Evaluation of Analgesic and Anti-inflammatory Activity of *Ficus racemosa* Linn. Stem Bark Extract in Rats and Mice

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Abstract

Present work was undergone to investigate analgesic and anti-inflammatory effect of *Ficus racemosa* Linn. Stem bark extract in rats and mice. *Ficus racemosa* Linn. was studied for its analgesic activity on acetic acid induced writhing test in mice, tail flick test in rats and hot plate method in mice. The anti-inflammatory effects were investigated by employing acute inflammatory model i.e carrageenan-induced hind paw oedema, egg albumin induced paw oedema in rats, also studied for its preliminary phytochemical screening and acute toxicity studies, revealed presence of flavonoids, tannins and polyphenolic compounds, triterpenoids, coumarins, phytosterols, carbohydrates.

The extract did not produce mortality up to 5000 mg/kg p.o. Ethanol extract at the maximum dose (500 mg/kg) showed comparatively significant ($p< 0.05$) activity in tail flick method, significant inhibition of the writhes in writhing test, showed more significant ($p< 0.05$) response at 90, 120 and 180 min in hot plate method, comparatively significant ($p< 0.01$) inhibition of paw volume in carrageenan and egg albumin induced paw oedema method to that of standard diclofenac sodium (100 mg/kg). Petroleum ether extract is non-significant in all the cases of analgesic and anti-inflammatory methods. While, hydro-alcoholic extract (100, 300, 500 mg/kg) showed quiet more significant ($p< 0.01$) response in analgesic and anti-inflammatory method to that of respective standard. The results obtained suggest marked analgesic and anti-inflammatory activity of ethanolic extract (500 mg/kg) and hydro-alcoholic extract (100, 300, 500 mg/kg). The results obtained support the stem bark is useful in inflammatory and painful conditions like leaves and unripe fruits of the same plant *Ficus racemosa* Linn.

Keywords: *Ficus racemosa*, Moraceae, Analgesic, Anti-inflammatory activity, Acute and Chronic models.

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INTRODUCTION

Ficus racemosa Linn. Syn. *Ficus glomerata* Roxb. is a moderate to large sized tree belonging to the family Moraceae. It is a very common plant distributed throughout the India. The different parts of the plant are employed in native medicine for treating diarrhea, dysentery and diabetes, hypocholesterolemic[1,2], spermatozoic. Unripe fruits and leaves of the same plant in treating inflammation, root bark as anti-oxidant[3,4]. *Ficus racemosa* also been reported to produce anti filarial, antibacterial activities[5,6], in treating wounds and ulcers[7,8]. Hence in the present investigation evaluation of anti-inflammatory activity of stem bark extract of *Ficus racemosa* Linn. (FRSE) was taken up.

MATERIAL AND METHODS

Plant Material and preparation of the extract

The stem bark of *Ficus Racemosa* Linn. was procured from botanical garden of Bhimashankar, Maharashtra, Pune, India. Plant was authenticated by Botanical Survey of India (BSI), Maharashtra, Pune, India where a voucher specimen is deposited for future reference. The grinded bark powder was exhaustively extracted for 24 hrs with respective solvent in soxhlet apparatus with frequent shaking. The extract was filtered and concentrated and made free from solvent using distillation assembly. It was further concentrated over low flame for about 15 min. to a thick consistency and dried in hot air oven for 48 hr at 50° C. The dried extract was kept in desiccator for further use.

Preliminary Phytochemical Screening9

Ficus Racemosa stem bark was studied for its preliminary Phytochemical screening for detection of the various plant constituents.

Preparation of the test drugs

All the test drugs were prepared by suspending the test drugs in 5 % gum acacia having water as vehicle. The doses of the test drugs were selected according to the assessment of the toxicity. All the test drugs were administered by oral route using catheter.

Experimental Animals

Healthy strain of Wister albino rats (150–250 g) and Swiss albino mice (18–20 g) of either sex were used as experimental models for testing of activity. The animals were housed in standard conditions of temperature (± 25°C), 12 hr light per day cycle and having relative humidity 45–55 % in the animal house. The animals were fed with the standard pellet diet (Hindustan Liver rats pellets), and water ad *libitum*. The all experimental protocol was approved by the institutional animal ethical committee (IAEC), and then experimental studies were undergone according to their rules and regulations. (1197/c/08/CPCSEA)

Acute oral toxicity[10]

For the testing of acute toxicity, the animals were kept on fasting for a night prior to the experimental procedure. The "Up and Down" or "Staircase" method was adopted and LD_{50}dose was found to be 5000 mg/kg and accordingly doses of hydro-alcoholic, alcoholic and petroleum ether extracts were fixed to 100, 300, 500 mg/kg for further study.

Mouse Writhing Assay[11,12]

The method of Ghule et al. was used FRSE petroleum ether, ethanolic, hydro-alcoholic extracts (100, 300, 500 mg/kg) p.o administered to respective groups and Aspirin at the dose of 25 mg/kg i.p administered to the standard group, Control group receives 5 % gum acacia and 30 min later the animals of these groups were administered with 0.5 ml of 1% acetic acid dissolved in 0.9 % saline by i.p. The number writhes were counted per 15 min.

Tail Flick Method [11,12]

To evaluate anti-nociceptive activity of herbal extract, rat tail flick apparatus (INCO, India) was used. In tail flick method Wister rats (150–250 g) randomly distributed in to eleven groups. The first group served as control and animal were administered with 5 % gum acacia, group II to group X animals received with FRSE petroleum ether, ethanolic, hydro-alcoholic extracts (100, 300, 500 mg/kg) p.o respectively. Group XI was standard and animals were received with ibuprofen (100 mg/kg), the reaction time was noted at 15, 30, 60, 90, 120 and 180 min time interval after drug administration.

Hot Plate Method [11,12]

In hot plate method Swiss Albino mice (18–25 g) randomly distributed in to eleven groups. The first group served as control and animal were administered with 5 % gum acacia, group II to group X animals received with FRSE petroleum ether, ethanolic, hydro-alcoholic extracts (100, 300, 500 mg/kg) p.o respectively. Group XI was standard and animals were received with Pentazocin (10 mg/kg), the basal reaction time was noted before and 30, 60, 90, 120 and 180 min after the administration of the test drug.

Carrageenan-induced Paw Oedema in rats [12,13,14]

For evaluation of acute inflammation, this model employed and method of *Winter et al*. was followed for the measurement of oedema volume by using and instrument plethysmograph. In this experiment healthy Wister rats of either sex were used having weight in the range of 150–250 g. The animals were starved overnight before previous to dosing. To ensure uniform hydration of the animals, the rats were provided with water *Ilibitum*. The first group served as control and animal were administered with 5 % gum acacia, group II to group X animals received with FRSE petroleum ether, ethanolic, hydro-alcoholic extracts (100, 300, 500 mg/kg) p.o respectively. Group XI was standard and animals were received with diclofenac sodium (100 mg/kg). The phlogestic agent carrageenan was prepared by suspending the drug (1%) into the normal saline vehicle and acute inflammation was produced by administration of 0.1 ml of the above suspension to all the animals of each five groups, through injection into the right hind paw at the sub plantar region.

A mark was made at the region of the malleolus of the paw and the paw was immediately immersed in the Plethysmometer, up to the mark, and the paw volume was measured, serves as reading for 0 hr. The readings were taken similarly and paw volumes were measured for 1, 2, 3 and 4 hr respectively. The average paw swelling in the groups of the test animals of different solvent

extracts treated were compared with control group which is treated with vehicle and standard group animals those have received diclofenac sodium. Mean increase in paw volume was determined.

Egg albumin-induced Paw oedema in rats[12,13,14]

This test was performed by *Winter et al*, The first group served as control and animal were administered with 5 % gum acacia, group II to group X animals received with FRSE petroleum ether, ethanolic, hydro-alcoholic extracts (100, 300, 500 mg/kg) p.o respectively. Group XI was standard and animals were received with diclofenac sodium (100 mg/kg). After 30 min, each group was injected with 0.5 ml raw egg albumin sub-plantar to the left hind paw. A plethysmometer was used to measure the volume of the paw oedema for the period of 120 min with readings take at 30 min interval, i.e. 30, 60 and 90 min after albumin administration.

Statistical Analysis

All data were expressed as Mean ± SEM and analyzed statistically by using Dunnett's 't-test'. Difference was considered significant at P value less than 0.05.

RESULTS

Acute Toxicity Studies

In the acute toxicity test, sign of toxicity included lethargy, jerk, convulsions and death not observed in acute dose of 5000 mg/kg p.o in mice. For further study of FRSE for the analgesic and anti-inflammatory activity the dose selected were 100, 300, 500 mg/kg.

Preliminary Phytochemical Screening

Preliminary Phytochemical screening of the FRSE showed the presence of tannins, triterpenoids, flavonoids, coumarins, phenolic compounds phytosterols and carbohydrates, proteins.

Analgesic Studies

The effect of FRSE on acetic acid induced writhing is demonstrated in Table 1. the ethanolic FRSE at higher dose 500 mg/kg showed significant (16.12 %) inhibition of writhes and 100, 300 mg/kg ethanolic extracts were insignificant. Hydro alcoholic FRLE showed dose dependent inhibition of writhes and maximum inhibition (41.66 %) was observed for 500 mg/kg. Standard aspirin (25 mg/kg) showed maximum inhibition of writhes (68.0 %). Petroleum ether FRSE was insignificant at all doses for inhibition of writhes induced by acetic acid in mice.

The analgesic activity of FRSE was evaluated using hot plate test. The results are presented in Table 2 shows the time course of analgesic effect of FRSE. Oral administration of hydro-alcoholic extracts at (100, 300, 500 mg/kg) and ethanolic FRSE (300, 500 mg/kg) resulted significant prolongation of latency time in hot plate test, except left over pet ether extracts. These effects reached their peak at 120 min after dose administration and then decreased. At 120 min mean latency time of ehanolic FRSE 500 mg/kg is 5.61, in case of hydro alcoholic FRSE 100, 300, 500 mg/kg is 8.91, 5.43, 10.25 second compared with control. Pet ether FRSE and ethanolic (100, 300 mg/kg) was non

Table 1. Effect of *Ficus racemosa* Linn. extracts in acetic acid induced writhing test

GROUP	TREATMENT	Number of writhes (per 15 min)	Inhibition (%)
I	Control	37.2±0.71	---
II	Aspirin (25 mg/kg)	11.9±0.57	68.01
III	Pet ether (100 mg/kg)	36.8±0.61	1.07
IV	Pet ether (300 mg/kg)	37.3±1.28	---
V	Pet ether (500 mg/kg)	36.7±0.40	1.34
VI	Ethanol (100 mg/kg)	35.8±0.83	3.76
VII	Ethanol (300 mg/kg)	33.6±0.87	9.677
VIII	Ethanol (500 mg/kg)	31.2±0.48	16.12
IX	Hydro-alcoholic(100 mg/kg)	29.4±0.56	20.96
X	Hydro-alcoholic(300 mg/kg)	24.6±0.55	33.87
XI	Hydro-alcoholic(500 mg/kg)	21.7±0.38	41.66

Values are mean ± SEM (n = 6), experimental group here compared with Control,

*, **, represent $p < 0.05$, $p < 0.01$ respectively, by Dunnett's test

Table 2. Effect of *Ficus racemosa* Linn. extracts in tail flick method

GROUP	TREATMENT	0 min	30 min	60 min	90 min	120 min	180 min.
I	Control	1.988±0.103	2.025±0.129	2.04±0.113	2.07±0.150	2.06±0.11	2.055±0.109
II	Ibuprofen100 mg/kg	2.056±0.118	6.511±0.222**	7.95±0.131**	8.78±0.162**	7.19±0.16**	5.87±0.10**
III	Pet ether100 mg/kg	2.14±0.021	2.16±0.025	2.02±0.045	2.16±0.048	2.24±0.068	2.12±0.071
IV	Pet ether300 mg/kg	2.32±0.45	2.23±0.13	2.17±0.81	2.21±0.26	2.08±0.29	2.56±0.31
V	Pet ether500 mg/kg	2.45±0.81	2.54±0.85	2.32±0.11	2.38±0.31	2.40±0.36	2.35±0.63
VI	Ethanol100 mg/kg	2.37±0.31	2.78±0.396	2.82±0.93	2.91±0.86	2.58±0.64	2.51±0.84
VII	Ethanol300 mg/kg	2.21±0.17	2.69±0.37	2.93±0.42	3.10±0.45	3.25±0.36	2.54±0.12
VIII	Ethanol500 mg/kg	2.18±0.71	3.76±0.12*	3.85±0.28*	3.99±0.31*	4.15±0.24*	3.14±0.28*
IX	Hydro-alcoholic100 mg/kg	2.008±0.132	4.438±0.248**	5.75±0.138**	6.77±0.137**	7.203±0.124**	5.96±0.24**
X	Hydro-alcoholic300 mg/kg	2.198±0.181	5.328±0.217**	6.71±0.21**	7.70±0.21**	8.48±0.17**	6.62±0.21**
XI	Hydro-alcoholic500 mg/kg	2.163±0.183	6.048±0.232**	7.58±0.26**	8.60±0.18**	9.21±0.15**	8.06±0.15**

Values are mean ± SEM (n = 6), experimental group here compared with Control,

* represent $p < 0.05$, by Dunnett's test

** represent $p < 0.01$, by Dunnett's test

Table 3. Effect of *Ficus racemosa* Linn. extracts in hot plate method

GROUP	TREATMENT	0 min	30 min	60 min	90 min	120 min	180 min
I	Control	1.72±0.088	1.73±0.092	1.731±0.095	1.753±0.085	1.906±0.096	1.966±0.13
II	Pentazocin	1.77±0.084	4.361±1.61**	8.341±0.33**	6.86±0.035**	5.23±0.12**	4.43±0.058**
III	Pet ether100 mg/kg	1.70±0.13	1.83±0.61	1.78±0.53	1.86±0.19	1.81±0.93	1.92±0.95
IV	Pet ether300 mg/kg	1.93±0.42	1.87±0.23	1.94±0.26	1.98±0.85	1.85±0.68	1.71±0.58
V	Pet ether500 mg/kg	1.87±0.32	1.82±0.97	1.91±1.21	1.84±1.01	1.93±0.56	1.88±0.25
VI	Ethanol100 mg/kg	1.56±0.123	1.72±0.67	1.96±1.68	1.96±1.34	1.84±0.87	1.76±0.167
VII	Ethanol300 mg/kg	1.63±0.11	2.34±0.76	2.89±0.45	2.11±0.23	3.08±0.81	2.1±0.15
VIII	Ethanol500 mg/kg	1.72±0.45	2.65±0.41	3.23±0.71*	5.61±0.78**	4.89±0.19**	4.78±0.68**
IX	Hydro-alcoholic 100 mg/kg	1.46±0.117	2.486±0.176*	4.32±0.098**	5.911±0.223**	4.33±0.12**	3.433±0.153 **
X	Hydro-alcoholic 300 mg/kg	1.66±0.107	1.748±0.248	2.33±0.123	8.43±0.17**	6.71±0.071**	4.195±0.061**
XI	Hydro-alcoholic 500 mg/kg	1.77±0.128	4.303±0.259**	5.088±0.176**	10.25±0.182**	7.65±0.074**	6.97±0.22**

Values are mean ± SEM (n = 6), experimental group here compared with Control,

* represent $p < 0.05$, by Dunnett's test

** represent $p < 0.01$, by Dunnett's test

significant. Pentazocin significantly increased response latency time of animal with maximum effect was observed at1h after treatment.

The results of analgesic activity evaluated by tail flick method are presented in Table 3. Hydro-alcoholic FRSE at all doses showed dose dependent increase in tail flick latency period and hydro-alcoholic FRSE (500 mg/kg) and ibuprofen (100 mg/kg) showed maximum increase in tail flick latency period. Ethanol FRSE 500 mg/kg showed significant increase in tail flick latency period, petroleum ether FRSE at all doses and ethanolic FRSE (100, 300 mg/kg) was found insignificant.

Anti—inflammatory studies

The activity of FRSE on against carrageenan induced paw edema is shown in Table 4. Hydro alcoholic FRSE 100,300, 500 mg/kg is highly significant, ehanolic FRSE 300, 500 mg/kg is quiet significant in reduction of paw oedema induced by carrageenan. While pet ether FRSE was not showed any significant effect. Maximum reductions in rat paw oedema at 2h, 4h after administration of hydro alcoholic FRSE and standard diclofenac sodium.

The effect of FRSE on egg albumin induced hind paw oedema in rats is shown in Table 5. The results showed

Table 4. Effect of *Ficus racemosa* Linn. extracts in carrageenan induced rat paw oedema

	Average volume of mercury displaced (ml)			
Treatment	**0 h**	**1 h**	**2 h**	**4 h**
Control	0.25±0.02	0.51±0.02	0.62±0.03	0.71±0.03
Petroleum ether (100mg/kg)	0.25±0.02	0.45±0.03	0.57±0.03	0.56±0.03
Petroleum ether (300mg/kg)	0.24±0.02	0.47±0.03	0.55±0.03	0.55±0.03
Petroleum ether (500mg/kg)	0.25±0.02	0.45±0.03	0.52±0.02	0.51±0.02
Ethanol (100mg/kg)	0.25±0.02	0.42±0.02	0.50±0.03	0.51±0.03
Ethanol (300mg/kg)	0.25±0.02	0.32±0.02	0.43±0.03	0.45±0.03
Ethanol (500mg/kg)	0.24±0.02	0.30±0.02*	0.41±0.02*	0.40±0.02*
Hydro-alcoholic (100mg/kg)	0.24±0.03	0.34±0.02*	0.32±0.02*	0.30±0.02**
Hydro-alcoholic (300mg/kg)	0.26±0.02	0.31±0.02**	0.29±0.04**	0.28±0.04**
Hydro-alcoholic (500mg/kg)	0.25±0.02	0.28±0.02**	0.26±0.03**	0.26±0.02**
Diclofenac Sodium (100mg/kg)	0.24±0.02	0.25±0.02**	0.27±0.02**	0.26±0.02**

Values are mean ± SEM (n = 6), experimental group here compared with Control,

* represent $p < 0.05$, by Dunnett's test

** represent $p < 0.01$, by Dunnett's test

Table 5. Effect of *Ficus racemosa* Linn. extracts in egg-albumin induced rat paw oedema

	Average volume of mercury displaced (ml)		
Treatment	**30 min**	**60 min**	**90 min**
Control	0.48±0.02	0.54±0.03	0.61±0.03
Petroleum ether (100mg/kg)	0.45±0.03	0.51±0.03	0.58±0.03
Petroleum ether (300mg/kg)	0.47±0.03	0.56±0.03	0.59±0.03
Petroleum ether (500mg/kg)	0.41±0.03	0.48±0.02	0.52±0.02
Ethanol (100mg/kg)	0.46±0.02	0.53±0.03	0.56±0.03
Ethanol (300mg/kg)	0.43±0.02	0.42±0.03	0.45±0.03
Ethanol (500mg/kg)	0.36±0.02*	0.39±0.02*	0.41±0.02*
Hydro-alcoholic (100mg/kg)	0.36±0.02*	0.33±0.02*	0.38±0.02*
Hydro-alcoholic (300mg/kg)	0.38±0.02*	0.37±0.04**	0.39±0.04*
Hydro-alcoholic (500mg/kg)	0.27±0.02**	0.26±0.03**	0.28±0.02**
Diclofenac Sodium (100mg/kg)	0.26±0.02**	0.28±0.02**	0.29±0.02**

Values are mean ± SEM (n = 6), experimental group here compared with Control,

* represent $p < 0.05$, by Dunnett's test

** represent $p < 0.01$ respectively, by Dunnett's test

that the hydro-alcoholic FRSE and diclofenac sodium caused dose dependent and significant inhibition of egg-albumin induced rat paw oedema over a period of 90 min and ethanolic FRSE 500 mg/kg also showed significant results while petroleum ether FRSE at all doses and ethanolic FRSE (100, 300 mg/kg) showed non significant inhibition of paw oedema.

DISCUSSION

The results presented here may help to establish the scientific basis for utilization of stem bark of *Ficus racemosa Linn*. For the treatment of pain and inflammation in folk medicine as that of leaves and fruit extracts of the same plant reported earlier research. In the present investigation here is study of stem bark for the claimed application of the same plant.

In this work, we have demonstrated the effect of petroleum ether, ethanolic and hydro-alcoholic stem bark extracts of *Ficus racemosa Linn*. (FRSE) at 100, 300, 500 mg/kg p. o doses on acetic acid induced writhing test, hot plate method and tail flick test for analgesic effect and carrageenan-induced paw oedema as well as egg albumin-induced paw oedema for anti-inflammatory effect of test drug. The hydro-alcoholic FRSE showed analgesic and anti-inflammatory effects in laboratory animals at the

dose dependent manner and ethanolic FRSE produced significant results at higher dose 500 mg/kg and 100, 300 mg/kg showed non significant results in all models. Petroleum ether FRSE found insignificant in analgesic and anti-inflammatory models.

