	IN/PA No.	
	Name	
	Mobile No.	
7. ADDRESS FOR SERVICE OF APPLICANT IN INDIA	Name	Prof. Dr. Deokule Subhash S.
	Postal Address	Department of Botany, Savitribai Phule Pune University (SPPU), Ganeshkhind Road, Pune- 411007. Maharashtra, India
	Telephone No.	020 2560 1217
	Mobile No.	09371088210
	Fax No.	020 2560 1217
	Email ID	deokule.ss@gmail.com

8. IN CASE OF APPLICATION CLAIMING PRIORITY OF APPLICATION FILED IN CONVENTION COUNTRY, PARTICULARS OF CONVENTION APPLICATION

- NOT APPLICABLE -

9. IN CASE OF PCT NATIONAL PHASE APPLICATION, PARTICULARS OF INTERNATIONAL APPLICATION FILED UNDER PATENT CO-OPERATION TREATY (PCT)

- NOT APPLICABLE -

10. IN CASE OF DIVISIONAL APPLICATION FILED UNDER SECTION 16, PARTICULARS OF ORIGINAL (FIRST) APPLICATION

- NOT APPLICABLE -

11. IN CASE OF PATENT OF ADDITION FILED UNDER SECTION 54, PARTICULARS OF MAIN APPLICATION OR PATENT

- NOT APPLICABLE -

4. DECLARATIONS

(i) Declaration by the inventor(s):

We, the above named inventors are the true & first inventors for this invention and declare that IRSHA, & RGBR of BVDU and Herbs Foundation herein our assignee.

Signature:

Name: Prof. Dr. Deokule Subhash S. Date: 01/01/2018

Signature:

Name: Dr. Batool Sadegi Date: 02/01/2018

Signature:

Name: Dr. Bipin Raj N.K Date: 01/01/2018

Signature:

Name: Dr. Mokat Digambar N. Date: 02/01/2018

Signature:

Name: Dr. Nangare Ninad B. Date: 01/01/2018

Signature:

Name: Dr. Nagarkar Bhagyashri E Date: 01/01/2018

Signature:

Name: Dr. Jagtap Suresh D. Date: 01/01/2018

(ii) Declaration by the applicant(s) in the convention country

- NOT APPLICABLE -

Declaration by the applicants:

We the applicants hereby declares that:-

√	We are in possession of the above-mentioned invention.
√	The complete specification relating to the invention is filled with this application.
√	The invention as disclosed in the specification uses the biological material from India and the necessary permission from the competent authority shall be submitted by us before the grant of patent to us.
√	There is no lawful ground of objection to the grant of the patent to us.
√	We are the true & first inventors.
√	We are the assignee of true and first inventors.
√	The application or each of the applications, particulars of which are given in Paragraph-8, was the first application in convention countries in respect of our inventions.
√	We claim the priority from the above mentioned application(s) filed in convention countries and state that no application for protection in respect of the invention had been made in a convention country before that date by us or by any person from which we derive the title.
X	Our application in India is based on international application under Patent Cooperation Treaty (PCT) as mentioned in Paragraph-9.
X.	The application is divided out of our application particulars of which is given in Paragraph-10 and pray that this application may be treated as deemed to have been filed on .....under section 16 of the Act.
X	The said invention is an improvement in or modification of the invention particulars of which are given in Paragraph-11.

13. FOLLOWING ARE THE ATTACHMENT WITH THE APPLICATION:

(a) Form 2

Item	Details	Fee	Remarks
Complete specification	No. of pages: 20	1750/-	
No. of Claims	No. of claims - 06 No. of pages - 01		
No of drawing sheets	10		
No. of figures	15		
No. of tables	04		
No of abstract pages	01		

(b) Complete specification (1 copies)

(c) Statement and undertaking on Form 3

(d) Declaration as to Inventorship on Form 5

(e) Total fee Rs. 1750/- in cash.

We hereby declare that to the best of my/our knowledge, information and belief the fact and matters stated herein are correct and I/we request that a patent may be granted to me/us for the said invention.

Dated this 04....day of ..January.....2018.

Signature:

Prof. Dr. Deokule Subhash S.
Department of Botany,
Savitribai Phule Pune University (SPPU),
Ganeshkhind Road,
Pune- 411007. Maharashtra, India.

To,

THE CONTROLLER OF PATENTS,

THE PATENT OFFICE, MUMBAI

E-2/28/2018

FORM 2

THE PATENT ACT 1970

(39 OF 1970)

&

The patents rules, 2003

200188621

COMPLETE SPECIFICATION

(See section 10 and rule 13)

1. TITLE OF THE INVENTION:

Development of new herbal lotion for the management of dermatophytic infections

2. APPLICANT (S):

a.	**Name**	:	Prof. Dr. Deokule Subhash S.
b.	**Nationality**	:	Indian
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a.	**Name**	:	Dr. Bipinraj. N. K
b.	**Nationality**	:	Indian
c.	**Address**	:	Department of Microbial Biotechnology, Bharati Vidyapeeth (Deemed to be University), Rajiv Gandhi Institute of Information technology and Biotechnology, Pune Satara Road, Pune-411 043, Maharashtra, India.
d.	**Name**	:	Dr. Mokat Digambar N.
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a.	**Name**	:	Dr. Nangare Ninad B.
b.	**Nationality**	:	Indian
c.	**Address**	:	Department of Dravyagun, Bharati Vidyapeeth (Deemed to be University), College of Ayurveda, Pune Satara Road, Pune 411 043, Maharashtra, India.
d.	**Name**	:	Dr. Nagarkar Bhagyashri E.
e.	**Nationality**	:	Indian
f.	**Address**	:	Herbs Foundation, A1-604, Dream City, Behind Telco Coloney, Dattanagar, Ambegaon, Pune 411 046, Maharashtra, India.
a.	**Name**	:	Dr. Suresh D. Jagtap
b.	**Nationality**	:	Indian
c.	**Address**	:	Herbal Medicine, Bharati Vidyapeeth (Deemed to be University), Interactive Research School for Health Affairs (IRSHA), Pune Satara Road, Pune – 411 043. Maharashtra, India.

3. PREAMBLE TO THE DESCRIPTION	
PROVISIONAL	**COMPLETE**
NOT APPLICABLE	The following specification describes the invention.

4. DESCRIPTION

Field of the Invention

Review of literature revealed that *Ixora brachiata Roxb.* (Rubiaceae) is having antidermatophytic activity. The present investigation includes *in-vitro* and *in-vivo* studies of antidermatophytic activity of *Ixora brachiata Roxb.* In *in-vivo* assay, the therapeutic effectiveness of *I. brachiata* root lotion showed significant results without any notable external effects. The results of this investigation will be helpful to treat skin diseases caused by dermatophytic fungi such as *Epidermophyton floccosum, Microsporum canis, Microsporum gypseum, Trichophyton mentagrophytes* and *Trichophyton rubrum.*

Background of the invention:

Mycotic infections are probably the most common cause of skin disease in developing countries of tropical regions. Dermatophytosis is the most frequent superficial fungal infection occurring in India. The most common dermatophytes in these areas are *Epidermophyton floccosum, Microsporum canis, Microsporum gypseum, Trichophyton mentagrophytes, Trichophyton rubrum* and *Trichophyton verrecusum, which* causes *Tinea pedis, Tinea corporis, Tinea cruris, Tinea capitis, Tinea faciei* and *Tinea manuum* respectively. Various drugs are being used for treating dermatophytosis, which exhibit several side effects and have limited efficacy (Gupta et al. 1998 and Carazo et al. 1999). Hence, there is a distinct need for the discovery of new safer and more effective antifungal agents. The use of herbal drugs in the treatment of skin diseases including mycotic infections is an age-old practice in many parts of the world (Irobi et al. 1993). This use has been supported by the isolation of active antifungal compounds from plant extracts (Costa et al. 2000).

***Ixora brachiata* Roxb.** (Rubiaceae)

Ixora brachiata Roxb. is a gigantic shrub or small tree found to be growing in rain forest. The woody stem of the plant is quadrangular when young. The leaves are opposite, stipulate, lanceolate, long and obtuse. Flowers are white and fragrant, borne in panicles.

Medicinal uses: It is used in the treatment of skin diseases, usually in many tribal communities. Annapurna and Raghavan (2003) has reported the presence of alkaloids, flavonoids, sapogenins, sterols and terpenes in ether extract and alkaloids, phenols and sterols in methanol extract of the plant.

Dermatophytes:

Dermatophytes are a closely interrelated group of keratinophilic fungi which cause infections of skin, hair, and nail known as dermatophytosis. Terms such as *Tinea*, ringworm, trichophytia, and athlete's foot are also used to refer to these infections (Rippon, 1988; Elewski and Kwon, 1992; Martin, 1993).

Dermatophytes can be classified according to their natural habitats into following categories: (1) *geophilic*, (2) *zoophilic*, and (3) *anthrophilic.*

The *geophilic* dermatophytes are normally live in the soil, contribute to the breakdown of the keratinous stroma of the fallen horns, feathers, and skin animals (English, 1962). Many of them are nonpathogenic, but some can infect both the animals and humans. They can be isolated from the soil by the hair-baiting technique.

The *Zoophilic* dermatophytes are primarily parasitizing the body surfaces of animals and then

transmitted to humans.

The *Anthropophilic* dermatophytes are generally infect humans and then transmitted between individuals.

Tinea corporis is dermatophytosis of the glabrous skin of the trunk and extremities (Drake et al. 1996), commonly referred as ringworm (Figure.2). This infection consists of a round, scaly patch that has a prominent, enlarging border and a relatively clear central portion (Rosen, 1997). The prominent edge often contains pustules or follicular papules, and multiple lesions can be present. Itching is variable and not diagnostic. *T rubrum* is the most common cause of *Tinea corporis*. A deep form of *Tinea corporis* known as trichophytic granuloma can develop when the fungus is driven down into the hair follicles. It typically develops after inappropriate topical corticosteroid therapy (Rosen, 1997). *Tinea corporis* may appear similar to the infection include eczema, plaque psoriasis and contact dermatitis. *Tinea corporis* (ringworm of glabrous skin of the trunk) characterized by both inflammatory lesions and non-inflammatory lesions.

Tinea pedis, also referred as athlete's foot or ringworm of the foot (Figure.3), is the most common dermatophytosis in warm and humid climate and may affect up to 70% of adult population of the world (Elewski, 1992). It is characterized by presence of inflammatory and non-inflammatory lesions on the plantar surface and interdigital spaces of the foot. Other causative agents of dermatophytoses are *Trichophyton rubrum, Epidermophyton floccosum Trichophyton mentagrophytes*, yeasts (e.g. *Candida albicans*) and bacteria (e.g. *Corynebacterium minutissimum*) (Hay and Mackenzie, 1992).

The three most common clinical forms of *Tinea pedis* are interdigital, Moccasin-type or hyperkeratotic. Interdigital infection often presents as white, macerated skin between the fourth and fifth toes but it may appear in any web space and it typically produces itching and a foul smell from bacterial superinfection with diphtheroids or *Pseudomonas* species. In moccasin-type or hyperkeratotic *Tinea pedis* usually presents as silvery white scales on a red, thickened base. Typically, moccasin-type *Tinea pedis* is a chronic condition (Kwon, 1992). Dry-type interdigital infection can mimic psoriasis (Hay and Mackenzie, 1992). Moccasin-type *Tinea pedis* may appear similar to long-standing contact dermatitis of the foot.

Tinea manuum (ringworm of the hand) is an unusual dermatophytic infection of the interdigital and palmar surfaces (Elewski, 1992), usually of one hand but sometimes of both (Figure.4 & 5). It may coexist with other fungal infections, such as *Tinea pedis*. The palm surface often has diffuse areas of dry, hyperkeratotic skin.

Tinea faciei (ring worm of the face) also known as *Tinea incognito* of its subtle appearance is dermatophytosis of the nonbearded area of the face (Drake et al. 1996). The condition may present as itchy, red skin without a discernible border or it may has a raised border as seen with *Tinea corporis*. Common causative organisms are *T. rubrum* and *T. mentagrophytes*. The infection often responds to topical therapy.

Antifungal Drugs and Their Action

Polyene Antibiotics

These act by binding tightly to sterols present in cell membranes. The resulting deformity of the membrane cause leakage of intracellular ions and enzymes, leading cell death. Those polyenes that have useful antifungal activity bind selectively to ergo sterol, the most important sterol in fungal (but not mammalian) cell walls.

Azoles

Antifungal azoles comprise the following:

- *Imidazoles* like ketoconazole, miconazole and clotrimazole interfere with fungal oxidative enzymes to cause lethal accumulation of hydrogen peroxide; they also reduce the formation of ergo sterol, an important constituent of the fungal cell wall, which thus becomes permeable to intracellular constituents. Every effective, but lack of selectivity in these actions results in important adverse effects.
- *Triazoles* (fluconazole, itraconazole) damage the fungal cell membrane by inhibiting a demethylase enzyme. They have greater selectivity against fungi, better penetration of the central nervous system (CNS), resistance to degradation and cause less endocrine disturbance than do the Imidazoles.

Ketoconazole

Ketoconazole is well absorbed from the gut. It is widely distributed in tissues but concentrations in cerebrospinal fluid (CSF) and urine are low; its action is terminated by metabolism by cytochrome P450 3A (CYP 3A) systemic mycoses. Impairment of steroid synthesis by ketoconazole has been put to other uses, e.g. inhibition of testosterone synthesis lessens bone pain in patients with advanced androgen-dependent prostatic cancer.

Adverse reactions of usage include nausea, giddiness, headache, pruritus and photophobia. Of particular concern is impairment of liver function, ranging from transient elevation of hepatic transaminases and alkaline phosphatase to severe injury and death.

Clotrimazole

Clotrimazole is an effective topical agent for dermatophyte, yeast and other fungal infections like intertrigo, athlete' s foot, ringworm, pityrasis versicolor, fungal rash.

Summary of the invention

The main aim of the present investigation is to screen the crude extract prepared in organic solvents of different polarity such as ethanol, methanol, acetone, Di-ethyl ether as well as water. In the present investigation, chemical composition and antidermatophytic activity of root of *Ixora brachiata,* used in traditional systems of medicine in India was evaluated against three different genera of dermatophytes like *Microsporum*, *Trichophyton* and *Epidermophyton* by Agar Dilution Method. It was further investigated using *in-vitro* and *in-vivo* assay for antidermatophytic activity.

In the present investigation, the ethanolic extracts of root and leaf of *Ixora brachiata* inhibited tested clinical isolates of dermatophytic species like *Trichophyton mentagrophytes*, *T. mentagrophytes, Microsporum canis*, *M. gypseum* and *Epidermophyton floccosum* with MIC & MFC values between 1.250-10 mg ml^{-1}medium.

The study also observed that *Trichophyton mentagrophytes* was the most susceptible and was completely inhibited by the extract while *Microsporum canis* was less susceptible than the other evaluated dermatophytic species. In the treatment, *Ixora brachiata* showed significant antifungal activity ($PV < 0.05$) in ethanolic extract.

Phytochemical tests of studied plant indicated that the root and leaf of *Ixora brachiate* gave positive test for saponins, reducing sugars and proteins. The leaf and root showed presence of glycosides.

In the present investigation, quantitative estimations were carried out for proteins, starch, total sugars, reducing sugars and phenols. Leaf of *Ixora brachiata* contains tannins, sugars, anthraquinones, phenols and coumarins and root of *Ixora brachiata* contains tannins, sugars, anthraquinones, glycosides and triterpenes. The leaf and root extracts were inactive in water, methanol and acetone while the ethanol and Di-ethyl ether extracts of this plant were active. Hence, antidermatophytic activity of this plant may attributed due to triterpenes or coumarins.

Further, it was tasted on thirty patients who were severely suffering from dermatophytic infections. The survey performed for comparing clinical mycological effectiveness and tolerability of phytopharmaceutical formulation in the form of lotion prepared from *Ixora brachiata* root. Final assay were carried out on experimental group by herbaceous drugs and control group by topical Clotrimazole1% Cream. Clinical diagnosis of thirty patients showed the results at the end of two weeks of treatment. In majority of the cases they showed negative mycological examination. The rate of therapeutic effectiveness showed satisfactorily percentage without any side effects. Therefore, the toleratibility percentage was 100% for the treatments.

Detailed description of the tables:

Table 1: MICs (mg ml^{-1}) of the extracts
Table 2: Phytochemical tests
Table 3: Proteins from different parts of plants
Table 4: Activity of extract made with various organic solvent against *T. mentagrophytes*

Detailed description of the figures

Figure 1: *Ixora brachiata Roxb.*(Rubiaceae)
Figure 2: *Tinea corporis,* pinkish annular plaque on skin (ring worm*)*
Figure 3: *Tinea pedis* with hyperkeratotic
Figure 4: *Tinea manuum*
Figure 5: *Tinea manuum*

Figure 6: The activity of medicinal plant extracts made in solvents of varied polarity against *T. mentagrophytes* by disk diffusion *Ixora brachiata* leaf

Figure 7: The activity of medicinal plant extracts made in solvents of varied polarity against *T. mentagrophytes* by disk diffusion *Ixora brachiata* root.

Figure 8: Inhibitory effects of ethanolic extract of I. brachiata leaf against T. mentagrophytes by Agar Dilution Method on Mycosel medium. Decreasing dilution ranging from 0.078-5.0 mg/ml medium. MIC=5.0 mg/ml medium.

Figure 9: Inhibitory effects of ethanolic extract of *I. brachiata* root against *T. mentagrophytes* by agar dilution method on mycosel medium. Decreasing dilution ranging from 0.078 -5.0 mg/ml medium. MIC=2.50 mg/ml medium

Figure 10: HPTLC study of *I. brachiata* root &leaf for detection and confirmation of coumarins and triterpenes

Figure 11: Chromatogram

Figure 12: Bioautogram

Figure 13: Cured patient with *I. brachiata* root extract lotion applied topical every day for 4 weeks

Detailed description of the Graphs:

Graph 1: Estimation of coumarins at 254 nm before derivation from *Ixora brachiata* leaf

Graph 2: Estimation of coumarins at 366 nm after derivation from *Ixora brachiata* leaf

Graph 3: Estimation of triterpenes at 254 nm before derivation from *Ixora brachiata* root

Graph 4: Estimation of triterpenes at 366 nm after derivation from *Ixora brachiata* root

Detailed description of the invention

Detailed experimental studies

Medicinal plants were selected on the basis of their antidermatophytic activity. They were screened for phytochemical components and antidermatophytic activity were tested by *In-vitro* and *in-vivo* assays. In *in-vivo* assay, the therapeutic effectiveness of *I. brachiata* root and leaf *lotions* showed satisfactorily percentage without any side effects. The results of this investigation will be helpful for skin diseases caused by dermatophytic fungi such as *E. floccosum, M. canis, M. gypseum, T. mentagrophytes* and *T. rubrum.*

The plant material was collected from various places of Western Ghats regions of Maharashtra, Ratnagiri District. The specimens were identified with the help of Cooke (1958) and authenticated by Botanical Survey of India, Western circle Pune-411001. The healthy and disease free plant parts were separated and dried in shade so as to avoid the decomposition of chemical constituents. These were powdered in grinder and stored in clean and dry airtight containers for future studies.
In dermatophytoses, it is necessary to collect a specimen while paying careful attention to the clinical features.
Skin lesions:
The lesions of *Tinea* show a circular pattern, with a large amount of the fungus distributed at the active border of the lesion and only a little in the center.
The lesion was first cleaned with 70% alcohol and then the scale on its borders, the roof of the vesicles, or the keratinous part of the papules was scraped off by using scalpel flamed before use, taking great care not to cause any bleeding.

Preparation of Extract:
The roots and leaves were extracted in the organic solvent of ethanol. To 10 g of each powdered material 100 ml organic solvent, ethanol 80% (drug/solvent ratio=1:10 w/v) was added in a conical flask for maceration, plugged with cotton and then kept on a rotary shaker at 190-220 rpm for 3×24h at room temperature (Fenner et al. 2005). Following filtration of the suspension through a Buckner funnel and Whatman filter paper #1, the crude ethanol extracts were evaporated to dryness in oven or water bath at 45^0C and dissolved in 100 % dimethyl sulfoxide (DMSO) to get final concentration of 1000 mg ml^{-1}.

***In-vitro* assay:**
Antifungal activity of medicinal plants extracts were evaluated against three different genera of dermatophytes (*Microsporum, Trichophyton,* and *Epidermophyton*) using following method.

i) Dermatophyte isolates:
In the present investigation for the antifungal evaluation, 3 strains obtained from the Persian type culture collection (PTCC) Tehran viz. *Trichophyton mentagrophytes* PTCC5054, *Microsporum canis* PTCC5069, *M. gypseum* PTCC5070, and in addition to these 13 strains isolated from different lesions of patients at the Medical diagnosis laboratory, Ahwaz, such as *Microsporum canis* (n=2): MC-1, MC-2, *M. gypseum* (n=3): MG-1, MG-2, MG-3, *Trichophyton rubrum* (n=2): TR-1, TR-2, *T. mentagrophytes* (n=3): TM-1, TM-2, TM-3 and *Epidermophyton floccosum* (n=3): EF-1, EF-2, EF-3 and identified by standard procedure (Rebell & Taplin 1970). Sabouraud's dextrose agar at 25°C was

used to maintain isolates. In antifungal assays, each dermatophyte isolate was sub cultured onto Sabouraud-dextrose agar (SDA; Hi Media- India) slants and incubated at 30^0C for 4 to 5 days and sub cultured every 15 days to prevent pleomorphic transformations (Fenner et al. 2005).

ii) Antifungal agents:

Standard antifungal used were griseofulvin (Sigma Chemical Company) andk eteconazole (Janssen pharmaceutical).

Stock solution of griseofulvin and keteconazole were prepared by dissolving the powder in 100% dimethyl sulfoxide to get stock solution of 4000 µg ml^{-1}. (Curtin Matheson Scientific Inc., Houston, Tex.) Jessup (2000) and Somchit (2003).

iii) Drug dilution:

Serial two-fold dilutions of Griseofulvin and Keteconazole used were as follows: 400, 200, 100, 50, 25, 12.5, 6.25, 3.12, 1.56, 0.78 and 0.39 µg ml^{-1}.

iv) Preparation of medium:

Mycosel (Sabouraud culture medium containing Cyclohexamid and Chloramphenicol) agar 36 gm (Difco, Detroit MI) was completely dissolved in 100 ml distilled water in a conical flask and autoclaved at 121^0C and 15 lb/cm^2 pressures for 20 min. and then cooled to 50^0C before pouring to make plates for susceptibility testing.

v) Preparation of fungal inoculum:

A standardized inoculum was prepared by counting the micro conidia microscopically. For this the suspension of conidia was prepared by using 0.85% sterile physiological saline which containing 0.05% Tween 80 (Sigma). The suspension was added to the slant tube culture and gently swabbing the colony surface with a sterile bent glass rod to dislodge the conidia from the hyphal mat. The suspension was transferred to a sterile centrifuges tube and the volume was adjusted to 5 to 10 ml with sterile physiological saline. The final suspension of conidia was counted with a hemocytometer cell counting chamber. The inoculum of cell or spore suspensions were obtained according to reported procedure (Shin and Lim 2004 & Wright et al. 1983) and adjusted to 10^4-10^5 colony-forming units ($CFUml^{-1}$) or dermatophyte suspension was prepared by using 0.85% sterile physiological saline with Tween 80 (0.05%) and adjusted with a spectrophotometer at 530 nm to obtain 90% transmission (Lima et al. 1993).

Phytochemistry:

In the present investigation phytochemical investigation was carried out, on selected plant which consists of qualitative tests for the presence of, starch, proteins, tannins, saponins, reducing sugars, anthraquinones, alkaloids, glycosides and flavonoids. Besides these, quantitative estimations were carried out for starch, reducing sugars, proteins, and alkaloids.

Different methods used for these studies are as given below:

i) Qualitative tests for starch:

The plant material was finely ground and extracted with boiling methanol (methanol removes fats, fatty acids, salts, chlorophyll and inactive enzymes). After drying, the plant tissues were centrifuged with cold water and tested with iodine in 2% aqueous potassium iodide (Peach and Tracy, 1955).

ii) Qualitative tests for proteins (Millon's test):
Millon's reagent is a solution of mercuric nitrate in nitric acid (it reacts specifically with any phenolic compound in which 3 and 5 positions are unsubstituted). Proteins give red colorations with Millon's reagent.
Procedure: 2 ml of the test solution was boiled with a few drops of millon's reagent and the colour was observed (Trease and Evans, 1972).

iii) Qualitative test for Tannins:
Powdered plant material was treated with Ferric Chloride (acidic) and observed for the presence of tannins (Trease and Evans, 1972).

iv) Qualitative tests for Saponins:
Water extract of the plant material was vigorously shaken (with few drops of neutral water). A permanent lather (foam) indicates the presence of saponins (Trease and Evans, 1972).
A portion of residue obtained after evaporating the ethanol extract was dissolved in water and shaken vigorously. A honey comb, froth persisting for 15 min indicated the presence of saponins. A portion was dissolved in chloroform and filtered. A few drops of concentrated sulphuric acid and 1 ml of acetic anhydride were added to 1 ml of iced filterate. The appearance of blue or bluish green or reddish brown colour showed the presence of saponins (Fransworth, 1960).

v) Qualitative tests for free Anthraquinones:
The plant extract of 5g material was shaken with 10ml of benzene and filtered. A 10% ammonium hydroxide solution (about 5ml) was added to the filtrate and the mixture was shaken. The presence of pink, red or violet colour in the ammonical phase indicated the presence of free anthraquinones (Fransworth, 1960).

vi) Qualitative estimation for reducing sugars (Benedict's test):
Reagent: Two stock solutions were prepared as follows:

(A) 17.3g of sodium citrate and 100g of anhydrous sodium carbonate were dissolved in 800 ml of warm distilled water. Filter and dilute the filtrate to 850 ml with distilled water.

(B) 17.3 g of pure crystalline copper sulphate was dissolved in 100 ml distilled water.

Working reagent: Solution B was slowly added to solution A, mixed thoroughly and was diluted to 1 liter with distilled water.
Procedure: To 5 ml of Benedict's reagent 5ml of the test solution was added. The tubes were incubated in boiling water on water bath for 10-30 minutes. The formation of brick red or an orange red precipitate indicated the presence of reducing sugars. (The principle is that when sugar has free or potentially free aldehyde or ketone group reduces copper ions in hot alkaline solution to form orange or red coloured Cu_2O).

vii) Test for flavonoids:
To 1 ml of ethanol extract, few drops of concentrated HCl and Mg turnings were added. The development of pink or magenta colour indicated the presence of flavonoids. (Fransworth, 1960).

viii) Qualitative estimation of Alkaloids:
Precipitation of alkaloids can be obtained with a variety of inorganic and organic reagents. Sometimes even from dilute solutions. Among the inorganic precipitating reagents, following are few reagents-potassium mercuric iodide (Mayer's reagent), bismuth potassium iodide (Dragendorff's reagent), iodine

potassium iodide (Wagner's reagent), potassium cadmium iodide (Marm's reagent), chloroplatanic acid, auric chloride in hydrochloric acid, phosphomolybdic acid (Sonnenschein's reagent), mercuric chloride, perchloric acid and rarely potassium ferric and ferrocyanide.

Some of the organics precipitating are reagents picric acid (Hager's reagent) styphnic acid, picrolonic acid and tannic acid. Characteristic colour reactions are obtained with the acid of dehydrating agents such as concentrated sulphuric acid, with oxidizing agent such as nitric acid, with a combination of these two or other reagents, which will dehydrate and oxidize simultaneously and finally by treating with aldehyde or like compounds in the presence of dehydrating agents. The exact mechanism of precipitation reactions of alkaloids is not clearly understood. However, these reactions have proved to be an efficient tool in detection of alkaloids in plant tissues.

Procedure: Plant material was crushed in mortar and pestle and the alkaloids were extracted in absolute alcohol. Filtered through Whatman No. 1 filter paper. Filterate served as a source of alkaloids. Similarly some of the plant material was dissolved in dilute H_2SO_4. The extract of alkaloids was tested with different reagents as follows.

For every test 2-3 ml of the alkaloid extract was used with 1-2 ml of reagent (Homersleg, 1950 and Cromwell, 1955).

ix) Test for Glycosides:

The plant material was extracted in absolute alcohol. Filtered through Whatman No. 1 filter paper. In 2 – 3 ml of filterate add equal volume of warm benzene slowly from the edges of test tube. The white precipitate develops at the edges of solution in the test tube indicates the presence of glycosides.

Factors Affecting Susceptibility Testing of Dermatophytes:

1. Incubation temperature for dermatophytic fungi has been recommended 34^0C since this temperature closely approximates skin surface temperature. But normally between 25-35^0C.
2. The minimum incubation period recommended between 4-14 days according Young et al. (1972).
3. The optimal of inoculum is 10^4-10^5 conidia/ml according to Shin et al. (2004).Because of the ability of dermatophytes to produce various kind of conidia like microconidia, macroconidia and clamidoconidia, for *in -vitro* susceptibility testing of this group of fungi agar media are better than broth media culture (Jessup, 2000).

Antifungal Susceptibility assay:

Assay for antifungal activity of different extracts were evaluated as per following two methods:

i) Disk diffusion Method:

Disk diffusion assays were carried out as described by Ficker et al. (2003). In this method 100 µl of the fungal cell suspension (10^5 $CFUml^{-1}$) was pipetted onto Sabouraud's dextrose agar plates and uniformly spread by using a sterile bent glass rod. Sterile filter blank disks (7mm diameter, Whatman #1) were impregnated with 20µl extract solution (40mg ml-1) with different dilutions of the extracts as follows: 20%, 40%, 60%, 80% provided with sterile distilled water and negative controls were performed with only equivalent volumes (20µl) of solvent (80% ethanol) without added extracts to paper disks and allowed to air dry. These were placed in the inoculated petri dishes (1 disk per plate). Petri dishes were inverted and incubated at 28-30^0C for 48 h before (Malheriros at el. 2005) antimycotic evaluation according to the method of Binns et al. (2000). Shortest distance of the clear zone from the outer edge of the filter disk to the edge of visible fungal growth were measured in mm as the zone of clearance and compared it with the standard.

ii) Agar Dilution Method:

The fungistatic activities of different extracts were evaluated by the Agar Dilution Method (Fenner et al. 2005; Mitscher et al. 1972 and Lucia et al. 2003) with the following modifications. For the assay, stock solutions of extracts were two-fold diluted with 0.85% sterile physiological saline to get dilutions ranging from 0.078 to 20 mg ml^{-1}. Mycosel agar medium (5 ml) with 1% yeast extract mixed with various dilutions of extract (100 µl) and 50µl the dermatophyte suspension of (10^5 $CUFml^{-1}$) removed from a seven days old culture of fungi, was poured in each petri dish (60mm diameter) under laminar flow condition. The plates were incubated at 28-30^{0}C in a moist, dark chamber, and MICs were visually recorded after 15 days. The antifungal agents, Keteconazole (Janssen pharmaceutical) and Griseofulvin (Sigma) were used as positive controls. Drug free solution (only with appropriate amount of DMSO) was also used as a blank control for verification of fungal growth. The minimal inhibition concentration (MIC) value was defined as the lowest extract concentration that inhibit the fungal growth, and the minimal fungicidal concentration (MFC) showing no visible fungal growth after incubation time. MIC50 and MIC90 values are the lowest extract concentration at which 50% and 90% of the clinical isolates were inhibited (Marco et al. 1998). Dermatophyte plates were examined visually for 50 and 80% growth inhibition as compared to growth control. MIC results were recorded in micrograms per milliliter. The experiments were performed in duplicates and replicated three times to get average value of the MIC and MFC.