The results supported the traditional use of this plant in some painful and inflammatory conditions. Because of presence of biologically active principles i.e flavonoids, tannins, phenolic compounds and phytosterols in the same plant from Phytochemical investigation suggests that one of above constituent or in combination together is responsible for producing the analgesic and anti-inflammatory effects. Further studies are in progress to isolate and characterize the active principle from the stem bark of the *Ficus racemosa Linn*.

The oral LD_{50} obtained with this plant extract also suggested that it may have a reasonable safety margin with regards to acute toxicity further justifying its wide application in various communities and lack of any reported side effect with the traditional use of this plant.

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Anthelmintic Potential of *Andrographis paniculata, Cajanus cajan* and *Silybum marianum*

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Abstract

Hydroalcoholic extracts of aerial parts of *Andrographis paniculata, Cajanus cajan* and *Silybum marianum* and their combinations were evaluated for anthelmintic properties using Indian adult earthworms (*Pheretima posthuma*) as a model, as they show physiological and anatomical resemblance with intestinal parasites and round worms. The results were compared with that of standard drug, piperazine citrate. *A. paniculata* showed better activity (paralysis and death time was 3.33 and 5.16 min respectively at a concentration of 40mg/ml), while its combination (1:1) with *S. marianum* extract was found to be the most potent (paralysis and death time was 2.83 and 6.33 min respectively at a concentration of 40mg/ml). This property is supposed to be due to the presence of phenolics (flavonoids and tannins) which are reported to have anthelmintic property.

Keywords: Anthelmintic property, *Andrographis paniculata, Cajanus cajan*, earthworm model, *Silybum marianum.*

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INTRODUCTION

Helmenthiasis is a highly prevalent disease mainly in the third world countries (1). Inadequate sanitation and poor hygiene may be the main reason behind the helmenthic infection (2). It is among the most important animal diseases inflicting heavy production losses. The gastro intestinal helmenthis has become resistant to the commonly used helmenthic drugs (3) and hence, there is a need to find the anthelmintic property from natural resources like medicinal plants, which may be effective against helmenthiasis and may have less or no side effects. *A. paniculata*, *C. cajan* and *S. marianum* belongs to family Acanthaceae, Compositae and Fabaceae respectively and shares some common medicinal properties such as antibacterial, antioxidant, hepatoprotective, etc (4–6).

MATERIAL AND METHODS

Plant material

The authenticated plants were collected from Natural Remedies Pvt. Ltd., Bangalore (sample invoice No. D119) and confirmed at Botany Department, Dr. H. S. Gour University, Sagar (M.P).

Chemicals and drugs

The following drugs and chemicals were used: Sodium chloride (HIMEDIA), Ethanol (RANKEM) and Piperazine citrate, a gift obtained from R. K. Pharmacy, Sagar.

Extract preparation

Dried and powdered plant materials were extracted with 70% ethanol using soxhlet apparatus. The extracts were concentrated and dried at 68°C and kept at 4°C for further studies.

Phytochemical test

Phytochemicals screening were performed to detect the presence or absences of various compounds such as tannins, flavonoids, alkaloids etc. as per standard methods (7).

Experimental model

The earthworms show physiological and anatomical resemblance with intestinal parasites and round worms (8) and were used for the anthelmintic assay. Indian adult

earthworms (*Pheretima posthuma*, 5–7cm in length) were freshly collected from organic farm of local earthworm breeding centre (N.H. 26, Sagar).

Experiment

The experiments were carried out by the method described by Jinu *et al*., 2009 (9). All the three extracts were dissolved in normal saline and diluted to get final concentrations of 20 and 40 mg/ml. Piperazine citrate (20 mg/ml) was used as a standard drug. Sixteen groups, each containing six earthworms were taken for the experiment. Extracts and piperazine citrate were dissolved separately in their respective concentration in 10ml normal saline. For combinational study, the different extracts were taken in the concentration ratio of 1:1. Time taken for paralysis and death of individual earthworm was noted. The paralysis occurs when the worms were placed in normal saline and were not able to move. The death was confirmed when the earthworms lost their motility and fading off their body colors (10).

RESULTS AND DISCUSSION

Biological activities of the plant extracts are due to the presence of various chemicals present in it. The activity of these phytochemicals depends upon the solvent used and the method of extraction (11). 70% ethanolic extracts showed maximum concentration of phenolic compounds (flavonoids). All the three plants analyzed for their anthelmintic potential showed a concentration depended activity. *A. paniculata* extract (40mg/ml) showed better activity (paralysis at 3.33 min and death at 5.16 min), while its combination with *S. marianum* extract (1:1) was found most potent (paralysis at 2.83 min and death at 6.33 min). The time taken by the standard drug (20mg/ml) for the paralysis and death of the worms was 7.0 and 14.83 min respectively. *C. cajan* extract showed the least activity, as it took longest time for paralysis (13.66 min) and death (17.0 min) of the worms (Table I).

Synthetic anthelmintic drugs like piperazine citrate, is known to cause paralysis of worms so that they are expelled in the feaces of men and animals. The extracts in the present study could cause paralysis as well as the death of the worms. These drugs may reach the target site in worms either orally or by diffusion and/or uptake through the cuticle, however the major uptake of the drug is through cuticle (12). All the three extracts when taken in combination, generally showed synergistically better activity as compared to individual effect. The variation in activity of the plant extract might be due to the difference in the proportion of the active compounds responsible for the anthelmintic property (13). The active constituents may be the phenolics such as flavonoids and tannins present in the plant extracts.

CONCLUSION

A. paniculata, C. cajan and S. marianum are well known for their medicinal properties (hepatoprotective and

"Table I: Anthelmintic activity hydroalcoholic extracts of three plants"

Extract	Concentration (mg/ml)	Paralysis time (min.)	Death time (min.)
A.paniculata (Ap)	20	5.33 ± 0.40	7.50 ± 0.20
	40	3.33 ± 0.18	5.16 ± 0.14
C. cajan (Cc)	20	20 ± 0.23	25.83 ± 0.33
	40	13.66 ± 0.50	17 ± 0.23
S.marianum (Sm)	20	6 ± 0.23	10.16 ± 0.43
	40	3.83 ± 0.14	7.5 ± 0.31
Ap + Sm (1:1)	20	5 ± 0.23	8.5 ± 0.20
	40	2.83 ± 0.14	6.33 ± 0.18
Ap + Cc (1:1)	20	8.33 ± 0.18	12 ± 0.23
	40	5.5 ± 0.20	8.5 ± 0.31
Cc + Sm (1:1)	20	10.50 ± 0.20	13.66 ± 0.18
	40	6.33 ± 0.18	10 ± 0.23
Ap + Cc + Sm (1:1:1)	20	6.66 ± 0.38	10.50 ± 0.45
	40	4 ± 0.23	8.16 ± 0.36
Piperazine	20	7 ± 0.33	14.83 ± 0.36
Control (Normal saline)	-	-	-

All the values are expressed as mean ± SEM, N=6

antioxidant). The present study on anthelmintic potential of these plants proves that their consumption may be good for health enhancement as well as intestinal problems.

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Pharmacognostical evaluation of *Cuscuta reflexa Roxb*

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Abstract

This paper gives the information about the pharmacognostical studies of *Cuscuta reflexa Roxb*. All the parameters were studied under the WHO and pharmacopoeial guidelines. Scientific parameters are not yet available to identify the exact plant material and to ascertain its quality and purity. The present investigation was therefore undertaken to determine the requisite pharmacognostical standards for evaluating the plant material. Various investigations like organoleptic or morphological characters, microscopic or anatomical studies, physicochemical evaluations, phytochemical screening, TLC finger print profiling and fluorescence analysis of powdered crude drug were carried out and the salient qualitative and quantitative parameters were reported. These studies provided referential information for correct identification and standardization of *Cuscuta reflexa Roxb*.

Keywords: *Cuscuta reflexa Roxb*., TLC finger print.

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INTRODUCTION

Cuscuta reflexa Roxb. (Family: Cuscutaceae) is commonly known as "Akashabela, Amarabela, Kasur" in "Hindi", also used in India. It grows as a large prickly shrub particularly in North-East India (Sikkim, Assam and Nagaland states). In India, the plant is traditionally used for various medicinal purposes. The seeds have a bitter bad taste; sedative, emmenagogue, diuretic; useful in disease of the liver and spleen, quartan fever, chronic fevers, griping, hiccough: purify the blood and cleanse the bowels; the infusion is given in ophthalmia, the decoction in biliousness as a purgative (1, 2). The stem is used as a purgative. The plant juice was given in combination with other purgative decoction (3). It has come to our notice that the rural people of India use the juice of plant as inhaled for treating jaundice and warm paste is applied in rheumatism and gout and other affected parts of the body, and the paste of whole plant is applied for relieving headache (4). However, no scientific standards or pharmacognostical parameters are yet available to ascertain the identity and to determine the quality of this crude drug. The pharmacognostical parameters are major and reliable criteria for confirmation of the identity and determination of quality and purity of the crude drugs. The present work therefore, attempts to report various necessary pharmacognostical standards of *Cuscuta reflexa Roxb*.

MATERIAL AND METHODS

Plant Material

The fully matured entire plants of *Cuscuta reflexa Roxb*. were collected during the month of November 2006 from Herbal Garden, Department of Life Sciences, Dibrugarh, Assam, India. The species was identified by Dr. M. Islam, Department of life sciences, Dibrugarh University, and specimen deposited in Department of Pharmaceutical Sciences, Dibrugarh University for future reference. All the branches were removed from the stems and branches carefully by using a sharp knife, without harming the stem. Then the stem was peeled off from the shoots. Longitudinal incisions were made by a sharp knife on the shoots and transverse markings were given so as to form the rings which also connect the longitudinal incisions producing the strips which were then peeled off. Then the stem was shade dried at 21–24°C.

Reagents and Chemicals

All reagents and chemicals used for testing were analytical grade obtained from Ranbaxy Fine Chemicals Ltd., New Delhi and Loba Chemie, Mumbai, India.

Organoleptic Evaluation

The freshly (just after collection) peeled stem of the plant were spreaded on a clean dry plastic sheet and investigated different organoleptic features by repeated observations using magnifying glass and ruler (where required) and recorded. Similarly the dried stem and root were also subjected to organoleptic evaluation.

Macroscopic Study

The macroscopically characters (i. e. Colour, Texture, Diameters, Taste, Odour) of the stem of *Cuscuta reflexa Roxb* were studied and reported in the results

Colour	Pale greenish yellow (Fresh Stem).
	Blackish brown (Light brown) colour (dried stem).
Texture	Filiform (Thread-like), Wiry and succulent.
Diameters	2 mm thick, glabrous.
Taste	Bitter and astringent taste.
Odour	Aromatic.

Microscopic Studies

The transverse sections (TS) of stem were obtained by usual techniques (5). Good sections were collected and placed on a grease free microscopic slide along with a drop of glycerine water (1:1). The sections were covered with clean cover slip and observed under the compound microscope at 40x magnification. A camera lucida was attached with the microscope and the sections were suitably traced out (6).

Physicochemical Evaluations

Physicochemical parameters such as the percentage of loss on drying (LOD), total ash, acid insoluble ash, water soluble ash were determined as per the Indian Pharmacopoeia (7). Water and alcohol soluble extractives

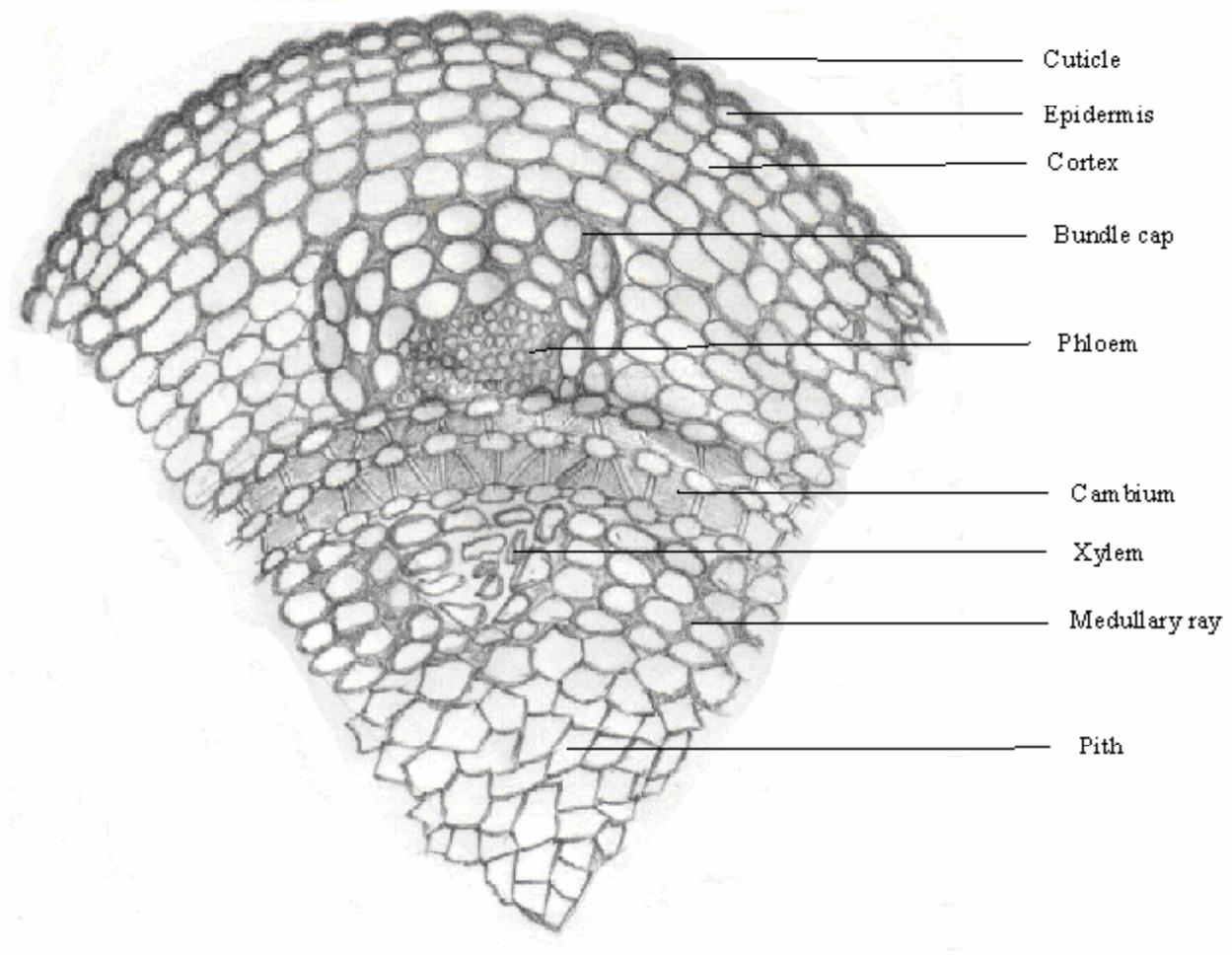


Figure 1 Schematic diagram illustrating various parts of stem.

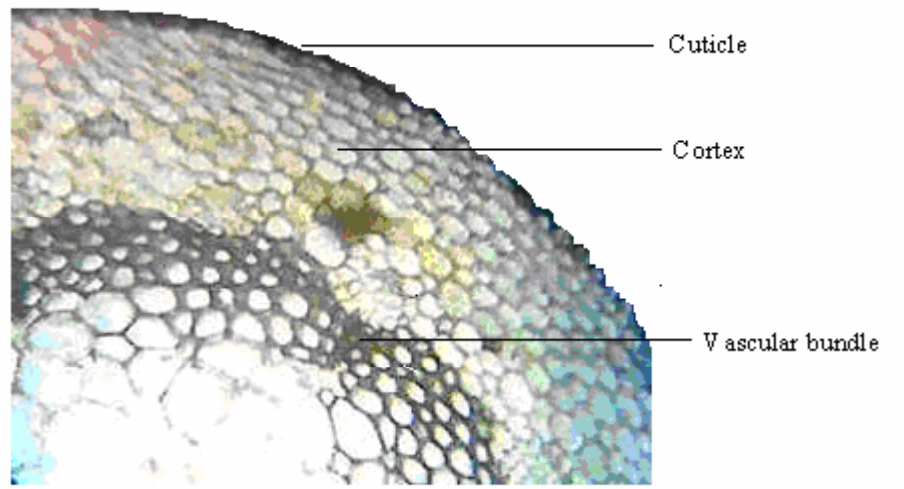


Figure 2 Schematic diagram of *Cuscuta reflexa* stem

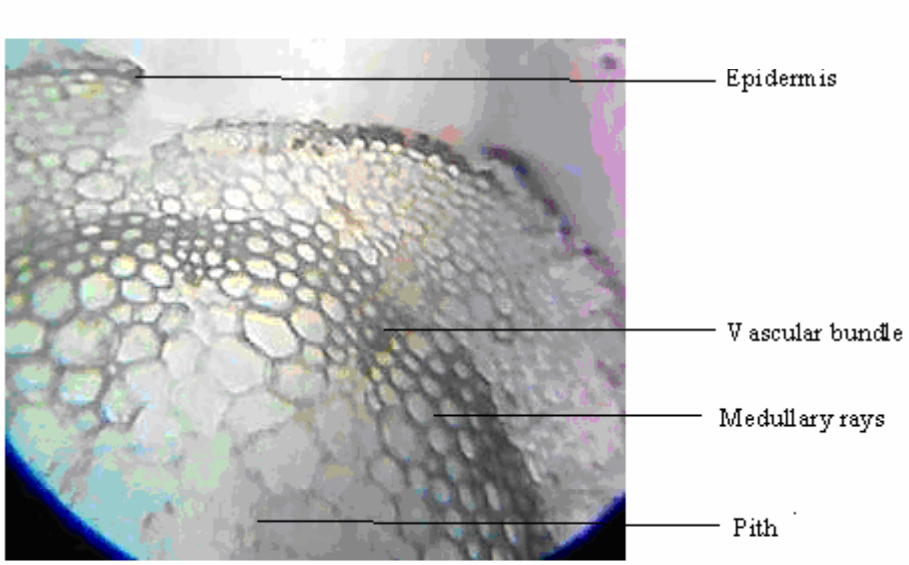
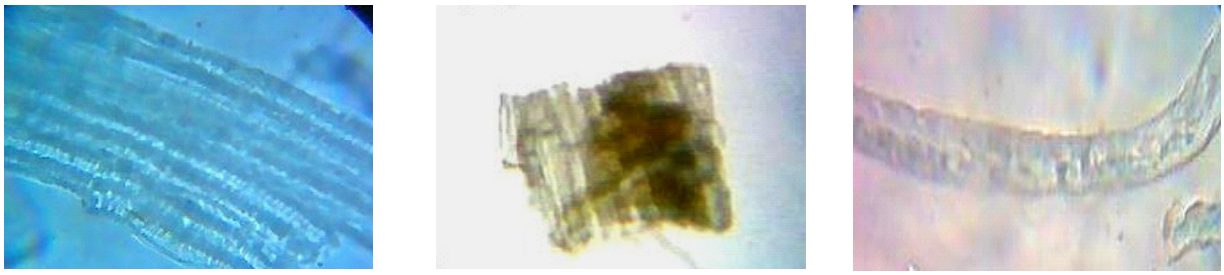


Figure 3 Schematic diagram of *Cuscuta reflexa* stem *Microscopical studies of powder drug*



(a) (b) (c)

Figure 4 (a) Reticulate vessels, (b) Xylem fibres, (c) Phloem fibres

were estimated by hot extraction and cold maceration according to the method prescribed by WHO (8). All determinations were performed in triplicate and the results are presented as mean ± standard deviation.

Phytochemical Screening (9,10)

The dried as well as fresh powdered stem was subjected to preliminary phytochemical screening for qualitative detection of phytoconstituents. The dried and coarsely powdered stem (100 g) was extracted successively with petroleum ether (40–60°C), chloroform (59.5–60°C), ethyl acetate (76.5–77.5°C), and ethanol (90%) in a soxhlet extractor by continuous hot percolation. Finally the marc was macerated with chloroform water. Each time before extracting with the next solvent of higher polarity the powdered drug (marc) was dried in a hot air oven below 50°C for 10 minutes. Each extract was concentrated by distilling off the solvent, which was recovered subsequently. The concentrated extracts were evaporated to dryness and the extracts obtained with each solvent were weighed. Their percentages were calculated in terms of initial air dried plant material. The colours of extracts were observed. The successive extracts, as mentioned above, were subjected to various qualitative phytochemical tests for the identification of chemical constituents present in the plant material.

Thin Layer Chromatographic Studies (11)

Approximately 1 g of dried coarsely powdered stem was taken in a 100 ml glass beaker and moistened with little amount (sufficient to moisten) of 25% liq. NH_3 with occasional stirring for 20 min. Then the beaker was kept on boiling hot water bath to dry the contents for a few minutes. The beaker was cooled at room temperature and added 10–15 ml of chloroform and extracted on boiling hot water bath for 10 min. Then the extract (1–2 ml) was collected in clean stoppered glass test tube and used for spotting the chromatographic plates. Silica gel G of particle size 10–40µ, applied as a thin layer on a clean glass plate support and activated just before use. Mobile phase were, $CHCl_3$: Ethyl acetate 60:40, Toluene: Dioxane: Glacial acetic acid 90:25:4. After development of TLC plates by one dimensional ascending method (7), visualization was performed by spraying with reagent. After visualization by spraying with reagent, dark and light spots were found. The Rf values of the spots were recorded carefully and the chromatogram was documented by graphical copying (12).

2.9. FLUORESCENCE ANALYSIS (13,14)

A small quantity of dried and finely powdered stem was placed on a grease free clean microscopic slide and added 1-2 drops of the freshly prepared reagent solution, mixed by gentle tilting the slide and waited for 1-2 minutes. Then the slide was placed inside the UV viewer chamber and viewed in day light, short (254 nm) and long (365 nm) ultraviolet radiations. The colours observed by application of different reagents in different radiations were recorded.

3. RESULTS

3.1. ORGANOLEPTIC EVALUATION:

The characters recorded are described below.

3.1.1. Fresh stem

Condition: Moist.

Shape of pieces: Flat strips.

Dimensions: Varies, 8–12 cm. long, 1–2.5 cm wide and 0.2–0.4 cm thick.

Colour: Outer surface pale greenish brown, inner surface dull greenish yellow.

Odour: Aromatic, characteristic.

Taste: Aromatic and bitter. Proper chewing leads to typical pungent and worm sensation of tongue lasting for 10–15 minutes.

3.1.2. Dried stem

Condition: Hard and contracted.

Shape of pieces: Recurved and channeled quills.

Dimensions: Varies, 6–8 cm long and 0.4–0.8 cm wide.

Colour: Outer surface blackish brown, inner surface brownish buff coloured.

Odour: Slight.

Taste: Same as fresh bark.

3.2. MICROSCOPIC STUDIES

The TS of stem is shown in Fig 1, 2, 3. The stem is an ascending axis of the plant developed from the plumule. It consists of nodes, internodes, and buds and it gives rise to branches, leaves and flowers. The stem may be aerial, sub-aerial, and underground. Depending upon the presence of mechanical tissues, the stem may be weak, herbaceous or woody. The primary stem shows the following structure: epidermis, cortex, medullary rays, medulla and a vascular system taking the form of a dicotyostele. The TS exhibited the cortex contained Cortex 5–6 layers with articulated laticifers. Including Hypodermis and General cortex. Hypodermis Just below the epidermis. There are 2–3 layers of collenchymatous cells larger in size than epidermis. The epidermis composed of a single layer of compactly arranged cells and bear stomata. General cortex 2–3 layers of radially elongated thin walled parenchymatous cells. There are conspicuous intracellular spaces in it.

Vascular Bundle

These are bicollateral, conjoint, open and arranged in a ring. Each bundle consists of xylem, phloem, and cambium.

Xylem

Xylem of vessels with simple end-walls, partially developed. The smaller vessels constituting the protoxylem and the bigger ones constituting the metaxylem lies away from the centre. The protoxylem consists of annular, spiral and scalariform vessels, and the metaxylem of reticulate and pitted vessels.

Phloem

Without intraxylary phloem.

Cambium

It consists of 2–3 layers of thin walled and rectangular, and is arranged in radial rows.

Medullary rays

Few layers of fairly big polygonal or readily elongated parenchymatous cell packed with yellow-brown masses (pigments).

Table 1. a. Results of phytochemical screenings of successive fresh extracts of *Cuscuta reflexa Roxb*. stem

	Extract Used				
Test Name	**Pet. Ether**	**Chloroform**	**Ethyl acetate**	**Methanol**	**Water**
Carbohydrates	–	–	–	–	–
Proteins	–	–	–	–	–
Amino acids	–	–	–	–	–
Steroids	–	–	–	–	–
Saponins	–	+	+	+	+
Alkaloids	–	–	–	–	–
Flavonoids	–	+	+	+	+
Phenolic compounds	–	–	–	–	–
Organic acids					
a) Citric acid	+	+	+	+	+
b) Malic acid	+	+	+	+	+

+ = Present, – = Absent

Table 1. b. Results of phytochemical screenings of successive dried extracts of *Cuscuta reflexa Roxb*. stem

Test Name	**Extract Used Successive extraction Direct extraction**						
	Successive extraction				**Direct extraction**		
	Pet. Ether	**Chloroform**	**Ethyl acetate**	**Methanol**	**Water**	**Methanol**	**Water**
Carbohydrates	–	–	–	–	–	–	–
Proteins	–	–	–	–	–	–	–
Amino acids	–	–	–	–	–	–	–
Steroids	–	–	–	–	–	–	–
Saponins	–	–	–	+	+	+	+
Flavonoids							
a) Lead acetate	–	–	–	+	–	+	+
b) NaOH	–	–	+	+	+	+	+
c) Ammonia Sol^n	–	–	–	+	+	+	+
d). Ammonia sol^n + Conc. H_2SO_4	–	–	–	+	+	+	+
Phenolic Compounds	–	–	–	–	–	–	–
Organic acid							
Citric acid	–	–	+	+	+	+	+

+ = Present, – = Absent

Pith

Large, thin walled, lignified big polygonal, rounded parenchymatous cells with intracellular space.

3.3. MICROSCOPIAL STUDIES OF POWDER DRUG

Powder shows mainly two types of elements, xylem fibres, and the phloem fibres.

Phloem fibres

Phloem fibres were lignified, thick walled and conical shaped. All are simple pitted found in groups and colour was brownish yellow.

Reticulate vessels

Reticulate fibre vessels showing numerous bordered pits with annular to spiral thickening and lignified.

Xylem fibres

Cells having a fibre-like form with living content and simple pits but which are really fusiform xylem parenchyma cell.

3.4. PHYTOCHEMICAL SCREENING

The results are shown in Table 1a and Table 1b. The results demonstrated presence of Saponins, Flavonoids, Organic acid such as Citric acid and Malic acid in fresh stem of *Cuscuta reflexa Roxb* and dried stem demonstrated presence of Saponins, Flavonoids, Organic acid such as Citric acid and Malic acid.

3.5. PHYSICO-CHEMICAL EVALUATIONS

The values of all determinations are summarized in Table 3.Water soluble ash was found to be quite lesser than acid insoluble ash value. The results showed greater extractive values (almost double) in hot extraction method. In both methods alcohol yielded higher extractives.

3.6. THIN LAYER CHROMATOGRAPHIC STUDIES

The stem extract yielded four orange brown spots of different intensity. The results are shown in Table 4.

3.7. FLUORESCENCE ANALYSIS

The results are summarized in Table 5.

4. DISCUSSION

To ensure reproducible quality of herbal products, proper control of starting material is utmost essential. Thus in recent years there has been an emphasis in standardization

Table 2. Loss on drying (LOD) and ash values of powdered stem bark of *Cuscuta reflexa Roxb*. stem

S. No.	Parameters	Values of 3 Replicates (%)	Mean ± SD
1	Ash Value		
	a) Total ash	4.05%	
		3.855	4.174±0.405
		4.63%	
	b) Acid-insoluble ash	0.50%	
		0.65%	0.533±0.104
		0.45%	
	c) water soluble ash	0.90%	
		1.25%	0.967±0.256
		0.75%	
2	Extractive Value		
	a) water soluble extract	29.40%	
		30.50%	30.45±1.025
		31.45%	
	b) Alcohol soluble extract	65.25%	
		63.42%	64.39±0.919
		64.50%	
3	Loss on drying	1.25%	
		1.50%	1.233±0.275
		0.95%	

SD = Standard Deviation

Table 3. Observations of thin layer chromatographic studies of *Cuscuta reflexa Roxb*. stem

Sample Used	Solvent system	Detecting agents	R_f value
MeOH (D)*	$CHCl_3$: Ethyl acetate 60:40	UV Long (366 nm)	0.53, 0.65, 0.81, 0.94
		As Reagents**	0.12
MeOH (S)***	$CHCl_3$: Ethyl acetate60:40	UV long (366nm)	0.53, 0.65, 0.81, 0.94
		As reagents	0.12
Ethyl acetate	$CHCl_3$: Ethyl acetate60:40	UV long (366nm)	0.63, 0.81, 0.95
MeOH (D)	Toluene:Dioxane: Glacial acetic acid 90:25:4	As reagent	0.53, 0.75, 0.89
MeOH (S)	Toluene:Dioxane: Glacial acetic acid 90:25:4	As reagent	0.51, 0.61, 0.76
Chloroform	Toluene:Dioxane: Glacial acetic acid 90:25:4	As reagent	0.34, 0.51, 0.69, 0.81, 0.91
Pet. ether	Toluene:Dioxane: Glacial acetic acid 90:25:4	As reagent	0.50, 0.56, 0.72, 0.85

Table 4. Fluorescence analysis of powdered *Cuscuta reflexa Roxb*. stem

Serial No.	Powder Crude Drug + Reagent	Day Light	UV (Short) 254 nm	UV (Long) 366 nm
1.	Powder Crude Drug as such	Brown	Light Brown	Black
2.	Drug + 1M NaOH	Black	Dark black	Charcoal black
3.	Drug + Picric Acid	Black brown	Henna Colour	Black
4.	Drug + 1M HCl	Brown	Green brown	Black
5.	Drug + Acetic acid	Brown	Greenish brown	Black
6.	Drug + dil. HNO_3	Brown	Yellowish black	Charcoal black
7.	Drug + 5% Iodine	Light brown	Dark brown	Black
8.	Drug + 5% $FeCl_3$	Black	Brown	Dark brown
9.	Drug + dil. Ammonia	Yellowish brown	Dark brown	Fluorescence brown
10.	Drug + Methanol	Dark brown	Greenish brown	Black
11.	Drug + 50% HNO_3	Brown	Yellowish black	Charcoal black
12.	Drug + Ammonia sol^n + HNO_3	Golden brown	Greenish brown	Black
13.	Drug + 1M H_2SO_4	Brown	Greenish brown	Black
14.	Drug + Conc. HNO_3	Reddish brown	Grey-brown	Black
15.	Drug + $K_2Cr_2O_7$	Black	Deep black	Charcoal black
16.	Drug + Glacial acetic acid	Brown black	Black	Dark black
17.	Drug + Ethanol	Dark brown	Grey brown	Black
18.	Drug + Toluene	Greenish Black	Deep black	Dark black

dil. - dilute, Conc. - concentrated, Sol^n - solution.

of medicinal plants of therapeutic potential. Despite the modern techniques, identification and evaluation of plant drugs by pharmacognostical studies is still more reliable, accurate and inexpensive means. According to World Health Organization (WHO) the macroscopic and microscopic description of a medicinal plant is the first step towards establishing its identity and purity and should be carried out before any tests are undertaken.

The organoleptic or macroscopic studies yielded important characteristics. The present study was focused on the structural features of the stem *Cuscuta reflexa* including macro and microscopic features and physiochemical parameters of roughly grinded stem. Dodders are obligate parasites, requiring both water and carbohydrate to be supplied by the host plant. To achieve the successful flow of both carbohydrate and water, the searching hyphae of the dodder must penetrate the host and transform into xylic or phloic hyphae, depending on the cell type that the hyphae encounter. The phloic hyphae develop a massive handlike appendage that surrounds the host phloem, whereas the xylic hyphae are directly connected to the host. These characteristics would be useful in identified and differentiating *C. reflexa* from its substitutes and adulterants.

On the basis of qualitative chemical test, it has been observed that chemically therapeutic compounds like organic acid, glycosides, and flavonoids are present in sufficient amounts in the stem of *Cuscuta reflexa Roxb* On

the basis of elemental analysis, it has been observed that sodium, iron and chloride are present in rich quantity. These are very helpful to nourish the body. The extracts were subjected to qualitative phytochemical tests to find out the active constituents. In the fresh stem extracts of *Cuscuta reflexa Roxb* with Chloroform, ethyl acetate, methanol and the aqueous extract saponin glycosides, flavonoids, and the organic acid like citric and malic acid were present. In the dried stem successive extraction with ethyl acetate, methanol and aqueous extract saponins, flavonoids, and citric acid were present. In direct methanolic and the aqueous extract saponins, flavonoids, and citric acid were identified.

The ash of any organic material is composed of their non-volatile inorganic components. Controlled incineration of crude drugs results in ash residue consisting of an inorganic material (metallic salts and silica). This parameters used for the determination of inorganic materials, such as carbonate, silicates, oxalates, and phosphates. Heating causes the loss of organic material in the form of CO_2 leaving behind the inorganic components. We can detect the extant of adulteration as well as establish the quality and purity of the drug by using this method. The Ash value determines the quality of the drug material. Here the value obtained for the stem of *Cuscuta reflexa roxb*. is around 4 percent as total ash. The acid insoluble ash determines the acid insoluble material present in the drug material and the value for the stem of *Cuscuta reflexa roxb*. Lies in the range from 0.5 to 0.6 and the water soluble ash determines the water soluble material specifically the water soluble inorganic salts. The value lies in the range from 0.75 to 1.25.

The extraction of any crude drug with particular solvent yields a solution containing different phyto-constituents. The composition of these phyto-constituents in that particular solvent depends upon the nature of the drug and solvent used. The use of a single solvent can be the means of providing preliminary information on the quality of a particular drug sample. The extractive value of the crude drug determines the quality as well as purity of the drug material. The ethanol and water soluble extractive value of the stem of *Cuscuta reflexa Roxb* is 64.39% and 30.45% respectively.

The loss on drying of stem powder was 1.233± 0.275. It signifies a considerable amount of moisture in the stem materials. The percentage of active chemical constituents in crude drugs is mentioned on air-dried basis. Hence, the moisture content of a drug should be determined and also be controlled to make the solution of definite strength. The moisture content of a drug should be minimised in order to prevent decomposition of crude drug either due to chemical change or due to microbial contamination. The objective of drying of fresh material is to fix their constituents i.e. to check enzymatic or hydrolytic reactions that might alter the chemical composition of the drug and to reduce their weight and bulk. Insufficient drying favours spoilage by mould and bacteria and makes possible the enzymatic destruction of the active principles. Not only is the ultimate dryness of the crude drug is important, equally important is the rate at which the moisture is removed and the conditions under which it is removed. If the rate is too slow, much spoilage may occur before the drying process is completed. Therefore, in general, drying should be accomplished as rapidly as is possible with good practices.

Thin layer chromatograms are produced with the aim of identifying the individual substances in a mixture and also for testing for purity or for separation of mixtures. They are particularly useful for checking the mixtures used for synthetic reactions or following the course of reactions. The Rf value indicates the position at which a substance is located in the chromatogram. It is appropriate to regard the Rf value as a guide for identification of medicinal plants it is difficult to obtain exactly reproducible Rf values as a result of the variety of influences operation during chromatography. For purposes of identification, it is necessary to relate the Rf values of the investigated substances and those of reference substances. If the Rf value agree, it is probable but not certain, that the two spots correspond to the same substance. Reliable identification is only possible by using spectroscopic investigation alongside with thin layer chromatography (e.g. IR spectrography, NMR spectroscopy, mass spectrometry or coupling there of with TLC).

The fluorescence character of powdered drug plays a vital role in the determination of quality and purity of the drug material. The powder drugs exhibit different fluorescence character in the presence of different chemical reagents under ultra-violet light. The change in the colour of stem powder under UV radiation in reference to day light was observed. The powder drug exhibit different fluorescence character due to presence of different functional groups in drug chemical constituents. The above table is about the fluorescence characteristics of stem powder of *Cuscuta reflexa Roxb* in the presence of different chemical reagents and ultra-violet light at 254nm and 365nm respectively.

Fluorescence is the phenomenon exhibited by various chemical constituents present in the plant material. Some constituents show fluorescence in the visible range in daylight. The ultra violet light produces fluorescence in many natural products (e.g. alkaloids like berberine), which do not visibly fluoresce in daylight. If the substances themselves are not fluorescent, they may often be

converted into fluorescent derivatives or decomposition products by applying different reagents. Hence, some crude drugs are often assessed qualitatively in this way and it is an important parameter of pharmacognostical evaluation.[14,18]

5. CONCLUSION:

After present investigation it can be concluded that the pharmacognostical study of *Cuscuta reflexa Roxb*. stem yielded a set of qualitative and quantitative parameters or standards that can serve as an important source of information to ascertain the identity and to determine the quality and purity of the plant material in future studies. As previously mentioned, *Cuscuta reflexa Roxb*. being a morphologically variable species, these information will also be helpful to differentiate *Cuscuta reflexa Roxb*. from the closely related other species and varieties of Cuscuta.

6. AKNOWLEDGEMENT:

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Pharmacognostic and Physicochemical investigation of *Pterospermum acerifolium Willd* leaves (Sterculiaceae)

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Abstract

Pterospermum acerifolium Willd, a large tree belonging to the family Sterculiaceae, is widely distributed in India particularly in sub-Himalayan tract and outer Himalayan valleys.
In present study pharmaconostic and physicochemical investigation of Pterospermum acerifolium has been carried out. Qualitative studies indicated presence of abundant covering and glandular trichomes, anmocytic stomata, 3–5 groups of oval shaped vascular bundles, dorsiventral type of leaf, calcium oxalate crystals, and heavy mucilage present, all physicochemical parameter are studied. These findings will be useful towards establishing pharmacognostic standards on identification, purity, quality and classification of the plant, which is gaining relevance in plant drug research, the identification and preparation of monograph of plant.

Keywords: *Pterospermum acerifolium Willd,* Pharmacognostic standardization, Quantitative estimation, leaf morphology..

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INTRODUCTION

Pterospermum acerifolium Willd (Sterculiaceae) having Muchkund as local Indian name, was evaluated for preliminary pharmacognostic, Phytochemical investigation.

Externally, the leaves are variable in shape and size, 25–35 by 15–30 cm, orbicular or oblong entire or various lobed, cordate and sometime peltate, it length globrous above, clothed beneath with whitish floccose tomentum.

Flowers are white, axillary having fragrant, solitary or in pairs. The sepals up to 10 cm long, linear- oblong, densely tomentose outside and petals are linear-oblong, somewhat obliquely, cuneate, slightly shorter than the sepals.

The seeds of plant are obliquely ovoid, compressed; wing large, thin. The ripe fruit remains for a long time on the tree and the bark is grayish- brown, sapwood pale white in colour but heart wood light pinkish red. (1–2)

In Phytochemical review chemical constituents present in seeds, leaves, flowers and in the bark of the plant. The seeds and bark contains amino acids and sugar while flowers possess 3, 7-diethyl-7methyl-1, 5-pentacosanolide, n-hexacosan-1, 26-diol and its dilignocerate, kaempferol, kaempferide, B-sitosterol, B-amyrin and friedelin also leaves has Luteolin and kaempferol 3-O-B-D galactoside and quercitin glycosides.

Therapeutically plant is employed for the leucorrhoea, inflammation, ulcer, and leprosy; small pox.(3–4)

MATERIAL AND METHOD

Plant material

Fresh leaves of *Pterospermum acerifolium Willd* collected in the month of August to September from Amravati District, Maharashtra, and the fresh leaves of *Pterospermum acerifolium* were dried under the shade & powdered in a mixture grinder. The powdered leaves packed in a paper bags & stored in air tight container until use.

Extraction

Hot continuous extraction, soxhletion method was used for the extraction of the plant material with solvent; choose according to increasing its polarity, like petroleum

ether, benzene, chloroform, acetone and ethanol. In cold maceration water is used.