Activity of medicinal plant extracts made with various organic solvents against *T. mentagrophytes* by disk diffusion method:

Procedure:

To 10 g of powdered material of *Ixora brachiata* (root and leaf) was added 100 ml organic solvent, ethanol 80% in a conical flask for maceration. Following filtration of the suspension through a Buckner funnel and Whatman filter paper #1. The crude ethanol extracts were evaporated to dryness in oven at 45^{0}C. Then added 5-10 ml different solvents with various polarities like diethyl ether, acetone, methanol and water to conical flasks contain dryness plant extracts respectively. Then conical flasks were stirred slowly for dissolving antifungal active fractions in those solvents. Finally four extracts were obtained by as mentioned above solvents. Then 100 µl of the fungal cell suspension (10^5 $CFUml^{-1}$) was pipetted onto petri dishes contain solid Sabouraud's dextrose agar and uniformly spreading by using a sterile bent glass rod. Sterile filter blank disks (7mm diameter, Whatman #1) were impregnated with 10-20µl obtained extracts with as mentioned various polarity solvents. Disks were placed on the surface of solid agar petri dishes that were inoculated with the *T. mentagrophytes* suspension (4 disks per plate).

Then after solidification medium agar petri dishes were inverted and incubated at 35^{0}C. Following an incubation period of 48-72 hours the diameter of the zone of inhibition around each disk was measured in millimeters. This work also carried out according to Agar Dilution Method.

High Performance Thin Layer chromatography (HPTLC):

HPTLC technique was followed for the qualitative analysis and the confirmation of chemicals present in the studied plant (Passera et al., 1964).

HPTLC is a versatile separation technique included various steps as given below:

1) Plant extraction
2) Selection of HPTLC plates and sorbent

3) Sample preparation
4) Application of sample
5) Development (separation)
6) Detection including post-chromatographic derivatization
7) Quantitation
8) Documentation

1) Plant extraction:

The first step in the phytochemical evaluation is extraction of the plant material. The choice of extraction method depends up on the nature of plant material and the compound (s) to be isolated.

Isolation and Purification of Antifungal Compounds from Medicinal Plant:
To 10 g of each powdered material was mixed in 100 ml ethyl acetate
Mixture kept on a rotary shaker at 150 rpm for 3 hours at room temperature
Centrifuged at 10,000 rpm for 5 minutes
Supernatant collected
Reduced the supernatant from 100 ml supernatant to 20 ml quantity
5-10 µl is used for HPTLC

2) Selection of HPTLC plates and sorbent:

Pre-coated plates with different support material (glass, aluminum, plastic) and with different sorbent layers are available in different format and thickness in various manufacturers. Usually plates with sorbent thickness of 100 – 250 µm are used for qualitative and quantitative analysis. However, for preparative TLC work, plates with sorbent thickness of 1.0 – 2.0 mm are available in addition to chemically modified layers. Aluminum sheet (0.1 mm thick) sheets as support offer the same advantage as polyester support but with increased temperature resistance. However, with eluents containing high concentration of mineral acids or concentrated ammonia. One may find problem, as they will chemically attack aluminum. Aluminum sheets are otherwise compatible with organic solvents and organic acids such as formic acid and acetic acid.

Plate size: Pre-coated TLC/ HPTLC plates in size of 20 × 20 cm with aluminum. It is always recommended to clear the plates before actual chromatography.

Activation of pre–coated plates: For the separation of compounds of herbal extracts pre-coated plates of silica gel G 60 are widely used especially the ones impregnated with phosphor (silica gel F_{254}, E. Merck). User UV at 254 nm, the resolved compounds whose absorption spectra overlap the excitation spectrum of phosphor are seen as dark bands against a yellow- green fluorescent background due to fluorescence quenching.

Freshly opened box of TLC/ HPTLC plates usually does not require activation. However, plates exposed to high humidity or kept on hand for long time may have to be activated by placing in oven at 110-120°C for 30 minutes prior to sample spotting (Sethi, 1996).

3) Sample Preparation:

Proper sample preparation is an important pre-requisite for success of thin layer chromatographic separation. The sample preparation procedure is to dissolve the dosage form with complete recovery of intact compound(s) of interest and minimum of matrix with a suitable concentration of analytic (s) for direct application on the HPTLC plate. Besides, maximizing the yield of analytic (s) in the selected solvent stability of analytics during extraction and analysis must be considered and ensured. Therefore,

the choice of a suitable solvent for a given analysis is very important. For normal phase chromatography using silica gel pre-coated plates (more than 80-90% HPTLC analysis is done using silica gel as sorbent) solvent for dissolving the sample should be non-polar and volatile as far as possible. It is preferable to keep the solvent as simple as possible and quantity employed is limited to ensure complete extraction of analytics and minimum of extraneous component. Sample and reference substances should be dissolved in the same solvent to ensure comparable distribution at starting zones (Stahl, 1969).

4) Application of sample:

Sample application is the most critical step for obtaining good resolution for quantification by HPTLC. The sample should be completely transferred to the layer, however, under no circumstances, the application process should damage the layer, as damaged layer results in unevenly shaped spots. Wherever possible use of automatic application devices is recommended for quantitative analysis. While using graduated capillaries, one must ensure that they fill and empty completely.

Usually application of 1–10 µl volume for TLC and 0.5–5 for HPTLC is recommended keeping the size of starting zone(s) down to minimum; 2–4 mm (TLC) and 0.5–1 mm (HPTLC) in the concentration range of 0.1–1 µg/µl for TLC/ HPTLC. However, volume and concentration primarily depend on the component under analysis and their sensitivity to various detection techniques.

5) Development (Mobile phase):

Poor grade of solvent used in preparing mobile phase have been found to decrease resolution, spot definition and R_f reproducibility. Mobile phase commonly called solvent system is traditionally selected by controlled process of trial and error and also based on one's own experience in the field. It is often possible that few layer-solvent combinations already reported in the literature for compounds of interest or similar compounds may be suitable in a given analytical problem with minor modifications. Nevertheless, it should not be forgotten that such conditions may have been chosen due to availability rather than suitability and often improvements are required. However, mobile phase should be chosen taking into consideration chemical properties of analytics and the sorbent layer. Use of mobile phase containing more than three or four components should normally be avoided, as it is often difficult to get reproducible rations of different components.

Pre-conditioning (Chamber saturation):

Chamber saturation has pronounced influence on the separation profile. When the plate is introduced into an unsaturated chamber, during the course of development, the solvent evaporates from the plate mainly at the solvent front. Therefore larger quantity of the solvent shall be required for a given distance hence, resulting is increase in R_f values. If the tank is saturated (by lining with filter paper) prior to development solvent vapors soon get uniformly distributed throughout the chamber. As soon as the plate is placed in such a saturated chamber, it soon gets pre-loaded with solvent vapors hence, less solvent shall be required to travel a particular distance resulting in lower R_f values. Time required for saturation will depend on the nature and composition of mobile phase and layer thickness (equilibration time increases with increase in layer thickness). Once the chromatogram is developed it should be handled with utmost care. Application of reagents if required has to be homogeneous ensuring uniform reaction and finally stabilizing of end reaction product.If heating of the plate after it is treated with the reagent is not uniform. There always exists risk of reaction in homogeneity on the plate. Usually drying cupboard or hot plates are employed. Hot plates with regulated range of temperature i.e. 50-190° C ± 2° C are extensively being employed for heating the chromatogram.

6) Detection and visualization:

As soon as the development process is completed the plate is removed from the chamber contains the mobile phase of chloroform solvent and evaporated to remove the mobile phase completely. The zones can be located by various physical, chemical and biological i.e. physiological methods. There is apparently no difficulty in detecting colored substances or colorless substances in short wave ultra-violet (UV) region 254 nm and 366 nm or with intrinsic fluorescence such as riboflavin quinine sulphate. The R_f values and the colors of the resolved bands are recorded and fingerprint profiles are established. Identification of the chemical marker is by comparison of R_f value, absorption spectra, response to derivatizing reagent *etc.* (Wagner et al. 1984).

(Relative factor) R_f = Distance travelled by the solute/ Distance travelled by the solvent

7) Quantitation:

Spraying and dipping techniques are used for applying detection reagents. However, in addition to other reasons as enumerated below dipping followed by evaporation which is essential for both the precision and repeatability in ultimate quantitative analysis. Sample and standard are chromatographed on the same plate under similar conditions.

Preparation of spraying reagents:

Anisaldehyde- Sulphuric Acid reagent:

Mix o.5 ml anisaldehyde in 10 ml glacial acetic acid, 85 ml methanol and 5 ml conc. sulphuric acid. Spray the plate and heat at 100^0 C for 5-10 min. Use freshly prepared reagent (Wagner et al. 1984).

Ninhydrin reagent:

Dissolve 30 mg of ninhydrin in 10 ml of n- butanol and 0.3 ml of glacial acetic. Spray the plate with the reagent and heat at 105^0 C till the spots appear.

Vanillin Sulphuric Acid reagent:

Solution 1: 1% Ethanolic Vanillin.

Solution 2: 10% Ethanolic Sulphuric Acid.

The plate is sprayed with 10 ml of solution 1 and then immediately with 10 ml of solution 2. Heat at 110^0 C for 5-10 min. (Wagner et al. 1984).

Detection of Coumarins:

- **UV-254 nm:** Distinct fluorescence quenching of coumarins.
- **UV-365 nm:** Intense blue or blue-green fluorescence (simple coumarins) yellow, brown, blue or blue-green fluorescence (furano-and pyranocoumarins).
- **Spraying reagents:**
  - Potassium Hydroxide (KOH) reagent. The fluorescence of the coumarins are intensified by spraying with 5%-10% ethanolic KOH. Concentrated ammonia vapour has the same effect.
  - Natural products-Polyethylene Glycol reagent (NP/PEG) intensifies and stabilizes the exiting fluorescence of the coumarins. Phenol Carboxylic Acid fluorescence blue or blue-green (e.g. chlorogenic or caffeic acid).

Detection of Triterpenes:

- **UV-254 nm:** Caffeic acid, derivatives and isoflavones show quenching.
- **UV-365 nm:** Caffeic acid, its derivatives and isoflavones fluorescence blue.
- **Anisaldehyde-Sulphuric Acid reagent:** The sprayed TLC is heated for 6 min. at 100^0 C.

Evaluation in vis.: Triterpenes blue-violet.

8) Documentation:

The use of application scheme and labeling every single chromatogram can avoid mistake in respect of order of application. It is preferable to apply each sample and reference solution twice by following data - pair method. A lead pencil can be used to write on the chromatoplate. The plate should never be marked below the starting point, as layer is likely to get damaged affecting chromatographic distribution of the substances under analysis which may ultimately lead to error in scanning. The best way to label the chromatoplate is to mark above the level of solvent point, immediately after development is completed, the solvent point should be marked both on left and right hand edges of the plate, this win facilitate calculation of R_f values. The practice of cutting a scratch across the whole layer is no longer in use. The type of plate, chamber system, composition of mobile phase, running time and detection method should all be recorded. HPTLC protocol format given in the text may be adopted for recording all the relevant data.

Bioautography Agar Overlay:

The bioautography agar overlay method is an improved version of a disc diffusion method. It is considered as one of the most efficient methods for the detection of antimicrobial compounds (Rahalison et al. 1991). It appears that bioautography is an important detection method for a new or unidentified antifungal compounds because it is based on the biological effects of the substances under study. In the bioautography agar overlay method the drug to be evaluated is adsorbed onto the Thin Layer Chromatography (TLC) plate and the inoculum is laid onto the plate as a very thin layer of about 1 mm in thickness. The bioautography agar overlay method uses very little amount of sample and since the crude extract is resolved into its different components it can identify and isolate the active compounds (Rahalison et al. 1991).

Bioautography agar overlay process:

The bioautography was performed for the ethyl acetate extracts of *I. brachiata,* against two dermatophytic species viz. *T. rubrum* and *T. mentagrophyts.*

Five microliters (5 µl) of the Ethyl acetate of extracts these plants were analyzed by thin layer chromatography (TLC) by using aluminum-backed TLC plates (Merck, silica gel 60 F254). The TLC plates were developed with systems viz. ethyl acetate/methanol/water (40:5.4:5): [EMW] (polar/neutral); chloroform/ethyl acetate/for-mic acid (5:4:1): [CEF] (intermediate polarity/ acidic); benzene/ethanol/ammonium hydroxide (90:10:1): [BEA] (non-polar/basic) and acetone/Ethyl acetate/petroleum ether (0.5: 0.5:2.0) [AEP]. The mobile phase was removed from the plate by drying in the room temperature. The developed plates were sprayed with Iodine reagent to check presentation of spots and were observed in UV light. Then developed TLC plates were sterilized by UV light and these were removed into Petri dishes which poured 5-10 ml Sabouraud's dextrose agar previously. To 5 ml non-solidified media with 45°C temperature was added 100 µl of the dermatophyte suspension inoculum with concentration of 10^5 $CUFml^{-1}$ and mixed evenly. About the 1 mm in thickness of this mixture was laid on the surface of developed TLC plates (Rahalison, 1999). The plates were incubated

at 35^{0}C in incubator. They were incubated for a period 48-72 hours after which the inhibition zones appeared colourless against a yellow background. Spots showing any inhibition were noted and their hR_f values measured. The tests were performed in duplicate.

Preparation of Herbal Formulation in the forms of Lotion

Since the effect of medicine in *in-vitro* is different from its *in-vivo* effect it is better to increase the concentration of the medicine compared if the patient can be tolerated it and it is not dangerous for patient.

Procedure:

Preparation of herbal medicine: viz. *Ixora bracliata* – lotion. The methanolic extract of *Ixora bracliata* root and leaf was accurately weighed (250mg). These are also known as Clotrimazole material.

Fatty material such as Vaseline and Ceto-estearyl Amine heated up to 70^0 C in a beaker. Then Sodium Loril Sulphate was mixed as surfactant to water and then heated up to 80^0 C in another beaker. Then Methyl and Prophyl Paraben were added in those as an antibacterial. These mixtures were added slowly but continuously in the mixture of Vaseline and Ceto-estearyl Amine and allowed to cool by stirring slowly to decrease temperature (as mixture No. A). Then those accurately weighed herbal extract or Clotrimazole material were mixed in Propylene glycol and was stirred until a smooth mixture obtained (as mixture No. B). Then mixture A and mixture B were mixed and heated up to 50-55^{0}C. Then the mixture was cooled by stirring slowly and the warm mixture drug packed into a special container or tube.

***In-vivo* assay:**

A double-blind *in-vivo* assay was performed on thirty patients who were severely suffering from dermatophytic infection. The survey and treatment were carried out in the Department of Mycoparacytology, Joundi-Shapur University Hospital and Medical Sciences, Ahwaz, Iran (Ethical approval no. JSUH/ HEC/2006-2007/27; dated 15/03/2006).

All patients were informed about the project and have signed consent forms for participation in this clinical investigation. Afterwards, they were randomized to one treatment.

It was performed for comparing clinical, mycological effectiveness and tolerability of phytopharmaceutical formulation i. e. lotions prepared from *Ixora brachita* root and leaf extract with concentration of 250 mg with the addition of topical antifungal Clotrimazole1% cream same formulated as phytodrug cream on the topical treatment of clinical features of dermatophytosis. Pharmacological studies have demonstrated antimycotic in vitro effect of *I. brachita* root and leaf against *T. mentagrpphytes, T. rubrum, M. canis, M. gypseum and E. floccosum,* while controlled clinical trials verified the effectiveness of these plants for treating of clinical features dermatophytosis without side effects. Both the formulations showed promising results. The final bioassays were carried out on thirty patients-15 in the experimental and 15 in the control group.

Experimental procedure:

The study was conducted over 4 weeks. At the beginning of the investigation every patient received a 30 ml plastic container with the instruction of treatment of how to use it (Directions: Apply 2-3 times daily for two to four weeks). The first evaluation was carried out for two weeks after the treatment began, and the patients received a fresh treatment by changing the empty container for a new one. The last evaluation took place for four weeks after first day of treatment. During the period of study at the beginning and after two and four weeks of treatment mycological tests include a direct examination

with potassium hydroxide and the culture of scrape scales of affected area of the body obtained on mycobiotic agar and incubated for 15 days at 25 0C.

Outcome variables:

The evaluated outcome variables were: a) Clinical effectiveness was declared when signs and symptoms attributed to the pathology under study were totally absent. b) Mycological effectiveness was detected as a negative direct examination and culture. c) Tolerabilility which was determined by the absence of local side effects such as erythema, stinging, blistering, peeling, edema, prurits, urticaria and irritation.

Results and discussion

Screening of medicinal plant extract for antidermatophytic activity:

The aim of the present investigation was to screen crude extract prepared in organic solvents such as ethanol, methanol, acetone and Di-ethyl ether as well as water also. In the present investigation ethanolic extract of root and leaf of Ixora *brachiata* selected based on the reputation in Ayurvedic and Indian traditional systems of medicine (Figure 1). These were investigated for their potential activity against 16 clinical and standardized dermatophytic species viz. *Trichophyton mentagrophytes, Microsporum canis, M. gypseum, Trichophyton rubrum* and *Epidermophyton floccosum* which were isolated from patients by both agar dilution and disk diffusion methods (Figure 6 & 7). Since disk diffusion method with many compounds particularly volatile compound did not show sharp demarcation between inhibition and growth on the agar plates. But this method is simple, cheaper and more convenient than the agar dilution technique. Previous studies reported that disk diffusion method showed clearly anti-Candida effects (Moore and Atkins, 1977). Hence, determination of the MIC and MFC carried out by agar dilution method.

The crude extracts in concentration up to 20 mg ml^{-1} were incorporated into the growth media according to material and method. The tested ethanolic extracts of medicinal plants were inhibited all tested dermatophytic species with MIC values between 1.250-10 mg ml^{-1} medium. MFC values of all tested dermatophytic species were ranged 1.250-10 mg ml^{-1} medium. The results are shown in Tables No. 1&4.

In the present investigation particularly plant species studied showed attractive antifungal activities. The plant *Ixora brachiata* which was studied for the first time and its antidermatophytic activities is reported in the present investigation. In the present investigation it was also observed that *T. mentagrophytes* the most susceptible, which completely inhibited the tested extract which is similar with the studies of Babal et al. (1999).

***In-vitro* Susceptibility Testing of Dermatophytes of *Ixora brachiata*:**

According to Annapurna and Raghavan (2003) activity against fungi was not significant but in our study, the crude extracts of *Ixora brachiata* root and leaf showed *in-vitro* antifungal properties and completely prevented the growth of tested dermatophytic species with MIC values IBL and IBR between 10-5 and 10-2.5 mg ml^{-1} medium, respectively. MIC90 and MIC50 values = 2.50 and 0.625 mg ml^{-1} medium for IBL and 1.250 and 0.312 for IBR. MIC90s and MIC50s were similar in all tested clinical and standardized dermatophytic species of *T. mentagrophytes, M. canis, M. gypseum, T. rubrum* and *E. floccosum*. MFC values IBL and IBR were also similar in all tested clinical and standardized dermatophytic species 5.0 and 2.5 mg ml^{-1} medium, respectively (Table 1, Figure 8, 9). In the present investigation, this kind of work has been carried out for the first time because antidermatophytic effects of *Ixora brachiata* have not been reported so far. Inhibitory effects of *Ixora*

brachiata have only studied for its antibacterial activity and exhibited good activity against gram-negative organisms like *Salmonella typhimorium*, *Pseudomonas aeruginosa* and *Escherichia coli* and also on gram-positive like *Staphylococcus aureus* and *Bacillus subtilis*. The MeOH extract of *Ixora brachiata* also showed moderate activity against all the tested organisms.

Results of Phytochemical Study:

Phytochemical tests of studied plant indicated that the root and leaf of *Ixora brachiata* gave positive test for saponins, reducing sugars and proteins. Among tested plants the leaf and root of *I. brachiata* gave positive for glycosides. In the present investigation, quantitative estimations were carried out for proteins, starch, total sugars, reducing sugars and phenols. Leaf of *Ixora brachiata* contains tannins, sugars, anthraquinones, phenols and coumarins and root of *Ixora brachiata* contains tannins, sugars, anthraquinones, glycosides and triterpenes (Table 2, 3).

Results of HPTLC Study:

It was found that chemicals such as terpenoids, triterpenes and coumarins are effective against pathogenic fungi (Cowan, 1999). Other studies regarding antifungal activity of chemical composition of tested plants reported that coumarins and triterpenoids were responsible for antimicrobial activity (Gray, 1994 and Govindachari, 1998). Hence, in the present investigation High Performance Thin Layer Chromatography (HPTLC) fingerprints of the root of *I. brachiata*, carried out for detection and confirmation of Phytochemical compounds like triterpenes and coumarins.

The ethyl acetate extract of *I. brachiata* leaf contains coumarin that it was showed in UV-254 nm with 8 peaks and in UV-366 nm with 9 peaks. The ethyl acetate extract of *I. brachiata* root contains triterpene that it was showed in visible and in UV-254 nm with 10 peaks and in UV-366 nm with 12 peaks (Figure 11, 12, Graph 1-4).

Finally after detection and confirmation of triterpenes and coumarins by HPTLC in plants mentioned above these compounds can be responsible for antidermatophytic activity of *I. brachiata* that gave positive test results by *in-vitro* assay previously.

Bioautography results:

After detection and confirmation triterpenes and coumarins by HPTLC method for showing the presence of compounds that these inhibited the growth of fungi, bioautography carried out for selected plants viz. *I. brachiata* that gave positive test results by *in-vitro* assay previously.

Bioautography was used to screen for finding out antifungal compounds present in the extract. Inhibition zones of antifungal compounds were observed as clear spots on a yellow background of media culture. The clear spots indicate presence of antifungal compounds that it inhibited the growth of tested fungi. In tested plant extract, *Ixora brachiata* root organisms did not grow too well that it was difficult to detect inhibition zones hence, no inhibition was observed. The non-activity of this plant extract in bioautography could be explained by evaporation of active compounds during removal of the TLC eluents or by the disruption of synergism between active constituents caused by TLC. Among of four separation systems were used, only the solvent system of AEP gave the best resolution of spots in TLC analysis because antifungal compounds were relatively semi-polar and therefore did not separate well in the polar eluent EMW (Figure 13).

The ethyl acetate extract of *Ixora brachiata* leaf separated with EAP possess few compounds with high activity against *M. gypseum* and *T. rubrum* at R_f values of the active compounds of 0.57–0.98.

***In-vivo* assay:** Experimental group were treated with plant extract lotion and control group were treated with topical commercial drug (i.e. Clotrimazol 1%). Clinical diagnosis of patients showed negative mycological examination the end of two weeks after the treatment. At the end of two and four weeks after the treatment, the rates of the therapeutic effectiveness showed toleratibility percentage without any side effects such as erythema, blistering, peeling, edema, itching and irritation (Figure 13).

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5. Claims

We claim:

1. An anti-fungal herbal formulation in the form of lotion, the said formulation comprising of root extract of *Ixora brachiata* Roxb.
2. Herbal formulation with its specific concentration as in claim 1, can be applied topically in the form of Lotion.
3. Herbal formulation with its specific concentration as in claim 1, and applied as in claim 2, have no toxic effects reported.
4. Herbal formulation with its specific concentration as in claim 1, and applied as in claim 2, is capable of substantially attenuate the fungal infection. Thus it has anti-fungal activity.
5. According to any of the proceeding claims, the said formulation in an embodiment is capable of reducing fungal strains from genus *Tinea* as mentioned in method.
6. According to any of the proceeding claims, the said formulation possesses anti-fungal activity including the fungal infection caused by *Tinea pedis, T. corporis, T. capitis, T. ungium, T. manuum, T. cruris* of skin.

Name : Prof. Dr. Deokule Subhash S Signature :

6. Date and Signature

Date: 01|01|2018

Place: Pune

Prof. Dr. Deokule Subhash S.
Department of Botany,
Savitribai Phule University of Pune (SPPU),
Ganeshkhind Road,
Pune- 411007. Maharashtra, India

TITLE: Development of new herbal lotion for the management of dermatophytic infections

7. ABSTRACT OF THE INVENTION

Mycotic infections, especially dermatophytosis are the most common cause of skin disease in developing countries of tropical regions. Owing to different side effects and low efficiency, there is a distinct need new safer and more effective antifungal agent. The use of medicinal herbal drugs in the treatment of skin diseases including mycotic infections is an age-old practice in many parts of the world, including India. Plant based drugs have the potential to cure mycotic infections without any side effects. Hence, after a detailed review of literature, plant selected for the present investigation was *Ixora brachiata*. The selected plant was collected from Ratnagiri of Western Ghat regions of Maharashtra state.

In the present investigation *in-vitro* and *in-vivo* assays have been used for the evaluation of antidermatophytic activity of *Ixora brachiata*. In addition, phytoconstituents of all selected medicinal plants were screened by using standard phytochemical methods to find out therapeutically active chemical constituent/s present in them.

Results of *in-vitro* and *in-vivo* assays demonstrated that fungi static and fungicidal properties of these plants.

Phytochemical screening of studied plant indicated that the leaf and root of *Ixora brachiata* shown positive test for saponins, reducing sugars and proteins. The root of this plant gave negative tests for tannins. HPTLC studies confirmed that the leaf of *I. brachiata* consists of coumarins while the root of *I. brachiata* contains triterpenes which may be responsible for antidermatophytic activity in this plant. This investigation will be an alternative source of herbal medicine in various systems of medicine such as Ayurved, Siddha, Unani, Homeopathy and pharmaceutical industries.

Table 1: MICs (mg ml $^{-1}$) of the extracts

Name of plant	Plant part used	MIC value (mg ml $^{-1}$)[a]				
		M. C.[b]	*M.g.*[c]	*E.f.*[d]	*T.r.*[e]	*T.m.*[f]
Ixora brachiata Roxb.	Leaf	5.00	5.00	5.00	5.00	5.00
	Root	2.50	2.50	2.50	2.50	2.50

Table 2: Phytochemical tests

Name of the Test carried out	Reagents used	*Ixora brachiata*	
		Leaf	**Root**
A. Water Extract			
Starch	I2-KI	**+ve**	**+ve**
Tannins	Acidic $FeCl_3$	**+ve**	**-ve**
Saponins	H_2SO_4 + Acetic unhydride	**+ve**	**+ve**
Proteins	Million's test	**+ve**	**+ve**
Anthraquinones	Benzene + 10%NH_4OH	**+ve**	**+ve**
Reducing sugars	Benedict's	**+ve**	**+ve**
B. Alcoholi Extracts			
Alkaloids	Mayer's	**-ve**	**-ve**
	Wagner's	**-ve**	**-ve**
	Dragendorff's	**-ve**	**-ve**
Flavonoids	HCl + Mg turnings	**-ve**	**+ve**
Glycosides	Benzene + hot ethanol	**+ve**	**+ve**

+ve: Present -ve: Absent

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Table 3: Proteins from different parts of plants

NAME OF THE PLANT	PLANT PART USED	TOTAL PROTEINS g/100g dry wt.
***Ixora brachiata* Roxb.**	Leaf	2.360
	Root	2.000

Table 4: Activity of extract made with various organic solvent against *T. mentagrophytes.*

Name of the Plant	Part used	Solvent with different polarity			
		Diethyl ether	Acetone	Methanol	Water
Ixora brachiata Roxb.	Root	45mm	–	–	–
	Leaf	15 mm	–	–	–

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Figure 1: *Ixora brachiata* Roxb.(Rubiaceae)

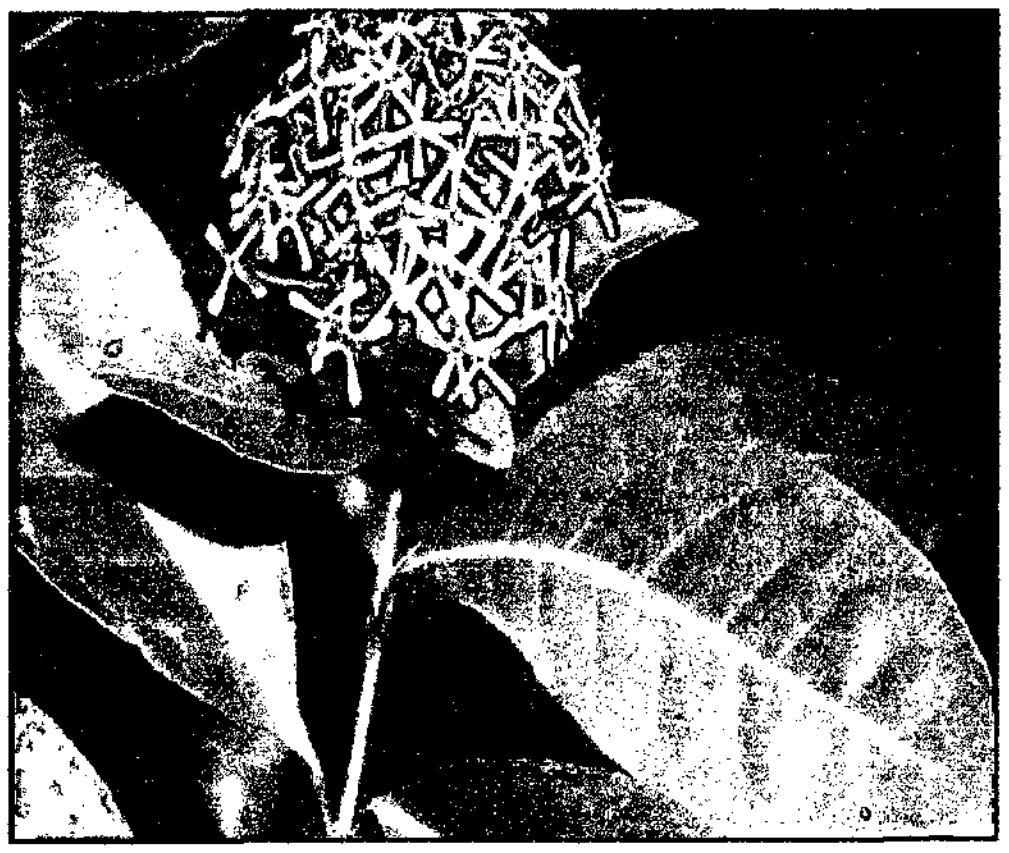

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Figure 2: ***Tinea corporis*****, pinkish annular**