Pharmacognostic investigation
Morphological investigation

External morphological characters of fresh collected leaves were study as per Treatise Indian medicinal plant.(5)

Colour -Green
Odour -Characteristic
Taste -Bitter
Shape -Leaves orbicular, peitate, irregular lobed or Entire
Size -Length 25 - 35 cm, Breadth 15 – 30 cm

Microscopical investigation

The qualitative features of the fresh leaves of *Pterospermum acerifolium willd* were determined using the methods of Evans and for quantitative study anatomical sections, surface preparations of the fresh leaves and the microscopy & chemo-microscopy of powdered samples were carried out according to methods outlined by Brain and turner and Evans.(6) (Table 1)

Table 1: Quantitative microscopy of the leaves

Sr.no.	Determinations	Range
1	Stomatal number (upper peel)	15–17
2	Stomatal index	25.2–26.6
3	Vein islet number	28–30
4	Vein termination number	60–65
5	Palisade ratio	1:6

Study of Midrib (Figure 1–2)

Epidermis - Epidermis single layer with cutinise & cuticuleise cork

Hypodermis - hypodermal cells with 6 to 7 layer thick collenchyma (Figure 3)

Cortex- Several mucilage glands in parenchymatous cells are present (Figure 4)

Vascular nature - Conjoint, collateral arranged in half circle

Pith- Oil canals are present. Pith is large containing several large resin canal, pith parenchymatous thin walled cells are isodimetric irregularly shape. Vascular bundle scatters in pith

Trichomes - Epidermis producing dense covering of trichomes

Mainly three types of trichomes are present

1. Unicellular long simple trichomes
2. Stellate trichomes with long arms.
3. Few short glands scatter here & there.

Glandular pairs containing oils. Glandular head four cells, cells of inner cortex contain spheraphides. (Figure 5)

Study of Lamina

It is hypostomatus and dorsiventral in nature.

Upper epidermis- Thick walled several cells containing spharaphides. (Figure 6)

Lower epidermis- Thin walled containing anomocytic stomata, glands are depressed between veins (Figure 7)

Mesophyll- Not clearly differentiated with spongy tissue & palisade. 2 to 3 layer of upper epidermis contains

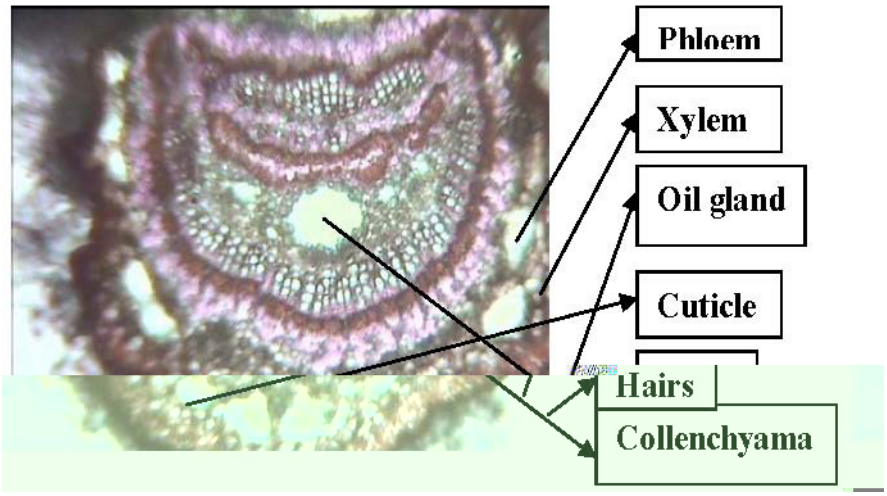


Figure 1 T. S. of Midrib with stain

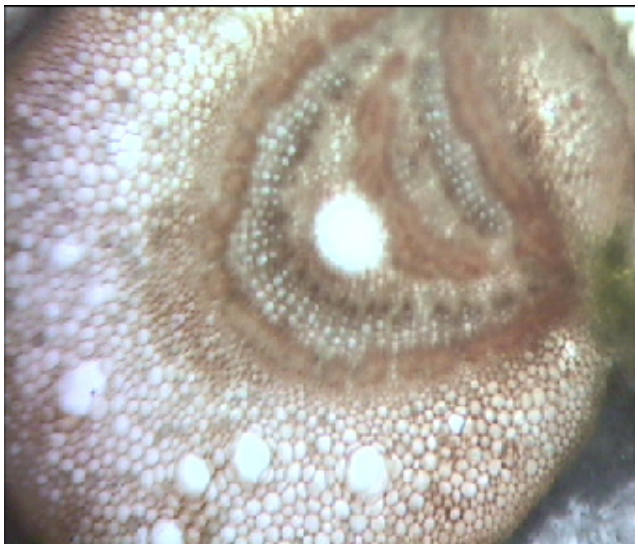


Figure 2 T. S. OF Midrib Without Stain

Figure 5 Covering Trichomes

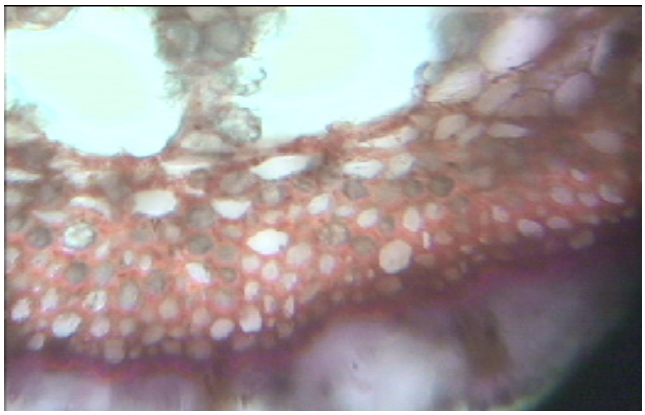


Figure 3 Collenchyma

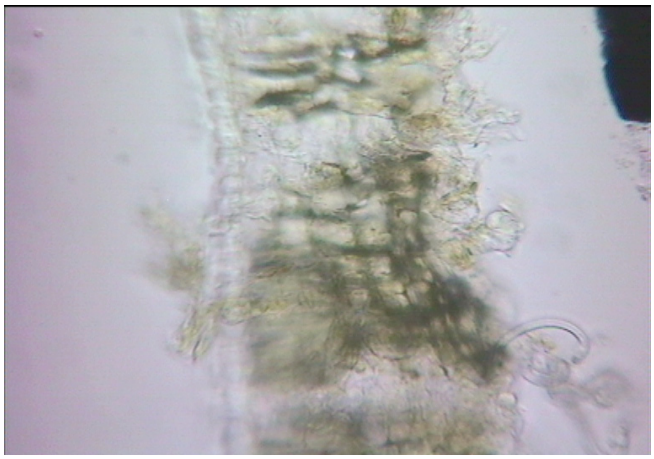


Figure 6 Epidermis with palisade cell

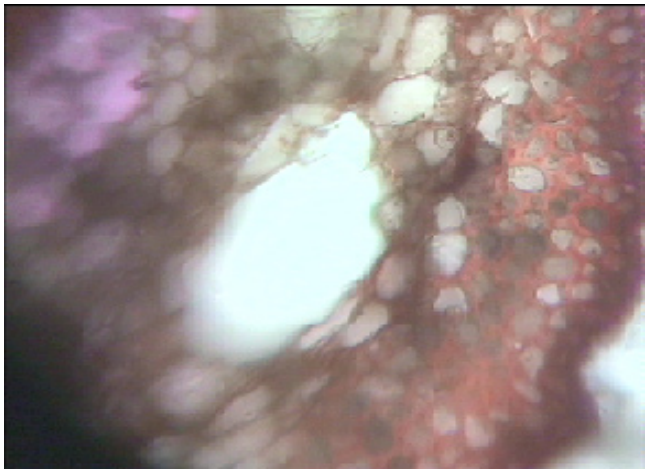


Figure 4 Mucilage canal

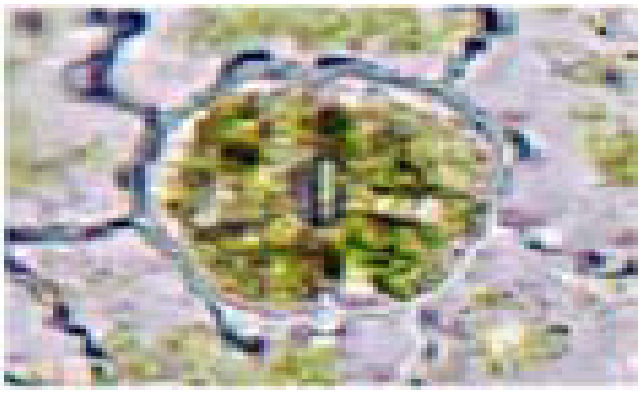


Figure 7 T. S. Stomata

columnar palisade cells while towards lower epidermis cells are relatively short some what loosely placed.

Study of Petiole

Circular in T. S., epidermis single layer, cutinize & cuticlelise hypodermis. (Figure 8)

1. Two to three layer thick walled collenchymas followed by 5 to 6 layer of cortical parenchyma.
2. Large air spaces present in cortex.
3. Endodermis & pericycle not distinct.
4. However inner cortex about three layered cell, regular, isodimetric in nature.
5. Vascular nature-Many congintal opening are conjoint, appears collateral. Open vascular bundle arrange in ring. Each bundle is capped by sclerenchyma on outer side.
6. Vascular bundle shows secondary growth to some extent, but this growth restricted to vascular bundle. Secondary conjunctive tissue lignified.
7. Pith- pith is large containing several large resin canal, pith parenchymatous thin walled cells are isodimetric irregularly shape. Vascular bundle scatters in pith.
8. Calcium oxalate crystal-in additions to specially cells of pith contain rectangular to polygonal crystal of calcium oxalate.

(Note-outer cortical cells contain unknown chemical which on exposure to air turns yellow orange similarly cells of phloem also turns radish orange on exposure to air)

Study of upper peel (Figure 9)

1. Cells are thick, polygonal.
2. Basal cells are present, for attachment of trichomes.
3. Stomata are absent.
4. Hairs are easily removed from upper peel.
5. When in leaves calcium oxalate crystals are accumulating, it converts to sphaeraphides due to these trichomes get easily detach.

Study of lower peel

1. Fully closed type venation present.
2. On lower side anomocytic stomata present with dense veination. (Figure 10a, 10b)
3. Glands are present on lower epidermis

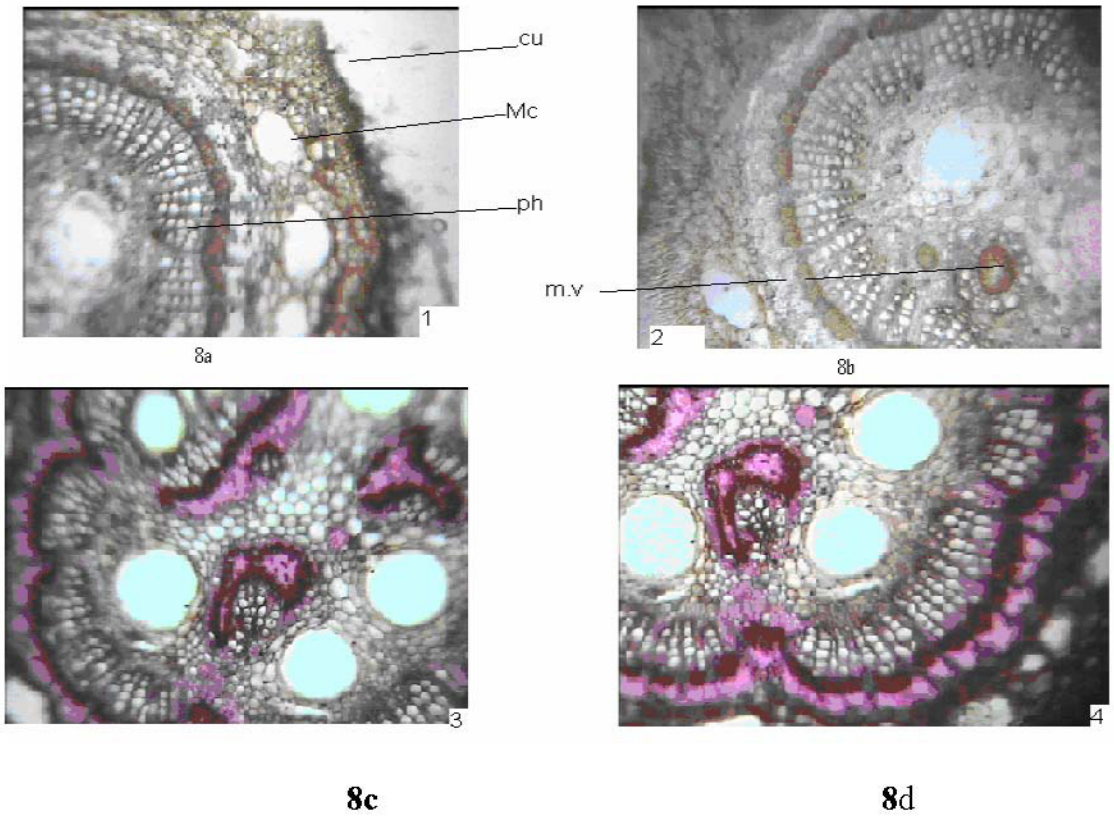
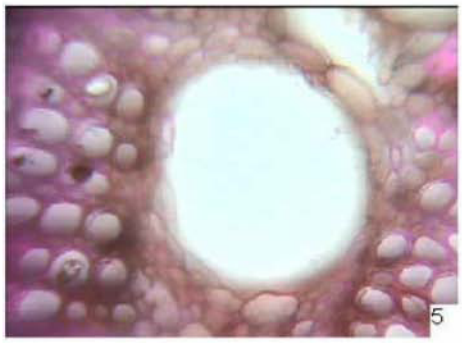


Figure 8 T. S. Petiole
(Cu-cuticle, Mc-mucilage canal, ph-phloem, mv-medullary vessels, upper figures are without staining while lower ones are with stain)

8e: Medullary vessels

8f: Xylem vessels



8g: Oil gland

8h: Xylem vessels with stain

8i: Collenchyma

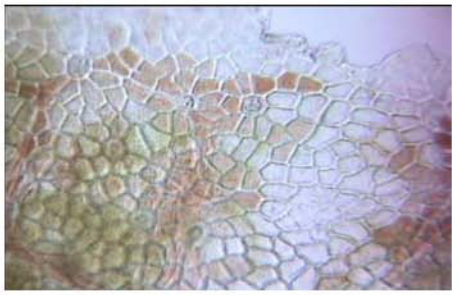


Figure 9: Upper peel with polygonal basal cell

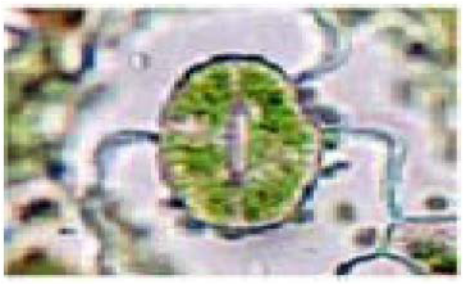


Figure 10a: Anomocytic stomata

Figure 10b: Stomata with dense Veination in lower peel

Table 2: Results of Physicochemical Parameter

Sr.no.	Evaluation parameters	Value (% w/w)
1	Moisture contains	9.09
2	Total ash	6.54
3	Acid insoluble ash	4.74
4	Water soluble ash	2.46

Physicochemical investigation (7)

The physicochemical investigation of leaves includes moisture content, ash value and extractives values and the results are shown in Table 2.

Micro-chemical testing: Table 3 showing observation of micro chemical tests.

Table 3: Results of Micro chemical tests

Sr.no.	Test	observation	Inference
1	TS/LS + phlouroglucinol + Conc. HCL	Lignified fibers Lignified sclerides	Fibers present Sclerides present
2	TS/LS/ + iodine	Blue coloured Granuals	Starch granules present
		Powder characterization	
3	Powder + few drops of water	No swelling	Mucilage absent
4	Powder + Phlouroglucinol + Conc. HCL	Lignified fibers Lignified sclerides	Fibers present Sclerides present
5	Powder + dil. Iodine solution	Blue starch grains observed	Starch grains present

Table 4: Results for Qualitative Study

Sr.no.	Chemical Test	Inference
1.	Tannins	+
2.	Cardiac Glycosides	+
3.	Saponin glycosides	+
4.	Steroids	+
5.	Flavonoids	+

(+ --> Presence)

Table 5: Inorganic constituents and their presence

Sr. No.	Test for Inorganic Elements	Inference
1.	Calcium	+
2.	Magnesium	+
3.	Sodium	–
4.	Potassium	–
5.	Sulphate	+
6.	Phosphate	–

(+ Presence, – absence)

Table 6: Extractive value of different solvents, Colour and consistency of extract

		Color of extract		
Type of solvent	Extractive value (%)	Day light	UV Light	Consistency
Pet. Ether	2.312	Greenish	Greenish brown	Sticky
Benzene	3.870	Greenish black	Black	Semisolid
Chloroform	5.084	Greenish black	Black	Semisolid
Acetone	11.64	Greenish black	Greenish yellow	Sticky
Ethanol	7.276	Greenish black	Black	Semisolid
Hydro-alcoholic (1:1)	4.526	Brownish	Black	Dry
Water	5.544	Brownish	Greenish brown	Dry

Phytochemical investigation(8)
Qualitative investigation

Qualitative Phytochemical studies were done for different types of plant secondary metabolite on powder leaves. The results of qualitative study were shown in Table 4

Inorganic testing: It showed the presence of magnesium, iron, sulphate, chloride, and carbonate. (Table 5)

Extractive values: Table 6 showing different solvent extractive values

RESULTS AND DISCUSSION

The leaves of *Pterospermum acerifolium Willd* were observed to be perennial, simple, petiolate, and ovate with obtuse apex. It shows pinnate-reticulate venation with pilose

surface, entire margin and symmetrical base. The leaves have green upper surface and ale-green underneath, with characteristic odour and bitter taste. Microscopically, the cells of the epidermis consist of anomocytic type of stomata, present only on the lower surface. Prism and clustered types of calcium oxalates crystals were also observed. Numerous unicellular covering and glandular trichomes observed. Chemo-microscopy revealed the presence of tannins, starch grains, proteins, calcium oxalate crystals, oil and mucilage. The results of the quantitative microscopy Table 1 and the evaluation of some pharmacognostic standards of the leaf of *Pterospermum acerifolium Willd* are presented in Tables 1 and 2, respectively. Different chemical compounds such as cardiac glycosides, tannins, flavonoids, triterpenoids among others were detected in *Pterospermum acerifolium Willd*, which could make the plant useful for treating different ailments and having a potential of providing useful drugs of human use. This is because the pharmacological activity of any plant is usually traced to a particular compound(s). The presence of tannins and other phenolic compounds, which have antiseptic properties, could explain the use of this plant for the treatment of sores and as anti-helmintics. The quantitative determination of some pharmacognostic parameters is useful for setting standards for crude drugs. The vein islet, and vein termination numbers and the other parameters determined in the quantitative microscopy, are relatively constant for plants and can be used to differentiate closely related species. The physical constant evaluation of the drugs is an important parameter in detecting adulteration or improper handling of drugs. The moisture content of the drug is not too high, thus it could discourage bacterial, fungi or yeast growth. Equally important in the evaluation of crude drugs, is the ash value and acid-insoluble ash value determination. The total ash is particularly important in the evaluation of purity of drugs, i.e. the presence or absence of foreign inorganic matter such as metallic salts and/or silica. Since the plant, *Pterospermum acerifolium Willd* is useful in traditional medicine for the treatment of some ailments, it is important to standardize it for use as a drug. The pharmacognostic constants for the leaves of this plant, the diagnostic microscopic features and the numerical standards reported in this work could be useful for the compilation of a suitable monograph for it proper identification.

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Stability-indicating RP-HPLC determination of Curcumin in Vicco Turmeric cream and Haridrakhand churna.

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Abstract

A simple, specific, precise and stability-indicating HPLC method of analysis of Curcumin both as a bulk drug and in cream and churna formulations was developed and validated. Chromatographic separation were achieved using Lachrom HPLC with Lichrospher, ODS, (250× 4.6) mm, 5 µ column at ambient temperature. Mixture of ACN: THF: 2%Aceticacid: Water (35: 30: 20:15) was used as mobile phase and delivered at constant flow rate of 0.5 ml/min. 429 nm was selected as wave length for detection of method. The Curcumin peak was obtained at RT 6.20. The linear regression analysis data for the calibration plots showed good linear relationship with r = 0.996, in the concentration range 1–4 µg/ml. The value of slope, intercept and correlation coefficient were found to be 3E+06, 478941 and 0.9985. The method was validated for specificity, precision and recovery. The limits of detection and quantitation were 52.9 ng/ml and 160 ng/ml respectively. Curcumin was subjected to acid and alkali hydrolysis, oxidation and photodegradation. The drug undergoes degradation under acidic, basic, light and oxidation conditions. This indicates that the drug is susceptible to acid, base hydrolysis, oxidation and photo oxidation. As the method could effectively separate the drug from its degradation product, it can be employed as a stability-indicating. The newly developed method can be used in pharmaceutical industry for routine analysis of Curcumin in cream and churna formulations.

Keywords: *Curcumin; stability indicating; RP-HPLC; Anti-inflammatory; Forced degradation*

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INTRODUCTION

Curcumin 1,7-bis-(4-hydroxy-3-methoxyphenyl)-1,6-heptadiene-2,5-dione (Fig. 1) is a yellow colored phenolic pigment obtained from powdered rhizome of *Curcuma longa* Linn. (Family: Zinziberaceae)[1], from ancient it was being used for relieving the pain and inflammation since ancient times in traditional medicine. Extensive researches have also revealed the potent anti-inflammatory effects of curcumin[2–4]. It blocks the synthesis of certain prostaglandins[5], reduces pro-inflammatory cytokine synthesis[6,7], inhibit pro-inflammatory arachidonic acid as well as neutrophils aggregation[8,9] when inflammatory conditions occurs. However the oxygen radical scavenging activity[10,11] of Curcumin has also been observed in its anti-inflammatory effects[12]. Curcumin is unstable at basic pH and undergoes alkaline hydrolysis in alkali/higher pH solution. Decomposition of Curcumin in Hydrolytic decomposition is reported in in vitro physiological condition (isotonic phosphate buffer, pH 7.2) [13–15]. It undergoes photodegradation while exposing to light in solution as well as in solid form[13]. There are already various methods developed for the analysis of Curcumin in the literature like UV[16], HPLC[17–19], TLC[20,21] and HPTLC[22], but there are very few reports on analytical methods for the estimation of curcumin in bulk and its dosage form. Stability-indicating method reported by Ansari *et al* [23] and in this paper pure curcumin was brought under stress condition. The International Conference on Harmonization (ICH) guideline entitled 'stability testing of new drug substances and products' requires the stress testing to be carried out to elucidate the inherent stability characteristics of the active substance[24]. Susceptibility to oxidation is one of the required tests. Also, the hydrolytic and the photolytic stability are required. An ideal stability-indicating method quantifies the percent drug and also resolves its degradation products. The aim of this work is to develop an accurate, specific, repeatable and stability-indicating method for the determination of curcumin in the presence of its degradation product(s).

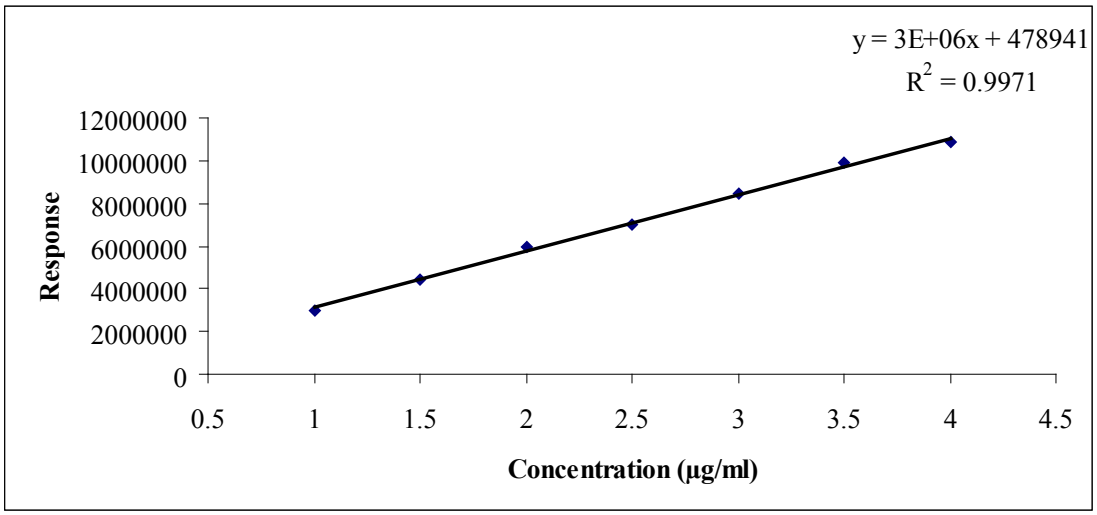

Figure 1 Calibration curve of Curcumin

2. Experimental

2.1. Materials

Curcumin was purchased from Loba Chemicals Bangalore, India. All chemicals and reagents used were of HPLC grade and were purchased from Merck Chemicals, India.

2.2. HPLC instrumentation

The chromatographic system consists of a L-7110 solvent delivery system (Merk Hitachi), L-7400 double beam UV detector (Merk Hitachi), L-7500 integrator and a rheodyne injector valve bracket fitted with a 20 µl sample loop.

2.3. Chromatographic conditions

Chromatographic separation were performed on a stainless steel lichroCART ODS Column, (250×4.6 mm) packed with 5 µ particle diameter, LichroCART HPLC guard cartridge system and a winchrom software. Mobile phase consisting of a mixture ACN, THF, 2 %Acetic acid and Water (35:30:20:15), was delivered at a flow rate of 0.5 ml/ min with detection at 429 nm.

2.4. Sample preparation

The stock solutions were prepared by dissolving 50 mg of Curcumin was dissolved in 50 ml methanol to get a concentration of 1000 µg/ml. Analytical standard solutions for linearity were prepared by diluting the stock solution with methanol immediately prior to use. All the preparations were made in borosilicate glass tubes.

2.5 Calibration curves of curcumin

A stock solution of curcumin (1 mg/ml) was prepared in methanol by transferring 50 mg of drug in 50 ml volumetric flask. 5 ml of this solution was transferred to 50 ml volumetric flask and volume was made up to the mark. From this solution concentration of 1, 1.5, 2.0, 2.5, 3, 3.5 and 4 µg/ml were prepared by diluting 0.1, 0.15, 0.2, 0.25, 0.3, 0.35 and 0.4 ml of previously prepared solution up to 10 ml with methanol in different 10 ml volumetric flask. The slope, intercept and correlation coefficient were found to by 3E+06, 478941 and 0.9985. Calibration curve of Curcumin has been shown in Figure 1.

No significance difference was observed between the slopes of the calibration curve (P>0.005).

3. Validation of Proposed Method

3.1 Specificity

Specificity of the stability indicating method was established by separation of the principle peak with the excipients peak in the Vicco cream and that of the degradant peak in the Curcumin pure, Vicco cream and Haridrakhand churna after degradation.

3.1.1 Acidic hydrolysis

1 ml of the stock solution of Curcumin (1 mg/ml) was transferred to a 10 ml volumetric flask. 1 ml 1 N HCl was added and kept for six hours at 90°C in dark to avoid the possible degradation effects of light. 1 ml of the solution was transferred in to 100 ml volumetric flask and diluted up to the mark with methanol to get 10 µg/ml. 2.5 ml of this solution was transferred to a 10 ml volumetric flask and diluted up to the mark with methanol to give 2.5 µg/ml solution.

500 mg Vicco cream was transferred to a 10 ml volumetric flask. 1 ml 0.05 N HCl was added to it and

kept for six hours at 90°C in dark to avoid the possible degradation effects of light.

500 mg Haridrakhand churna was transferred to a 10 ml volumetric flask. 1 ml 0.5 N HCl was added to it and kept for six hours at 90°C in dark to avoid the possible degradation effects of light.

3.1.2. Alkali degradation

1 ml of the standard stock solution of Curcumin (1 mg/ml) was transferred to a 10 ml volumetric flask. 1 ml 1 M NaOH was added to it and kept for six hours at 90 °C in dark to avoid the possible degradation effects of light. 1 ml of the solution was transferred in to 100 ml volumetric flask and diluted up to the mark with mobile phase to get 10 µg/ml. 2.5 ml of this solution was transferred to a 10 ml volumetric flask and diluted up to the mark with mobile phase to get test concentration of 2.5 µg/ml.

500 mg Vicco cream was transferred to a 10 ml volumetric flask. 1 ml 0.01 M NaOH was added to it and kept for six hours at 90 °C in dark to avoid the possible degradation effects of light.

3.1.3. Direct sunlight

The 50 mg of Curcumin, 500 mg Vicco cream and 500 mg Haridrakhand Churna were separately dissolved in 10 ml of methanol in 10 ml volumetric flask and exposed to direct sunlight and UV chamber at 254 nm for 24 h. The resultant solutions were then further diluted with methanol to get test concentration of 2.5 µg/ml.

3.1.4. Oxidative degradation by peroxide

1 ml of standard stock solution of Curcumin (1 mg/ml) was transferred in a 10 ml volumetric flask. 1 ml of 30 % H_2O_2 was added to it and kept for 6 hours on boiling water bath. Volume was made up to the mark with methanol. 1 ml was transferred to 10 ml volumetric flask and volume was made up to the mark. Further 2.5 ml was transferred to 10 ml volumetric flask and volume was made up to the mark and 20 µl was injected.

500 mg Vicco cream was weighed and transferred to a 10 ml volumetric flask. 1 ml 30 % H_2O_2 was added to it and kept on boiling water bath for 6 hours.

500 mg Haridrakhand churna was weighed and transferred to a 10 ml volumetric flask. 1 ml 30 % H_2O_2 was added to it and kept on boiling water bath for 6 hours. It was diluted to 10 ml with methanol.

3.1.5. Degradation by UV light

The 50 mg of Curcumin, 500 mg Vicco cream and 500 mg Haridrakhand Churna were separately dissolved in 10 ml of methanol in 10 ml volumetric flask and exposed to direct sunlight and UV chamber at 254 nm for 24 h. The resultant solutions were then further diluted with methanol to get test concentration of 2.5 µg/ml.

3.1.6. Thermal Degradation

The 50 mg of Curcumin, 500 mg Vicco cream and 500 mg Haridrakhand Churna were separately kept in porcelain dish at 90°C in hot air oven for 5 hours. It was dissolved in 10 ml of methanol in 10 ml volumetric flask and the resultant solutions were then further diluted with methanol to get test concentration of 2.5 µg/ml.

3.2. Linearity and Range

Linearity is accessed by visualizing the graph of calibration curve. The points in the calibration curve distributed equally above and below the trend line show linearity.

3.3. Precision
3.3.1 Repeatability

Repeatability was accessed by six replicate injections of 2.5 µg/ml solution of drug prepared for standard stock solution. 20 µl volume was injected.

3.3.2 Intraday

2 ml, 2.5 ml and 3 ml was taken out from the 10 µg/ml Curcumin solution and diluted to 10 ml to make 2 µg/ml, 2.5 µg/ml and 3 µg/ml respectively. Three replicates were injected three times a day.

3.3.3. Interday

Same procedure was followed and three replicates were injected in three days.

3.4 Accuracy
3.4.1 Recovery of Vicco cream

300 mg cream was accurately weighed and transferred to a 10 ml volumetric flask directly with butter paper. 5 ml methanol was added to it and sonicated for 15 minutes to extract the Curcumin from the cream. Then the volume was made up to the mark and centrifuged for 2 minutes at 2000 rpm. The centrifuged solution was filtered with 0.45 µ syringe filter. Three replicate samples were prepared and spiked with 1 µg/ml, 1.5 µg/ml and 2 µg/ml pure Curcumin respectively. Three injections (20 µl) of each sample were injected. Data of recovery of Vicco cream has been shown in Table 1.

3.4.2 Recovery of Haridrakhand Churna

100 mg churna was accurately weighed and transferred to a 10 ml volumetric flask and 5 ml methanol was added and

sonicated for 5 minutes to extract the Curcumin. Then the volume was made up to the mark and centrifuged for 1.5 minutes at 2000 rpm. The centrifuged solution was filtered with 0.45 µ syringe filter. 0.3 ml was taken and diluted to 10 ml. and 20 µl were injected. Three replicate samples were prepared and spiked with 1 µg/ml, 1.5 µg/ml and 2 µg/ml pure Curcumin respectively. Three injections (20 µl) of each sample were injected. Data of recovery of Vicco cream has been shown in Table 2.

3.5. Limit of detection (LOD) and Limit of quantification (LOQ)

LOD and LOQ were calculated through linear regression method.

3.6. System suitability testing

System suitability testing was performed by using six replicates of test concentrations. Variations in Tailing factor, asymmetry factor and R.T. were calculated. Number of theoretical plates (N) and HETP were calculated. Results have been shown in the Table 3.

4. Estimation of Curcumin in formulation
4.1 Optimization of extraction time for the method

Four samples of the Vicco cream were prepared according to the method given in the above section and sonicated for 5, 10, 15 and 20 minutes and 20 µl were injected. Centrifugation for 2 minutes at 2000 rpm was sufficient to settle the undissoved matter.

Four samples of the Haridrakhand churna were prepared in the mobile phase and sonicated for 5,10, 15 and 20 minutes and 20 µl was injected immediately. Centrifugation for 1.5 minute at 2000 rpm was sufficient to settle the undissoved matter.

4.2 Estimation of Curcumin in Vicco cream

500 mg Vicco cream was accurately weighted and transferred to a 10 ml volumetric flask directly with butter paper. 5 ml methanol was added to it and sonicated for 15 minutes to extract the Curcumin from the cream. Then the volume was made up to the mark and centrifuged for 2 minutes at 2000 rpm. The centrifuged solution was filtered with 0.45 µm syringe filter and 20 µl was injected.

4.3 Estimation of Curcumin in Haridrakhand churna

500 mg Haridrakhand churna was accurately weighted and transferred to a 10 ml volumetric flask and 5 ml methanol was added sonicated for 10 minutes to extract the Curcumin. Then the volume was made up to the mark and centrifuged for 1.5 minutes at 2000 rpm. The centrifuged solution was filtered with 0.45 µ syringe filter.

Table 1: Recovery study of Vicco cream

Conc. found before spiking (µg/ml) C_1	Conc. of Std. added (µg/ml) C_2	Conc. found after Spiking (µg/ml) C_3	% Recovery $(C_3-C_1) * 100/C_2$	Mean ± SD	RSD
1.68	0.49	2.18	102.04	100.49±0.023	2.31
		2.17	100		
		2.19	104.08		
1.68	1.024	2.69	98.63		
		2.71	100.58		
		2.68	97.65		

Table 2: Recovery study of Haridrakhand churna

Conc. found before spiking (µg/ml) C_1	Conc. of Std. added (µg/ml) C_2	Conc. found after Spiking (µg/ml) C_3	% Recovery $(C_3-C_1) * 100/C_2$	Mean ± SD	RSD
1.86	0.48	2.35	102.08	100.16±0.026	2.653
		2.36	104.17		
		2.34	96		
1.86	1.035	2.88	100		
		2.89	98.55		
		2.86	96.62		

Table 3: Summary of system suitability parameters

S. No.	Parameters	Inference
1.	Number of theoretical plates(N)	17500
2.	Height equivalent to theoretical plate (HETP)	1.42×10^{-3}
3.	Retention time	6.44
4.	Capacity factor	2.078
5.	Tailing factor	0.87–0.90
6.	Asymmetry factor	0.85–0.88

0.3 ml was taken and diluted to 10 ml and 20 µl were injected.

5. RESULTS AND DISCUSSION

5.1 Validation of the proposed method

5.1.1 Specificity

The method separates the peak of excipients in the Vicco cream with resolution 2.4 and Haridrakhand churna shows no extra peak except Curcumin peak. Also the method separates the peaks of potential degradants formed after forced degradation studies with the resolution more than 2. Hence it can be concluded that the method is specific in nature. The results of the forced degradation study.

5.1.2 Linearity

Two points exists above the calibration curve, two points exists below the calibration curve and one point on calibration curve shows the linearity.

5.1.3 Range

Linearity range: 1 – 4 µg/ml.
Target range: 2, 2.5, 3 µg/ml.
Working range: 0.16–4 µg/ml.
Target concentration: 2.5 µg/ml.

5.1.4 Precision

5.1.4.1 Repeatability

RSD of six replicates injection of test concentration (2.5 µg/ml) was 0.438, hence method is repeatable because the calculated RSD is less then one.

5.1.4.2 Intraday and interday

The mean RSD for intraday and interday precision was 0.704 and 0.672 respectively which is less than two concluding method is precise.

5.1.5 Accuracy

5.1.5.1 Recovery study of Vicco cream (Table 2)
5.1.5.2 Recovery study of Haridrakhand churna (Table 3)

5.1.6 Limit of quantification (LOQ) and limit of detection (LOD)

LOD and LOQ of the method were found to be 52.9 ng/ml and 160 ng/ml respectively.

5.1.7 System suitability

Summary of system suitability parameters is given in Table 3.

Table 4: Summary of validation parameters

S.No.	Parameters	Results	
1.	Specificity	Resolution of degradants and excipients with drug peak > 1.5, hence method is specific.	
2.	Linearity	Method shows linearity between 1–4 µg/ml	
3.	Range	Linearity range	1–4 µg/ml
		Target range	2, 2.5, 3 µg/ml
		Working range	0.16–4 µg/ml
		Target concentration	2.5 µg/ml
4.	Accuracy (%recovery)	Cream	97.65–104.08 %
		Churna	96–104.17 %
5.	Precision (RSD)	Repeatability	0.438
		Intraday	0.704
		Interday	0.672
6.	LOD and LOQ	52.9 and 160 ng/ml respectively	

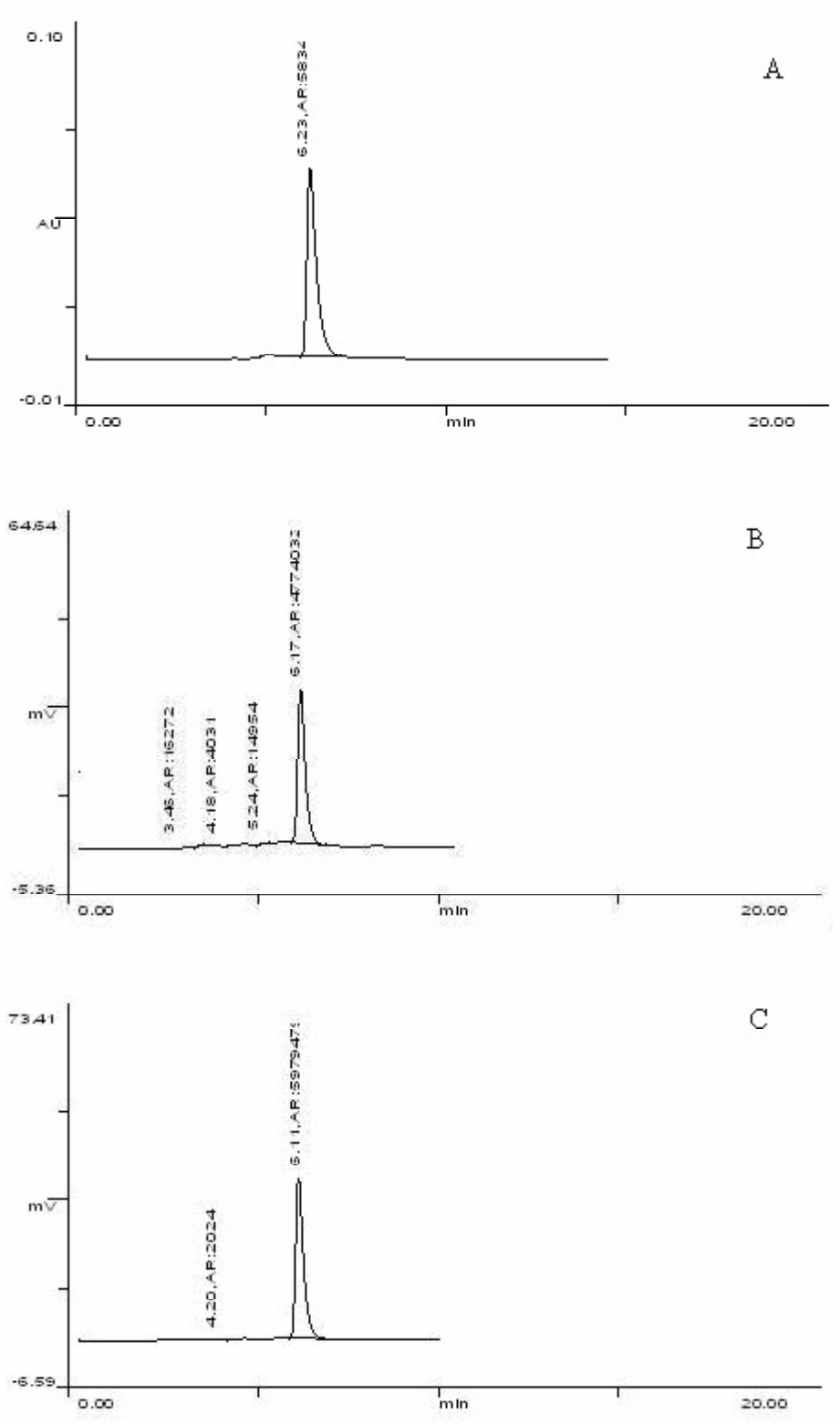

Figure 2 Chromatogram obtained after acidic hydrolysis of A) Pure Curcumin B) Vicco cream and C) Hridrakhand churna