Figure 3: ***Tinea pedis with hyperkeratotic***

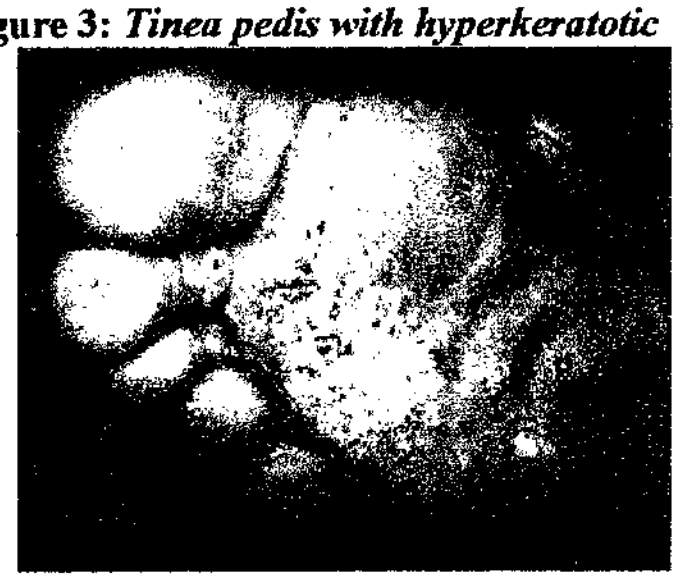

Figure 4: ***Tinea manuum***

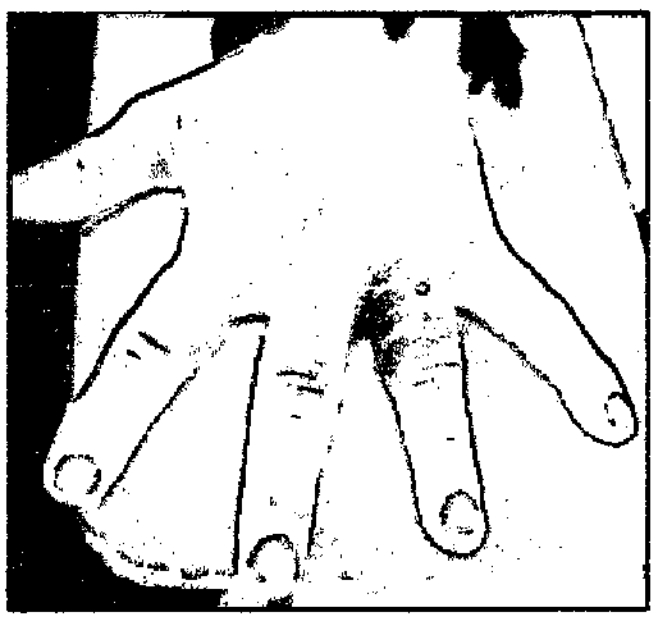

Figure 5: ***Tinea manuum***

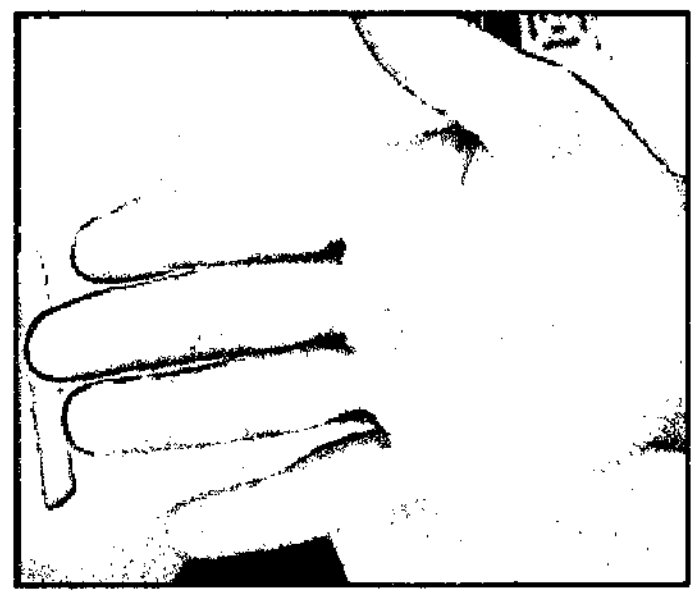

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Figure 6: Activity of medicinal plant extracts made in solvents of varied polarity against *T. mentagrophytes* by Disk Diffusion *Ixora brachiata* Leaf

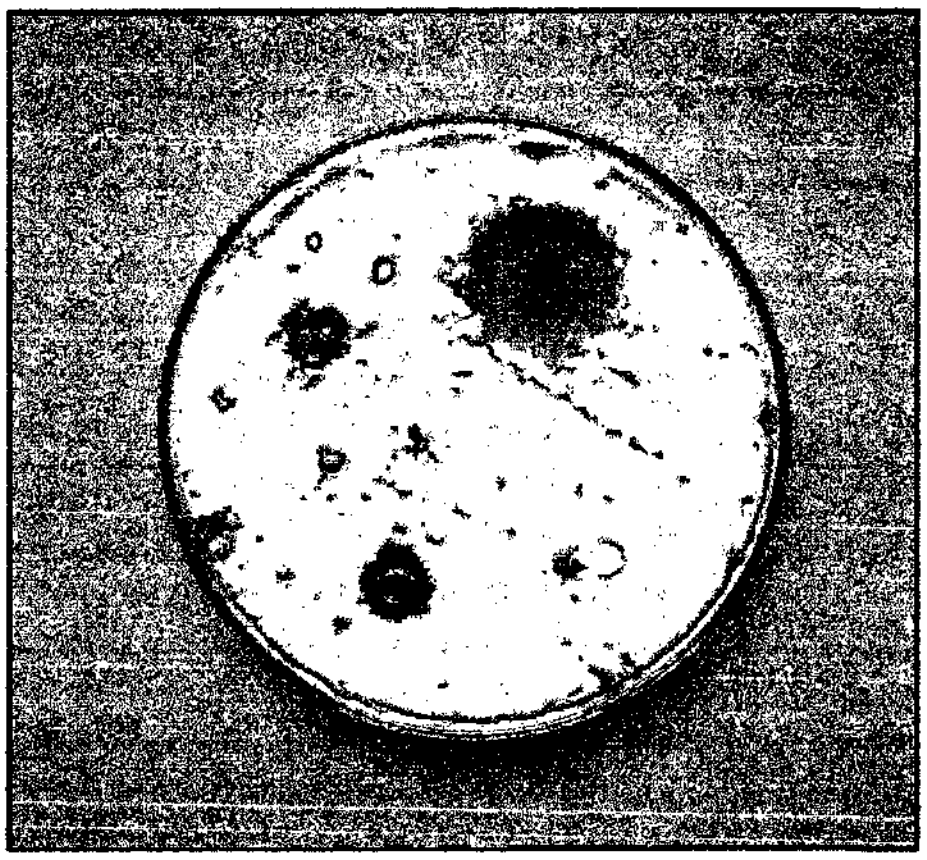

Figure 7: Activity of medicinal plant extracts made in solvents of varied polarity against *T. mentagrophytes* by Disk Diffusion *Ixora brachiata* Root

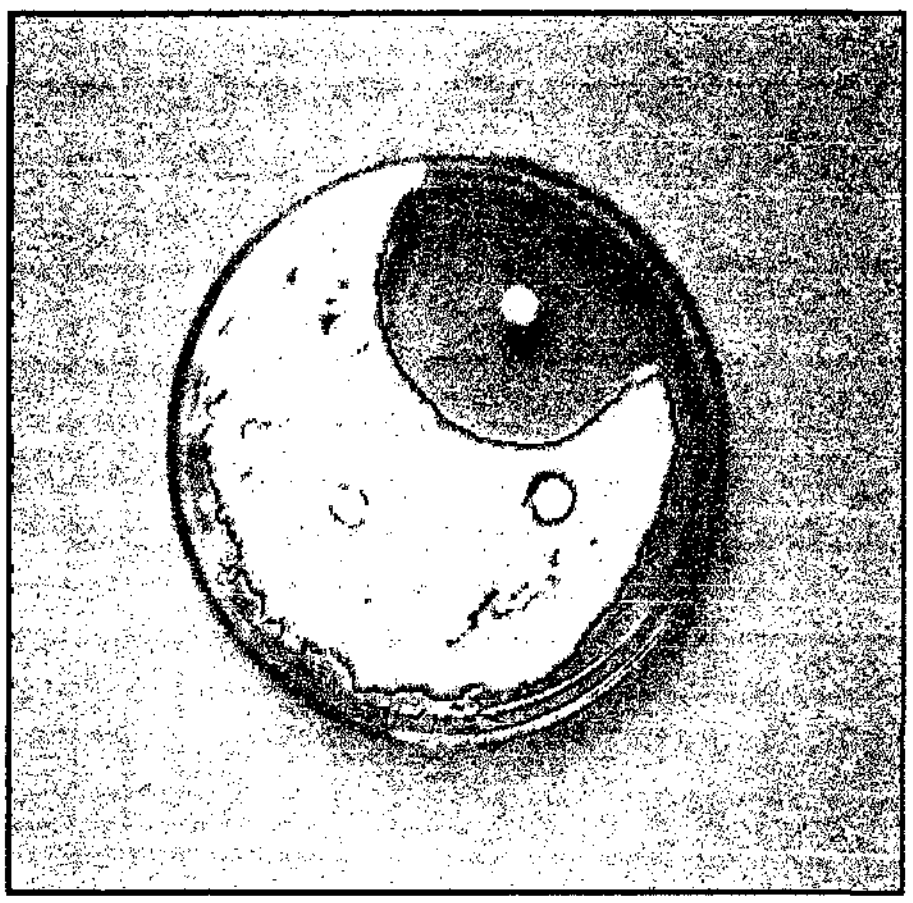

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Figure 8: Inhibitory effects of ethanolic extract of I. brachiata leaf against T. mentagrophytes by Agar Dilution Method on Mycosel medium. Decreasing dilution ranging from 0.078-5.0 mg/ml medium. MIC=5.0 mg/ml medium.

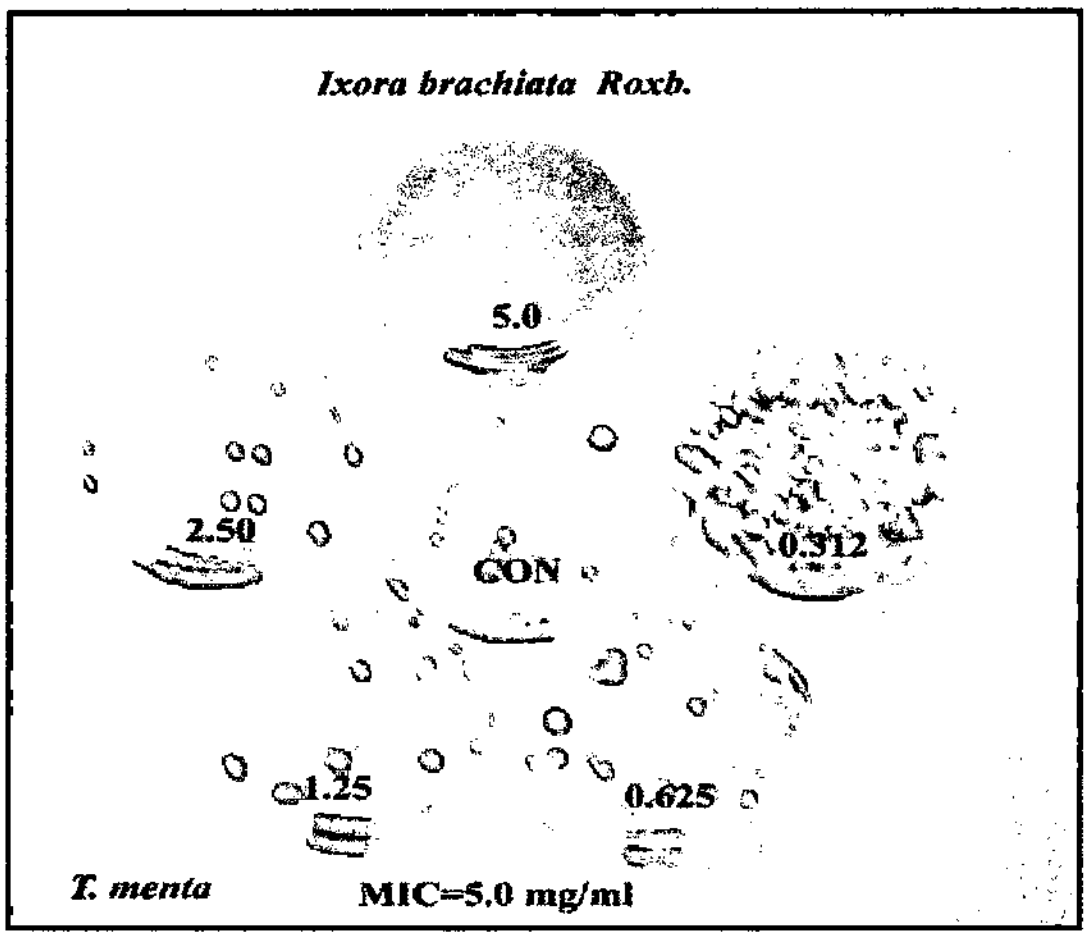

Figure 9: Inhibitory effects of ethanolic extract of *I. brachiata* root against *T. mentagrophytes* by Agar Dilution Method on Mycosel medium. Decreasing dilution ranging from 0.078 -5.0 mg/ml medium. MIC=2.50 mg/ml medium

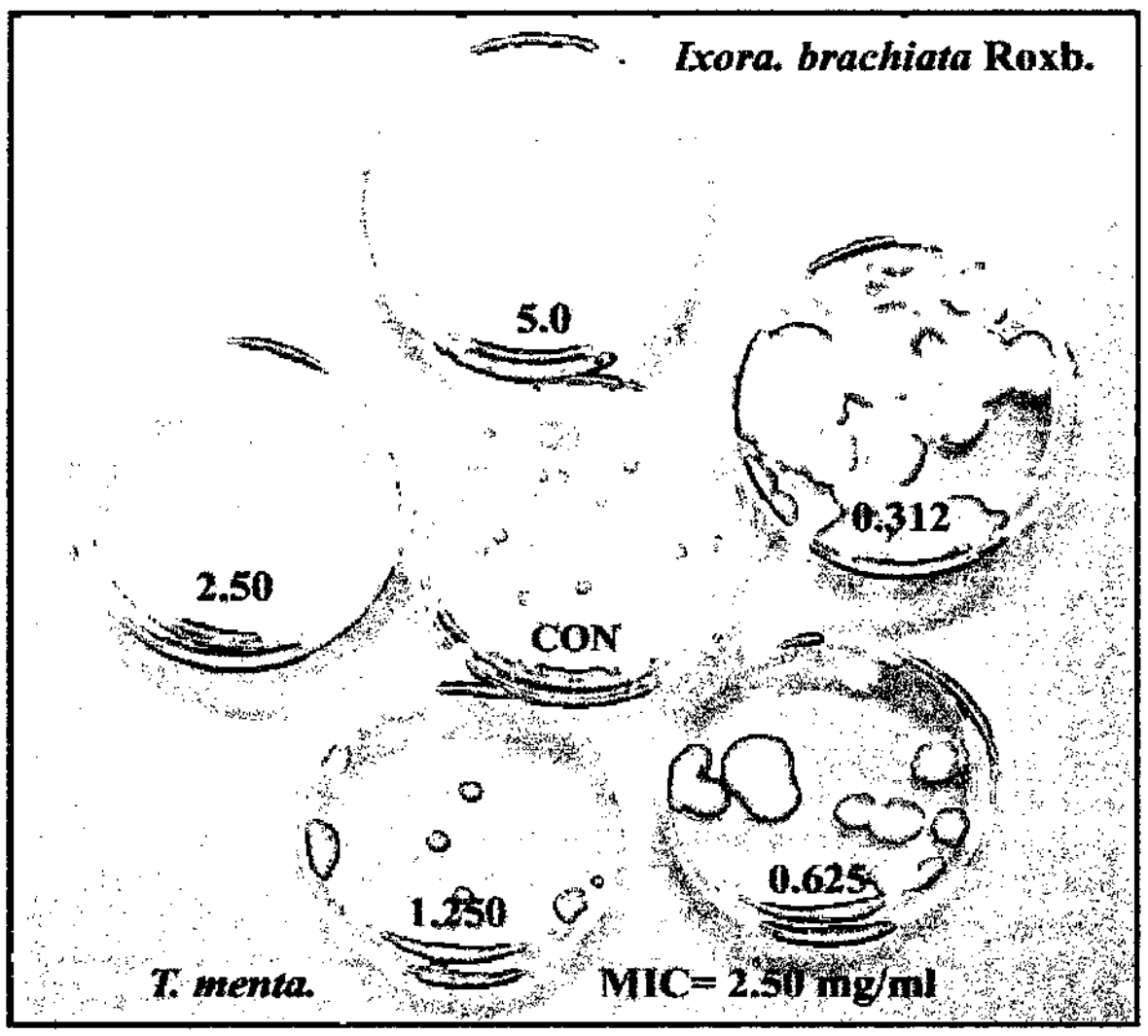

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Figure 10: HPTLC study of *I. brachiata* Root & Leaf for Detection and confirmation of Coumarins and Triterpenes

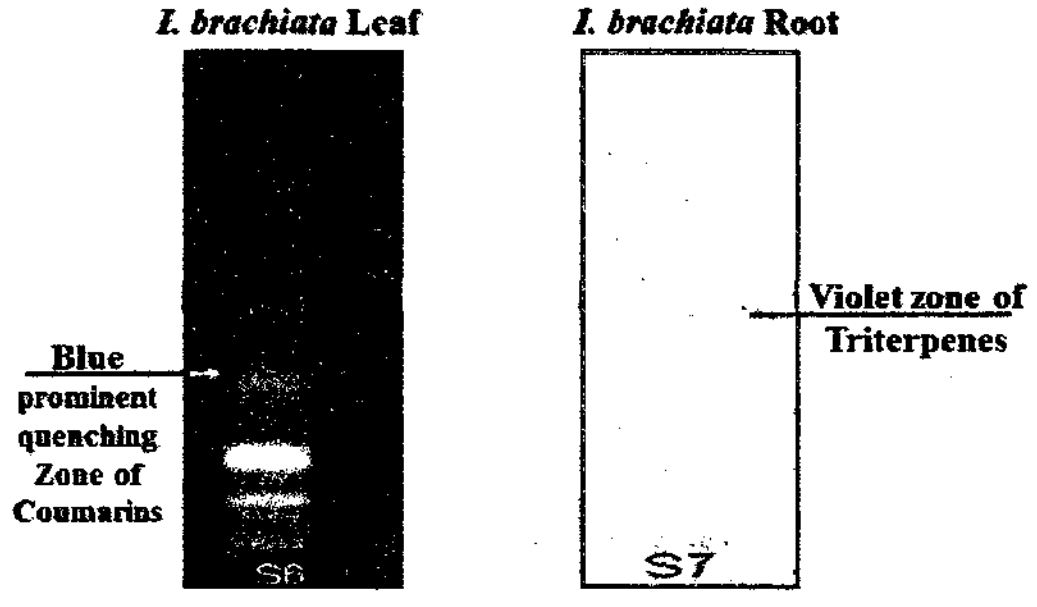

Figure 11: Chromatogram (c and d)

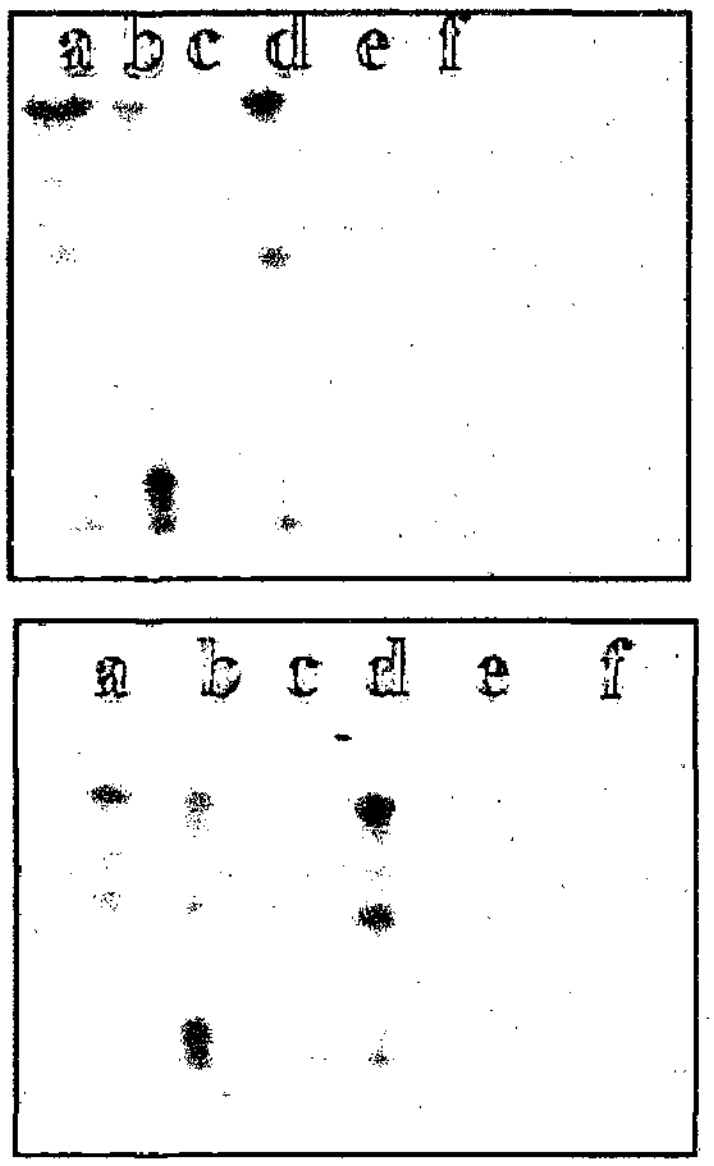

Figure 12: Bioautogram (c and d)

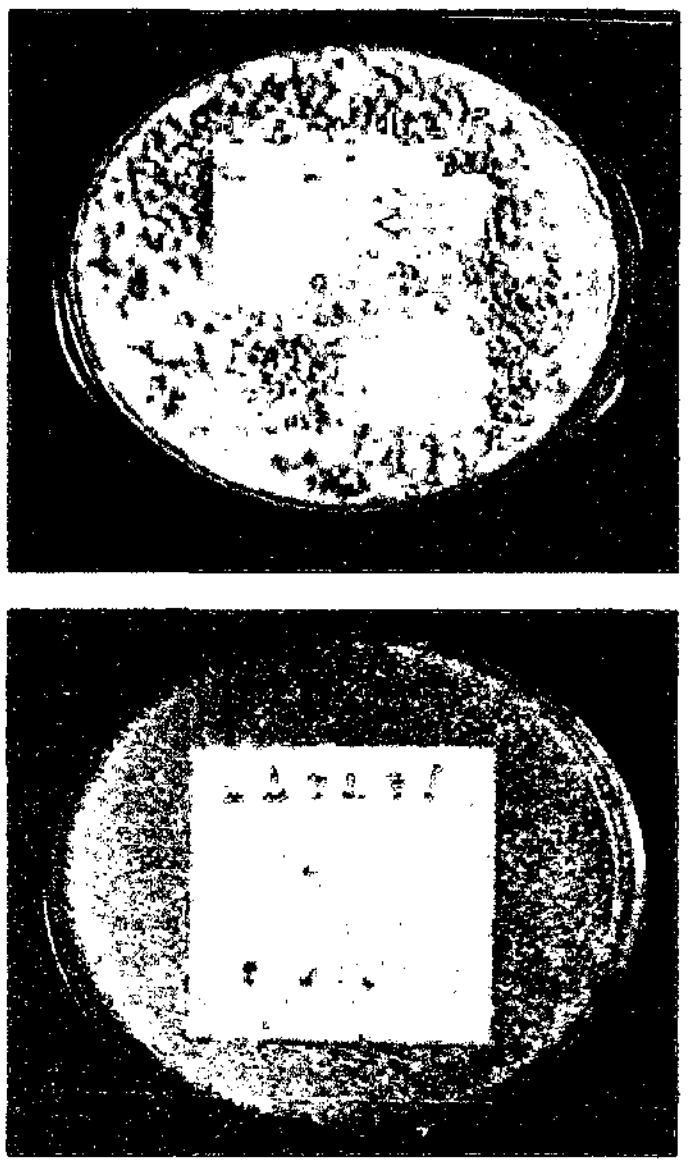

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Figure 13: Cured Patient With *I. brachiata* Root extract lotion applied topical every day for 4 weeks

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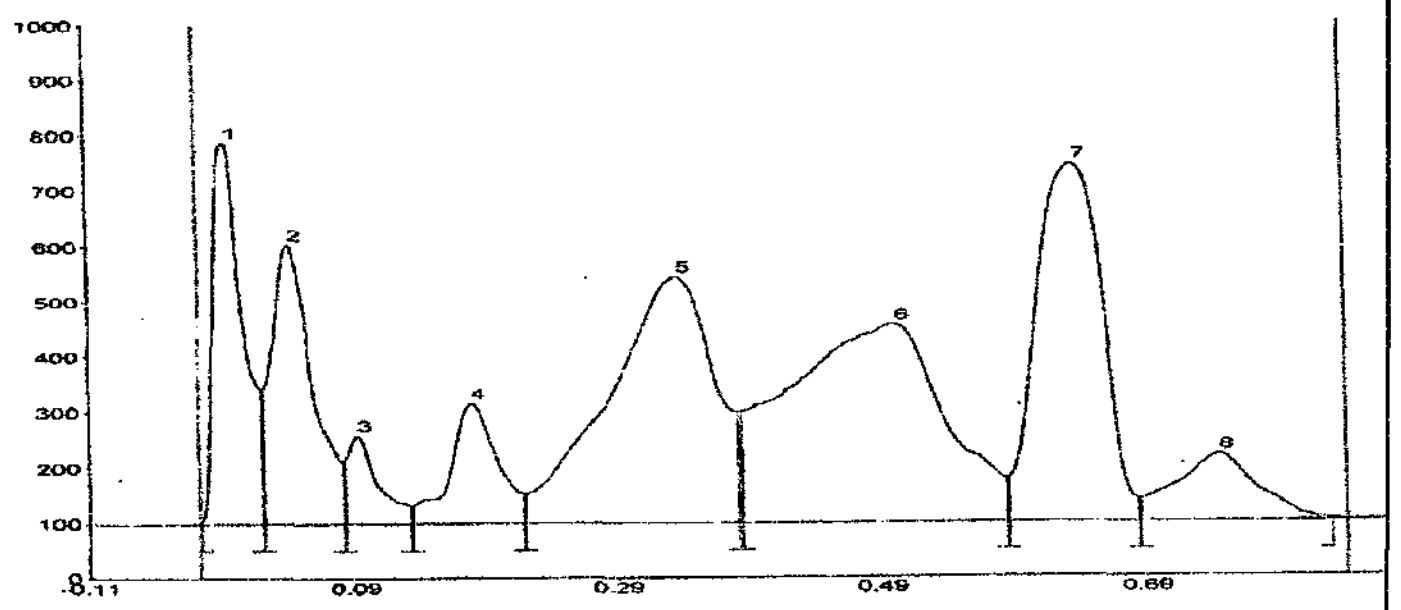
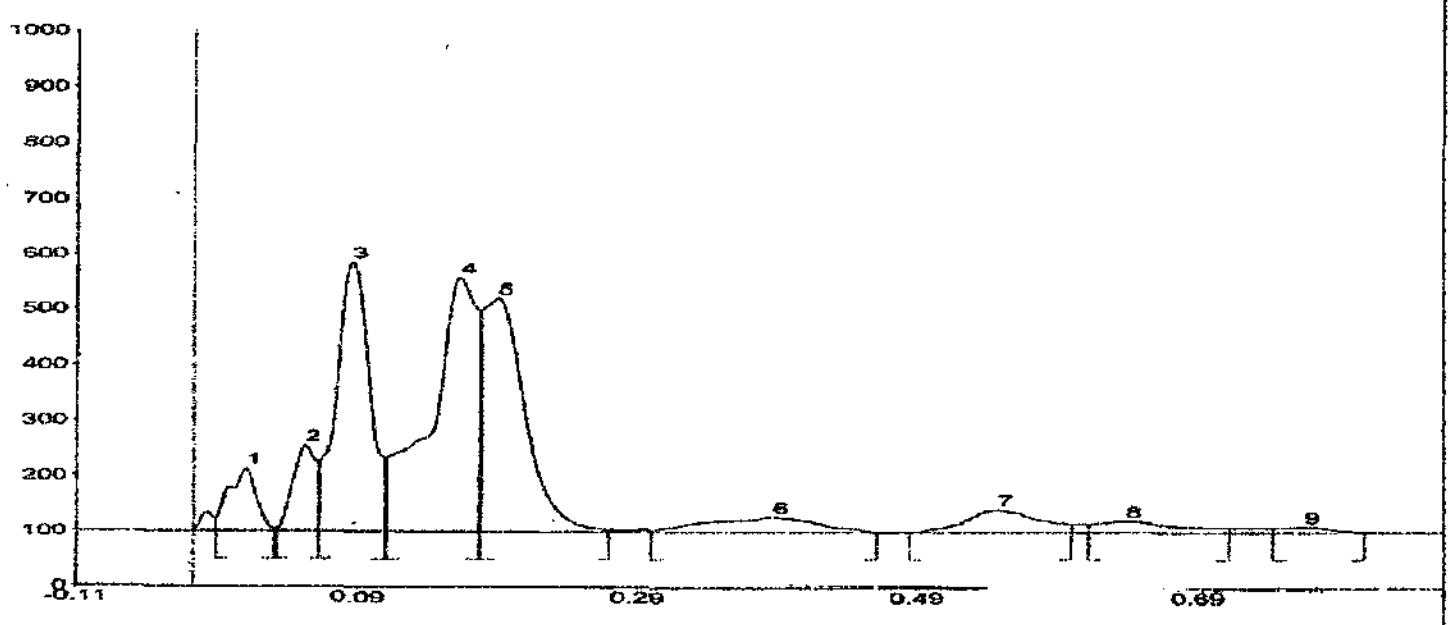
Graph 1: Estimation of Coumarins at 254 nm before Derivation from *Ixora brachiata* Leaf



Peak	Start Rf	Start Height	Max Rf	Max Height	Max %	End Rf	End Height	Area	Area %
1	-0.03	4.8	-0.01	687.4	21.93	0.02	241.3	13060.2	9.74
2	0.02	242.0	0.04	504.7	16.10	0.08	111.2	13173.0	9.82
3	0.08	114.3	0.09	159.7	5.10	0.13	33.1	3162.9	2.36
4	0.13	33.6	0.18	217.0	6.92	0.22	54.8	6602.1	4.92
5	0.22	54.9	0.34	445.3	14.21	0.38	199.6	29605.1	22.07
6	0.38	201.0	0.50	356.1	11.36	0.58	79.2	35393.5	26.38
7	0.59	80.3	0.64	643.6	20.53	0.68	40.6	26887.9	20.04
8	0.69	41.2	0.75	120.5	3.84	0.83	2.0	6272.2	4.68

Graph 2: Estimation of Coumarins at 366 nm after Derivation from *Ixora brachiata* Leaf



Peak	Start Rf	Start Height	Max Rf	Max Height	Max %	End Rf	End Height	Area	Area %
1	-0.01	24.6	0.01	114.0	6.57	0.03	3.4	1869.7	4.42
2	0.03	5.1	0.05	155.6	8.97	0.06	126.1	2165.1	5.11
3	0.06	128.5	0.08	486.9	28.07	0.11	133.9	9681.5	22.86
4	0.11	134.9	0.16	458.7	26.45	0.18	399.9	12987.2	30.67
5	0.18	400.0	0.19	420.2	24.22	0.27	3.0	10580.2	24.99
6	0.30	4.2	0.39	27.6	1.59	0.46	1.0	1846.3	4.36
7	0.48	0.7	0.55	40.6	2.34	0.60	15.7	1814.3	4.28
8	0.61	15.9	0.64	20.2	1.16	0.71	8.8	1067.1	2.52
9	0.74	7.9	0.77	10.7	0.62	0.81	1.6	331.3	0.78