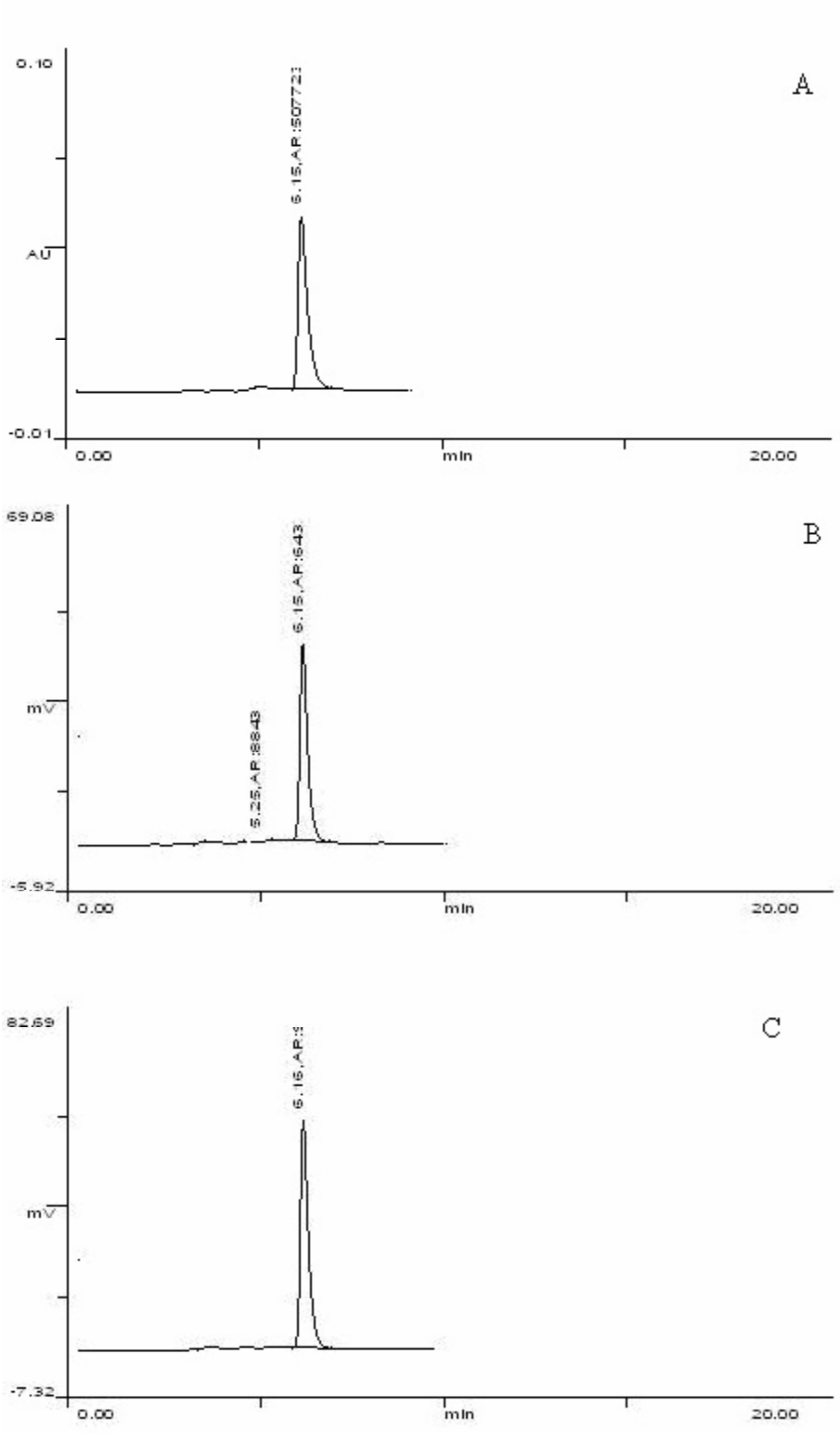

Figure 3 Chromatogram obtained after alkali degradation of A) Pure Curcumin B) Vicco cream and C) Hridrakhand churna

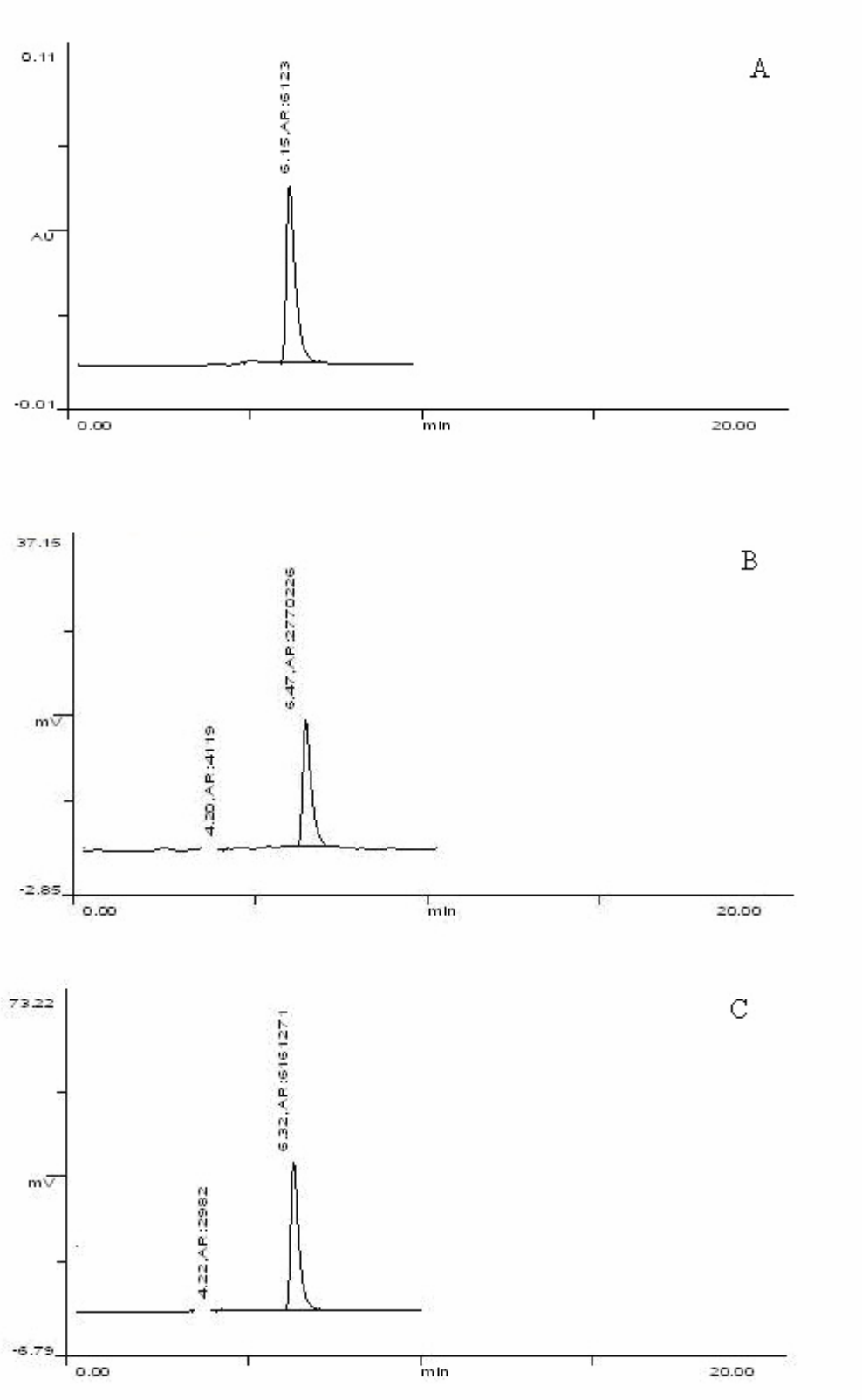

Figure 4 Chromatogram obtained after degradation from direct sunlight of A) Pure Curcumin B) Vicco cream and C) Hridrakhand churna

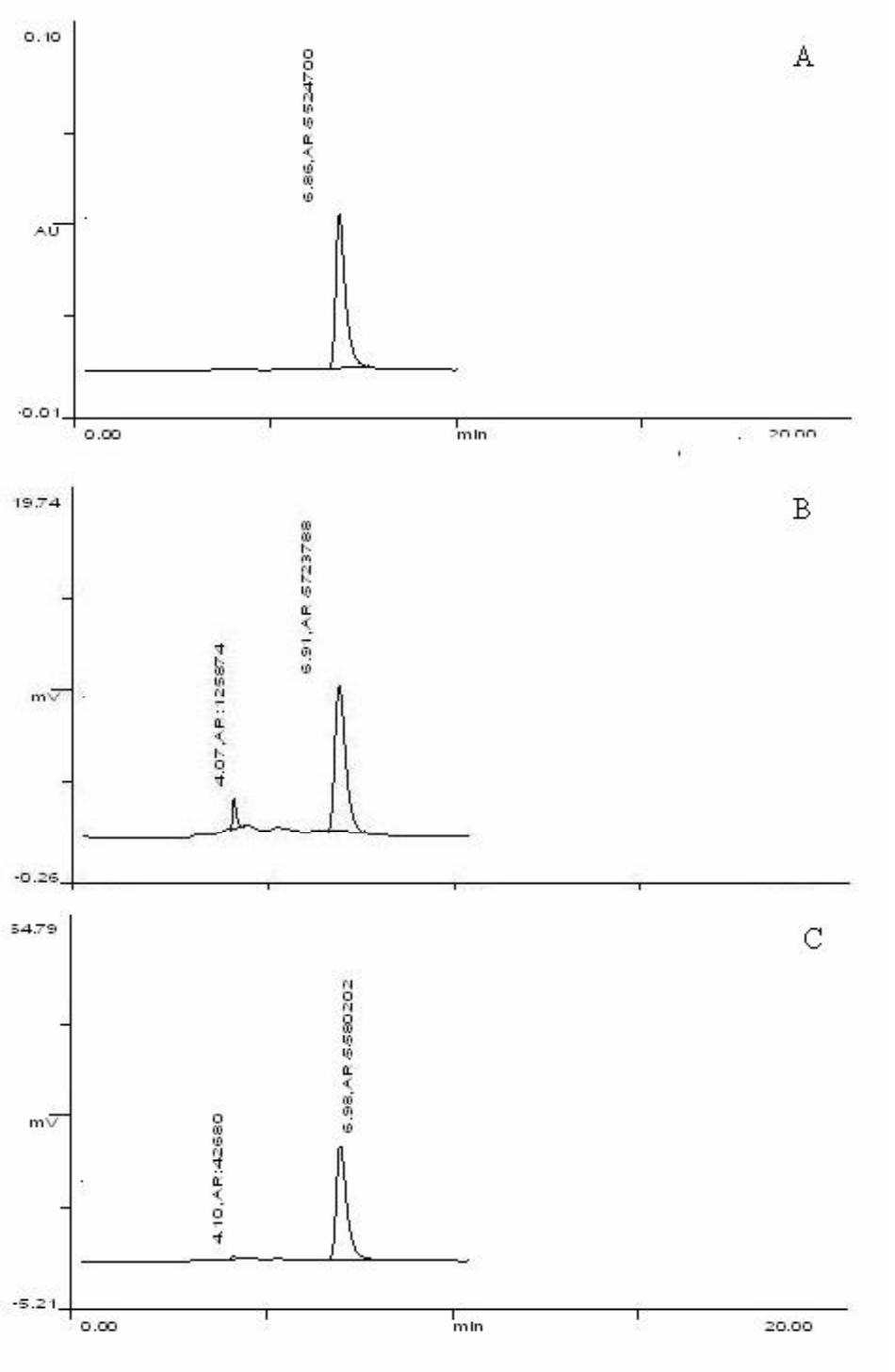

Figure 5 Chromatogram obtained after oxidative degradation of A) Pure Curcumin B) Vicco cream and C) Hridrakhand churna

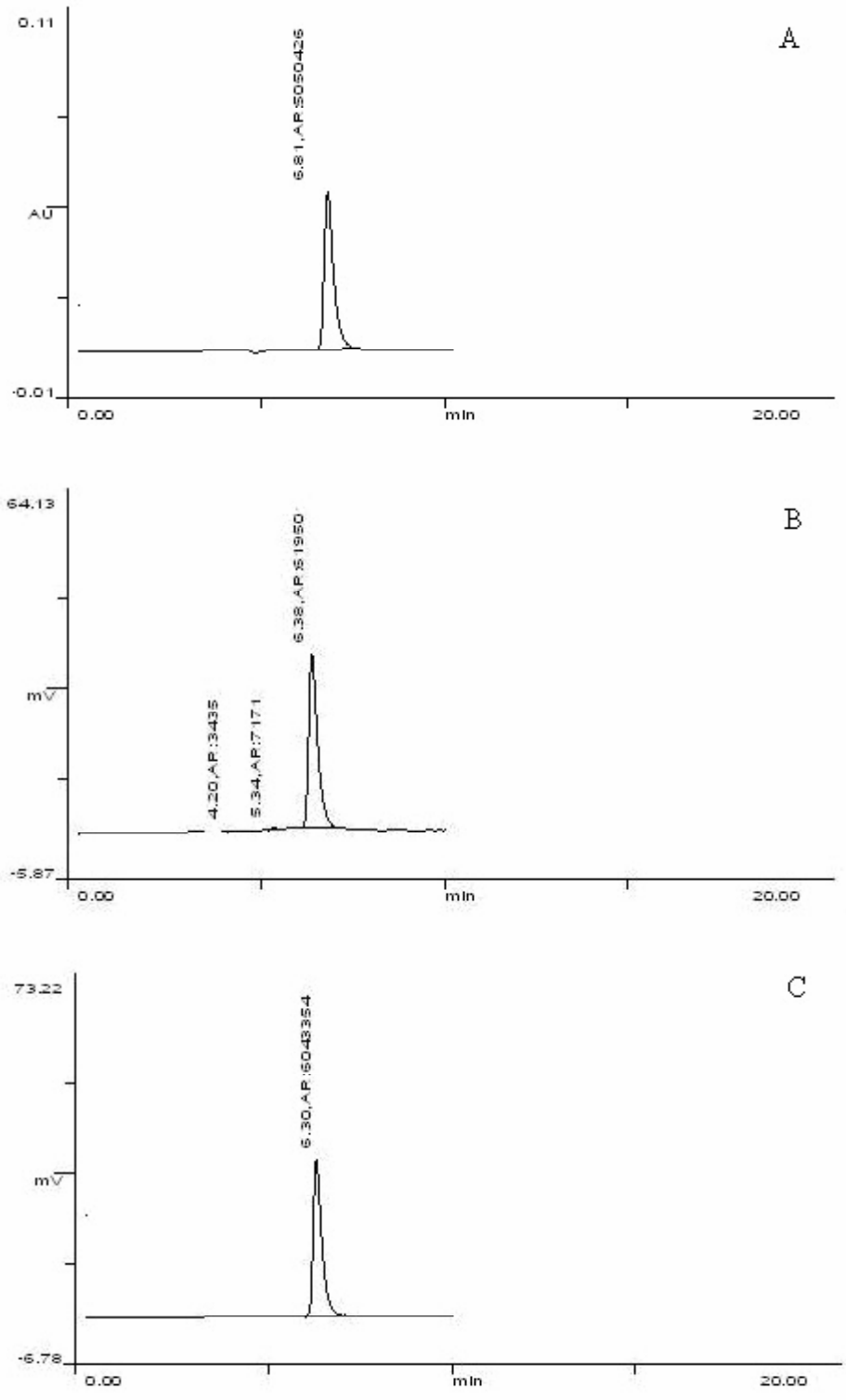

Figure 6 Chromatogram obtained after degradation by UV of A) Pure Curcumin B) Vicco cream and C) Hridrakhand churna

Table 5: Summary of degradation study

S.No.	Degradation parameters	Formulation	Degradants RT	Resolution	% Recovery
1.	Acidic hydrolysis	Pure	3.45, 5.24	2.9, 1.8	76.7
		Cream	-	-	75.4
		Churna	-	-	80.1
2.	Alkali hydrolysis	Pure	-	-	82.1
		Cream	5.25	1.8	89.2
		Churna	-	-	75.3
3.	Direct sunlight	Pure	-	-	83.1
		Cream	-	-	84.6
		Churna	-	-	85.9
4.	Oxidative degradation	Pure	-	-	70
		Cream	4.07	2.6	4.2
		Churna	4.10	2.2	70.1
5.	UV degradation	Pure	-		81.6
		Cream	5.34	1.9	84.6
		Churna	-	-	81.4
6.	Thermal degradation	Pure	-	-	99.5
		Cream	-	-	99.8
		Churna	-	-	99.9

Summary of validation parameters has been given in the Table 4

5.2 Estimation of Curcumin in formulation

Extraction time was optimized to 12 min. for cream and 10 min for churna and content of Curcumin was found to be 5.608 and 18.59 µg/ml for cream and churna formulation respectively.

5.3 stability indicating property

The chromatograms of the samples degraded with acid, base, hydrogen peroxide and light showed well-separated spots of pure Curcumin as well as some additional peaks at different RT values. The spots of degraded product were well resolved from the drug spot as shown in Figure 2 – 6. The number of degradation products with their RT values, resolution with Curcumin peak remained and percentage recovery were calculated and listed in Table 5.

5.4 CONCLUSIONS

In the present work the RP-HPLC method for the estimation of Curcumin in Vicco cream and Haridrakhand churna has been developed. The method is simple, precise, accurate and specific. The method doesn't suffer any interference due to common ingredients of the cream and churna formulations. Forced degradation studies also show that degradants doesn't interfere with the drug peak. The newly developed method can be used in pharmaceutical industry for routine analysis of Curcumin in cream and churna formulations.

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In vitro antioxidant activity of *Entada pursaetha, Toddalia aculeata,* and *Ziziphus mauritiana.*

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Abstract

The antioxidant activity of the crude methanolic extract of the seeds of *Entada pursaetha*, the stem of *Toddalia aculaeta*, and the fruit of *Ziziphus mauritiana* was investigated. The total phenolic composition of methanolic extract was calculated to be 5.5mg catechol equivalents/g of sample. Antioxidant activity of the extract was evaluated on the basis of ability of scavenging free radical and hydroxyl radical with the Ic 50 values 2.12mg/ml and 1.034mg/ml respectively. Total antioxidant capacity of crude plant extract was found to be 1.43mg ascorbic acid equivalents at 250µg/ml extract concentration. The reducing power of the extract increased dose dependently and the extract reduced the most Fe^{3+} ions to the extent less than the standard ascorbic acid.

Keywords: Antioxidants, Entada *pursaetha, Toddalia aculeata, Ziziphus mauritiana*, DPPH, Hydroxyl radical, Reducing power

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INTRODUCTION

Reactive oxygen species(ROS) are highly reactive molecules derived from the metabolism of oxygen causing damage to DNA, proteins, carbohydrates and lipids.ROS include super oxide anion, hydrogen peroxide, hydroxyl radical and singlet molecular oxygen(1). All these radicals exert oxidative stress towards the cells of human body and this leads to a number of physiological disorders. Antioxidants are of great importance in terms of reducing oxidative stress that is thought to cause damage to biological molecules(2).Phenolic compounds have been reported to play key antioxidant roles using the mechanism of delocalization of the single electron of the radical(3).

The commonly used synthetic antioxidants such as butylated hydroxyl anisole (BHA) and butylated hydroxy toluene (BHT) are suspected to have some toxic effects and possible carcinogens (4). Therefore development and utilization of more effective antioxidants of natural origins are desired. A great number of natural medicinal plants have been tested for their antioxidant activities and results have shown that the raw extracts or isolated pure compounds from them were more effective antioxidants in vitro than BHT or vitamin E(5).

The plants used for the present study were *Entada pursaetha, Toddalia aculeata, and Ziziphus mauritiana. Entada pursaetha* is a gigantic creeper with giant pods among legumes and is an endangered species belonging to the family Fabaceae. It can be used as a narcotic or as a tonic or used in curing liver troubles, allaying body pains, in warding of cold, curing eye diseases, arthritis and paralysis(6). *Toddalia aculeata* is a thorny large shrub belongs to the family Rutaceae. It has been used by traditional health practitioners in East Africa for management of diseases (7). *Ziziphus mauritiana* is a tropical fruit tree species belonging to the family Rhamnaceae. The fruits are sweet, cooling, anodyne purgative, mucilaginous, pectoral. Styptic, aphrodisiac, invigorative, depurative, appetizer and tonic. They are useful in encephalopathy, opthalmopathy, dipsia, cough, asthma. Wound vitiated conditions of pitta burning sensation, diarrhea, vomiting, leucorrhoea, general debility and insomnia (8).

MATERIALS AND METHOD

Extraction of plant materials

The seeds of *Entada pursaetha*, the stems of *Toddalia aculeata* and the fruits of *Ziziphus mauritiana* were collected from Kolli hills, Namakkal, Salem District, Tamilnadu, India. The samples were air dried in shade at room temperature and then ground to a fine powder in a mechanic grinder.10 g of the mixed powdered plant material was extracted

with 400ml of methanol in a soxhlet extractor for 24hrs. The resultant crude methanolic extract was evaporated to dryness and then stored in the freezer until ready for use.

Quantification of total phenolic compound.

Total phenolics were quantified and expressed as catechol equivalents according to a method proposed by Singleton et al 1999 (9).To 0.1ml of methanolic extract added 3.9ml of distilled water and 0.5ml of Folin –Ciocalteau reagent.

The tubes were incubated at room temperature for 30 min. To this added 2ml of 20% sodium carbonate and kept at boiling water bath for 3 min.The blue colour formed was read at 650 nm with spectrophotometer. The standard graph was prepared with a plot of various concentrations against corresponding absorbance values for catechol .Total phenol values are expressed as catechol equivalents (mg/g dry mass).

Determination of Total antioxidant activity of plant extracts.

Antioxidant activity of plant extracts was determined by the method of Prieto et al 1999 (10). The tubes containing 0.2 ml of plant extract, 1.8 ml of distilled water and 2 ml of phosphomolybdenum reagent solution (0.6M sulphuric acid,28 mM sodium phosphate and 4 mM ammonium molybdate) were incubated at 95°C for 90 minutes. The mixture was cooled to room temperature and the absorbance was measured at 695 nm using an UV/VIS spectrophotometer. The antioxidant activity is expressed as number of equivalents of ascorbic acid.

Measurement of free radical scavenging activity.

The ability of the extract to scavenge free radical was assayed with use of a synthetic free radical compound 2, 2-diphenyl-1-picrylhydrazyl (DPPH). The method of Brand Williams et al 1995 (11). has been adapted. 200 µl of the extracts of concentration between 50–250 µg were mixed with 2 ml of DPPH reagent (0.1 mM DPPH in methanol). The disappearance of pink color of DPPH was read spectrophotometrically at 517 nm after 30 mins of incubation at room temperature in the dark. The same solvent was used as a control. The same procedure was repeated with methanolic solutions of synthetic antioxidant Quercetin as positive control. Free radical scavenging capacity was expressed as percentage inhibition of DPPH radical and was calculated by the following equation;

% inhibition = 100 × (1 – Absorbance of sample/ Absorbance of control).

Measurement of hydroxyl radical scavenging activity.

The hydroxyl radical scavenging activity was measured by a modified deoxyribose method determining thiobarbituric acid reactive substances (TBARS) proposed by Res at Apak et al 2006 (12). To a test tube added 3 ml of phosphate buffer (pH 7.0) , 1 ml of 10 mM 2-deoxy-D-ribose, 0.5 ml of 20 mM Na_2 EDTA , 0.5 ml of 20mM $Fecl_3$ solution , 3.8 ml distilled water , 0.2 ml of plant extract (200-1000 µg) and 1 ml of 10mM of H_2O_2 in the given order and the mixture of incubated for 4 hours at 37°C in a water bath . At the end of the period the reaction was arrested by adding 5 ml of 2.8% TCA. To this added 5 ml of 1% TBA and the reaction mixture was kept in a boiling water bath for 10 minutes. The mixture was cooled and the absorbance was measured at 520 nm. Hydroxyl radical scavenging capacity was expressed as percentage inhibition of Hydroxyl radical and was calculated by the following equation;

% inhibition = 100 × (1 – Absorbance of sample/ Absorbance of control).

Determination of reducing power of plant extracts.

The reducing power of the methanolic plant extract was determined according to the method of Oyaizu 1986 (13). To 1 ml of plant extract 2.5 ml of phosphate buffer of pH 6.6 and 2.5 ml of 1% potassium fericyanide were added. The mixture was incubated at 50°C for 20 min 2.5 ml of 10% Trichloroacetic acid was added to the mixture and the mixture was centrifuged at 3000 rpm for 10 min. 2.5 ml of the supernatant was mixed with 2.5 ml distilled water and 0.5 ml 0.1% $Fecl_3$. Absorbance was measured at 700 nm using spectrophotometer.

RESULTS

10 g of powdered sample yielded 1.6g of extract after concentration and drying with rotary evaporator. Percentage yield was calculated to be 16 %.

Total Phenolic content of methanolic plant extract was estimated by Folin-Ciocalteu method as 5.5 mg catechol equivalent /gm of sample. Fig –1 shows the antioxidant activity exhibited by the plant extract of various concentrations (50–250 µg). The study reveals that antioxidant activity of the plant extract was increased in a dose dependent manner. Total antioxidant capacity of plant extract is expressed as the mg equivalents of ascorbic acid. Total antioxidant capacity of plant extract was found to be 1.430 mg ascorbic acid equivalents at 250 µg/ ml of extract concentration. The DPPH free

radical scavenging activity of the plant extract is showed in fig–2. The extract exhibited a notable dose dependent inhibition of the DPPH activity with a 50% inhibition (Ic50) at a concentration of 2.12 mg/ml while the Ic50 value of the positive control quercetin was found to be 0.75 mg/ml.Hydroxyl radical scavenging activity of crude plant extract is given in Fig–3.The ability of the extract to scavenge these radicals was evaluated by the Fenton mediated 2-deoxy ribose assay. The Ic 50 value of the crude extract was found to be 1.034mg/ml while the Ic

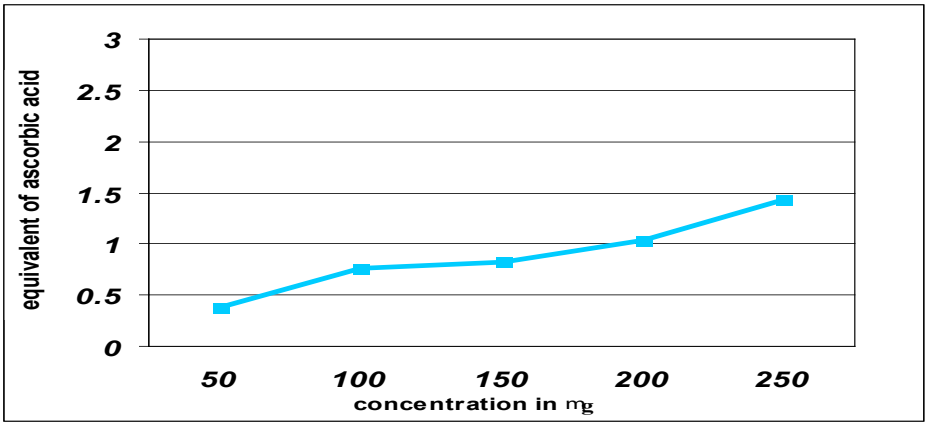

Figure 1 TOTAL ANTIOXIDANT ACTIVITY.

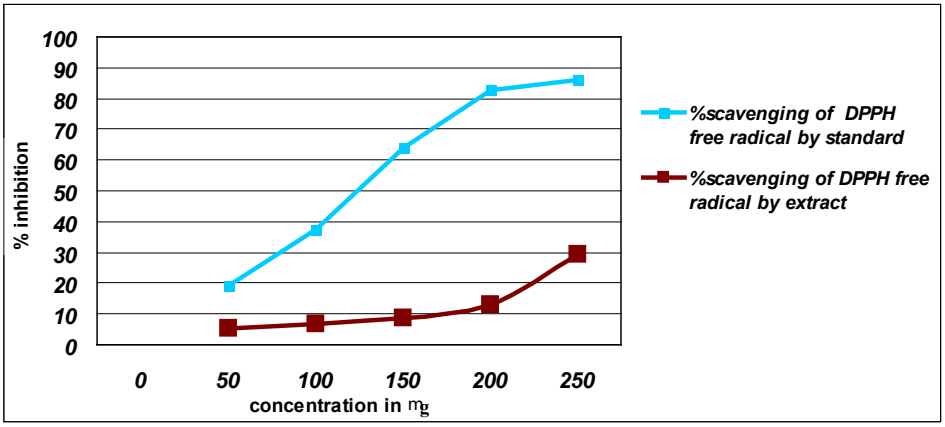

Figure 2 SCAVENGING POTENTIAL AGAINST DPPH FREE RADICAL.

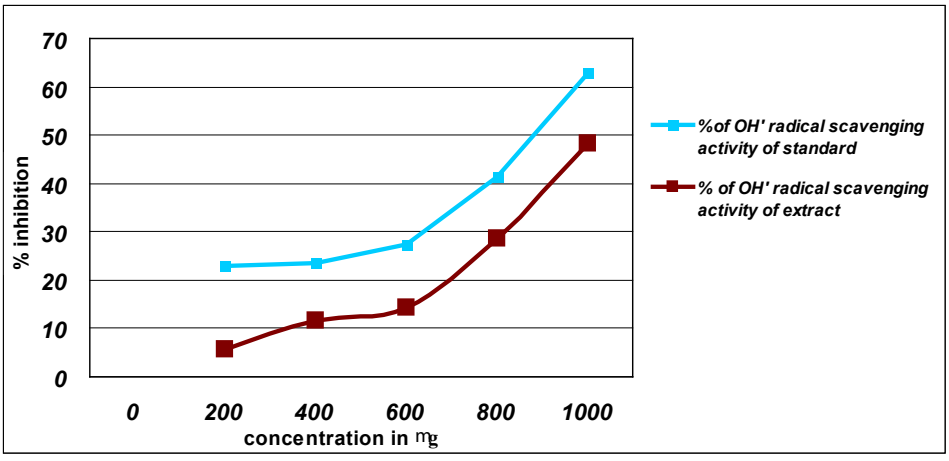

Figure 3 HYDROXYL RADICAL SCAVENGING ACTIVITY.

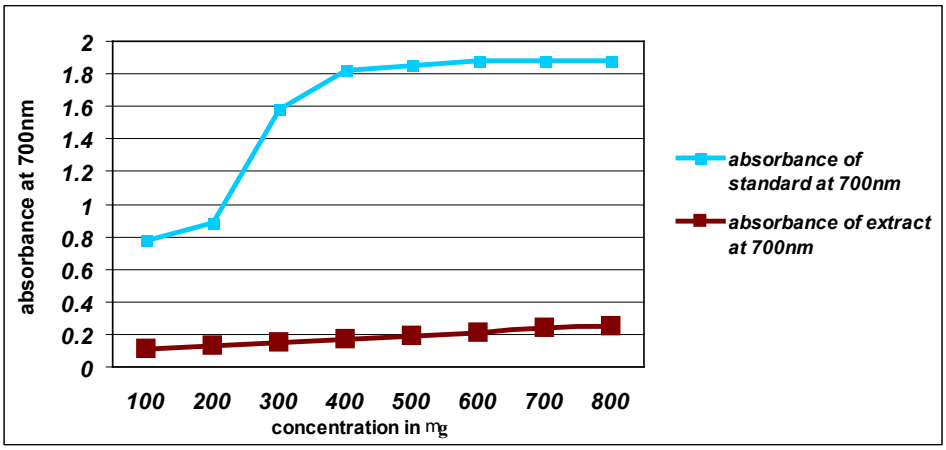

Figure 4 REDUCING POWER.

50 value of standard quercetin was found to be 0.793mg/ml.The reducing power of the crude plant extract is given in fig–4. The reducing power of the extract was increased with concentration of the sample. The plant extract could reduce the most Fe^{3+} ions which had a lesser reductive activity than the standard ascorbic acid.

DISCUSSION

Total phenolic content of plant extract was estimated to be 5.5 mg catechol equivalent /gm of sample. Phenolic compounds present in the molecular structure of natural antioxidants help in enhancing their antioxidant activity (14–15). Phenolic acids have implicated as natural antioxidants in fruits, vegetables and other plants. For ex- Caffeic acid, Ferulic acid and vanillic acid are widely distributed in plant kingdom (16). Phosphomolybtenum assay used to determine the total antioxidant capacity is based on the reduction of Mo (VI) to Mo (V) by the extract and subsequent formation of a green phosphate /Mo (V) complex at acid pH (17). Total antioxidant capacity of crude plant extract was found to be 1.430 mg ascorbic acid equivalent at 250 µg/ml extract concentration. The study reveals that the antioxidant activity of the extract exhibits increasing trend with increasing concentration of the plant extract. The DPPH antioxidant assay is based on the ability of 1, 1 diphenyl 1-2 picryl – hydrazyl, a stable free radical to be decolorized in the presence of antioxidants (18). The DPPH radical contains an odd electron which is responsible for the absorbance at 517 nm and for deep purple colour.When DPPH accepts an electron donated by an antioxidant compound the DPPH is decolorized which can be quantitatively measured from the changes in absorbance. The extract exhibited a notable dose dependent inhibition with Ic50 value of 2.12mg /ml while the Ic50 value of standard quercentin was found to be 0.75 mg/ml. The higher Ic50 value of extract found in the experiment was because the sample used was a crude extract with the compound (s) react as antioxidant. Oxidative DNA damage has been implicated to be involved in various degenerative diseases including Alzheimer's disease, Parkinson's disease, and Hodgkin's disease (19). In view to make ascertain hydroxyl radical scavenging activity of crude extract, Fenten reaction generated hydroxyl radical damaging assay to deoxy ribose was studied. In the present study the Ic50 value of the crude plant extract was found to be 1.034mg/ml. The Ic50 value of Euphorbia hirta for hydroxyl radical scavenging activity was found to be 0.162mg/ml(20). In comparison to this earlier work our plant extract showed good hydroxyl radical scavenging activity. The reducing ability of a compound depends on the presence of reductants which exhibit antioxidant activity by breaking the free radical change through donation of a hydrogen atom (21). The reducing power of the extract increased dose dependently. However the extract reduced most Fe^{3+} ions to the extent less than ascorbic acid.

CONCLUSION

This study suggested that the crude plant extract of Entada *pursaetha, Toddalia aculeata and, Ziziphus mauritiana* possess antioxidant activity which might be helpful in preventing or slowing the progress of various oxidative stress related diseases. Further works may be performed for the isolation and characterization of antioxidant component(s) in the plants.

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Antioxidant potential of *Trichosanthes dioica* Roxb (fruits)

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Abstract

Objective: The study was undertaken to evaluate the antioxidant activity of fruits of *Trichosanthes dioica* (Cucurbitaceae) and compared with ascorbic acid (Standard).

Materials and Methods: Anti-oxidant activity of aqueous extract of *Trichosanthes dioica* (TSD) fruits was studied for its free radical scavenging property in different in vitro methods as 1, 1 diphenyl-2- picryl hydrazyl, nitric oxide, reducing power assay and hydrogen peroxide radical method. Different concentrations of aqueous extract of TSD were prepared and evaluated by standard methods.

Results: The IC_{50} values of aqueous extract of TSD were compared with ascorbic acid (Standard) and it was noted that, the extract showed significant concentration dependent free radical scavenging property in all the methods.

Conclusion: Results from the study showed that aqueous extract of TSD possess *in vitro* free radical scavenging activity. The findings could justify the inclusion of this plant in the management of antioxidant activity.

Keywords: *Trichosanthes dioica*, antioxidant, DPPH, Reducing power assay

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INTRODUCTION

Free radicals can be described as chemical species that have an unpaired electron and play very important role in human health and beneficial in combating against several diseases. These free radicals are highly unstable and when the amount of these free radicals exceed in the body, it can damage the cells and tissues and may involved in several diseases like cardiovascular disorders, lung damage, inflammation etc. Thus there is the need of antioxidant of natural origin because they can protect the human body from the diseases caused by free radicals (12).

Trichosanthes dioica (family- Cucurbitaceae) is a well known plant commonly called as Parwal in Hindi, Potol in Bengal, and Palwal in Punjab. The medicinal attributes *T. dioica* have been known since time immemorial. The plant is alternative, tonic, useful in obstinate fevers, boils etc. The roots are cathartics. The leaves are anthelmentic. The fruits are sweet, cardiotonic, appetizer and stomachic. The plant *T. dioica* possesses many medicinal properties including laxative, cooling, febrifuge, cathartic etc. (3–5).

However relevant experimental work has not yet been explored. Therefore, the aim of this study was to evaluate the antioxidant activity of aqueous extract of *Trichosanthes dioica* Roxb fruits.

MATERIAL AND METHODS

Plant material and extraction

Fresh unripe fruits of *Trichosanthes dioica* (2 kg) were purchased from the local market of Bhopal, India, in the month of August 2008. The plant was authenticated by Dr. A.S.Yadav, Professor, Government MVM College, Bhopal. The collected fruits were cut into small pieces and were shade dried. The dried pieces were pulverized into moderately coarse powder and stored in well closed container. The shade dried powder of TSD fruits (125g) were macerated with water for 72 hrs then concentrated and dried under reduced pressure to semisolid mass and

residue was obtained (16.96g yield w/w). The residue was stored in a desiccator.

Qualitative test analysis

Qualitative test analysis was performed to determine chemical constituents present in the dried aqueous extract of TSD. The extract was tested for the presence of various phytoconstituents viz. alkaloids, carbohydrates, glycosides, flavonoids, phenolic compounds and tannins (6–7).

DPPH scavenging activity

The procedure of Brand-Williams(8) has been adapted for evaluation of the free radical scavenging capacity of the aqueous extract. Different concentrations (05–45µg/ml) of aqueous extract of TSD were prepared in suitable solvent and 3ml of each solution was mixed with 1 ml of a 0.1mM DPPH solution. The decrease in absorbance was measured at 515 nm after 30 minutes of incubation period at room temperature using a UV Visible spectrophotometer 1700 (Shimadzu). The scavenging activity of sample extract was expressed as the inhibition of DPPH radical and calculated according to the following formula with as the control:

Scavenging Activity (%) = [(A control – A sample) / A control]*100, where $A_{control}$ (containing DPPH solution) and A_{sample} is the Absorbance with different dilutions of drug extract. Ascorbic acid was used as reference standard.

Reducing power assay

Reducing power of aqueous extract of TSD was estimated using the protocol reported by Oyaizu (9). Different concentrations of aqueous extract of TSD (5–25µg/ml) were prepared and 1ml of each solution was mixed with phosphate buffer (2.5ml, 0.2M, pH 6.8) and potassium ferricyanide (2.5ml, 1%). The mixture was incubated at 50°C for 20 min. To this mixture, 2.5 ml of 10% trichloroacetic acid (TCA) was added and then centrifuged at 3000 rpm for 10 min. The upper layer of the solution (2.5 ml) was mixed with distilled water (2.5ml) and $Fecl_3$(0.5 ml, 0.1%) was added and the absorbance was measured at 700 nm.

The percentage scavenging was calculated by using the formula

$(A_{control} - A_{sample})/ A_{control} \times 100$, where $A_{control}$ is the absorbance of solution without extract and A_{sample} is the Absorbance with different dilutions of drug extract. Ascorbic acid was used as reference standard.

Nitric oxide scavenging activity

Nitric oxide scavenging activity was evaluated by the method of Gupta (10).1ml of Sodium nitroprusside (10mM) in phosphate-buffered saline (PBS) was mixed with 3.0 ml of different concentrations (20 – 120µg/ml) of the aqueous extract of TSD dissolved in water and incubated at 25°C for 180 min. The samples from the above were reacted with equal volume of Greiss reagent (1% sulphanilamide, 0.1% napthylethylenediamine hydrochloride and 3% of phosphoric acid). The absorbance of the chromophore formed during the diazotization of nitrite with sulphanilamide and subsequent coupling with napthylethylenediamine was read at 546 nm. The percentage scavenging was calculated by using the formula

$(A_{control} - A_{sample})/ A_{control} \times 100$, where $A_{control}$ is the absorbance of solution without extract and A_{sample} is the absorbance with different dilutions of drug extract. Ascorbic acid was used as reference standard.

Hydrogen peroxide radical scavenging activity

The ability of the aqueous extract of TSD to scavenge hydrogen peroxide was determined according to the method of Ruch (11). A solution of hydrogen peroxide (40 mM) was prepared in phosphate buffer (pH 7.4). The concentration of hydrogen peroxide was determined by absorption at 230 nm using a UV Visible spectrophotometer 1700. Then hydrogen peroxide solution (0.6 ml, 40 mM) was mixed to different concentrations (30 – 180µg/ml) of the extract dissolved in water. The absorbance of hydrogen peroxide at 230 nm was determined after ten minutes against a blank solution containing phosphate buffer without hydrogen peroxide.

The percentage scavenging was calculated by using the formula

$(A_{control} - A_{sample})/ A_{control} \times 100$, where $A_{control}$ is the absorbance of solution without extract and A_{sample} is the Absorbance with different dilutions of drug extract. Ascorbic acid was used as reference standard.

Statistical Analysis

Data are presented as the mean ± SEM of each triplicate test. The analysis was performed by using Dunnett vs. Control test and by ANOVA. $P<0.05$ were considered to be statistically significant.