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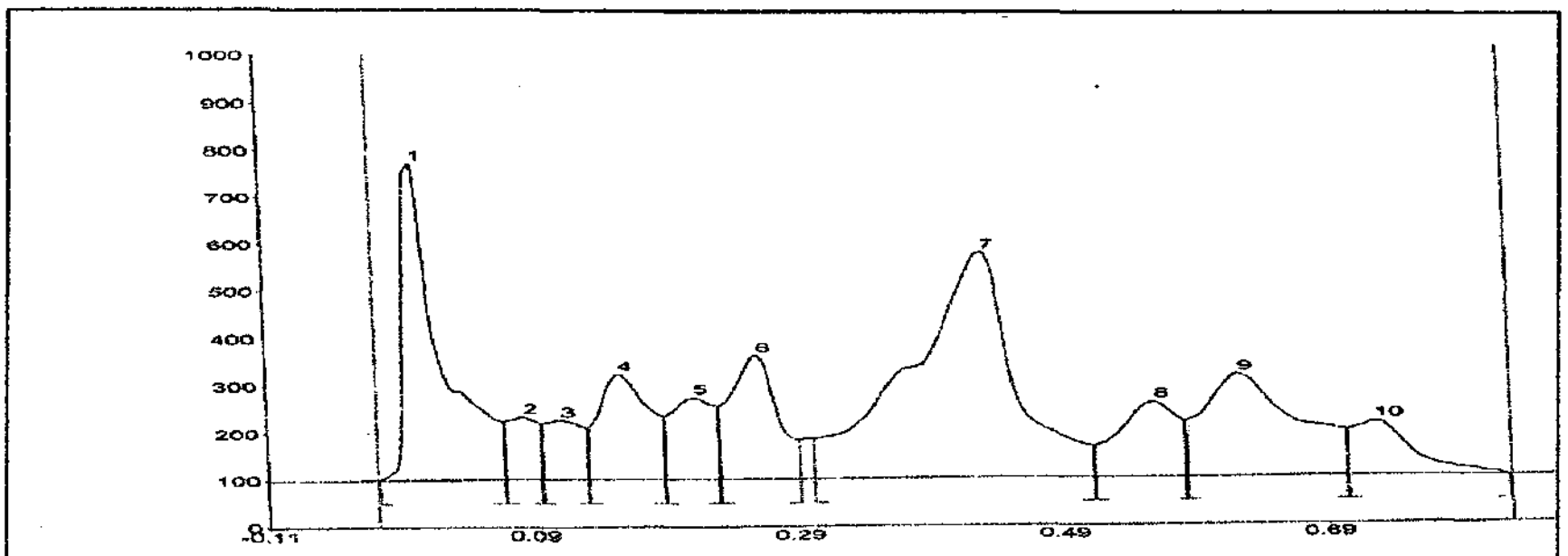
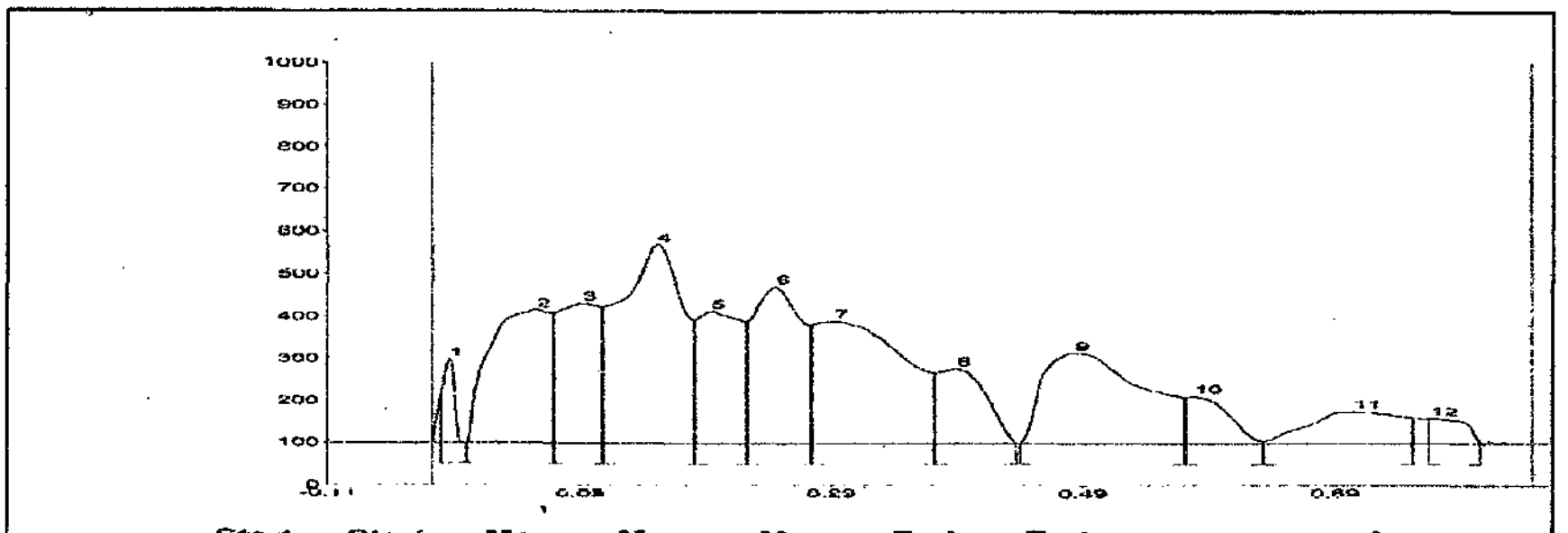
Graph 3: Estimation of Triterpenes at 254 nm before Derivation from *Ixora brachiata* Root



Peak	Start Rf	Start Height	Max Rf	Max Height	Max %	End Rf	End Height	Area	Area %
1	-0.03	0.7	0.00	671.2	26.27	0.07	125.7	16194.5	16.73
2	0.07	126.1	0.08	134.1	5.25	0.10	122.0	2578.3	2.66
3	0.10	122.2	0.11	127.0	4.97	0.13	110.2	3047.7	3.15
4	0.13	111.3	0.16	222.8	8.72	0.19	137.6	7234.2	7.47
5	0.19	137.7	0.21	174.3	6.82	0.23	156.9	4799.9	4.96
6	0.23	157.4	0.26	260.9	10.21	0.29	84.4	7889.6	8.15
7	0.30	86.4	0.43	474.6	18.58	0.51	68.7	31618.6	32.67
8	0.52	68.8	0.56	159.8	6.25	0.58	120.0	6041.7	6.24
9	0.58	120.0	0.63	215.2	8.42	0.71	99.3	12998.9	13.43
10	0.71	99.3	0.73	114.8	4.49	0.83	0.3	4389.2	4.53

Graph 4: Estimation of Triterpenes at 366 nm after Derivation from *Ixora brachiata* Root



Peak	Start Rf	Start Height	Max Rf	Max Height	Max %	End Rf	End Height	Area	Area %
1	-0.02	112.9	-0.01	197.7	6.75	-0.00	0.0	1546.1	1.39
2	0.00	1.6	0.06	314.9	10.76	0.07	309.8	12601.4	11.36
3	0.07	310.1	0.09	330.9	11.30	0.11	323.5	9058.8	8.17
4	0.11	323.7	0.15	469.5	16.04	0.18	293.9	19879.7	17.92
5	0.18	294.2	0.19	314.2	10.73	0.22	290.5	9080.1	8.18
6	0.22	290.6	0.25	371.1	12.68	0.27	282.3	12113.0	10.92
7	0.28	282.9	0.29	290.3	9.92	0.37	168.0	17424.0	15.71
8	0.37	168.1	0.39	176.4	6.03	0.44	0.5	5655.8	5.10
9	0.44	0.8	0.48	215.5	7.36	0.57	110.5	14356.5	12.94
10	0.57	110.5	0.58	111.7	3.82	0.63	6.0	3130.3	2.82
11	0.63	6.1	0.71	76.0	2.60	0.75	60.6	4658.7	4.20
12	0.77	58.8	0.77	59.0	2.01	0.81	2.2	1440.8	1.30

Name: Prof. Dr. Deokule Subhash S.

Signature:

E-3/691/2018

FORM 3
THE PATENTS ACT, 1970
[39 OF 1970]
and
THE PATENTS RULES, 2003
STATEMENT AND UNDERTAKING UNDER SECTION 8
(See Section 8; Rule 12)

200188619

1. Name of the applicants	I, Deokule Subhash S., Department of Botany, Savitribai Phule Pune University, Pune-411 007, hereby declare:
2. Name, address and nationality of the joint applicant	Dr. Jagtap Suresh D., Herbal Medicine, IRHSA, Bharati Vidyapeeth (Deemed to be university), Pune – 411 043. (i) that we have not made any application for the same/substantially the same invention outside India
3. Name and address of the assignee	(ii) that the rights in the applications have been assigned to Prof. Deokule, Department of Botany, Savitribai Phule Pune University, Ganesh Khind road, Pune-7, Maharashtra, India. that we undertake that up to the date of grant of the patent by the Controller, we would keep him informed in writing the details regarding corresponding applications for the date of filling of such application Dated this 04 day of January, 2018.
4. To be signed by the applicant or his authorized registered patent agent	Deokule
5. Name of the natural person who has signed	Prof. Dr. Deokule Subhash S. Professor (Rtd.) Department of Botany, Savitribai Phule Pune University, Ganeshkhind road, Pune-7, Maharashtra, India.
	To, The Controller of Patents, The Patent Office, at Mumbai

RECD MUMBAI 04-01-2018 16:34

E-5|16|2018

FORM 5
THE PATENTS ACT, 1970
(39 of 1970)
&
The patents rules, 2003
DECLARATION AS TO INVENTORSHIP
[See section 10 (6) and rule 13(6)]

200188620

1. NAME OF APPLICANTS	(i) Prof. Dr. Deokule Subhash S.

Hereby declare that the true and first inventor(s) of the invention disclosed in the complete specification filed in pursuance of our application numbered..... Dated....04\01\2018.....are

2. INVENTOR(S)

a.	**Name**	:	Prof. Dr. Deokule Subhash S.
b.	**Nationality**	:	Indian
c.	**Address**	:	Department of Botany, Savitribai Phule Pune University, Ganeshkhind Road, Pune-411007. Maharashtra, India.
a.	**Name**	:	Dr. Batool Sadegi
b.	**Nationality**	:	Iranian
c.	**Address**	:	Department of Botany, Savitribai Phule Pune University, Ganeshkhind Road, Pune-411007, Maharashtra, India
a.	**Name**	:	Dr. BipinRaj N. K.
b.	**Nationality**	:	Indian
c.	**Address**	:	Department of Microbial Biotechnology, Bharati Vidyapeeth (Deemed to be University), Rajiv Gandhi Institute of Information technology and Biotechnology, Pune – 411 043. Maharashtra, India.
a.	**Name**	:	Dr. Mokat Digambar N.
b.	**Nationality**	:	Indian
c.	**Address**	:	Department of Botany, Savitribai Phule Pune University, Ganeshkhind Road, Pune-411007. Maharashtra, India.
a.	**Name**		Dr. Nangare Ninad B.
b.	**Nationality**		Indian
c.	**Address**		Department of Dravyaguna, Bharati Vidyapeeth (Deemed to be University), College of Ayurved, Pune Satara Road, Pune – 411 043. Maharashtra, India.

a.	**Name**	:	Dr. Nagarkar Bhagyashri E
b.	**Nationality**	:	Indian
c.	**Address**	:	Herbs Foundation, A1-604, Dream City, Behind Telco Coloney, Dattanagar, Ambegaon, Pune – 411 046, Maharashtra, India.
a.	**Name**	:	Dr. Jagtap Suresh D.
b.	**Nationality**	:	Indian
c.	**Address**	:	Herbal Medicine, Bharati Vidyapeeth (Deemed to be University), Interactive Research School for Health Affairs (IRSHA), Pune Satara Road, Pune – 411 043. Maharashtra, India.

Dated this ..04.... day of ..January.....2018

Savitribai Phule Pune University ★Dept. of Botany★

Signature:

Prof. Dr. Deokule S. S.

Retd. PROFESSOR
Department of Botany
Savitribai Phule Pune University
Pune - 411 007.

3. DECLARATION TO BE GIVEN WHEN THE APPLICATION IN INDIA IS FILED BY THE APPLICANTS IN THE CONVENTION COUNTRY:-

-NOT APPLICABLE-

4. STATEMENT (to be signed by the additional inventors not mentioned in the application form)

-NOT APPLICABLE-

To,

The Controller of Patent

The Patent Office, at Mumbai

200293029

FORM 18 THE PATENTS ACT 1970 (39 of 1970) & The Patents Rules, 2003 REQUEST/EXPRESS REQUEST FOR EXAMINATION OF APPLICATION FOR PATENT [See section 11B and rule 20(4)(ii), 24B(1)(i)]	(FOR OFFICE USE ONLY) RQ No: R202020312 86 Filing Date: 08/10/2020 Amount of Fee Paid: CBR No: 4400/ रूपये (नकद/चेक/मनी ऑर्डर द्वारा Signature CBR संख्या. 26226 ..दि..08/10/2020 के तहत प्राप्त हुए।

1. APPLICANTS

(a)	Name:	Deokule Subhash Sadhu
(b)	Nationality:	Indian
(c)	Address:	Department of Botany, Savitribai Phule Pune University (SPPU), Pune- 411 007, Maharashtra, India.

2. Statement in case of request for examination made by the applicants

We hereby request that our application for patent no.: **201821000396** filed on **04/01/2018** for the invention titled **"Development of new herbal lotion for the management of Dermatophytic infections"** shall be examined under sections 12 and 13 of the Act.

3. Statement in case of request for examination made by any other interested person

I/We the interested person request for the examination of the application no._____dated _____filed by the applicant_____titled_____under sections 12 and 13 of the Act.

As an evidence of my/our interest in the application for patent following documents are submitted.

(a) _____

Not applicable

4. ADDRESS FOR SERVICE

Department of Botany, Savitribai Phule Pune University (SPPU), Pune- 411 007, Maharashtra, India. **Email:** deokule.ss@gmail.com. **Mobile:** 9371088210.

Dated this 8 day of October 2020

Deokule

Deokule Subhash Sadhu

To,
The Controller of Patents
The Patent Office,
Mumbai

National Biodiversity Authority

राष्ट्रीय जैव विविधता प्राधिकरण

(Statutory body of Ministry of Environment, Forest and Climate Change, Government of India)

J. Justin Mohan, IFS
Secretary
+91 44 2254 1071
+91 44 2254 1074
secretary@nba.nic.in www.nbaindia.org

5th Floor, CSIR Road, TICEL Bio Park,
Taramani, Chennai - 600 113, Tamil Nadu, India.
5 वां तल, सीएसआईआर रोड, टाइसल बायो पार्क,
तरमणि, चेन्नई - 600113 तमिल नाडु, भारत.

NBA/IPR-Gen/33/17-1/18-19/2155

30 .10.2019

To

Shri. O P Gupta, IAS
Controller General of Patents, Designs & Trade Marks
Intellectual Property India,
Patents/Designs/Trade Marks/Geographical Indications,
Boudhik Sampada Bhavan,
Antop Hill, S.M. Road,
Mumbai-400037

Sir,

Sub: Patent application number 201821000396 –Requirement of Prior-approval from NBA- reg.

This has reference to patent application no.; 201821000396 titled "Development of new herbal lotion for the management of dermatophytic infections" filed by Prof Dr. Deokule Subhash. S and published by IPO. As you are aware, Section 6 of the Biological Diversity Act, 2002 mandates that any person applying for any Intellectual Property Right for an invention based on any research or information on a biological resource obtained from India, shall obtain prior approval of NBA.

In the patent application referenced above, the applicant has used *Ixora brachiata* for developing the claimed invention, the source of which is mentioned as Ratnagiri district. The applicant has also given a declaration in the Form I that the invention disclosed uses biological material from India and necessary permission from the competent authority will be obtained. However, the applicant has not obtained requisite approval under Section 6 of the Biological Diversity Act, 2002. Hence, it is requested that the applicant may be instructed to seek approval under section 6 and the patent shall not be granted until approval is obtained from National Biodiversity Authority.

Yours faithfully,

(J. Justin Mohan)
Secretary, NBA

Copy to:
Prof Dr. Deokule Subhash S, Department of Botany, Savitribai Phule Pune University (SPPU), Ganeshkhind Road, Pune-411 007, Maharashtra. E-mail: deokule.ss@gmail.com

D.NO. 384

SAVITRIBAI PHULE PUNE UNIVERSITY

(formerly University of Pune)

DEPARTMENT OF BOTANY
SAVITRIBAI PHULE PUNE UNIVERSITY
Ganeshkhind, Pune-411 007
Tele. No. : (020) 25601439, 25601438
Email : .....@unipune.ac.in

Ref. No. : Bot/ E-101/147/2018 **Date :** 04/01/2018

To,
The Controller of Patents,
The Patent Office,
Boudhik Sampada Bhavan,
S. M. Road, Antop Hill, Mumbai,
Maharashtra- 400037

200188617

Subject: Submission of Complete Patent Specification

Dear Sir,

Please find herewith documents for a complete specification application entitled **"Development of new herbal lotion for the management of dermatophytic infections"** along with the prescribed fee of Rs. 1750/-.

The Learned Controller is requested to take the same on record and issue the official filing receipt for the same.

Yours faithfully,

Prof. Dr. Deokule Subhash S.
Department of Botany,
Savitribai Phule Pune University (SPPU),
Ganeshkhind Road,
Pune- 411007. Maharashtra, India

Enclosures:
1. Application for Grant of Patent [Form 1]
2. Complete specification [Form 2]
3. Statement and undertaking under section 8 [Form 3]
4. Declaration as to inventorship [Form 5]
5. Fees of Rs. 1750/- (by cash)

ई-पोच मिळाली 04-01-2018 १६:३१

"FORM 1 THE PATENTS ACT 1970 (39 of 1970) and THE PATENTS RULES, 2003 **APPLICATION FOR GRANT OF PATENT** (See section 7, 54 and 135 and sub-rule (1) of rule 20)	(FOR OFFICE USE ONLY) 200188613

	Application No.	201821000395
	Filing date	04-01-2018
	Amount of Fee paid	Rs. 1750/-
	CBR No	160
	Signature	

1. APPLICANT'S REFERENCE / IDENTIFICATION NO. (AS ALLOTTED BY OFFICE)

1750 रूपये नकद/चेक/मनी ऑर्डर द्वारा CBR संख्या 160 दि. 04/01/2018 के तहत भरा हुए।

2. TYPE OF APPLICATION [Please tick (√) at the appropriate category]

Ordinary (√)		Convention ( )		PCT-NP ( )	
Divisional ( )	Patent of Addition ( )	Divisional ( )	Patent of Addition ( )	Divisional ( )	Patent of Addition ( )

3A. APPLICANTS

Name in Full	Nationality	Country of Residence	Address of the Applicant	
Prof. Dr. Deokule Subhash S.	Indian	India	House No.	Department of Botany, Savitribai Phule Pune University (SPPU)
			Street	Ganeshkhind Road,
			City	Pune
			State	Maharashtra
			Country	India
			Pin code	411 007.

3B. CATEGORY OF APPLICANT [Please tick (√) at the appropriate category]

Natural Person (√)	Other than Natural Person		
	Small Entity ( )	Startup ( )	Others ( )

4. INVENTORS [Please tick (√) at the appropriate category]				
Are all the inventors same as the applicants named above?			Yes ( )	No (√)
If "No", furnish the details of the inventor(s)				
Name in Full	Nationality	Country of Residence	Address of the Inventor	
Prof. Dr. Deokule Subhash S.	Indian	India	House No.	Department of Botany, Savitribai Phule University of Pune (SPPU).
			Street	Ganeshkhind Road
			City	Pune
			State	Maharashtra
			Country	India
			Pin code	411007
Dr. Batool Sadegi	Iranian	Iran	House No.	Department of Botany, Savitribai Phule Pune University (SPPU).
			Street	Ganeshkhind Road
			City	Pune
			State	Maharashtra
			Country	India
			Pin code	411007
Mr. Veer Sanjay Laxman	Indian	India	House No.	Herbs Foundation, A1-604, Dream City, Behind Telco Coloney, Dattanagar, Ambegaon, Pune.
			Street	Jambulwadi Road
			City	Pune
			State	Maharashtra
			Country	India
			Pin code	411 046.
Mr. Jagtap Nitin Suresh	Indian	India	House No.	At Post Kothale, Taluka- Purandhare, District –Pune.
			Street	Pune Jejuri Road
			City	Pune
			State	Maharashtra
			Country	India
			Pin code	412 301
Dr. Bhongale Chetan Jagdish	Indian	India	House No.	School of Energy Studies, Savitribai Phule Pune University (SPPU).
			Street	Ganeshkhind Road
			City	Pune
			State	Maharashtra
			Country	India
			Pin code	411 007
Mr. Bhongale Suraj Rajendra	Indian	India	House No.	Department of Microbial Biotechnology, Bharati Vidyapeeth (Deemed to be University), Rajiv Gandhi Institute of Information Technology and Biotechnology.
			Street	Pune Satara Road
			City	Pune
			State	Maharashtra
			Country	India
			Pin code	411 043

Dr. Jagtap Suresh D.			House No.	Herbal Medicine, Interactive Research School for Health Affairs (IRSHA), Bharati Vidyapeeth University.
			Street	Pune Satara Road
			City	Pune
			State	Maharashtra
			Country	India
			Pin code	411 043

5. TITLE OF THE INVENTION

Development of new herbal Ointment for the management of dermatophytic infections

6. AUTHORISED REGISTERED PATENT AGENT(S)	IN/PA No.	
	Name	
	Mobile No.	
7. ADDRESS FOR SERVICE OF APPLICANT IN INDIA	Name	Prof. Dr. Deokule Subhash S.
	Postal Address	Department of Botany, Savitribai Phule Pune University (SPPU), Ganeshkhind Road, Pune- 411007. Maharashtra, India
	Telephone No.	020 2560 1217
	Mobile No.	09371088210
	Fax No.	020 2560 1217
	Email ID	deokule.ss@gmail.com

8. IN CASE OF APPLICATION CLAIMING PRIORITY OF APPLICATION FILED IN CONVENTION COUNTRY, PARTICULARS OF CONVENTION APPLICATION

- NOT APPLICABLE -

9. IN CASE OF PCT NATIONAL PHASE APPLICATION, PARTICULARS OF INTERNATIONAL APPLICATION FILED UNDER PATENT CO-OPERATION TREATY (PCT)

- NOT APPLICABLE -

10. IN CASE OF DIVISIONAL APPLICATION FILED UNDER SECTION 16, PARTICULARS OF ORIGINAL (FIRST) APPLICATION

- NOT APPLICABLE -

11. IN CASE OF PATENT OF ADDITION FILED UNDER SECTION 54, PARTICULARS OF MAIN APPLICATION OR PATENT

- NOT APPLICABLE -

12. DECLARATIONS

(i) Declaration by the inventor(s):

We, the above named inventors are the true & first inventors for this invention and declare that IRSHA, BVDU herein our assignee.

Signature:

Name: Prof. Dr. Deokule Subhash S. Date: 01/01/2018

Signature:

Name: Dr. Batool Sadegi Date: 02/01/2018

Signature:

Name: Mr. Veer Sanjay L. Date: 01/01/2018

Signature:

Name: Mr. Jagtap Nitin S. Date: 01/01/2018

Signature:

Name: Dr. Bhongale Chetan J. Date: 01/01/2018

Signature:

Name: Mr. Bhongale Suraj R. Date: 03/01/2018

Signature:

Name: Dr. Jagtap Suresh D. Date: 01/01/2018

(ii) Declaration by the applicant(s) in the convention country

- NOT APPLICABLE -

Declaration by the applicants:

We the applicants hereby declares that:-

√	We are in possession of the above-mentioned invention.
√	The complete specification relating to the invention is filled with this application.
√	The invention as disclosed in the specification uses the biological material from India and the necessary permission from the competent authority shall be submitted by us before the grant of patent to us.
√	There is no lawful ground of objection to the grant of the patent to us.
√	We are the true & first inventors.
√	We are the assignee of true and first inventors.
√	The application or each of the applications, particulars of which are given in Paragraph-8, was the first application in convention countries in respect of our inventions.
√	We claim the priority from the above mentioned application(s) filed in convention countries and state that no application for protection in respect of the invention had been made in a convention country before that date by us or by any person from which we derive the title.
X	Our application in India is based on international application under Patent Cooperation Treaty (PCT) as mentioned in Paragraph-9.
X	The application is divided out of our application particulars of which is given in Paragraph-10 and pray that this application may be treated as deemed to have been filed on .....under section 16 of the Act.
X	The said invention is an improvement in or modification of the invention particulars of which are given in Paragraph-11.

13. FOLLOWING ARE THE ATTACHMENT WITH THE APPLICATION:

(a) Form 2

Item	**Details**	**Fee**	**Remarks**
Complete specification	No. of pages: 19	1750/-	
No. of Claims	No. of claims - 6 No. of pages - 1		
No of drawing sheets	07		
No. of figures	09		
No. of tables	06		
No of abstract pages	01		

(b) Complete specification (1 copies)

(c) Statement and undertaking on Form 3

(d) Declaration as to Inventorship on Form 5

(e) Total fee Rs. 1750/- in cash.

We hereby declare that to the best of my/our knowledge, information and belief the fact and matters stated herein are correct and I/we request that a patent may be granted to me/us for the said invention.

Dated this ..04....day of .January.....2018.

Signature:

Prof. Dr. Deokule Subhash S.
Department of Botany,
Savitribai Phule Pune University (SPPU),
Ganeshkhind Road,
Pune- 411007. Maharashtra, India

To,

THE CONTROLLER OF PATENTS,

THE PATENT OFFICE, MUMBAI

FORM 2

THE PATENT ACT 1970

(39 OF 1970)

&

The patents rules, 2003

COMPLETE SPECIFICATION

(See section 10 and rule 13)

1. TITLE OF THE INVENTION:

Development of new herbal ointment for the management of dermatophytic infections

2. APPLICANT (S):

a.	**Name**	:	Prof. Dr. Deokule Subhash S.
b.	**Nationality**	:	Indian
c.	**Address**	:	Department of Botany, Savitribai Phule Pune University, Ganeshkhind Road, Pune-411007, Maharashtra, India.
a.	**Name**	:	Dr. Sadegi Batool
b.	**Nationality**	:	Iranian
c.	**Address**	:	Department of Botany, Savitribai Phule Pune University, Ganeshkhind Road, Pune-411007, Maharashtra, India.
a.	**Name**	:	Mr. Veer Sanjay L.
b.	**Nationality**	:	Indian
c.	**Address**	:	Herbs Foundation, A1-604, Dream City, Behind Telco Coloney, Dattanagar, Ambegaon, Pune- 411 046, Maharashtra, India.
a.	**Name**	:	Mr. Jagtap Nitin S.
b.	**Nationality**	:	Indian
c.	**Address**	:	At post Kothale, Taluka - Purandhar, District-Pune, Jejuri Road, Pune-41230, Maharashtra, India.
a.	**Name**	:	Dr. Bhongale Chetan J.
b.	**Nationality**	:	Indian
c.	**Address**	:	School of Energy Studies, Savitribai Phule Pune University (SPPU), Ganeshkhind Road, Pune – 411 007, Maharashtra, India.
a.	**Name**	:	Mr. Bhongale Suraj R.
b.	**Nationality**	:	Indian
c.	**Address**	:	Department of Microbial Biotechnology, Bharati Vidyapeeth (Deemed to be University) Rajiv Gandhi Institute of Information Technology and Biotechnology, Pune Satara Road, Pune- 411 043, Maharashtra, India.
a.	**Name**	:	Dr. Jagtap Suresh D.
b.	**Nationality**	:	Indian
c.	**Address**	:	Herbal Medicine Bharati Vidyapeeth (Deemed to be University), Interactive Research School for Health Affairs (IRSHA), Pune Satara Road, Pune – 411 043. Maharashtra, India.

3. PREAMBLE TO THE DESCRIPTION	
PROVISIONAL NOT APPLICABLE	**COMPLETE** The following specification describes the invention.

4. DESCRIPTION

Field of the Invention

There are several reports on antidermatophytic activity of *Satureja khuzestanica* Jamzad. (Labiatae). The present investigation includes the in-*vitro* and *in-vivo* studies of antidermatophytic activity of *Satureja khuzestanica* Jamzad. In *in-vivo* assay, the therapeutic effectiveness of *Satureja khuzestanica* leaf ointment showed significant results without any notable external effects. The results of this investigation will be helpful for skin diseases caused by dermatophytic fungi such as *Epidermophyton floccosum, Microsporum canis, Microsporum gypseum, Trichophyton mentagrophytes* and *Trichophyton rubrum.*

Background of the invention:

Mycotic infections are probably the most common cause of skin disease in developing countries among them dermatophytosis is the most frequent superficial fungal infection in India. The following are the common infection in these area *Tinea pedis, Tinea corporis, Tinea cruris, Tinea capitis, Tinea faciei* and *Tinea manuum* caused by *Epidermophyton floccosum, Microsporum canis, Microsporum gypseum, Trichophyton mentagrophytes, Trichophyton rubrum* and *Trichophyton verrecusum* respectively. Many drugs are available to treat these infections, however, they exhibit several side effects and have limited efficacy. Owing to the side effects, there is a distinct need for the discovery of new safer and more effective antifungal agents. The use of herbal drugs in the treatment of skin diseases including mycotic infections is an age-old practice in many parts of the world (Irobi et al. 1993). This use has been supported by the isolation of active antifungal compounds from plant extracts (Costa et al. 2000).

Satureja khuzestanica* Jamzad (Labiatae). *S. khuzestanica (*Marzeh khuzestanica* in Persian). It is a branched shrub with ± 30 cm high and densely leafy stem (Figure 1). The plant has a pleasant fragrant especially in December - April during flowering. The plant materials were collected from Khoaramabad of Lorestan province of southern part of Iran. It is being used as antiseptic in folk medicine and tonic, carminative, astringent and expectorant in treating stomach and intestinal disorders. (Zargari, 1990). It possesses antibacterial and antifungal activity (Azaz, 2002 and Sokovic, 2002). Phytochemical screening of two Iranian *Satureja* species viz. *S. edmondi* and *S. isophylla* were carried out and showed that the oil of *S. edmondi* with p-cymene, gamma-terpinene, thymol and alpha-terpineol and while the oil of *S. isophylla* contains alpha, beta and gamma-eudesmol, camphor, beta-caryophyllene geranial respectively (Sefidkon and Jamzad, 2004). Recently (Farsam, 2004 and Sefidkon, 2000) have reported chemical composition of the essential oils of wild as well as cultivated *S. khuzestanica.*

Medicinal uses are as an analgesic and antiseptic in folk medicine (Zargari, 1990). It possesses antiviral (Abad, 1999); antibacterial and antifungal activity (Azaz, 2002 and Sokovic, 2002). Also reported as antispasmodic & antidiarrhea (Hajhashemi, 2000) and vasodilatory (Sanchez, 1999).

Dermatophytes:

Dermatophytes are a closely interrelated group of keratinophilic fungi that cause infections of skin, hair, and nail known as dermatophytoses. Terms such as *Tinea*, ringworm, trichophytia, and athlete's foot are also used to refer to these infections (Rippon, 1988; Elewski, 1992; Martin, 1993).

Dermatophytes can be classified according to their natural habitats into following categories: (1) *geophilic*, (2) *zoophilic*, and (3) *anthrophilic.*

The *geophilic* dermatophytes are normally live in the soil, contribute to the breakdown of the keratinous stroma of the fallen horns, feathers, and skin animals. Many of them are nonpathogenic, but some can infect both the animals and humans. They can be isolated from the soil by the hair-baiting technique.

The *Zoophilic* dermatophytes are primarily parasitizing the body surfaces of animals and then transmitted to humans.

The *Anthropophilic* dermatophytes are generally infect humans and then transmitted between individuals.

Tinea corporis is dermatophytosis of the glabrous skin of the trunk and extremities (Drake et al. 1996), commonly referred as ringworm. This infection consists of a round, scaly patch that has a prominent, enlarging border and a relatively clear central portion (Rosen, 1997). The prominent edge often contains pustules or follicular papules, and multiple lesions can be present. Itching is variable and not diagnostic. *T rubrum* is the most common cause of *Tinea corporis*. A deep form of *Tinea corporis* known as trichophytic granuloma can develop when the fungus is driven down into the hair follicles. It typically develops after inappropriate topical corticosteroid therapy (Rosen, 1997). *Tinea corporis* may appear similar to the infection include eczema, plaque psoriasis and contact dermatitis. *Tinea corporis* (ringworm of glabrous skin of the trunk) characterized by both inflammatory lesions and non-inflammatory lesions.

Tinea pedis, also referred as athlete's foot or ringworm of the foot, is the most common dermatophytosis in warm and humid climate and may affect up to 70% of adult population of the world (Elewski, 1992). It is characterized by presence of inflammatory and non-inflammatory lesions on the plantar surface and interdigital spaces of the foot. Other causative agents of dermatophytosis are *Trichophyton rubrum, Epidermophyton floccosum Trichophyton mentagrophytes*, yeasts (e.g. *Candida albicans*) and bacteria (e.g. *Corynebacterium minutissimum*) (Hay and Mackenzie, 1992).

The three most common clinical forms of *Tinea pedis* are interdigital, Moccasin-type or hyperkeratotic. Interdigital infection often presents as white, macerated skin between the fourth and fifth toes but it may appear in any web space and it typically produces itching and a foul smell from bacterial super infection with diphtheroids or *Pseudomonas* species. In moccasin-type or hyperkeratotic *Tinea pedis* usually presents as silvery white scales on a red, thickened base. Typically, moccasin-type *Tinea pedis* is a chronic condition (Kwon, 1992). Dry-type interdigital infection can mimic psoriasis (Hay and Mackenzie, 1992). Moccasin-type *Tinea pedis* may appear similar to long-standing contact dermatitis of the foot.

Tinea manuum (ringworm of the hand) is an unusual dermatophytic infection of the interdigital and palms surfaces (Elewski, 1992), usually of one hand but sometimes of both. It may coexist with other fungal infections, such as *Tinea pedis*. The palm surface often has diffuse areas of dry, hyperkeratotic skin.

Tinea faciei (ring worm of the face) also known as *Tinea incognito* of its subtle appearance is dermatophytosis of the nonbearded area of the face (Drake et al. 1996). The condition may present as itchy, red skin without a discernible border or it may has a raised border as seen with *Tinea corporis*. Common causative organisms are *T. rubrum* and *T. mentagrophytes* (Emanuel et al. 1996). The infection often responds to topical therapy.

Antifungal Drugs and Their Action

Polyene Antibiotics

These act by binding tightly to sterols present in cell membranes. The resulting deformity of the membrane allows leakage of intracellular ions and enzymes, causing cell death. Those polyenes that have useful antifungal activity bind selectively to ergo sterol, the most important sterol in fungal (but not mammalian) cell walls.

Azoles

Antifungal azoles comprise the following:

- *Imidazoles* like ketoconazole, miconazole and clotrimazole interfere with fungal oxidative enzymes to cause lethal accumulation of hydrogen peroxide; they also reduce the formation of ergo sterol, an important constituent of the fungal cell wall, which thus becomes permeable to intracellular constituents. Every effective, but lack of selectivity in these actions results in important adverse effects.
- *Triazoles* (fluconazole, itraconazole) damage the fungal cell membrane by inhibiting a demethylase enzyme. They have greater selectivity against fungi, better penetration of the central nervous system (CNS), resistance to degradation and cause less endocrine disturbance than do the imidazoles.

Ketoconazole

Ketoconazole is well absorbed from the gut. It is widely distributed in tissues but concentrations in cerebrospinal fluid (CSF) and urine are low; its action is terminated by metabolism by cytochrome P450 3A (CYP 3A) systemic mycoses. Impairment of steroid synthesis by ketoconazole has been put to other uses, e.g. inhibition of testosterone synthesis lessens bone pain in patients with advanced androgen-dependent prostatic cancer.

Adverse reactions include nausea, giddiness, headache, pruritus and photophobia. Of particular concern is impairment of liver function, ranging from transient elevation of hepatic transaminases and alkaline phosphatase to severe injury and death.

Clotrimazole

Clotrimazole is an effective topical agent for dermatophyte, yeast and other fungal infections like intertrigo, athlete' s foot, ringworm, pityrasis versicolor, fungal rash.

Summary of the invention

The main aim of the present investigation is to screen the crude extracts prepared in organic solvents of different polarity such as ethanol, methanol, acetone, di-ethyl ether as well as water. In the present investigation, chemical composition and antidermatophytic activity of medicinal plant used in traditional systems of medicine in Iran were evaluated against three different genera of dermatophytes like *Microsporum*, *Trichophyton* and *Epidermophyton* by agar dilution method. Plant materials were collected from Andimesh to Khorramabad (Khuzestan and Lorestan provinces) southern part of Iran. These are investigated *in-vitro* and *in-vivo* assay for evaluation of the antidermatophytic activity. In addition, phytoconstituents from plants were screened by using standard phytochemical methods to find out therapeutically active chemical constituent/s present in them.

***In-vitro* assay:**

In the present investigation, the ethanolic extract of selected medicinal plant inhibited tested clinical isolates of dermatophytic species like *Trichophyton mentagrophytes*, *Microsporum canis*, *M. gypseum*, *T. mentagrophytes* and *Epidermophyton floccosum*, which was isolated from patients who were suffering from skin disease with MIC & MFC values between 1.250-10 mg ml^{-1} medium. In the present investigation it was observed that *T. mentagrophytes* was the most susceptible, it has completely inhibited the tested extract while *Microsporum canis* was less susceptible than the other evaluated dermatophytic species. In the present investigation *Satureja khuzestanica* showed most promising antifungal activity. The ethanolic extract of tested medicinal plant showed significant antifungal activity ($PV < 0.05$).

***In-vivo* assay:**

It is performed on thirty patients who were severely suffering from dermatophytic infections. The survey performed for comparing clinical mycological effectiveness and tolerability of phytopharmaceutical formulation of cream prepared from *Satureja khuzestanica* leaf. Final assay were carried out on experimental group by herbaceous drugs and control group by topical Clotrimazole1% Cream. Clinical diagnosis of thirty patients showed the results at the end of two weeks after the treatment. In majority of the cases they showed negative mycological examination. The rate of therapeutic effectiveness showed satisfactorily percentage without any side effects. Therefore, the toleratibility percentage was 100% for the treatments.

Detailed description of the tables:

Table 1: MICs (mg ml^{-1}) of the extracts.

Table 2: Minimum inhibitory concentration (MIC) and minimum fungicidal concentration SKL, Griseofulvin (GRS) and Keteconazole (KTZ) against dermatophytes by Agar Dilution Method.

Table 3: Phytochemical tests of plant.

Table 4: Proteins from leaf of plant.

Table 5: Phenols from leaf of plant.

Table 6: Alkaloids from leaf of plant.

Detailed description of the figures

Figure 1: *Satureja khuzestanica* Jamzad

Figure 2: Inhibitory effects of ethanolic extract of *S. khuzestanica* leaf against *M. gypseum* by Agar Dilution Method on Mycosel medium. Decreasing dilution ranging from 0.078-5.0 mg/ml medium.

Figure 3: Cured Patient With *S. khuzestanica* Leaf Extract Cream Applied Topical Every Day For 4 Weeks.

Figure 4: Cured Patient With *S. khuzestanica* Leaf Extract Cream Applied Topical Every Day For 4 Weeks.
Figure 5: Cured Patient With *S. khuzestanica* Leaf Extract Cream Applied Topical Every Day For 4 Weeks.
Figure 6: Cured Patient With *S. khuzestanica* Leaf Extract Cream Applied Topical Every Day For 4 Weeks.
Figure 7: HPTLC study of *Satureja khuzestanica* leaf for detection and confirmation of Triterpenes
Figure 8: Chromatogram (f)
Figure 9: Bioautogram (f)

Detailed description of the Graphs:
Graph 1: Estimation of Triterpenes at 254 nm before Derivatization from *Satureja khuzestanica* leaf.
Graph 2: Estimation of Triterpenes at 366 nm after Derivatization from *Satureja khuzestanica* leaf.

Detailed description of the invention
Detailed experimental studies
The plant material was collected from southern part of Iran, Andimesh to Khorramabad (Andimesh of Khuzestan and Khorramabad of Lorestan province). Efforts were made to collect these plants in flowering and fruiting conditions for the correct Botanical identification. The healthy and disease free plant parts were separated and dried in shade so as to avoid the decomposition of chemical constituents. These were powdered in grinder and stored in clean and dry airtight containers for future studies.
In dermatophytoses, it is necessary to collect a specimen while paying careful attention to the clinical features.
Skin lesions:
The lesions of *Tinea* show a circular pattern, with a large amount of the fungus distributed at the active border of the lesion and only a little in the center.
The lesion was first cleaned with 70% alcohol and then the scale on its borders, the roof of the vesicles, or the keratinous part of the papules was scraped off by using scalpel flamed before use, taking great care not to cause any bleeding.

Preparation of Extract:
The leaves, stems, roots, rhizomes, flowers and fruits were extracted in the organic solvent of ethanol. To 10 g of each powdered material of air dried was added 100 ml organic solvent, ethanol 80% (drug/solvent ratio=1:10 w/v) in a conical flask for maceration, plugged with cotton and then kept on a rotary shaker at 190-220 rpm for 3×24h at room temperature (Fenner et al. 2005). Following filtration of the suspension through a Buckner funnel and Whatman filter paper #1, the crude ethanol extracts were evaporated to dryness in oven or water bath at 45^0C. Then 1 gm dryness extract dissolved in 1 ml 100 % dimethyl sulfoxide (DMSO). Final concentration of each extract adjusted to1000 mg ml^{-1}.

***In-vitro* assay:**
Evaluated of the antifungal activity of medicinal plants extracts against three different genera of dermatophytes (*Microsporum, Trichophyton,* and *Epidermophyton*) using following methods-

i) Dermatophyte isolates:
In the present investigation for the antifungal evaluation, 3 strains obtained from the Persian type culture collection (PTCC) Tehranviz. *Trichophyton mentagrophytes* PTCC5054, *Microsporum canis* PTCC5069, *M. gypseum* PTCC5070, and in addition to these 13 strains isolated from different lesions of patients at the Medical diagnosis laboratory, Ahwaz, such as *Microsporum canis* (n=2): MC-1, MC-2, *M. gypseum* (n=3): MG-1, MG-2, MG-3, *Trichophyton rubrum* (n=2): TR-1, TR-2, *T. mentagrophytes* (n=3): TM-1, TM-2, TM-3 and *Epidermophyton floccosum* (n=3): EF-1, EF-2, EF-3

and identified by standard procedure. Sabouraud's dextrose agar at 25°C was used to maintain isolates. In antifungal assays, each dermatophyte isolate was sub cultured onto Sabouraud-dextrose agar (SDA; Hi Media- India) slants and incubated at 30^0C for 4 to 5 days and sub cultured every 15 days to prevent pleomorphic transformations (Fenner et al. 2005).

ii) Antifungal agents:

Standard powders of two antifungal were obtained from the respective manufacturer's viz. Griseofulvin (Sigma Chemical Company) and Keteconazole (Janssen pharmaceutical).

Stock solution of Griseofulvin and Keteconazole were prepared by dissolving 4 mg of the antimycotic drugs in 1 ml 100% dimethyl sulfoxide (Curtin Matheson Scientific Inc., Houston, Tex.) (Jessup, 2000) and Somchit 2003). Antimycotic drugs were prepared as stock solution of 4000 $\mu g\ ml^{-1}$.

iii) Drug dilution:

Serial two-fold dilutions of Griseofulvin and Keteconazole used were as follows: 400, 200, 100, 50, 25, 12.5, 6.25, 3.12, 1.56, 0.78 and 0.39 $\mu g\ ml^{-1}$.

iv) Preparation of medium:

Mycosel, 36 gm. (Sabouraud culture medium containing Cyclohexamid and Chloramphenicol) agar (Difco, Detroit MI) was dissolved in a conical flask, after dissolving completely conical flask was autoclaved at 121^0C and 15 lb/cm^2 pressures for 20 min. and then cooled to 50^0C and use for susceptibility testing.

v) Preparation of fungal inoculum:

A standardized inoculum was prepared by counting the micro conidia microscopically. For this the suspension of conidia was prepared by using 0.85% sterile physiological saline which containing 0.05 % Tween 80 (Sigma). The suspension was added to the slant tube culture and gently swabbing the colony surface with a sterile bent glass rod to dislodge the conidia from the hyphal mat. The suspension was transferred to a sterile centrifuges tube and the volume was adjusted to 5 to 10 ml with sterile physiological saline. The final suspension of conidia was counted with a hemocytometer cell counting chamber. The inoculum of cell or spore suspensions were obtained according to reported procedure of (Shin and Lim 2004 & Wright et al. 1983) and adjusted to 10^4-10^5 Cells/spores with colony-forming units ($CFUml^{-1}$) or dermatophyte suspension was prepared by using 0.85% sterile physiological saline with Tween 80 (0.05%) and adjusted with a spectrophotometer at 530 nm to obtain 90% transmission (Lima et al. 1993).

Phytochemistry:

In the present investigation chemical investigation on these plants were carried out, which consists of qualitative tests for the presence of, starch, proteins, tannins, saponins, reducing sugars, Anthraquinones, alkaloids, glycosides and flavonoids. Besides these, quantitative estimations were carried out for starch, reducing sugars, proteins, and alkaloids.

Different methods used for these studies are as given below:

i) Qualitative tests for starch:

The plant material was finely ground and extracted with boiling methanol (methanol removes fats, fatty acids, salts, chlorophyll and inactive enzymes). After drying, the plant tissues were centrifuged with cold water and tested with iodine in 2% aqueous potassium iodide (Peach and Tracy, 1955).

ii) Qualitative tests for proteins (Million s test):

Million's reagent is a solution of mercuric nitrate in nitric acid (it reacts specifically with any phenolic compound in which 3 and 5 positions are unsubstituted). Proteins give red colorations with Millon's reagent.

Procedure: 2 ml of the test solution was boiled with a few drops of Millon's reagent and the color was observed (Trease and Evans, 1972).

iii) Qualitative test for Tannins:

Powdered plant material was treated with Ferric Chloride (acidic) and observed for the presence of tannins (Trease and Evans, 1972).

iv) Qualitative tests for Saponins:

Water extract of the plant material was vigorously shaken (with few drops of neutral water). A permanent lather (foam) indicates the presence of saponins (Trease and Evans, 1972).

A portion of residue obtained after evaporating the ethanol extract was dissolved in water and shaken vigorously. A honey comb, froth persisting for 15 min indicated the presence of saponins. A portion was dissolved in chloroform and filtered. A few drops of concentrated sulphuric acid and 1 ml of acetic anhydride were added to 1 ml of iced filtrate. The appearance of blue or bluish green or reddish brown color showed the presence of saponins (Farnsworth, 1960).

v) Qualitative tests for free Anthraquinones:

The plant extract of 5g material was shaken with 10ml of benzene and filtered. A 10% ammonium hydroxide solution (about 5ml) was added to the filtrate and the mixture was shaken. The presence of pink, red or violet color in the ammonical phase indicated the presence of free Anthraquinones (Farnsworth, 1960).

vi) Qualitative estimation for reducing sugars (Benedict test):

Reagent: Two stock solutions were prepared as follows:

(A) 17.3g of sodium citrate and 100g of anhydrous sodium carbonate were dissolved in 800 ml of warm distilled water. Filter and dilute the filtrate to 850 ml with distilled water.

(B) 17.3 g of pure crystalline copper sulphate was dissolved in 100 ml distilled water.

Working reagent: Solution B was slowly added to solution A, mixed thoroughly and was diluted to 1 liter with distilled water.

Procedure: To 5 ml of Benedict's reagent 5ml of the test solution was added. The tubes were incubated in boiling water on water bath for 10-30 minutes. The formation of brick red or an orange red precipitate indicated the presence of reducing sugars. (The principle is that when sugar has free or potentially free aldehyde or ketone group reduces copper ions in hot alkaline solution to form orange or red colored Cu_2O).

vii) Test for flavonoids:

To 1 ml of ethanol extract, few drops of concentrated HCl and Mg turnings were added. The development of pink or magenta color indicated the presence of flavonoids. (Farnsworth, 1960).

viii) Qualitative estimation of Alkaloids:

Precipitation of alkaloids can be obtained with a variety of inorganic and organic reagents. Sometimes even from dilute solutions. Among the inorganic precipitating reagents, following are few reagents- potassium mercuric iodide (Mayer's reagent), bismuth potassium iodide (Dragendorff's reagent), iodine potassium iodide (Wagner's reagent), potassium cadmium iodide (Marm's reagent), chloroplatanic acid, auric chloride in hydrochloric acid, phosphomolybdic acid (Sonnenschein's reagent), mercuric chloride, perchloric acid and rarely potassium ferric and ferrocyanide.

Some of the organics precipitating are reagents picric acid (Hager's reagent) styphnic acid, picrolonic acid and tannic acid. Characteristic color reactions are obtained with the acid of dehydrating agents such

as concentrated sulphuric acid, with oxidizing agent such as nitric acid, with a combination of these two or other reagents, which will dehydrate and oxidize simultaneously and finally by treating with aldehyde or like compounds in the presence of dehydrating agents. The exact mechanism of precipitation reactions of alkaloids is not clearly understood. However, these reactions have proved to be an efficient tool in detection of alkaloids in plant tissues.

Procedure: Plant material was crushed in mortar and pestle and the alkaloids were extracted in absolute alcohol. Filtered through Whatman No. 1 filter paper. Filtrate served as a source of alkaloids. Similarly some of the plant material was dissolved in dilute H_2SO_4. The extract of alkaloids was tested with different reagents as follows.

For every test 2-3 ml of the alkaloid extract was used with 1-2 ml of reagent (Homersleg, 1950 and Cromwell, 1955).

ix) Test for Glycosides:

The plant material was extracted in absolute alcohol. Filtered through Whatman No. 1 filter paper. In 2 – 3 ml of filtrate add equal volume of warm benzene slowly from the edges of test tube. The white precipitate develops at the edges of solution in the test tube indicates the presence of glycosides.

Factors Affecting Susceptibility Testing of Dermatophytes:

1. Incubation temperature for dermatophytic fungi has been recommended 34^0C since this temperature closely approximates skin surface temperature. But normally between 25-35^0C.
2. The minimum incubation period recommended between 4-14 days according Young et al. (1972).
3. The optimal of inoculum is 10^4-10^5 conidia/ml according to Shin and Lin (2004). Because of the ability of dermatophytes to produce various kind of conidia like micro conidia, macro conidia and clamidoconidia, for *in -vitro* susceptibility testing of this group of fungi agar media are better than broth media culture (Jessup, 2000).

Antifungal Susceptibility assay:

Assay for antifungal activity of different extracts were evaluated as per following two methods:

i) Disk diffusion Method:

Disk diffusion assays were carried out as described by Ficker et al. (2003). In this method 100 µl of the fungal cell suspension (105 CFUml-1) was pipetted onto Sabouraud's dextrose agar plates and uniformly spread by using a sterile bent glass rod. Sterile filter blank disks (7mm diameter, Whatman #1) were impregnated with 20µl extract solution (40mg ml-1) with different dilutions of the extracts as follows: 20%, 40%, 60%, 80% provided with sterile distilled water and negative controls were performed with only equivalent volumes (20µl) of solvent (80% ethanol) without added extracts to paper disks and allowed to air dry. These were placed in the inoculated petri dishes (1 disk per plate). Petri dishes were inverted and incubated at 28-300C for 48 h before (Malheriros at el. 2005) antimycotic evaluation according to the method of Binns et al. (2000). Shortest distance of the clear zone from the outer edge of the filter disk to the edge of visible fungal growth were measured in mm as the zone of clearance and compared it with the standard.

ii) Agar Dilution Method:

The fungistatic activities of different extracts were evaluated by the Agar Dilution Method (Fenner et al. 2005; Mitscher et al. 1972; Lucia et al. 2003) with the following modifications. For the assay, stock solutions of extracts were two-fold diluted with 0.85% sterile physiological saline to get dilutions ranging from 0.078 to 20 mg ml-1. Mycosel agar medium (5 ml) with 1% yeast extract mixed with

various dilutions of extract (100 µl) and 50µl the dermatophyte suspension of (105 CUFml-1) removed from a seven days old culture of fungi, was poured in each petri dish (60mm diameter) under laminar flow condition. The plates were incubated at 28-300C in a moist, dark chamber, and MICs were visually recorded after 15 days. The antifungal agents, Keteconazole (Janssen pharmaceutical) and Griseofulvin (Sigma) were used as positive controls. Drug free solution (only with appropriate amount of DMSO) was also used as a blank control for verification of fungal growth. The minimal inhibition concentration (MIC) value was defined as the lowest extract concentration that inhibit the fungal growth, and the minimal fungicidal concentration (MFC) showing no visible fungal growth after incubation time. MIC50 and MIC90 values are the lowest extract concentration at which 50% and 90% of the clinical isolates were inhibited (Marco et al. 1998). Dermatophyte plates were examined visually for 50 and 80% growth inhibition as compared to growth control. MIC results were recorded in micrograms per milliliter. The experiments were performed in duplicates and replicated three times to get average value of the MIC and MFC.

Activity of Medicinal Plant Extracts Made with Various Organic Solvents against *T. mentagrophytes* by Disk Diffusion Method:

Procedure:

To 10 g of powdered material plant, *Satureja khuzestanica*, was added 100 ml organic solvent, ethanol 80% in a conical flask for maceration. Following filtration of the suspension through a Buckner funnel and Whatman filter paper #1. The crude ethanol extracts were evaporated to dryness in oven at 45^0C. Then added 5-10 ml different solvents with various polarities like diethyl ether, acetone, methanol and water to conical flasks contain dryness plant extracts respectively. Then conical flasks were stirred slowly for dissolving antifungal active fractions in those solvents. Finally four extracts were obtained by as mentioned above solvents. Then 100 µl of the fungal cell suspension (10^5 $CFUml^{-1}$) was pipetted onto petri dishes contain solid Sabouraud's dextrose agar and uniformly spreading by using a sterile bent glass rod. Sterile filter blank disks (7mm diameter, Whatman #1) were impregnated with 10-20µl obtained extracts with as mentioned various polarity solvents. Disks were placed on the surface of solid agar petri dishes that were inoculated with the *T. mentagrophytes* suspension (4 disks per plate).
Then after solidification, medium agar Petri dishes were inverted and incubated at 35^0C. Following an incubation period of 48-72 hours, the diameter of the zone of inhibition around each disk was measured in millimeters. This work was also carried out according to Agar Dilution Method.

High Performance Thin Layer chromatography (HPTLC):

HPTLC technique was followed for the qualitative analysis and the confirmation of chemicals present in the studied plant (Passera et al., 1964).
HPTLC is a versatile separation technique included various steps as given below:

1) Plant extraction
2) Selection of HPTLC plates and sorbent
3) Sample preparation
4) Application of sample
5) Development (separation)
6) Detection including post-chromatographic derivatization
7) Quantitation
8) Documentation

1) Plant extraction:

The first step in the phytochemical evaluation is extraction of the plant material. The choice of extraction method depends up on the nature of plant material and the compound (s) to be isolated.

Isolation and Purification of Antifungal Compounds from Medicinal Plant:

To 10 g of each powdered material was mixed in 100 ml ethyl acetate. Mixture kept on a rotary shaker at 150 rpm for 3 hours at room temperature. Centrifuged at 10,000 rpm for 5 minutes. Supernatant was collected; Reduced the supernatant from 100 ml supernatant to 20 ml quantity and 5-10 µl is used for HPTLC.

2) Selection of HPTLC plates and sorbent:

Pre-coated plates with different support material (glass, aluminum, plastic) and with different sorbent layers are available in different format and thickness in various manufacturers. Usually plates with sorbent thickness of 100 – 250 µm are used for qualitative and quantitative analysis. However, for preparative TLC work, plates with sorbent thickness of 1.0 – 2.0 mm are available in addition to chemically modified layers. Aluminum sheet (0.1 mm thick) sheets as support offer the same advantage as polyester support but with increased temperature resistance. However, with eluents containing high concentration of mineral acids or concentrated ammonia. One may find problem, as they will chemically attack aluminum. Aluminum sheets are otherwise compatible with organic solvents and organic acids such as formic acid and acetic acid.

Plate size: Pre-coated TLC/ HPTLC plates in size of 20 × 20 cm with aluminum. It is always recommended to clear the plates before actual chromatography.

Activation of pre–coated plates: For the separation of compounds of herbal extracts pre-coated plates of silica gel G 60 are widely used especially the ones impregnated with phosphor (silica gel F_{254}, E. Merck). User UV at 254 nm, the resolved compounds whose absorption spectra overlap the excitation spectrum of phosphor are seen as dark bands against a yellow- green fluorescent background due to fluorescence quenching.

Freshly opened box of TLC/ HPTLC plates usually does not require activation. However, plates exposed to high humidity or kept on hand for long time may have to be activated by placing in oven at 110-120°C for 30 minutes prior to sample spotting (Sethi, 1996).

3) Sample Preparation:

Proper sample preparation is an important pre-requisite for success of thin layer chromatographic separation. The sample preparation procedure is to dissolve the dosage form with complete recovery of intact compound(s) of interest and minimum of matrix with a suitable concentration of analytic (s) for direct application on the HPTLC plate. Besides, maximizing the yield of analytic (s) in the selected solvent stability of analytics during extraction and analysis must be considered and ensured. Therefore, the choice of a suitable solvent for a given analysis is very important. For normal phase chromatography using silica gel pre-coated plates (more than 80-90% HPTLC analysis is done using silica gel as sorbent) solvent for dissolving the sample should be non-polar and volatile as far as possible. It is preferable to keep the solvent as simple as possible and quantity employed is limited to ensure complete extraction of analytics and minimum of extraneous component. Sample and reference substances should be dissolved in the same solvent to ensure comparable distribution at starting zones (Stahl, 1969).

4) Application of sample:

Sample application is the most critical step for obtaining good resolution for quantification by HPTLC. The sample should be completely transferred to the layer, however, under no circumstances, the application process should damage the layer, as damaged layer results in unevenly shaped spots. Wherever possible use of automatic application devices is recommended for quantitative analysis. While using graduated capillaries, one must ensure that they fill and empty completely.

Usually application of 1–10 µl volume for TLC and 0.5–5 for HPTLC is recommended keeping the size of starting zone(s) down to minimum; 2–4 mm (TLC) and 0.5–1 mm (HPTLC) in the concentration range of 0.1–1 µg/µl for TLC/ HPTLC. However, volume and concentration primarily depend on the component under analysis and their sensitivity to various detection techniques.

5) Development (Mobile phase):

Poor grade of solvent used in preparing mobile phase have been found to decrease resolution, spot definition and R_f reproducibility. Mobile phase commonly called solvent system is traditionally selected by controlled process of trial and error and also based on one's own experience in the field. It is often possible that few layer-solvent combinations already reported in the literature for compounds of interest or similar compounds may be suitable in a given analytical problem with minor modifications. Nevertheless, it should not be forgotten that such conditions may have been chosen due to availability rather than suitability and often improvements are required. However, mobile phase should be chosen taking into consideration chemical properties of analytics and the sorbent layer. Use of mobile phase containing more than three or four components should normally be avoided, as it is often difficult to get reproducible rations of different components.

Pre-conditioning (Chamber saturation):

Chamber saturation has pronounced influence on the separation profile. When the plate is introduced into an unsaturated chamber, during the course of development, the solvent evaporates from the plate mainly at the solvent front. Therefore larger quantity of the solvent shall be required for a given distance hence, resulting is increase in R_f values. If the tank is saturated (by lining with filter paper) prior to development solvent vapors soon get uniformly distributed throughout the chamber. As soon as the plate is placed in such a saturated chamber, it soon gets pre-loaded with solvent vapors hence, less solvent shall be required to travel a particular distance resulting in lower R_f values. Time required for saturation will depend on the nature and composition of mobile phase and layer thickness (equilibration time increases with increase in layer thickness). Once the chromatogram is developed it should be handled with utmost care. Application of reagents if required has to be homogeneous ensuring uniform reaction and finally stabilizing of end reaction product.

If heating of the plate after it is treated with the reagent is not uniform. There always exists risk of reaction in homogeneity on the plate. Usually drying cupboard or hot plates are employed. Hot plates with regulated range of temperature i.e. 50-190° C ± 2° C are extensively being employed for heating the chromatogram.

6) Detection and visualization:

As soon as the development process is completed the plate is removed from the chamber contains the mobile phase of chloroform solvent and evaporated to remove the mobile phase completely. The zones can be located by various physical, chemical and biological i.e. physiological methods. There is apparently no difficulty in detecting colored substances or colorless substances in short wave ultra-violet (UV) region 254 nm and 366 nm or with intrinsic fluorescence such as riboflavin quinine sulphate. The R_f values and the colors of the resolved bands are recorded and fingerprint profiles are

established. Identification of the chemical marker is by comparison of R_f value, absorption spectra, response to derivatizing reagent *etc.* (Wagner et al. 1984).
(Relative factor) R_f = Distance travelled by the solute/ Distance travelled by the solvent

7) Quantitation:

Spraying and dipping techniques are used for applying detection reagents. However, in addition to other reasons as enumerated below dipping followed by evaporation which is essential for both the precision and repeatability in ultimate quantitative analysis. Sample and standard are chromatographed on the same plate under similar conditions.

Preparation of spraying reagents:

Anisaldehyde- Sulphuric Acid reagent:
Mix o.5 ml anisaldehyde in 10 ml glacial acetic acid, 85 ml methanol and 5 ml conc. sulphuric acid. Spray the plate and heat at 100^0 C for 5-10 min. Use freshly prepared reagent (Wagner et al. 1984).

Ninhydrin reagent:
Dissolve 30 mg of ninhydrin in 10 ml of n- butanol and 0.3 ml of glacial acetic. Spray the plate with the reagent and heat at 105^0 C till the spots appear.

Vanillin Sulphuric Acid reagent:
Solution 1: 1% Ethanolic Vanillin.
Solution 2: 10% Ethanolic Sulphuric Acid.
The plate is sprayed with 10 ml of solution 1 and then immediately with 10 ml of solution 2. Heat at 110^0 C for 5-10 min. (Wagner et al. 1984).

Detection of Coumarins:

- **UV-254 nm:** Distinct fluorescence quenching of coumarins.
- **UV-365 nm:** Intense blue or blue-green fluorescence (simple coumarins) yellow, brown, blue or blue-green fluorescence (furano-and pyranocoumarins).
- **Spraying reagents:**
  - Potassium Hydroxide (KOH) reagent. The fluorescence of the coumarins are intensified by spraying with 5%-10% ethanolic KOH. Concentrated ammonia vapour has the same effect.
  - Natural products-Polyethylene Glycol reagent (NP/PEG) intensifies and stabilizes the exiting fluorescence of the coumarins. Phenol Carboxylic Acid fluorescence blue or blue-green (e.g. chlorogenic or caffeic acid).

Detection of Triterpenes:

- **UV-254 nm:** Caffeic acid, derivatives and isoflavones show quenching.
- **UV-365 nm:** Caffeic acid, its derivatives and isoflavones fluorescence blue.
- **Anisaldehyde-Sulphuric Acid reagent:** The sprayed TLC is heated for 6 min. at 100^0 C.

Evaluation in vis.: Triterpenes blue-violet.

8) Documentation:

The use of application scheme and labeling every single chromatogram can avoid mistake in respect of order of application. It is preferable to apply each sample and reference solution twice by following data - pair method. A lead pencil can be used to write on the chromatoplate. The plate should never be marked below the starting point, as layer is likely to get damaged affecting chromatographic distribution of the substances under analysis which may ultimately lead to error in scanning. The best way to label the chromatoplate is to mark above the level of solvent point, immediately after development is completed, the solvent point should be marked both on left and right hand edges of the plate, this win facilitate calculation of R_f values. The practice of cutting a scratch across the whole layer is no longer in use. The type of plate, chamber system, composition of mobile phase, running time and detection method should all be recorded. HPTLC protocol format given in the text may be adopted for recording all the relevant data.

Bioautography Agar Overlay:

The bioautography agar overlay method is an improved version of a disc diffusion method. It is considered as one of the most efficient methods for the detection of antimicrobial compounds (Rahalison et al. 1991). It appears that bioautography is an important detection method for a new or unidentified antifungal compounds because it is based on the biological effects of the substances under study. In the bioautography agar overlay method the drug to be evaluated is adsorbed onto the Thin Layer Chromatography (TLC) plate and the inoculum is laid onto the plate as a very thin layer of about 1 mm in thickness. The bioautography agar overlay method uses very little amount of sample and since the crude extract is resolved into its different components it can identify and isolate the active compounds (Rahalison et al. 1991).

Bioautography agar overlay process:

The bioautography was performed for the ethyl acetate extracts of *S. khuzestanica* against two dermatophytic species viz. *T. rubrum* and *T. mentagrophytes.*

Five microliters (5 µl) of the Ethyl acetate of extracts these plants were analyzed by thin layer chromatography (TLC) by using aluminum-backed TLC plates (Merck, silica gel 60 F254). The TLC plates were developed with systems viz. ethyl acetate/methanol/water (40:5.4:5): [EMW] (polar/neutral); chloroform/ethyl acetate/for-mic acid (5:4:1): [CEF] (intermediate polarity/ acidic); benzene/ethanol/ammonium hydroxide (90:10:1): [BEA] (non-polar/basic) and acetone/Ethyl acetate/petroleum ether (0.5: 0.5:2.0) [AEP] (Kotze and Eloff (2002). The mobile phase was removed from the plate by drying in the room temperature. The developed plates were sprayed with Iodine reagent to check presentation of spots and were observed in UV light. Then developed TLC plates were sterilized by UV light and these were removed into Petri dishes which poured 5-10 ml Sabouraud's dextrose agar previously. To 5 ml non-solidified media with 45°C temperature was added 100 µl of the dermatophyte suspension inoculum with concentration of 10^5 $CUFml^{-1}$ and mixed evenly. About the 1 mm in thickness of this mixture was laid on the surface of developed TLC plates (Rahalison, 1999). The plates were incubated at 35^0C in incubator. They were incubated for a period 48-72 hours after which the inhibition zones appeared colourless against a yellow background. Spots showing any inhibition were noted and their hR_f values measured. The tests were performed in duplicate.

Preparation of Herbal Formulation in the forms of Ointment

From experimental herbal medicine preparations were made *Satureja khuzestanica* – Ointment.

The methanolic extract of *Satureja kuzestanica* leaf was accurately weighed (125mg). These are also known as Clotrimazole material.

Fatty material such as Vaseline and Ceto-estearyl Amine heated up to 70^0 C in a beaker. Then Sodium Loril Sulphate was mixed as surfactant to water and then heated up to 80^0 C in another beaker. Then Methyl and Prophyl Paraben were added in those as an antibacterial. These mixtures were added slowly but continuously in the mixture of Vaseline and Ceto-estearyl Amine and allowed to cool by stirring slowly to decrease temperature (as mixture No. A). Then those accurately weighed herbal extracts or Clotrimazole material were mixed in Propylene glycol and was stirred until a smooth mixture obtained (as mixture No. B). Then mixture A and mixture B were mixed and heated up to 50-55^{0}C. Then the mixture was cooled by stirring slowly and the warm mixture drug packed into a special container or tube.

***In-vivo* assay:**

A double-blind *in-vivo* assay was performed on thirty patients who were severely suffering from dermatophytic infection. The survey and treatment were carried out in the Department of Mycoparacytology, Joundi-Shapur University Hospital and Medical Sciences, Ahwaz, Iran (Ethical approval no. JSUH/ HEC/2006-2007/27; dated 15/03/2006).

All patients were informed about the project and have signed consent forms for participation in this clinical investigation. Afterwards, they were randomized to one treatment.

It was performed for clinical, mycological effectiveness and tolerability of phytopharmaceutical formulation i. e. lotion prepared from leaf extract of *Satureja khuzestanica* with concentration of 250 mg and cream prepared from 125 mg with the addition of topical antifungal Clotrimazole1% cream same formulated as phytodrug cream on the topical treatment of clinical features of dermatophytosis. Pharmacological studies in this survey have demonstrated antimycotic *in vitro* effect of the leaves *S. khuzestanica* against *T. mentagrophytes, T. rubrum, M. canis, M. gypseum and E. floccosum,* while controlled clinical trials verified the effectiveness of these plants for treating of clinical features dermatophytosis without side effects. The prepared formulations showed promising results. The final bioassays were carried out on thirty patients-15 in the experimental and 15 in the control group.

Experimental procedure:

The study was conducted over 4 weeks. At the beginning of the investigation every patient received a 30 ml plastic container with the instruction of treatment of how to use it (Directions: Apply 2-3 times daily for two to four weeks). The first evaluation was carried out for two weeks after the treatment began, and the patients received a fresh treatment by changing the empty container for a new one. The last evaluation took place for four weeks after first day of treatment. During the period of study at the beginning and after two and four weeks of treatment mycological tests include a direct examination with potassium hydroxide and the culture of scrape scales of affected area of the body obtained on mycobiotic agar and incubated for 15 days at 25 0C .

Outcome variables:

The evaluated outcome variables were: a) Clinical effectiveness was declared when signs and symptoms attributed to the pathology under study were totally absent. b) Mycological effectiveness was detected as a negative direct examination and culture. c) Toleratibility which was determined by the absence of local side effects such as erythema, stinging, blistering, peeling, edema, prurits, urticaria and

irritation.

Results and discussion

Screening of medicinal plant extract for antidermatophytic activity:

In the present investigation ethanolic extracts from different parts of one Iranian plant species i.e. *Satureja kuzestanica* were selected based on their reputation in Ayurvedic and Indian traditional systems of medicine.

This was investigated for their potential activity against 16 clinical and standardized dermatophytic species viz. *Trichophyton mentagrophytes*, *Microsporum canis*, *M. gypseum*, *Trichophyton rubrum* and *Epidermophyton floccosum*, which were isolated from patients by both agar dilution and disk diffusion methods. Since, disk diffusion method with many compounds particularly volatile compound did not show sharp demarcation between inhibition and growth on the agar plates. Previous studies reported that disk diffusion method showed clearly anti-Candida effects (Moore. and Atkins, 1977). Hence, determination of the MIC and MFC carried out by agar dilution method.

The crude extracts in concentration up to 20 mg ml^{-1} were incorporated into the growth media according to material and method. MIC values $\leq$ 20 mg ml^{-1} were found to be active. The extract of the plant species which is seen as reduced growth of the colony of dermatophytes as compared with the control treatment. The tested ethanolic extracts of medicinal plants were inhibited all tested dermatophytic species with MIC values between 1.250-10 mg ml^{-1} medium. MFC values of all tested dermatophytic species were ranged 1.250-10 mg ml^{-1} medium. The results are shown in Tables No. 1 & 2.

In the present investigation *Satureja kuzestanica* showed remarkable antifungal activities. This is studied for the first time and its antidermatophytic activities are reported in the present investigation. It was also observed that *T. mentagrophytes* the most susceptible, which completely inhibited by the tested extract, which is similar with the studies of Baba et al. (1999).

***In-vitro* Susceptibility Testing of Dermatophytes to *S. khuzestanica*:**

Review of literature revealed that this kind of study has not been reported so far. This the first report on the antifungal activity of the ethanolic extract of *S. khuzestanica* leaf on dermatophytic species. It is observed that the ethanolic extract of *S. khuzestanica* leaf showed the most active against tested dermatophytic species with MIC values between 10 and 1.250 mg ml^{-1} medium as compared to other studied plants. MIC90 and MIC50 values=0.625-1.250 and 0.156-0.312 mg ml^{-1} medium, respectively. The lowest MIC90s and MIC50s were concerned the clinical and standardized dermatophytic species of *T. mentagrophytes, M. gypseum*, *T. rubrum* and *E. floccosum* and the most MIC90s and MIC50s were concern *M. canis* strains. The MFCs values of this plant were in the range of 1.250-2.50 mg ml^{-1} medium (Figure 2).

It was also observed that *M. canis* was the most resistant among the five evaluated dermatophytic species against inhibitory effects of *S. kuzestanica* and *T. rubrum*, *T. mentagrophytes, M. gypseum* while *E. floccosum* was more sensitive than other tested dermatophytic species. This is remarkable that *E. floccosum* produces arthroconidia which survive for a longer time than other dermatophytic species. This may be because of environmental source of contagion sometimes leading to recurrent outbreaks of dermatophytosis in individuals and in institutions (Di Domenico, 1999).

On the basis our results it is shown that the ethanolic extract of *S. khuzestanica* leaf possesses antifungal properties not only against tested standardized dermatophytic species but also against clinical dermatophytic species. Previous studies reported the antifungal activities of essential oil *S. khuzestanica* and components carvacrol, camphor and 1, 8-cineole against 13 fungal species. The presence of oil showed various degrees of inhibition against all the investigated fungi. The highest and broadest activity

showed by the carvacrol content oil of *S. thymbra*. Carvacrol exhibited the highest and 1, 8-cineole the lowest level of antifungal activity among the tested components (Sokovic and Couladis, 2002) and essential oil of *Satureja parnassica* tested against *Helicobacter pyloria* and six aerobically growing bacteria. Both the samples showed a moderate activity against gram-positive bacteria. The present investigation, it has been demonstrated the antifungal properties of *Satureja parnassica* extracts in *in-vitro* assays.

Results of Phytochemical Study:

Phytochemical tests of studied plant indicated that the root of *Satureja kuzestanica* gave positive test for saponins, reducing sugars and proteins (Table 3, 4, 5 & 6). Leaves and stems of aromatic plant, *S. kuzestanica* showed positive test for alkaloids. In the present investigation, quantitative estimations were carried out for proteins, starch, total sugars, reducing sugars and phenols.

Results of HPTLC Study:

It was found that chemicals such as terpenoids, triterpenes and coumarins are effective against pathogenic fungi (Cowan, 1999). Other studies regarding antifungal activity of chemical composition of tested plant reported that coumarins and triterpenoids were responsible for antimicrobial activity (Gray, 1994 and Govindachari, 1998). Hence, in the present investigation High Performance Thin Layer Chromatography (HPTLC) fingerprints of leaves, *S. kuzestanica* carried out for detection and confirmation of Phytochemical compounds like triterpenes and coumarins.

The ethyl acetate extract of *S. khuzestanica* leaves contain triterpene that they showed in visible and in UV-254 nm with 7, 7, 10, 7, 9, 12 peaks respectively (Graphs No.1; figure 7) and in UV-366 nm with 14, 10, 14, 9, 13, 12 peaks respectively (Graphs No. 2). Finally after detection and confirmation of triterpenes and coumarins by HPTLC in plant mentioned above these compounds can be responsible for antidermatophytic activity of, *S. kuzestanica* that gave positive test result by *in-vitro* assay previously.

Bioautography results:

After detection and confirmation triterpenes and coumarins by HPTLC method for showing the presence of compounds that these inhibited the growth of fungi, bioautography carried out for *S. kuzestanica* that gave positive test result by *in-vitro* assay previously.

Bioautography was used to screen for finding out antifungal compounds present in the extract. Inhibition zones of antifungal compounds were observed as clear spots on a yellow background of media culture. The clear spots indicate presence of antifungal compounds that it inhibited the growth of tested fungi. In tested plant extract *S. kuzestanica* organisms did not grow too well that it was difficult to detect inhibition zones hence, no inhibition was observed. The non-activity of this plant extract in bioautography could be explained by evaporation of active compounds during removal of the TLC eluents or by the disruption of synergism between active constituents caused by TLC. Among the four separation systems used, only the solvent system of AEP gave the best resolution of spots in TLC analysis because antifungal compounds were relatively semi-polar and therefore did not separate well in the polar eluents EMW (Figure 8 and 9).

***In-vivo* assay:** Experimental group were treated with plant extract ointment and control group were treated with topical commercial drug (i.e. Clotrimazol 1%). Clinical diagnosis of patients showed negative mycological examination the end of two weeks after the treatment (Figure 3). At the end of two and four weeks after the treatment, the rates of the therapeutic effectiveness showed toleratibility percentage without any side effects such as erythema, blistering, peeling, edema, itching and irritation.

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5. Claims

We claim:

1. An anti-fungal herbal formulation in the form of ointment was developed, the said formulation comprising of leaf extract of *Satureja kuzestaica* Jamzad.
2. Herbal formulation with its specific concentration as in claim 1, can be applied topically in the form of Ointment.
3. Herbal formulation with its specific concentration as in claim 1, and applied as in claim 2, have no toxic effects reported.
4. Herbal formulation with its specific concentration as in claim 1, and applied as in claim 2, is capable of substantially attenuate the fungal infection. Thus it has anti-fungal activity.
5. According to any of the proceeding claims, the said formulation in an embodiment is capable of reducing fungal strains from genus *Tinea* as mentioned in method.
6. According to any of the proceeding claims, the said formulation showed anti-fungal activity by curing the fungal infection caused by *Tinea pedis, T. corporis, T. capitis, T. ungium, T. manuum, T. cruris* of skin.

Name : Prof. Dr. Deokule Subhash S Signature :

6. Date and Signature

Date: 01|01|2018

Place: Pune

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Department of Botany,
Savitribai Phule University of Pune (SPPU),
Ganeshkhind Road,
Pune- 411007. Maharashtra, India

TITLE:

Development of new herbal ointment for the management of dermatophytic infections

7. ABSTRACT OF THE INVENTION

Mycotic infections are the most common cause of skin disease in developing countries of tropical regions. Dermatophytosis is the most frequent superficial fungal infection occurring in India. For these ailments various drugs are being used which exhibits several side effects and have limited efficacy. So that there is a distinct need for the discovery of new safer and more effective antifungal agents. The use of medicinal herbal drugs in the treatment of skin diseases including mycotic infections is an age-old practice in many parts of the world. *Satureja khuzestanica* Jamzad is one of the important plant reported traditional medicine. The selected plant was collected from Andimesh to Khorramabad (Khuzestan and Lorestan provinces) southern part of Iran.

In the present investigation *in-vitro* and *in-vivo* assays have been used for the evaluation of the antidermatophytic activity. In addition, phytoconstituents of selected medicinal plant were screened by using standard phytochemical methods to find out therapeutically active chemical constituent/s present in them.

As a result, *in-vitro* and *in-vivo* assays demonstrated the remarkable fungi static and fungicidal properties of new formulation in the form of ointment using leaves *Satureja kuzestanica*. This investigation will be an alternative source as an herbal medicine in various systems of medicines.

Phytochemical screening of studied plant indicated that the leaves of *Satureja khuzestanica,* gave positive test for saponins, reducing sugars and proteins. The leaves of the mentioned above plant showed positive test for starch. All the parts of the mentioned above plants gave positive tests for tannins.

HPTLC studies also confirmed that the leaves of *S. khuzestanica* contain triterpene which may be responsible for antidermatophytic activity in these plants.

Table 1: MICs (mg ml $^{-1}$) of the extracts

Name of plant	Plant part used	MIC value (mg ml $^{-1}$)				
		M.C.[a]	*M.g.*[b]	*E.f.*[c]	*T.r.*[d]	*T.m.*[e]
***Satureja khuzestanica* Jamzad**	**Leaf**	**2.500**	**1.250**	**1.250**	**1.250**	**1.250**
a. *Microsporum canis*, b. *M. gypseum*, c. *Epidermophyton floccosum*, d, *Trichophyton rubrum*, e, *Trichophyton mentagrophytes*						

Table 2: Minimum inhibitory concentration (MIC) and minimum fungicidal concentration SKL, Griseofulvin (GRS) and Keteconazole (KTZ) against dermatophytes by Agar Dilution Method

	Antifungal Compounds	MFC[a] , MIC*& MFC				Geometric Mean MIC
		Range	50%	90%	MFC	
***T. mentagrophytes* (3)**	**KTZ**	**0.78-6.25**	1.56	6.25	12.5	3.52
T.mentagrophytes	**GRS**	**12.5-100**	25	100	200	56.25
PTCC5054	**SKL**	**0.078-0.625**	0.156	0.625	1.250	0.352
***M. gypseum* (3)**	**KTZ**	**0.78-6.25**	1.56	6.25	12.5	3.52
***Mgypseum* (PTCC5070)**	**GRS**	**12.5-100**	25	100	200	56.25
	SKL	**0.078-0.625**	0.156	0.625	1.250	0.352

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Table 3: Phytochemical tests of plant

Name of the Test carried out	Reagents used	*Satureja khuzestanica* Leaf
A. Water Extract		
Starch	I2-KI	+ve
Tannins	Acidic $FeCl_3$	+ve
Saponins	H_2SO_4 + Acetic unhydride	+ve
Proteins	Millon's test	+ve
Anthraquinones	Benzene + 10%NH_4OH	-ve
Reducing sugars	Benedict's	+ve
B. Alcoholic Extracts		
Alkaloids	Mayer's	-ve
	Wagner's	+ve
	Dragendorff's	+ve
Flavonoids	HCl + Mg turnings	+ve
Glycosides	Benzene+hot ethanol	-ve

ve: Present -ve: Absent+

Table 4: Proteins from leaf of plant

NAME OF THE PLANT	PLANT PART USED	TOTAL PROTEINS g/100g dry wt.
Satureja khuzestanica Jamzad	Leaf	1.060

Table 5: Phenols from leaf of plant

NAME OF THE PLANT	PLANT PART USED	TOTAL PHENOLS g/100g dry wt.
Satureja khuzestanica Jamzad	Leaf	0.198

Table 6: Alkaloids from leaf of plant

NAME OF THE PLANT	PLANT PART USED	TOTAL ALKALOIDS g/100g dry wt.
***Satureja khuzestanica* Jamzad**	**Leaf**	**2.6 %**

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Figure 1: ***Satureja khuzestanica*** **Jamzad**

Figure 2: Inhibitory effects of ethanolic extract of *S. khuzestanica* leaf against *M. gypseum* by Agar Dilution Method on Mycosel medium. Decreasing dilution ranging from 0.078-5.0 mg/ml medium.

MIC=1.250 mg/ml medium.

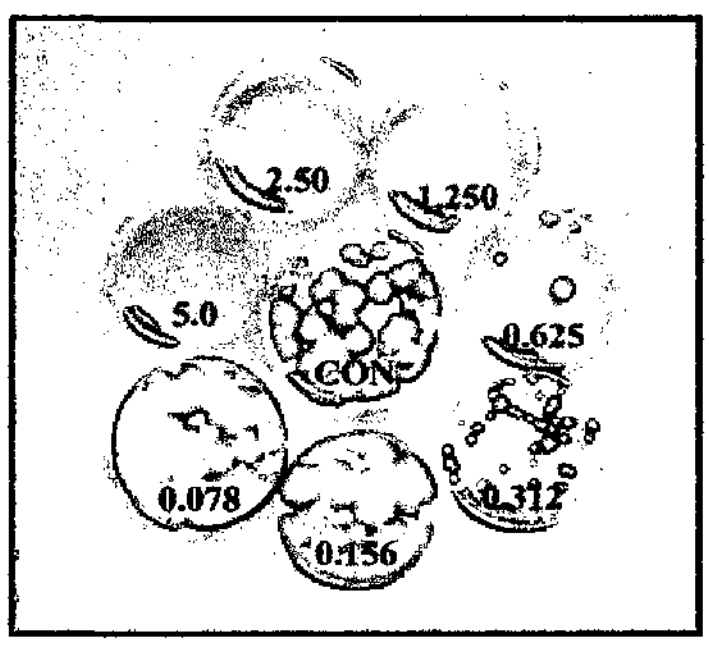

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Figure 3: Cured Patient with *S. khuzestanika* Leaf Extract Cream Applied Topical Every Day For 4 Weeks

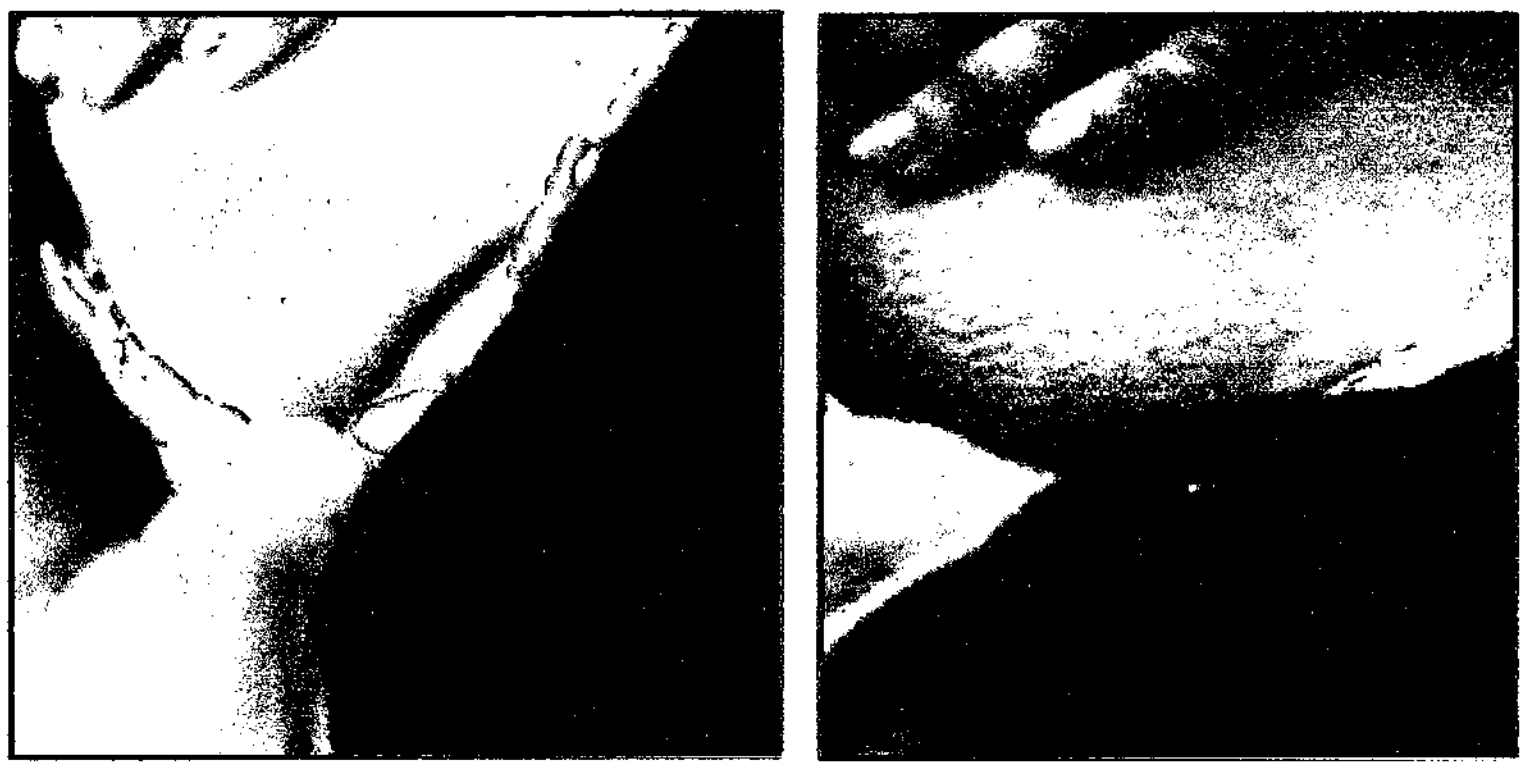

Figure 4: Cured Patient With *S. khuzestanika* Leaf Extract Cream Applied Topical Every Day For 4 Weeks

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Figure 5: Cured Patient With *S. khuzestanika* Leaf Extract Cream Applied Topical Every Day For 4 Weeks

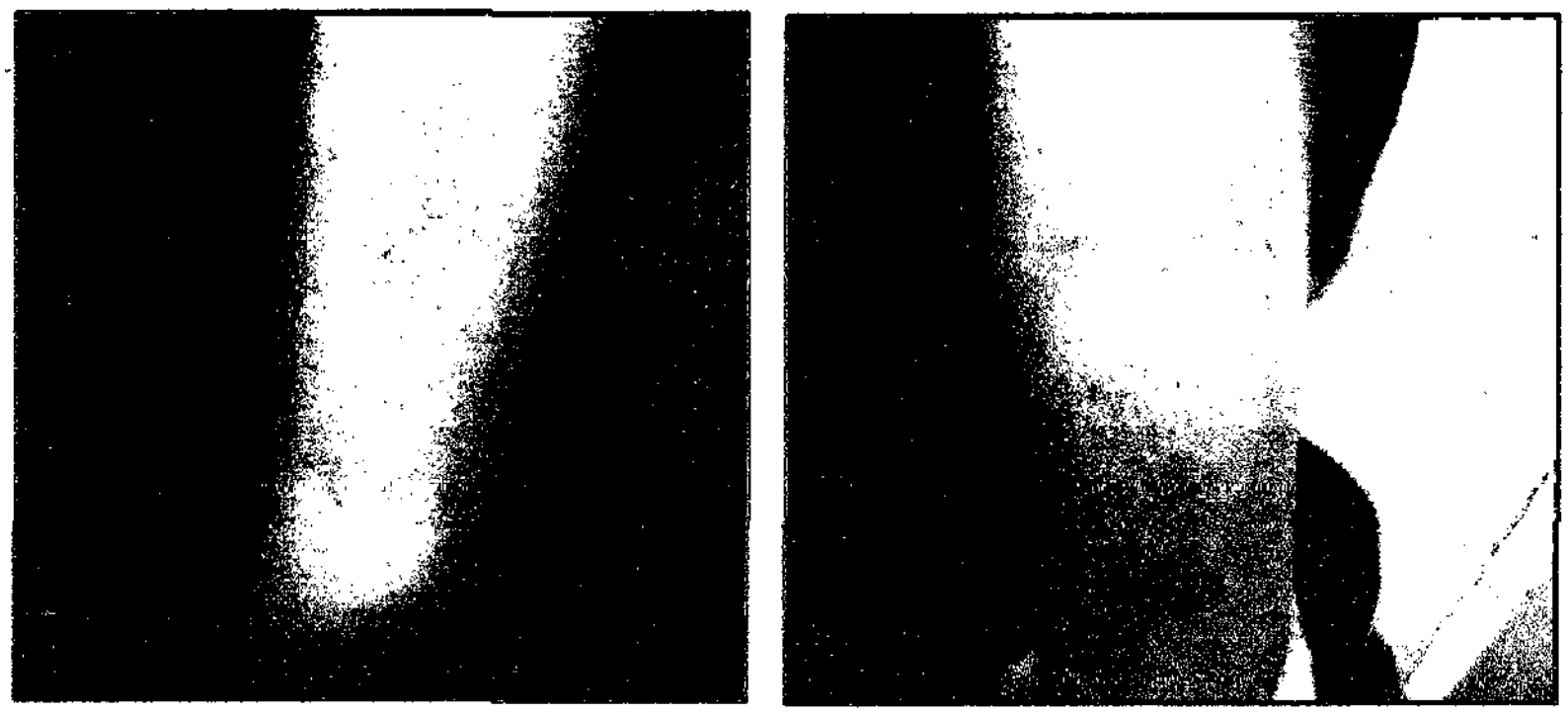

Figure 6: Cured Patient With *S. khuzestanika* Leaf Extract Cream Applied Topical Every Day For 4 Weeks .

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Figure 7: HPTLC study of *Satureja khuzestanica* leaf for detection and confirmation of Triterpenes

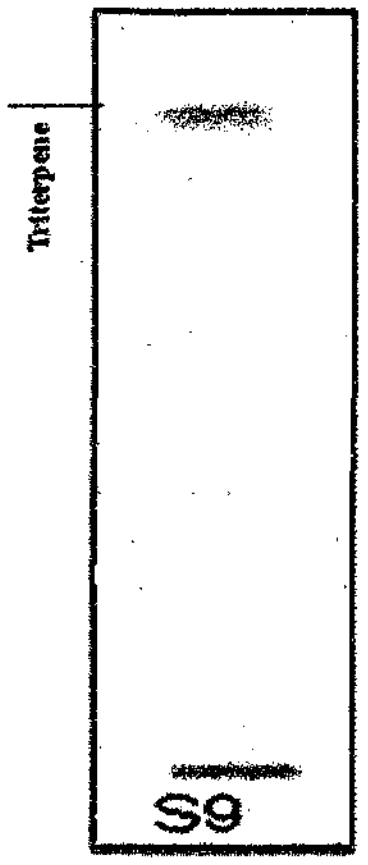


Figure 8: Chromatogram (f)

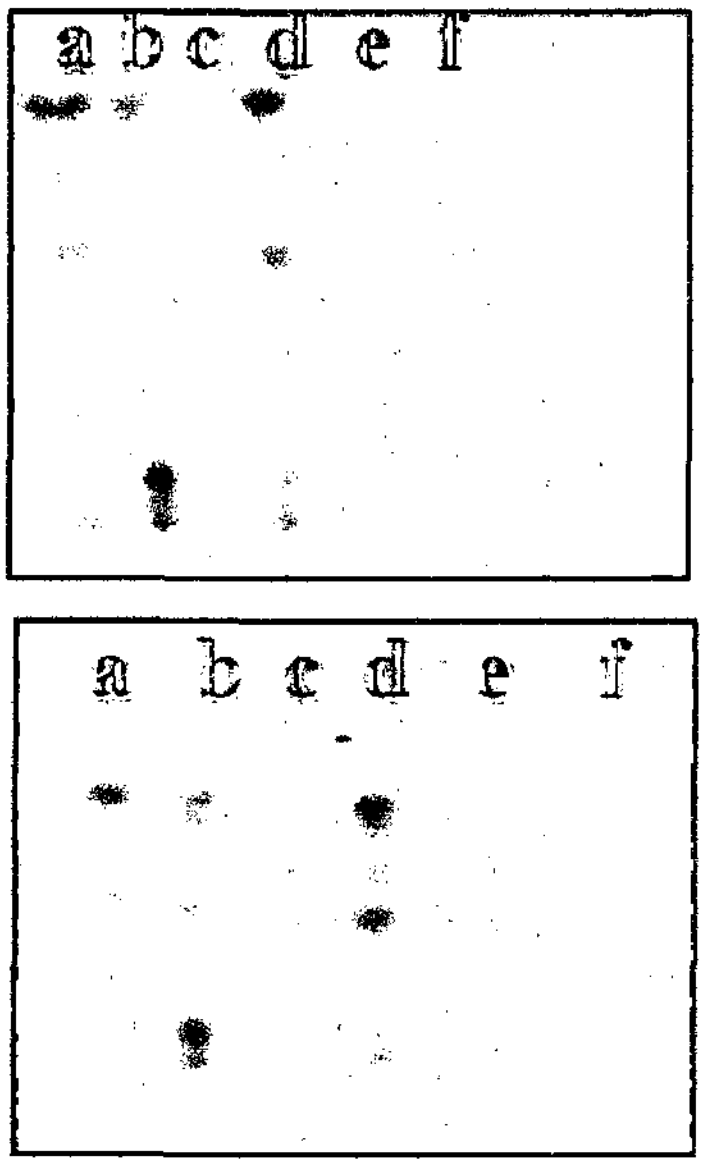
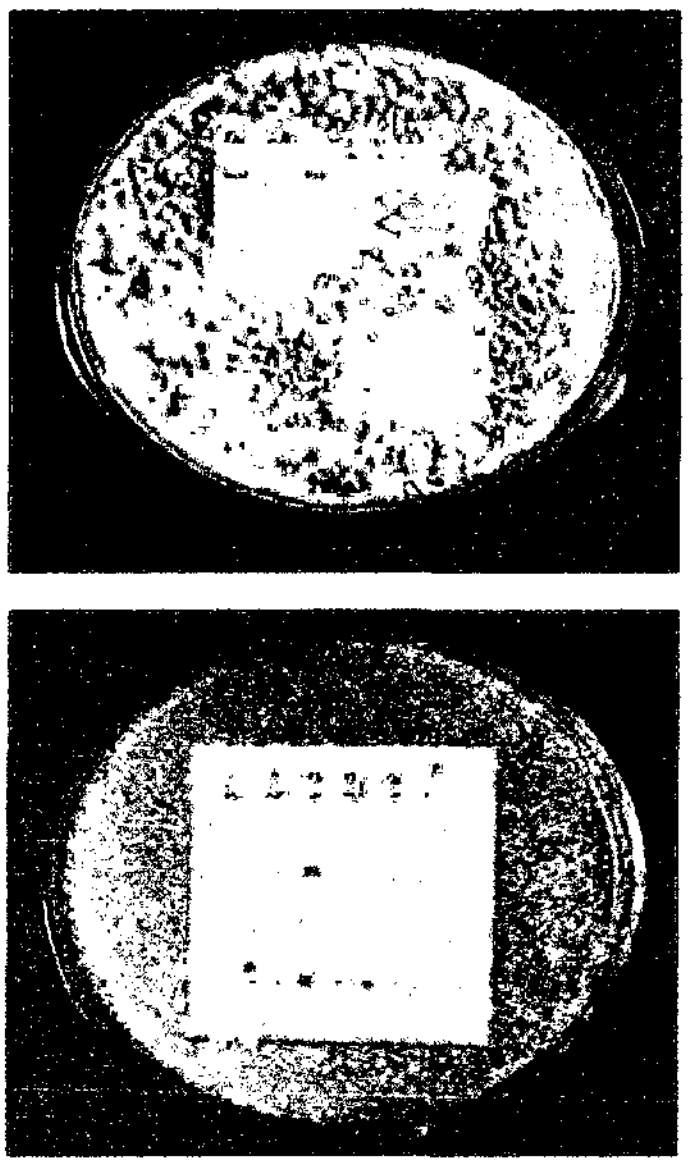


Figure 9: Bioautogram (f)



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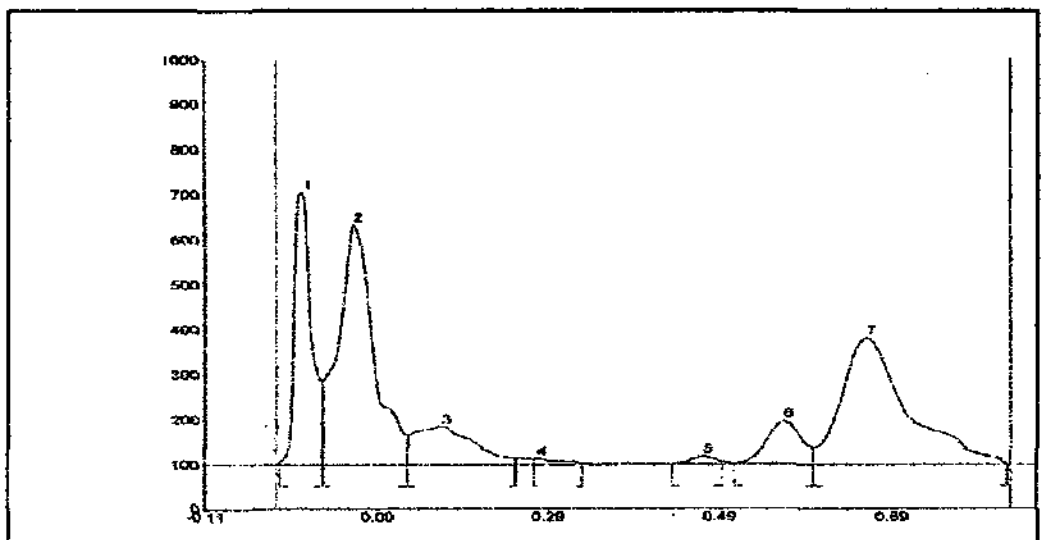
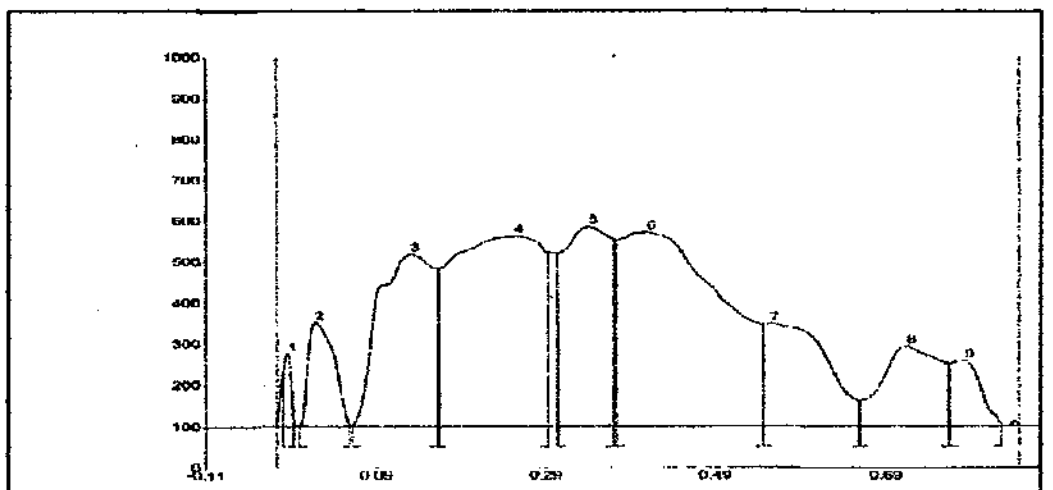
Graph 1: Estimation of Triterpenes at 254 nm before derivatization from *Satureja khuzestanica* leaf



Peak	Start Rf	Start Height	Max Rf	Max Height	Max %	End Rf	End Height	Area	Area %
1	-0.02	6.6	0.00	606.3	37.05	0.03	188.9	10169.6	18.10
2	0.03	187.4	0.06	533.5	32.61	0.13	56.0	18790.5	33.44
3	0.13	56.2	0.17	85.8	5.24	0.25	13.2	5060.4	9.00
4	0.28	12.3	0.28	13.6	0.83	0.33	3.6	373.2	0.66
5	0.44	0.1	0.47	17.7	1.08	0.49	7.0	442.1	0.79
6	0.51	1.4	0.57	97.3	5.95	0.60	38.4	3310.5	5.89
7	0.60	38.5	0.66	282.2	17.24	0.83	2.4	18051.8	32.12

Graph 2: Estimation of Triterpenes at 366 nm after derivatization from *Satureja khuzestanica* leaf



Peak	Start Rf	Start Height	Max Rf	Max Height	Max %	End Rf	End Height	Area	Area %
1	-0.02	128.7	-0.02	175.9	6.13	-0.01	13.9	1237.5	0.72
2	-0.00	5.9	0.02	253.0	8.82	0.06	0.9	6404.7	3.71
3	0.06	1.2	0.13	418.8	14.59	0.16	383.4	22802.3	13.21
4	0.16	383.7	0.25	463.2	16.14	0.29	423.0	41307.4	23.92
5	0.30	422.0	0.34	485.7	16.93	0.37	456.4	22815.0	13.10
6	0.37	454.8	0.41	471.8	16.45	0.55	246.8	47594.4	27.56
7	0.55	246.7	0.56	248.4	8.66	0.66	63.2	15122.3	8.76
8	0.66	63.3	0.72	194.0	6.76	0.76	151.6	10957.6	6.35
9	0.77	151.9	0.78	158.6	5.53	0.83	2 3	4637.5	2.69

Name: Prof. Dr. Deokule Subhash S.

Signature:

E-3/68/2018.

FORM 3
THE PATENTS ACT, 1970
[39 OF 1970]
and
THE PATENTS RULES, 2003
STATEMENT AND UNDERTAKING UNDER SECTION 8
(See Section 8; Rule 12)

200188614

1. Name of the applicants	I, Deokule Subhash S., Department of Botany, Savitribai Phule Pune University, Pune-7, hereby declare:
2. Name, address and nationality of the joint applicant	Dr. Jagtap Suresh D. Herbal Medicine, Interactive Research School for Health Affairs (IRSHA), Bharati Vidyapeeth University 411 043. (i) that we have not made any application for the same/substantially the same invention outside India
3. Name and address of the assignee	(ii) that the rights in the applications have been assigned to Prof. Deokule, Department of Botany, Savitribai Phule Pune University, Ganesh Khind road, Pune-7, Maharashtra, India. that we undertake that up to the date of grant of the patent by the Controller, we would keep him informed in writing the details regarding corresponding applications for the date of filling of such application Dated this 04 day of January. 2018.
4. To be signed by the applicant or his authorized registered patent agent	Deokule
5. Name of the natural person who has signed	Prof. Dr. Deokule Subash S. Professor (Rtd.) Department of Botany, Savitribai Phule Pune University, Ganeshkhind road, Pune-7, Maharashtra, India.
	To, The Controller of Patents, The Patent Office, at Mumbai

E-5/1/2018

FORM 5
THE PATENTS ACT, 1970
(39 of 1970)
&
The patents rules, 2003

200188615

DECLARATION AS TO INVENTORSHIP
[See section 10 (6) and rule 13(6)]

1. NAME OF APPLICANTS	(i) Prof. Dr. Deokule Subhash S.

Hereby declare that the true and first inventor(s) of the invention disclosed in the complete specification filed in pursuance of our application numbered..... Dated...04/01/2018.....are

2. INVENTOR(S)

a.	**Name**	:	Prof. Dr. Deokule Subhash S.
b.	**Nationality**	:	Indian
c.	**Address**	:	Department of Botany, Savitribai Phule Pune University, Ganeshkhind Road, Pune-411007, Maharashtra, India.
a.	**Name**	:	Dr. Batool Sadegi
b.	**Nationality**	:	Iranian
c.	**Address**	:	Department of Botany, Savitribai Phule Pune University, Ganeshkhind Road, Pune-411007, Maharashtra, India.
a.	**Name**	:	Mr. Veer Sanjay L.
b.	**Nationality**	:	Indian
c.	**Address**	:	Herbs Foundation, A1-604, Dream City, Behind Telco Coloney, Dattanagar, Ambegaon, Pune 411 046. Maharashtra, India.
a.	**Name**	:	Mr. Jagtap Nitin S.
b.	**Nationality**	:	Indian
c.	**Address**	:	At post Kothale, Taluka- Purandhar, District- Pune, Pin.-412301. Maharashtra, India.
a.	**Name**	:	Mr. Bhongale Chetan J.
b.	**Nationality**	:	Indian
c.	**Address**	:	School of Energy Studies, Savitribai Phule Pune University (SPPU)– 411 007. Maharashtra, India.

a.	**Name**	:	Mr. Suraj Bhongale
b.	**Nationality**	:	Indian
c.	**Address**	:	Rajiv Gandhi Institute of Biotechnology, Bharati Vidyapeeth University, Pune Satara Road, Pune – 411 043. Maharashtra, India.
a.	**Name**	:	Dr. Suresh D. Jagtap
b.	**Nationality**	:	Indian
c.	**Address**	:	Herbal Medicine, Interactive Research School for Health Affairs (IRSHA), Bharati Vidyapeeth University, Pune Satara Road, Pune – 411 043. Maharashtra, India.

Dated this ..04.... day of ..January.....201

Signature:

Prof. Dr. Deokule S. S.
Rtd. PROFESSOR
Department of Botany
..ibai Phule Pune University
Pune - 411 007.

3. DECLARATION TO BE GIVEN WHEN THE APPLICATION IN INDIA IS FILED BY THE APPLICANTS IN THE CONVENTION COUNTRY:-

-NOT APPLICABLE-

4. STATEMENT (to be signed by the additional inventors not mentioned in the application form)

-NOT APPLICABLE-

To,

The Controller of Patent

The Patent Office, at Mumbai

National Biodiversity Authority

राष्ट्रीय जैव विविधता प्राधिकरण

(Statutory body of Ministry of Environment, Forest and Climate Change, Government of India)

J. Justin Mohan, IFS
Secretary
☎ +91 44 2254 1071
+91 44 2254 1074
secretary@nba.nic.in www.nbaindia.org

5th Floor, CSIR Road, TICEL Bio Park,
Taramani, Chennai - 600 113, Tamil Nadu, India.
5 वां तल, सीएसआईआर रोड, टाइसल बायो पार्क,
तरमणि, चेन्नई - 600113 तमिल नाडु, भारत

NBA/IPR-Gen/33/17-1/18-19/2134

30.10.2019

To

Shri. O P Gupta, IAS
Controller General of Patents, Designs & Trade Marks
Intellectual Property India,
Patents/Designs/Trade Marks/Geographical Indications,
Boudhik Sampada Bhavan,
Antop Hill, S.M. Road,
Mumbai-400037

Sir,

Sub: Patent application number: 201821000395 – Requirement of Prior-approval from NBA- reg.

This has reference to patent application no.: 201821000395 titled "Development of new herbal ointment for the management of dermatophytic infections" filed by Prof Dr. Deokule Subhash S and published by IPO. As you are aware, Section 6 of the Biological Diversity Act, 2002 mandates that any person applying for any Intellectual Property Right for an invention based on any research or information on a biological resource obtained from India, shall obtain prior approval of NBA.

In the patent application referenced above, the applicant has used *Satureja khuzestanica* for developing the claimed invention and the applicant has given a declaration in the Form I that the invention disclosed uses biological material from India and necessary permission from competent authority will be obtained. However, the applicant has not obtained requisite approval under Section 6 of the Biological Diversity Act, 2002. Hence, it is requested that the applicant may be instructed to seek approval under section 6 and the patent shall not be granted until approval is obtained from National Biodiversity Authority.

Yours faithfully,

(J. Justin Mohan)
Secretary, NBA

Copy to:

Prof Dr. Deokule Subhash S, Department of Botany, Savitribai Phule Pune University (Sppu), Ganeshkhind Road, Pune-411 007, Maharashtra. E-mail: deokule.ss@gmail.com

SAVITRIBAI PHULE PUNE UNIVERSITY

(formerly University of Pune)

DEPARTMENT OF BOTANY
SAVITRIBAI PHULE PUNE UNIVERSITY
Ganeshkhind, Pune-411 007
Tele. No. : (020) 25601439, 25601438
Email : .....@unipune.ac.in

Ref. No. : Bot/ D-381 **Date :** 04/01/2018.

To,
The Controller of Patents,
The Patent Office,
Boudhik Sampada Bhavan,
S. M. Road, Antop Hill, Mumbai,
Maharashtra- 400037

201/127/2018

200188612

Subject: Submission of Complete Patent Specification

Dear Sir,

Please find herewith documents for a complete specification application entitled "**Development of new herbal ointment for the management of dermatophytic infections**" along with the prescribed fee of Rs. 1750/-.

The Learned Controller is requested to take the same on record and issue the official filing receipt for the same.

Yours faithfully,

Prof. Dr. Deokule Subhash S.
Department of Botany,
Savitribai Phule Pune University (SPPU),
Ganeshkhind Road,
Pune- 411007. Maharashtra, India

Enclosures:

6. Application for Grant of Patent [Form 1]
7. Complete specification [Form 2]
8. Statement and undertaking under section 8 [Form 3]
9. Declaration as to inventorship [Form 5]
10. Fees of Rs. 1750/- (by cash)

EPO MUMBAI 04-01-2018 16:39

D No. 57750

E-101|4994|2020

SAVITRIBAI PHULE PUNE UNIVERSITY

(Formerly University of Pune)

Dr. S. S. Deokule
M.Sc.Ph.D.(Pharmac.)D.Sc.FABSc.
Ex-Prof. & Head
Email - deokule.ss@gmail.com
Mob. +91 - 9371088210

Department Of Botany
Savitribai Phule Pune University
Ganeshkhind, Pune - 411 007
Mob. +91 - 9371088210

Ref.No.

Date : 06/10/2020

To
The Controller of Patents,
The Patent Office,
Boudhik Sampada Bhavan,
S. M. Road, Antop Hill, Mumbai,
Maharashtra – 400 037.

200293024

Dear Sir,

Re: Submission of form 18 for examination of Patent

We are submitting a request for examination on Form 18 along with the prescribed fee of Rs. 4400/-. Patent Application numbers along application dates are: (2509/MUM/2014).

You are respectfully requested to carry out examination of the above application and first examination report may please be issued to us.

Yours faithfully,

Deokule

Dr. Subhash Sadhu Deokule
Retired Professor & Head
Department of Botany,
Savitribai Phule Pune University, Ganeshkhind Road,
Pune-411 007

Enclosures:

1. Form 18 (07 applications)
2. Fees of Rs. 4400 x 07 applications=30,800 (by cash)

IPO MUMBAI 08-10-2020 15:57

"FORM 1 THE PATENTS ACT 1970 (39 of 1970) and THE PATENTS RULES, 2003 **APPLICATION FOR GRANT OF PATENT** (See section 7, 54 and 135 and sub-rule (1) of rule 20)	(FOR OFFICE USE ONLY) 200207860

1750/- रूपये रोकडे/चेक/मनी ऑर्डर द्वारा CBR संख्या 18878 दि. 20/08/2018 के तहत प्राप्त हुए।	Application No.	201821031061
	Filing date	20-08-2018
	Amount of Fee paid	₹. 1750/-
	CBR No	18878
	Signature	

1. APPLICANT'S REFERENCE / IDENTIFICATION NO. (AS ALLOTTED BY OFFICE)	

2. TYPE OF APPLICATION [Please tick (√) at the appropriate category]

Ordinary (√)		Convention ( )		PCT-NP ( )	
Divisional ( )	Patent of Addition ( )	Divisional ( )	Patent of Addition ( )	Divisional ( )	Patent of Addition ( )

3A. APPLICANTS

Name in Full	Nationality	Country of Residence	Address of the Applicant	
Prof. Dr. Deokule Subhash S.	Indian	India	House No.	Department of Botany, Savitribai Phule Pune University (SPPU)
			Street	Ganeshkhind Road,
			City	Pune
			State	Maharashtra
			Country	India
			Pin code	411 007.

3B. CATEGORY OF APPLICANT [Please tick (√) at the appropriate category]

Natural Person (√)	Other than Natural Person		
	Small Entity ( )	Startup ( )	Others ( )

4. INVENTORS [Please tick (√) at the appropriate category]				
Are all the inventors same as the applicants named above?			Yes ( )	No (√)
If "No", furnish the details of the inventor(s)				
Name in Full	Nationality	Country of Residence	Address of the Inventor	
Prof. Dr. Deokule Subhash S.	Indian	India	House No.	Department of Botany, Savitribai Phule University of Pune (SPPU).
			Street	Ganeshkhind Road
			City	Pune
			State	Maharashtra
			Country	India
			Pin code	411007
Dr. Kabnoorkar Panchashilla S.	Iranian	Indian	House No.	Department of Botany, Savitribai Phule Pune University (SPPU).
			Street	Ganeshkhind Road
			City	Pune
			State	Maharashtra
			Country	India
			Pin code	411007
Ms. Sandecha Eitisha K.	Indian	India	House No.	576, Laxmi Narayan Apartment, Rasta Peth, Near Daruwala Pool.
			Street	Phadke Haud
			City	Pune
			State	Maharashtra
			Country	India
			Pin code	411 011.
Mr. Jadhav Suraj D.	Indian	India	House No.	64, Ganesh House, Khandoba Mal Coloney, Sambhajinagar
			Street	Sambhaji Nagar Road
			City	Satara
			State	Maharashtra
			Country	India
			Pin code	415 004.
Mr. Wagh Ranjit H.	Indian	Indian	House No.	Shirwal, Tal.- Khandala
			Street	Pune Shirval Road
			City	Pune
			State	Maharashtra
			Country	India
			Pin code	412 201
Dr. Bhongale Chetan J.	Indian	Indian	House No.	D-703, Bravuria Society, Balewadi
			Street	Banrer Balewadi Road
			City	Pune
			State	Maharashtra
			Country	India
			Pin code	411 045

Dr. Jagtap Suresh D.	Indian	Indian	House No.	Herbal Medicine, Interactive Research School for Health Affairs (IRSHA), Bharati Vidyapeeth University.
			Street	Pune Satara Road
			City	Pune
			State	Maharashtra
			Country	India
			Pin code	411 043.

5. TITLE OF THE INVENTION

Development of new cultivation technique using bio-elicitation for *Asparagus racemosus* (Shatavari)

6. AUTHORISED REGISTERED PATENT AGENT(S)	IN/PA No.	-
	Name	-
	Mobile No.	-
7. ADDRESS FOR SERVICE OF APPLICANT IN INDIA	Name	Prof. Dr. Deokule Subhash S.
	Postal Address	Department of Botany, Savitribai Phule Pune University (SPPU), Ganeshkhind Road, Pune- 411007. Maharashtra, India
	Telephone No.	020 2560 1217
	Mobile No.	09371088210
	Fax No.	020 2560 1217
	Email ID	deokule.ss@gmail.com

8. IN CASE OF APPLICATION CLAIMING PRIORITY OF APPLICATION FILED IN CONVENTION COUNTRY, PARTICULARS OF CONVENTION APPLICATION

- NOT APPLICABLE -

9. IN CASE OF PCT NATIONAL PHASE APPLICATION, PARTICULARS OF INTERNATIONAL APPLICATION FILED UNDER PATENT CO-OPERATION TREATY (PCT)

- NOT APPLICABLE -

10. IN CASE OF DIVISIONAL APPLICATION FILED UNDER SECTION 16, PARTICULARS OF ORIGINAL (FIRST) APPLICATION

- NOT APPLICABLE -

11. IN CASE OF PATENT OF ADDITION FILED UNDER SECTION 54, PARTICULARS OF MAIN APPLICATION OR PATENT

- NOT APPLICABLE -

12. DECLARATIONS

(i) Declaration by the inventor(s):

We, the above named inventors are the true & first inventors for this invention and declare that IRSHA, BVDU herein our assignee.

Signature:

Name: Prof. Dr. Deokule Subhash S. Date: 14/8/2018

Signature:

Name: Dr. Kabnoorkar Panchashilla S. Date: 14/8/2018

Signature:

Name: Ms. Sandecha Eitisha K. Date: 14/08/2018

Signature:

Name: Mr. Jadhav Suraj D. Date: 13/08/2018

Signature:

Name: Mr. Wagh Ranjit H. Date: 13/08/2018

Signature:

Name: Dr. Bhongale Chetan J. Date: 16/08/2018

Signature:

Name: Dr. Jagtap Suresh D. Date: 14/08/2018

(ii) Declaration by the applicant(s) in the convention country

- NOT APPLICABLE -

Declaration by the applicants:

We the applicants hereby declares that:-

√	We are in possession of the above-mentioned invention.
√	The complete specification relating to the invention is filled with this application.
√	The invention as disclosed in the specification uses the biological material from India and the necessary permission from the competent authority shall be submitted by us before the grant of patent to us.
√	There is no lawful ground of objection to the grant of the patent to us.
√	We are the true & first inventors.
√	We are the assignee of true and first inventors.
√	The application or each of the applications, particulars of which are given in Paragraph-8, was the first application in convention countries in respect of our inventions.
X	We claim the priority from the above mentioned application(s) filed in convention countries and state that no application for protection in respect of the invention had been made in a convention country before that date by us or by any person from which we derive the title.
X	Our application in India is based on international application under Patent Cooperation Treaty (PCT) as mentioned in Paragraph-9.
X	The application is divided out of our application particulars of which is given in Paragraph-10 and pray that this application may be treated as deemed to have been filed on 20th August 2018 under section 16 of the Act.
X	The said invention is an improvement in or modification of the invention particulars of which are given in Paragraph-11.

13. FOLLOWING ARE THE ATTACHMENT WITH THE APPLICATION:

(a) Form 2

Item	Details	Fee	Remarks
Complete specification	No. of pages: 10	1750	
No. of Claims	No. of claims - 07 No. of pages – 01		
No of drawing sheets	00		
No. of figures	06		
No. of tables	01		
No of abstract pages	01		

(b) Complete specification (1 copies)

(c) Statement and undertaking on Form 3

(d) Declaration as to Inventorship on Form 5

(e) Total fee Rs.....in cash.

We hereby declare that to the best of my/our knowledge, information and belief the fact and matters stated herein are correct and I/we request that a patent may be granted to me/us for the said invention.

Dated this 14th day of August ,2018.

Signature:

Prof. Dr. Deokule Subhash S.
Department of Botany,
Savitribai Phule Pune University (SPPU),
Ganeshkhind Road,
Pune- 411007. Maharashtra, India

To,

THE CONTROLLER OF PATENTS,

THE PATENT OFFICE, MUMBAI

FORM 2
THE PATENT ACT 1970
(39 OF 1970)
&
The patents rules, 2003
COMPLETE SPECIFICATION
(See section 10 and rule 13)

200207861

1. TITLE OF THE INVENTION:

Development of new cultivation technique using bio-elicitation for *Asparagus racemosus* Willd. (Shatavari)

2. APPLICANT (S):

a. Name : Prof. Dr. Deokule Subhash S.
b. Nationality : Indian
c. Address : Department of Botany, Savitribai Phule Pune University, Ganeshkhind Road, Pune-411007, Maharashtra, India.

a. Name : Dr. Kabnoorkar Panchashila S.
b. Nationality : Iranian
c. Address : Department of Botany, Savitribai Phule Pune University, Ganeshkhind Road, Pune-411007, Maharashtra, India.

a. Name : Ms. Sandecha Eitisha K.
b. Nationality : Indian
c. Address : 576, Laxmi Narayan Apartment, Rasta Peth, Near Daruwala Pool. Pune-411011, Maharashtra, India.

a. Name : Mr. Jadhav Suraj D.
b. Nationality : Indian
c. Address : 64, Ganesh House, Khandoba Mal Coloney, Sambhajinagar, Satara – 415 004, Maharashtra, India.

a. Name Mr. Wagh Ranjit H.
b. Nationality Indian
c. Address Shirwal, Tal.- Khandala, District – Satara – 412 201, Maharashtra, India.

a. Name Dr. Bhongale Chetan J.
b. Nationality Indian
c. Address D-703, Bravuria Society, Balewadi, Banrer Balewadi Road, Pune – 411 045, Maharashtra, India.

a. Name : Dr. Jagtap Suresh D.
b. Nationality : Indian
c. Address : Herbal Medicine, Bharati Vidyapeeth (Deemed to be University), Interactive Research School for Health Affairs (IRSHA), Pune Satara Road, Pune – 411 043. Maharashtra, India.

3. PREAMBLE TO THE DESCRIPTION

PROVISIONAL	**COMPLETE**
NOT APPLICABLE	The following specification describes the invention.

4. DESCRIPTION

Field of the Invention.

There are reports on activity of *Asparagus racemosus* Willd. which includes numerous therapeutic application viz. antioxidant, diuretic, antidepressant, antiepileptic, antitussive, anti-HIV, immunostimulant, hepatoprotective, cardio-protective, antibacterial, antiulcerative, neurodegenerative activities (Singla *et al.*, 2014).

Despite *Asparagus racemosus* Willd. being highly demanded in the market, but its availability of raw drug is comparatively less. It is also under cultivation but there are certain limitations to meet the ratio of demand and supply.

The present investigation includes modified cultivation technique by using Bio-elicitation, field trials such as soil, land preparation, propagation, transplanting material, irrigation, manuring, weeding and harvesting. Shatavarin IV is one of the major therapeutically active compounds responsible in various ailments of human. Therefore, its quantity is confirmed with the help of HPTLC study. Comparative infection of fungus was also studied using Blotter test method.

The results of this investigation lead to vigorous growth of tuberous root as well as they were free from fungal infections when after a year Neem leaves mixed with Farm Yard Manure (FYM) and green manure were added in the field. Another benefit of Shatavari crop, grown under red soil mixed with Farm Yard Manure (FYM) and green manure showed maximum production of the tuberous roots and benefit cost ratio is also doubled. Results also showed that concentration of Shatavarin IV which is bioactive secondary metabolite has been enhanced.

Background of the invention:

***Asparagus racemosus* Willd. :** It is an indigenous medicinal plant of the family Liliaceae (Chopra et al., 1956; Anonymous, 1976) is important for its sapogenin content (Rao, 1952; Subramanian and Nair, 1968). It is regarded as a 'rasayana' which means plant drugs promoting general well-being by increasing cellular vitality and resistance. (Singla *et al.*, 2014)

Plant part used: Tuberous Roots, Leaves, flowers and fruits.

Habitat: This climber is found to be growing throughout India; especially in Northern India. (Nadkarni, 1954). The plant is a climber growing upto 1-2m in length found all over India (Jarald & Jarald, 2007).

Description: *Asparagus racemosus* Willd. is plant with a woody stem that sends runners out, has needle like leaves with small white flowers, (Aviva Romm, 2010). It is scandant, much branched spinous under shrub with numerous tuberous, short roots. The single root stock bearing numerous fusiform tuberous roots 30-100cm thick. Leaves are reduced to minute chaffy scales & spines. Cladodes are acicular 2-6 hate, falcate finely acuminate flower white. Berries are globose, 7mm in diameter and 1-seeded, red (Sharma et al., 2000).

Distribution: Throughout India, Tropical and subtropical parts including Andamans and ascending in the Himalayas up to an altitude of 1500m (Sharma et al., 2000).

Bioelicitation

It is a process followed for the enhancement of quantity of chemical compounds. There are two types of elicitors such as Biotic and Abiotic elicitors. Generally, the biotic elicitors such as *Yeast, Aspergillus flavus, Aspergillus niger,* and *Mucor* species are been used. Here, bioelicitation was performed for Shatavarin IV. Generally *A. racemosus* contains several secondary metabolites in which shatavarin I-IV are promienent but Shatavarin I and IV are major constituents. Shatavarin IV plays major role in various medicinal activities of plant *A. racemosus* (Alok et al., 2013).

Propagation and cultivation: The plants can be successfully grown in variety of soil, but it prefers sandy well drained soil. They can be propagated by seeds and divisions of rhizomatous disc. Seedling should be planted preferably on ridges, 60 in to 60 cm apart. Application of 12 tons per hectare (Red Soil + FYM + Green Manure 1:1:1) increases the yield of roots considerably. Harvesting is recommended 40 months after plantation. Shatavari can also be propagated by shoot tip culture on MS medium supplemented with BAP (0.5mg/l). Shoot tip proliferates into a number of offshoots supplemented with NAA (1.0 mg/l) + Kinetin (0.5 mg/l) (Sharma et al., 2000).

In wild condition, this plant is grown in variety of soils where the yield is reaching upto 30 tuberous roots/plant wherever it is found to be growing. The review of literature revealed that the plant can be successfully grown in black cotton soil mixed with river sand and yield is about 50-70 tuberous roots/plants. As the plant is age old it has indiscriminately exploited for its medicinal use in different systems of medicine such as Ayurveda, Siddha, Unani, Tibbi, Amachi and by pharmaceuticals. Hence, in the present investigation, modification of cultivation technique was tried.

Summary of the invention

Considering the present scenario, there are certain limitations to obtain more yield and concentration of secondary metabolites which is Shatavarin IV responsible for particular activity. The main aim of the present investigation is to develop the new modified methods for the maximum production of tuberous roots of Shatavari. In the present investigation, modified cultivation technique was used in such a way that Bio-elicitation + Black soil mixed with Red soil + Farm Yard Manure (FYM) + Green manure (1:1:1), which gave tremendous yield upto 8-140 tuberous roots/plant as in this combination porosity of Black cotton soil increases while humidity decreases and for vigorous growth of tuberous roots plant is getting maximum amount of NPK. Various cultivation techniques were followed using soil, land preparation, propagating material, transplanting material, irrigation, manuring, weeding and harvesting. Also, different field stages of Shatavari were observed. As a result, we have observed increased yield of tuberous roots, increased percentage of active secondary metabolite and less or no fungal infection. In brief, we have developed new cultivation technique using bioelicitation method.

Detailed description of the tables:

Table 1: Shatavari HPTLC analysis report

Detailed description of the figures:

Figure 1: Cultivation plot of *A. racemosus*

Figure 2 : After 1 year used ring method to addition of formulation

Figure 3 : Plot wise yield of tuberous roots

Figure 4 : Difference between tuberous roots of plot A and B

Figure 5 : Estimation of fungal infection

Figure 6 : qualitative analysis of Shatavari

Detailed description of the invention:

Detailed experimental studies

The plant seeds were procured from Rahuri Krishi Vidyapeeth, Ahmednagar.

Seed: Micropropagation

Preparation of culture media and inoculation of explants: The asparagus seeds were surface sterilized and inoculated on Sterile MS basal medium, hypocotyls from seed were used to produce callus (Lomror et al., 2018). The above obtained callus culture was further used for bio-elicitation process.

Preparation of biotic elicitors and elicitation medium

The maintained culture of *Aspergillus niger* fungal species in the laboratory was inoculated in potato dextrose broth (PDB) and incubated at 24 ± 2° C. Mycelia mat was developed in the culture vessels on the surface of the PDB after 12 days of incubation. The culture vessels were autoclaved at 121° C for 10 mins before harvesting the dense mycelial mats. Mycelia mats were harvested, filtered and collected. This collected biomass was thoroughly washed with sterile double distilled water to remove medium components. The obtained biomass was air dried and grounded to a fine powder. This fine powder was added to the liquid MS media with the varied concentrations ranging from 0–100 mg/100 mL medium (0, 25, 50, 75 and 100 mg/100 mL medium) as per research paper (Vasudeva, 2016). The biomass-free fungal culture filtrate (ECF) was sterilized using 0.45 lM syringe filter (Millipore, USA) and then added to the media as biotic elicitor with the concentrations ranging from 0–20 mL/ 100 mL medium (0, 5, 10, 15 and 20 mL/100 mL medium). The powder was supplied solely and in consortium for the elicitation in the cell suspension cultures and biotic elicitors were added to the actively growing cell suspension cultures.

Micropropagation and transplantation

The developed calli were sub cultured to regeneration medium containing various concentrations of growth regulators. The elongated shoots were transplanted on half strength MS medium having various concentration of IBA. All the cultures were incubated in a growth room with standard photoperiod except callus culture. *In vitro* regenerated plantlets were planted on plastic tray containing sterile sand, soil and farmyard manure in equal ratio (1:1:1), the plantlets were covered with transparent polythene bag to maintain humidity for initial ten days. After this the plantlets were shifted to green house under less humid environment and natural sunlight. Finally, these plants were transplanted in the cultivation field (Patel and Patel, 2015).

Cultivation Techniques

Plants were planted in two plots i.e. Plot A: Black soil used as control and Plot B: Black soil mixed with red soil + FYM + green manure. Observation made regularly to study the different stages of vegetative growth (Figure. 1, 2).

a. **Soil-** The Shatavari was cultivated in isolated plots in the area of Department of Botany, Pune University, Pune. Plots were taken in specialized field having black soil and pH 8.

b. **Land preparation**- Before planting, the field was ploughed for 2-3 times. Then in one plot red soil + FYM + Green manure (1:1:1) combination were added and another plot was kept as Control - Black soil.

c. **Propagating material**- Seeds were used for propagation.

d. **Transplanting material-** Transplanting is done at a distance of 60 cm × 60 cm in a row and supported by bamboo stakes when the plants attained a certain height.

e. **Irrigation-** After transplanting the seedlings, watering was done in the field. Initially 2-3 irrigations were required within a week till the plants are established. After establishment of plants, light irrigation was done in 3-4 days of interval.

f. **Manuring-** Organic manures like Farm Yard Manure (FYM), Green manure, etc. were used as per the requirement of plants.

g. **Weeding-** Frequent weeding was done during its early period of growth. Weeding should be done as per need during rainy season.

h. **Harvesting-** When the above ground parts start turning pale yellow then it's the best time for harvesting tuberous roots. Plants were harvested after 18 or 20 months after sowing.

Antifungal assay:

Agar Plate Method:

Agar Plate Method has been used for identification and detection of microorganisms associated with seed based on the growth and colony characteristics on a nutrient agar. Sterile Potato Dextrose Agar (P.D.A.) medium was prepared for inoculation. Roots were treated with 0.1% mercuric-chloride for two minutes and washed three times with sterilized water. The plates were incubated at 28 ± 1°C with 12 hours alternating cycles of light and darkness and examine the plates after 8 days of incubation. Characteristics of fungal colonies were noted; slides were prepared and examined under compound microscope. Percentages of infection were recorded.

Standard Blotter Method: (Doyer, 1938)

The blotter method is widely used for detecting fungi which are able to produce mycelial growth and fruiting structures under the incubation conditions available in the test. In standard blotter method (SBM), roots were surface sterilized with 2% sodium hypochlorite for 2 min followed by repeated wash with distilled water. Roots were placed in petri plates on three layers of moistened blotters and incubated at 24oC for 12 h with alternating cycles of light and darkness for 7 days (ISTA, 2005). Single spore isolation was made on potato dextrose agar (PDA) slants, incubated at 250C for 5-6 days and the pure fungal colonies were subjected to further observation. The percentage frequency of each fungal colony was calculated (Shakoor *et al.*, 2011).

Extraction and Isolation of Shatavarin IV: (Gohel *et al.* 2015)

Root powder of *A. racemosus* (250 g) collected for plot A and B, defatted with Hexane and extracted with methanol at room temperature for 24 h. After evaporation of methanolic extract concentrated liquid was dissolved in 10% methanol. The resulting solution was partitioned with Chloroform, Ethyl acetate and n-Butanol, successively. The n-Butanol extract was dried on a water bath and the dried fraction was dissolved in minimum quantity of 90% methanol to load on the column as the sample. Silica gel G (60-120) was used for packing the column and eluted with Ethyl acetate: Methanol: Water (8:1:1 v/v) as mobile phase. Fractions were collected (each of 27 mL) and eluted, showed presence of Shatavarin IV. Shatavarin IV containing fractions was confirmed with Vanillin-Sulfuric acid reagent by TLC using Ethyl acetate: Methanol: Water (7.5:1.5:1 v/v). The dried, isolated compound was obtained and characterized by using HPTLC method compared to the reference standard.

Estimation of Shatavarin IV by HPTLC:
Shatavarin IV was estimated using HPTLC for both plot A and B. Shatavarin IV standard and isolated compound was dissolved in methanol individually and prepared 100 µg/ml solutions. 16 µl of sample and standards were applied to a precoated Silica gel (60 F254 plate of 0.25 mm thickness). The plates were then developed with the mobile phase consisting of Ethyl acetate: Methanol: Water (7.5:1.5:1 v/v). The spots were visualized by dipping the plate in Vanillin Sulphuric acid reagent and heating at 105°C for 5 min. The plates were scanned using CAMAG TLC Scanner IV at 425 nm to estimate the concentration of Shatavarin IV.

Benefit Cost Ratio (BCR):
A benefit cost ratio (BCR) is an indicator used in cost benefit analysis, to show the relationship between the costs and benefits of a proposed project, in monetary or qualitative terms.

$$\text{Benefit Cost Ratio} = \frac{\text{Cost of gross monitoring returns}}{\text{Cost of production}}$$

Results and discussion

Bioelicitation and transplantation:
Callus obtained from the seeds were used for bio-elicitation and plantlets developed using bioelicitation were sub cultured, micro propagated and after hardening they were transferred in cultivation field.

Harvesting and yield:
In the present investigation, plants were planted in two plots viz. A and B (Figure 1,2). After 18 months, Shatavari crop starts turns to yellow which is an indication of harvesting time (Figure 1). Crop was harvested. The crop grown in plot A showed less tuberous root production as compare to the grown in plot B (Figure 3 & 4). Each harvested tuberous roots were measured.
Plot A: A single plant yield about 300-400 g. of fresh root.
Plot B: A single plant yield about 700-1000 g. of fresh root.

Screening of the extract for Antifungal Assay:
Growth of fungus *Aspergillus niger* was observed in the roots harvested from Plot A whereas, there was no fungal infection in roots of Plot B. Similarly, growth fungus *Aspergillus niger* was not observed in the plot treated with Neem extract (Figure 5).

Quantification of Shatavarin IV:
In the present study, isolation of Shatavarin IV of plot A and plot B was performed by proposing a column chromatographic method using. Yellow colour fractions showed spot at the Rf of standard Shatavarin IV which confirmed the presence of the Shatavarin IV. The physical characteristics of the isolated compound were observed with standared Shatavarin IV which indicates isolated compound as Shatavarin IV (Figure 6). Peak purity was established through spectral detection of peaks. The % yield of isolated Shatavari IV was 0.25 % for Plot A (control) where as 0.38 % for Sample B (treated). Overall there is 0. 13 % increase in Shatavarin IV using this new technology, especially bioelicitation (Table 1).

Benefit cost ratio:

Benefit cost ratio was calculated for easy understanding of enhanced yield. In which the cost of gross monitoring returns was 130000 where as cost per production is 60000. The ratio is 2.16 /acre which is more significant.

Conclusion:

After one year Neem leaves + FYM + Green manure added in the field, it helped for the vigorous growth of tuberous root as well as the roots become free from fungal infection. Shatavari coops, grown under red soil + FYM + Green manure showed maximum production of the tuberous roots of Shatavari as well as benefit cost ration is also doubled. In brief the present technology is useful for the enhanced growh of tuberous roots of Shatavari and can be implemented for commercial cultivation of Shatavari. Bioelicitation method has found to be more effective for the enhancement of Shatavarin IV.

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5. Claims

We claim:

1. A novel technique developed with combination of different cultivation methods for *Asparagus racemosus* Willd.(Shatavari).
2. New technique of cultivation with its specific combination of different method as in claim 1 can be used for cultivation of Shatavari.
3. Method applied for bioelicitation is as in claim 1, can be used for enhancement of active secondary metabolites.
4. Novel technique with its specific concentrations of manures as in claim 1, and applied as in claim 2, have no adverse effects reported.
5. Novel technique with its specific combination as in claim 1, and specific concentration as in claim 2, and using technique as in claim 3, is capable of giving substantially increased yield. Thus it has remarkable capacity to enhance yield.
6. According to any of the proceeding claims, the said technology in an embodiment is capable of increasing yield as mentioned in method.
7. According to any of the proceeding claims, the said technology of bioelicitation showed remarkable increase in the concentration of active secondary metabolite in roots of *A. racemosus* Willd.

Name : Prof. Dr. Deokule Subhash S. Signature :

6. Date and Signature

Date: 14/8/2018

Place: Pune

Prof. Dr. Deokule Subhash S.
Department of Botany,
Savitribai Phule Pune University (SPPU),
Ganeshkhind Road, Pune- 411007.
Maharashtra, India.

TITLE: Development of new cultivation technique using bio-elicitation for *Asparagus racemosus* (Shatavari)

7. ABSTRACT OF THE INVENTION

In present investigation, developed a new method for the maximum production of tuberous roots of Shatavari using bioelicitation technique. Modified cultivation techniques were used in such a way that Black soil mixed with Red soil + Farm Yard Manure (FYM) + Green manure (1:1:1), which gave tremendous yield upto 8-140 tuberous roots/plant as in this combination porosity of Black cotton soil increases while humidity decreases and for vigorous growth of tuberous roots plant is getting maximum amount of NPK. Along with bioelicitation, various cultivation techniques were followed using soil, land preparation, propagating material, transplanting material, irrigation, manuring, weeding and harvesting. Considering these limitations, to obtain more yield and concentration of secondary metabolites responsible for biological activity, we have developed new cultivation techniques using Bio-elicitation. This overall study resulted into increase in yield and concentration of secondary metabolites which was confirmed using HPTLC techniques.

Table 1: Shatavari HPTLC analysis report

Sr. No.	Name of Sample	Shatavarin IV
1.	Sample I (Control) A	0. 25%
2.	Sample II B	0. 38%

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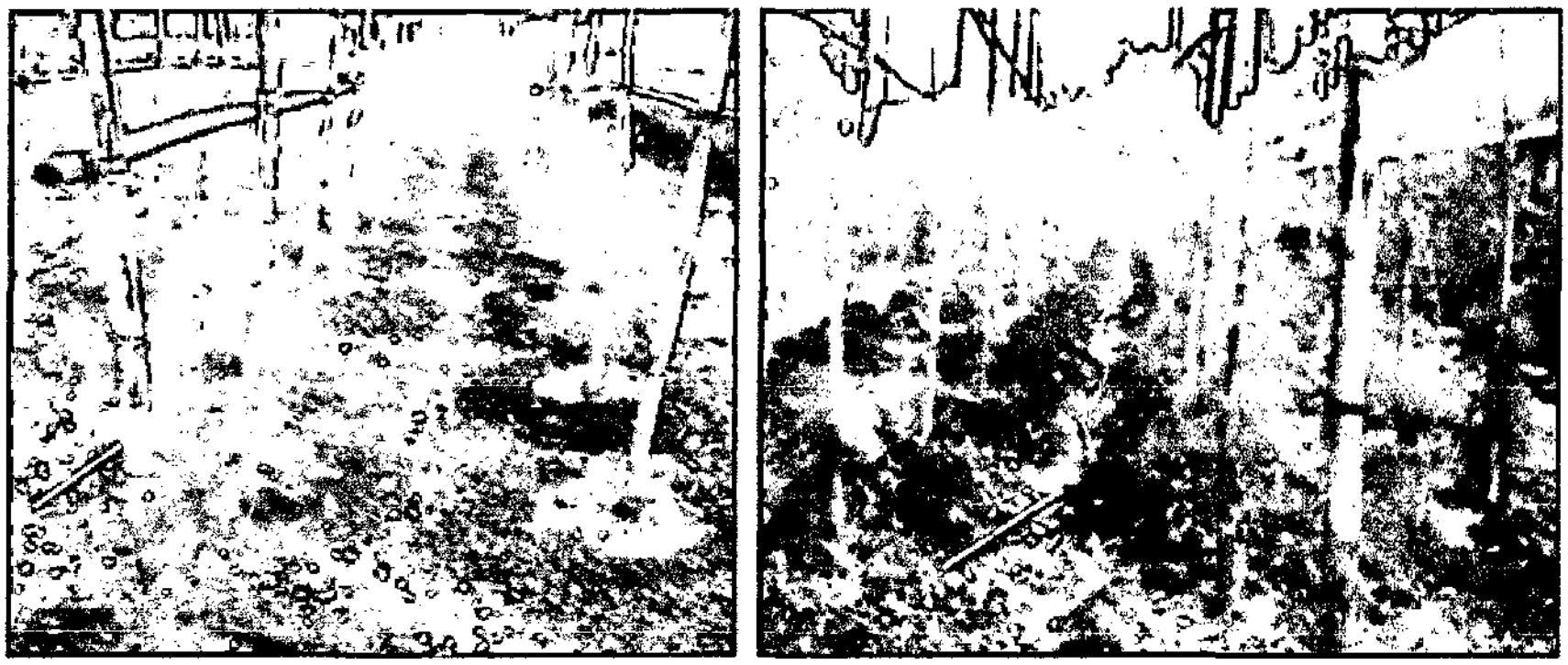
Signature:

Figure 1: Cultivation plot of *A. racemosus*

3 months old seedlings | 6 months | 12 months | 15 months

18 months | Plants Turn to yellow at the time of harvesting | Flowering and Fruiting twigs

Figure 2 : After 1 year used ring method to addition of formulation



Name: Prof. Dr. Deokule Subhash S.

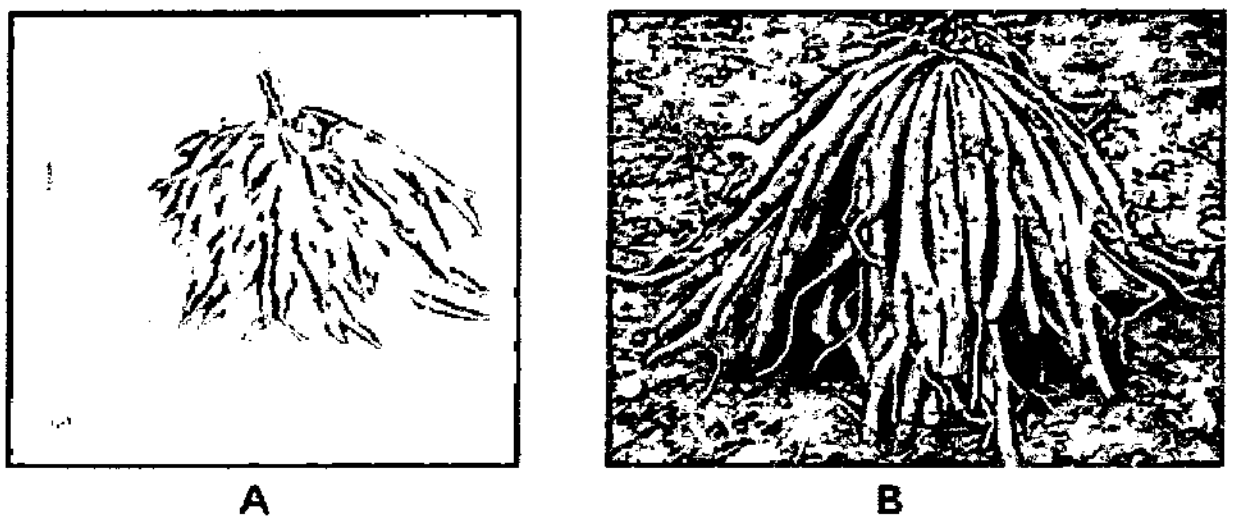
Signature:

Figure 3 : Plot wise yield of tuberous roots

Control (Plot A)

Test (Plot B)

Figure 4 : Diference between tuberous roots of plot A and B



A

B

After harvesting tuberous roots of Shatavari

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Signature:

Figure 5: Estimation of fungal infection

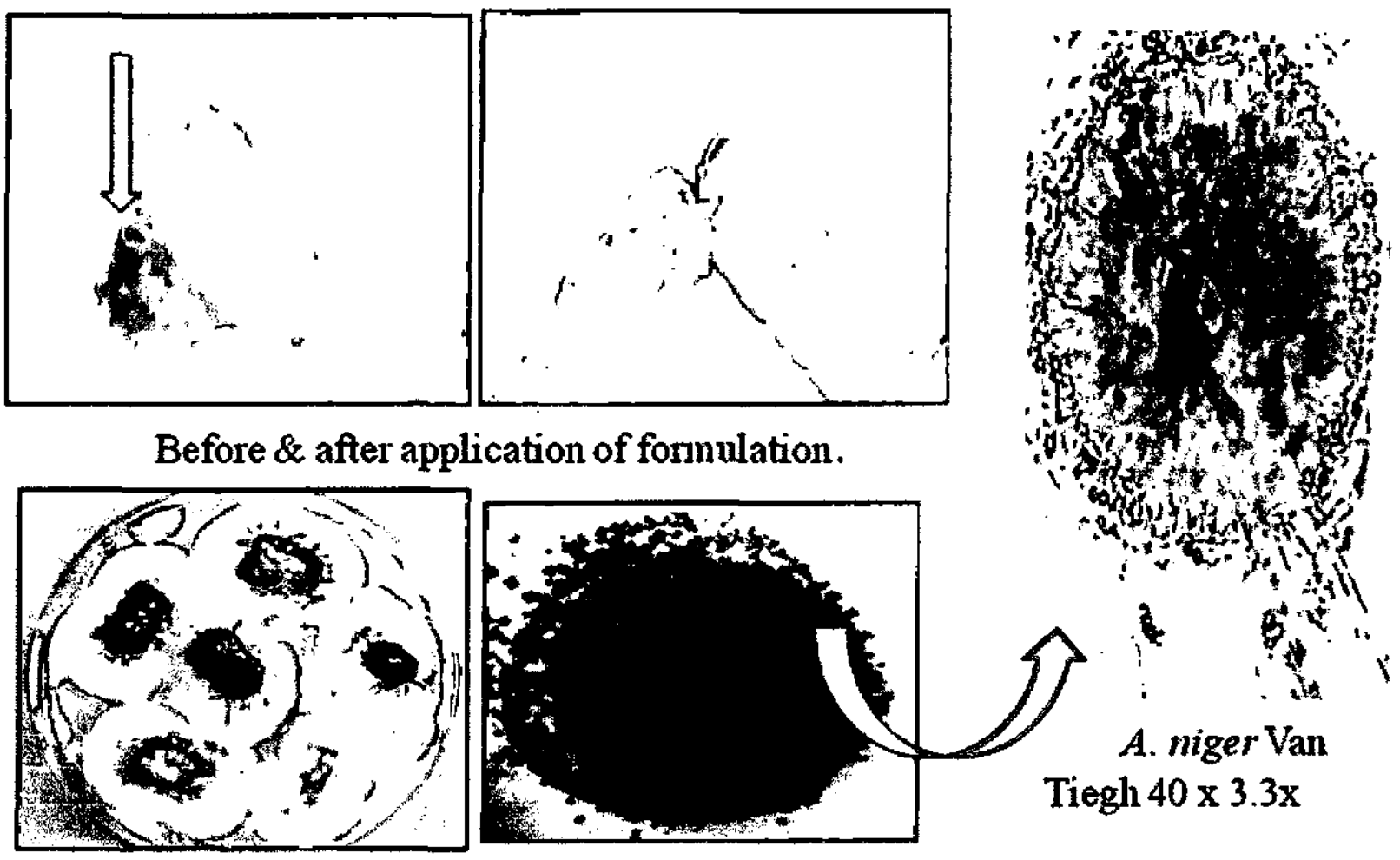
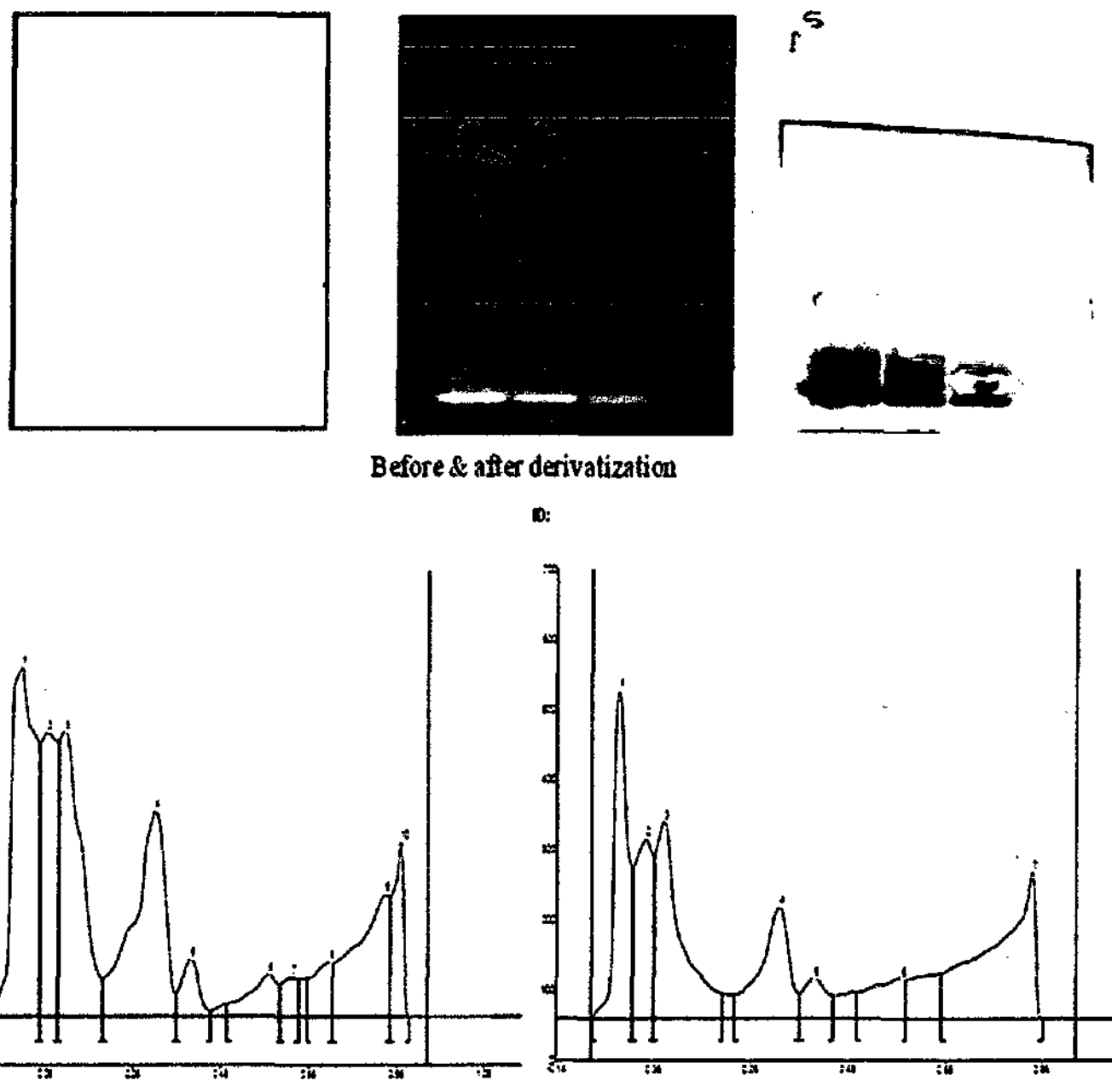


Figure 6 : qualitative analysis of Shatavari



Name: Prof. Dr. Deokule Subhash S.

Signature

E-3/10271/2018

FORM 3
THE PATENTS ACT, 1970
[39 OF 1970]
and
THE PATENTS RULES, 2003
STATEMENT AND UNDERTAKING UNDER SECTION 8
(See Section 8; Rule 12)

200207862

1. Name of the applicants	I, Deokule Subhash S., Department of Botany, Savitribai Phule Pune University, Pune-7, hereby declare:
2. Name, address and nationality of the joint applicant	Dr. Jagtap Suresh D. Herbal Medicine, Interactive Research School for Health Affairs (IRSHA), Bharati Vidyapeeth University 411 043. (i) that we have not made any application for the same/substantially the same invention outside India
3. Name and address of the assignee	(ii) that the rights in the applications have been assigned to Prof. Deokule, Department of Botany, Savitribai Phule Pune University, Ganesh Khind road, Pune-7, Maharashtra, India. that we undertake that up to the date of grant of the patent by the Controller, we would keep him informed in writing the details regarding corresponding applications for the date of filling of such application Dated this 20th day of August 2018.
4. To be signed by the applicant or his authorized registered patent agent	Deokule
5. Name of the natural person who has signed	Prof. Dr. Deokule Subash S. Professor (Rtd.) Department of Botany, Savitribai Phule Pune University, Ganeshkhind road, Pune-7, Maharashtra, India.
	To, The Controller of Patents, The Patent Office, at Mumbai

E-5/1352/2018.

FORM 5
THE PATENTS ACT, 1970
(39 of 1970)
&
The patents rules, 2003
DECLARATION AS TO INVENTORSHIP
[See section 10 (6) and rule 13(6)]

1. NAME OF APPLICANTS	(i) Prof. Dr. Deokule Subhash S.

Hereby declare that the true and first inventor(s) of the invention disclosed in the complete specification filed in pursuance of our application numbered…….. Dated……..are

2. INVENTOR(S)

a.	**Name**	:	Prof. Dr. Deokule Subhash S.
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Dated this ..14th.... day of ....August.....2018.

Signature:

Savitribai Phule Pune University • Dept. of Botany •

Prof. Dr. Deokule S. S.

Dr. S. S. Deokule
Rtd. Professor & Head
Department of Botany
Savitribai Phule Pune University,
Pune- 411 007.

3. DECLARATION TO BE GIVEN WHEN THE APPLICATION IN INDIA IS FILED BY THE APPLICANTS IN THE CONVENTION COUNTRY:-

-NOT APPLICABLE-

4. STATEMENT (to be signed by the additional inventors not mentioned in the application form)

-NOT APPLICABLE-

To,

The Controller of Patent

The Patent Office, at Mumbai

200293023

FORM 18
THE PATENTS ACT 1970
(39 of 1970)
&
The Patents Rules, 2003
REQUEST/EXPRESS REQUEST FOR EXAMINATION OF APPLICATION FOR PATENT

[See section 11B and rule 20(4)(ii), 24B(1)(i)]

(FOR OFFICE USE ONLY)

RQ No: R2020203|280
Filing Date: 08|10|2020
Amount of Fee Paid: रूपये नकद/चेक/मनी ऑर्डर द्वारा
CBR No: 44009 CBR संख्या. 26226 ..दि.08|10|2020
Signature के नहत प्राप्त हुए।

रोकड़ीय

1. APPLICANTS

(a) Name: Deokule Subhash Sadhu

(b) Nationality: Indian

(c) Address: Department of Botany, Savitribai Phule Pune University (SPPU), Pune- 411 007, Maharashtra, India.

2. Statement in case of request for examination made by the applicants

We hereby request that our application for patent no.: **201821031061** filed on **20/08/2018** for the invention titled **"Development of new cultivation technique using Bio-elicitation for *Asparagus racemosus* Willd. (Shatavari)"** shall be examined under sections 12 and 13 of the

3. Statement in case of request for examination made by any other interested person

I/We the interested person request for the examination of the application no._____dated _____filed by the applicant_____titled_____under sections 12 and 13 of the Act.

As an evidence of my/our interest in the application for patent following documents are submitted.

(a) _____

Not applicable

4. ADDRESS FOR SERVICE

Department of Botany, Savitribai Phule Pune University (SPPU), Pune- 411 007, Maharashtra, India. **Email:** deokule.ss@gmail.com. **Mobile:** 9371088210.

Dated this 8 day of October 2020

Deokule

Deokule Subhash Sadhu

To,
The Controller of Patents
The Patent Office,
Mumbai

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08-Oct-2020/57750/201821031061/Form 18

SAVITRIBAI PHULE PUNE UNIVERSITY

(Formerly University of Pune)

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Ref.No. Bot / 512 / 2018

D-40122

Date : 16|8|2018

To,
The Controller of Patents,
The Patent Office,
Boudhik Sampada Bhavan,
S. M. Road, Antop Hill, Mumbai,
Maharashtra- 400037

E-101/9924/2018

200207859

Subject: Submission of Complete Patent Specification

Dear Sir,

Please find herewith documents for a complete specification application entitled **"Development of new cultivation technique using bio-elicitation for *Asparagus racemosus* (Shatavari)"** along with the prescribed fee of Rs. 1750/-.

The Learned Controller is requested to take the same on record and issue the official filing receipt for the same.

Yours faithfully,

Deokule
16|8|2018

Prof. Dr. Deokule Subhash S.
Department of Botany,
Savitribai Phule Pune University (SPPU),
Ganeshkhind Road,
Pune- 411007. Maharashtra, India

Enclosures:

1. Application for Grant of Patent [Form 1]
2. Complete specification [Form 2]
3. Statement and undertaking under section 8 [Form 3]
4. Declaration as to inventorship [Form 5]
5. Fees of Rs. 1750/- (by cash)

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