RESULTS AND DISCUSSION

Phytochemicals

The phytochemical screening of aqueous extract of TDS revealed the presence of alkaloids, carbohydrates,

glycosides, flavonoids, phenolic compounds and tannins.

DPPH radical scavenging activity

The results of DPPH radical scavenging activity of the aqueous extract of TSD with IC_{50} (% Inhibition) are shown in fig 1.1. The IC_{50} value of aqueous extract of TSD and standard (ascorbic acid) were found to be 33µg/ml, and 11µg/ml, respectively. The results showed a significant ($p < 0.01$) decrease in the concentration of DPPH radical due to the scavenging ability of aqueous extract as compared to standard (ascorbic acid).

Reducing power assay

The Reducing power of aqueous extract of TSD and ascorbic acid were shown in the fig 1.2. The IC_{50} value of aqueous extract of TSD and ascorbic acid (standard) were found to be 25µg/ml and 12µg/ml, respectively.

Nitric oxide scavenging activity

The nitric oxide scavenging activity of aqueous extract of TSD and ascorbic acid were shown in the fig 1.3 which illustrates the % inhibition of nitric oxide generation by aqueous extract of TSD. The IC_{50} value of aqueous extract and ascorbic acid (standard) were found to be 112µg/ml and 85µg/ml, respectively. The results indicate significant ($p<0.01$) decrease in the concentration of nitric oxide radical due to the scavenging ability of aqueous extract as compared to standard.

Hydrogen peroxide radical scavenging activity

Hydrogen peroxide scavenging activity of aqueous extract of TSD and ascorbic acid were shown in the fig 1.4. It showed significant scavenging activity of hydroxyl radical generated from H_2O_2 system. The IC_{50}value for aqueous extract was 171µg/ml, whereas 167µg/ml was the value of ascorbic acid. The results indicated that aqueous extract of TSD possessed significant antioxidant activity ($p<0.01$).

The main characteristic of an antioxidant is its ability to trap free radicals. Highly reactive free radicals and oxygen species are present in biological systems from a wide variety of sources. These free radicals may oxidize nucleic acids, proteins, lipids or DNA and can initiate degenerative disease.

Aqueous extract of TSD showed the presence of flavonoids and phenolic compounds. Since the antioxidant activities of these constituents from plant origin have already been established (12), 22 it can speculate that these constituents may be responsible for the observed antioxidant effects.

DPPH scavenging activity has been used by various researchers as a quick and reliable parameter to assess the *in vitro* antioxidant activity of crude plant extracts (13–14). A DPPH radical scavenging ability of the extract was significantly lower than those of ascorbic acid. It was evident that the extract did show the proton donating ability and could serve as free radical inhibitor or scavenger, acting possibly as primary antioxidants.

In the present study, the reductive capacity of the aqueous extract of TSD was compared with ascorbic

Fig 1.1 Effect of aqueous extract of Trichosanthes dioica on DPPH scavenging activity

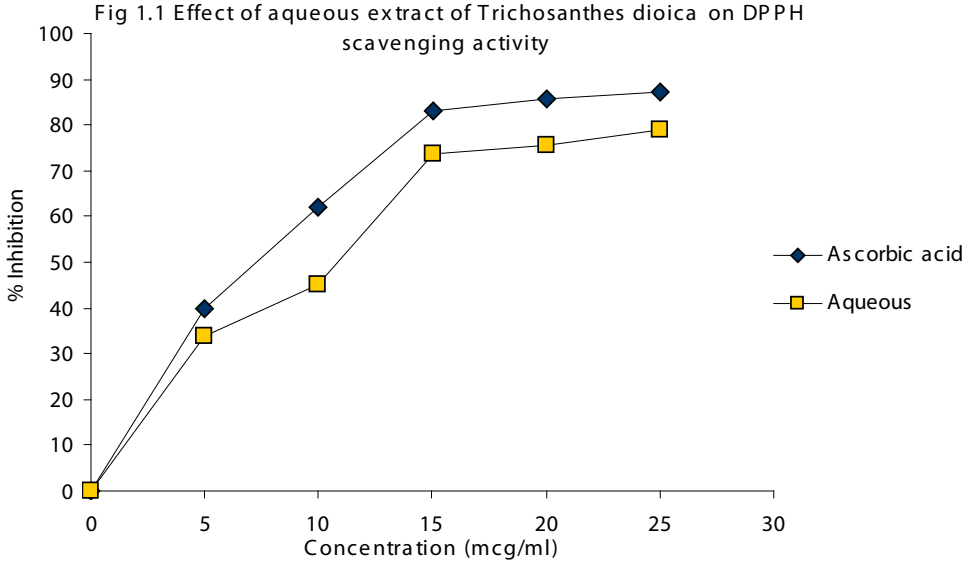


Fig 1.2 Effect of aqueous extract of Trichosanthes dioica on reducing power assay

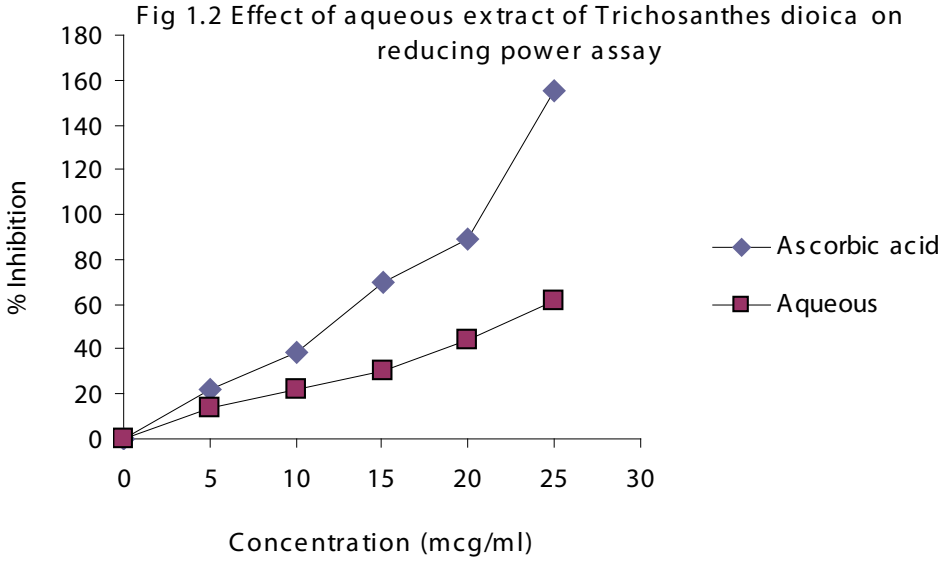
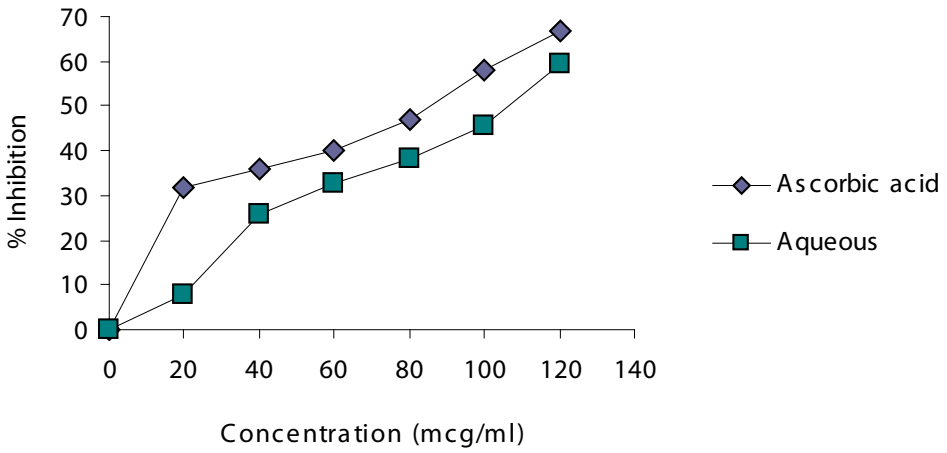


Fig 1.3 Effect of aqueous extract of Trichosanthes dioica on NO scavenging activity



acid (Standard). Significant antioxidant potential of any compound depends on its reducing capacity (15). The reducing capacity of the aqueous extract of TSD was found to be concentration dependent and showed significant potential.

Nitric oxide is a short-lived (half-life 3–30 s) colorless gas that is moderately soluble in water highly soluble in organic solvents (16). It is an important chemical mediator or essential bioregulatory molecule which is generated by neurons, endothelial cells etc and required for several physiological processes like immune response, neural signal transmission and control of blood pressure. Several diseases occur due to excess concentration of nitric oxide (17–18). Oxygen reacts with the excess nitric oxide to generate nitrites and anions which act as free radicals (19–20). In this study, the fruits of plant TSD compete with oxygen to react with nitric oxide and thus inhibit generation of anions.

Hydroxyl radicals are the major active species causing lipid oxidation and enormous biological and cellular damages (21). Its ability to produce active oxygen species is due to its ability to generate highly reactive hydroxyl

radical through the Fenton reaction (22). As the aqueous extract scavenged hydrogen peroxide radical similar to the standard (ascorbic acid) so it reflects that the plant extract could possibly inhibit the formation of hydroxyl radical.

CONCLUSION

In this study, the present results indicates that the aqueous extract of TSD possess antioxidant properties due to the presence of phenolic acids, polyphenols and flavonoids scavenge free radicals such as peroxide, hydroperoxide or lipid peroxyl and thus inhibit the oxidative mechanisms that lead to degenerative diseases and could serve as free radical inhibitors or scavengers, acting possibly as primary antioxidants. This study has to some extent validated the medicinal value of the fruits of *Trichosanthes dioica*.

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Antioxidative activity and Phytochemical investigation on a High Altitude Medicinal Plant *Dracocephalum heterophyllum* Benth.

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Abstract

The antioxidant activity and phytochemical investigation on *Dracocephalum heterophyllum* Benth. used in the traditional medicine of Ladakh was carried out. The studies on ethnomedicine keenly represent the search for new economic plants for developing modern medicines. The methanol extract was subjected to antioxidative assay using 2,2'- diphenylpicryl-1-hydrazyl (DPPH) and various biochemical tests of aqueous and chloroform extracts were performed. The results indicated that the plant was rich in tannins, flavonoids, saponins, steroids and alkaloids. The free radical scavenging and antioxidant activities may be attributed to the presence of phenolic and flavonoid compounds present in the extract. The results obtained in the present study indicate that *Dracocephalum heterophyllum* Benth. is a potential source of natural antioxidants.

Keywords: *Dracocephalum heterophyllum*, traditional medicine, DPPH, antioxidative activity, phytochemical investigation.

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INTRODUCTION

The early attempts of traditional medicine usage reveal the use of native plants or their extracts and many of these herbal remedies proved successful (1). Green plants posses the broadest spectrum of synthetic activity and have been the source of many useful compounds (2). The medicinal value of these plants lies in some chemical substances that produce a definite physiological action on the human body. The northern part of India harbors a great diversity of medicinal plants because of the majestic Himalayan range. Ladakh, the cold desert located in the northern most part of trans- Himalaya in Jammu and Kashmir State is well known for its rich ethno botanical wealth and harbours a great diversity of medicinal plants Fig.1. Health care of the tribal population is mainly dependent on traditional system of medicine which is popularly known as Amchi system of medicine (3). The most important of these bioactive constituents of plants are alkaloids, tannins, flavonoids and phenolic compounds (4). The scientific studies available on a good number of medicinal plants indicates that promising phytochemicals can be developed for many human health problems (5,6) including diabetes, cancer and infectious diseases.Because of the possible toxicities of the synthetic antioxidants like butylated hydroxyl anisole (BHA), butylated hydroxyl toluene (BHT), increased attention is being paid on natural antioxidants (7). The use of essential oils or plant extracts in foods may act as natural antioxidant preservatives influencing the health of the consumers and prolonging the shelf life of the food products (8–10). Consumption of foods containing natural essential oils or aromatic plant extracts is expected to prevent risks of many free radical-mediated diseases (11,12). Due to depletion of immune system natural antioxidants in different maladies,consuming antioxidants as free radical scavengers may be necessary (13–16).

Dracocephalum heterophyllum

Benth. belonging to the family Lamiaceae is a small perennial aromatic herb and has been valued in Ayurveda, Tibetan and aromatic systems of medicine for its therapeutic properties Fig 1. The habitat pertains to open and moist slopes. The plant used for the study is extensively used in Amchi system of medicine in the Ladakh region of the Himalaya for long period. The decoction of dried flowers and leaves are used for cold, cough and head ache (17). The plant yield essential oil from the flowers. Since the middle ages, essential oils have been widely used for bactericidal, virucidal, fungicidal,

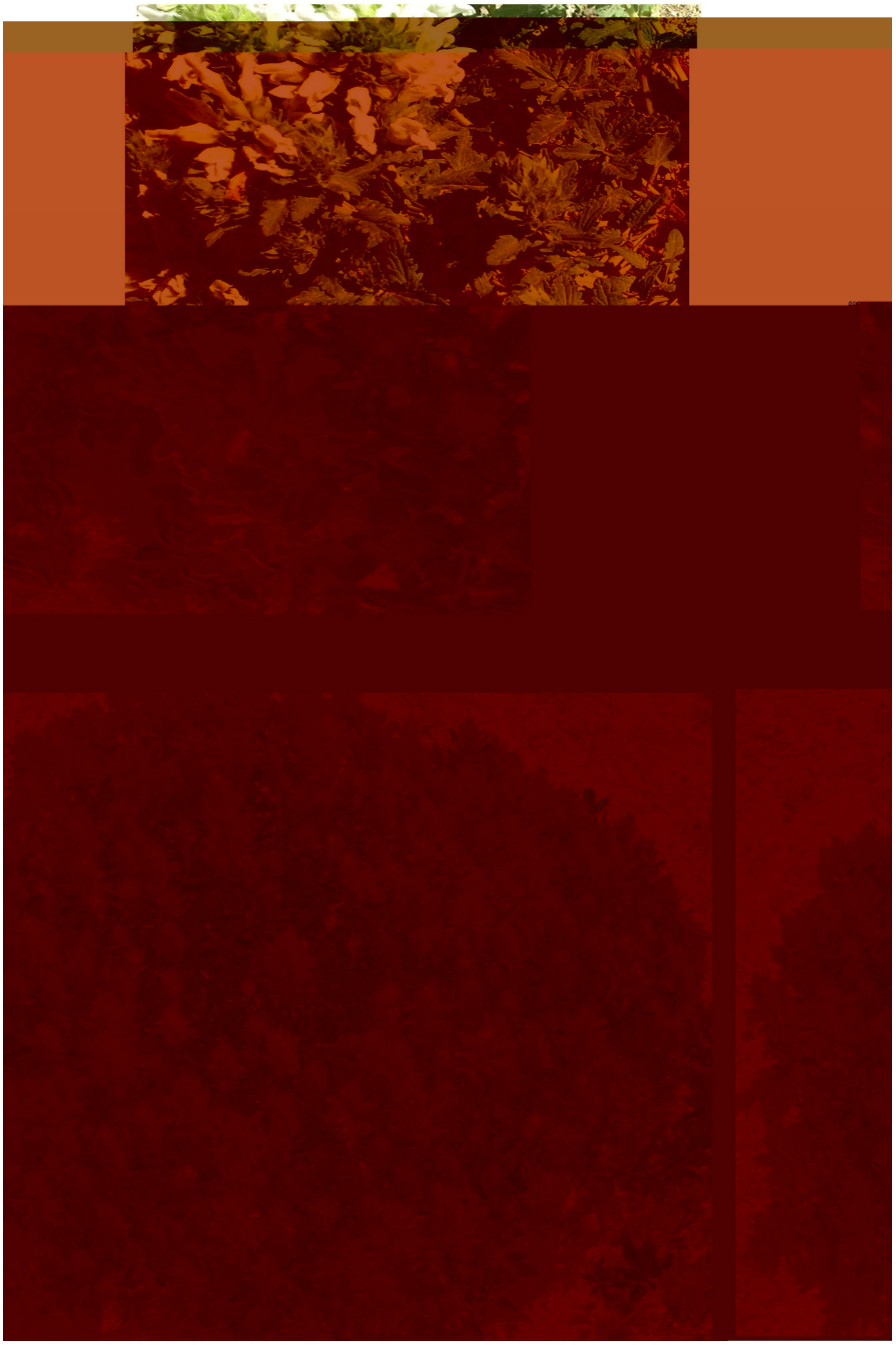

Figure 1: *Flowering plant of Dracocephalum heterophyllum Benth*

antiparasitical, insecticidal, medicinal and cosmetic applications, especially nowadays in pharmaceutical, sanitary, cosmetic, agricultural and food industries. Because of the mode of extraction, mostly by distillation from aromatic plants, they contain a variety of volatile molecules such as terpenes and terpenoids, phenol-derived aromatic components and aliphatic components. *In vitro* physicochemical assays characterise most of them as antioxidants (18). Considering the importance of the phytochemicals responsible for the medical properties *Dracocephalum heterophyllum* Benth. The investigation was carried out for antioxidative activity and the presence of phytochemicals with biological activity.

MATERIAL AND METHODS

Collection of Plant material:

The flowers, leaves and roots of *Dracocephalum heterophyllum* Benth. were collected and identified by the

authors from uncultivated lands and the herbal garden, DIHAR of Ladakh during May, 2008.

Preparation of extracts: The plant samples were air-dried and ground into uniform powder using pestle and mortar. Three types of extracts namely the aqueous, chloroform and methanol were prepared. The aqueous extracts were prepared by soaking 5 g of dried powdered samples in 20 ml of distilled water for 12 hours. The chloroform extracts were prepared by dissolving 5 g of dried powder in 20 ml of chloroform for 6 hours. The solution was extracted with dilute H_2SO_4 or dilute HCl and the acid layer was used for tests. The methanol extracts were prepared by dissolving 5 g of dried powder in 85% methanol .The solvents were concentrated using rotavapor at temperature not higher than 50 °C and kept in dark at 4°C until used.

Antioxidative activity

DPPH assay

The hydrogen atom- or electron donation ability of the corresponding extracts and some pure compounds were measured from the bleaching of the purple coloured methanol solution of 2,2'- diphenylpicryl-1-hydrazyl (DPPH). This spectrophotometric assay used stable radical DPPH as a reagent (19,20). 5 ml of various concentrations such as 20,40,60,80 and 100 ppm of the extracts in methanol were added to 5 ml of 0.0002% DPPH solution. After 30 minutes incubation at room temperature in dark, the absorbance was read against 517 nm. The blank used was methanol and the control was 5 ml methanol and 5 ml DPPH solution (0.002%). Inhibition of free radical DPPH in percent (I%) was calculated as

$$I\% = [(A_{blank} - A_{sample})/ A_{blank}] \times 100$$

Where $A_{blank}$is the absorbance of the control reaction (containing all the reagents except the test compound) and A_{sample} is the absorbance of the test compound. Extract concentration providing 50% inhibition was calculated using the graph by plotting inhibition percentage against extract concentration. Ascorbic acid (AA) and the synthetic antioxidant reagent butylated hydroxyl toluene (BHT) were used as positive controls and all the tests were replicated thrice.

Statistics and IC_{50}

Decolorisation was plotted against the sample extract concentration and a linear regression curve was established in order to calculate IC_{50}(µg/ml), which is the amount of sample required to decrease the absorbance of the DPPH free radical by 50%. All the analyses were carried out in triplicate and the results are expressed as mean ± SD. Statistical analysis were performed using SPSS 11.5

Phytochemical screening

Chemical tests were carried out on the aqueous extract and chloroform extracts using standard procedures to identify the constituents as described by (21–23). All the biochemical tests were performed to test the qualitative presence of tannins, saponins, terpenoids, flavonoids and alkaloids were carried out thrice and were found to be replicable.

Test for Tannins

0.5 g of the dried powdered samples was boiled in 20 ml of water in a test tube and then filtered. A few drops of 0.1 percent ferric chloride was added and observed for browrish green or a blue-black colouration.

Test for Flavonoids: Five ml of dilute ammonia solution were added to a portion of the aqueous filtrate of each plant extract followed by addition of concentrated H_2S0_4. A yellow colouration observed in each extract indicated the presence of flavonoids. The yellow colouration disappeared on standing.

Test for Saponins: About 2 g of the powdered sample was boiled in 20 ml of distilled water in a water bath and filtered. 10ml of the filtrate was mixed with 5 ml of distilled water and shaken vigorously for a stable persistent froth. The frothing was mixed with 3 drops of olive oil and shaken vigorously, then observed for the formation of emulsion.

Test for Cardiac glycosides (Keller-Killani test): Five ml of each extracts was treated with 2 ml of glacial acetic acid containing one drop of ferric chloride solution. This was underlayed with 1 ml of concentrated sulphuric acid. A brown ring of the interface indicates a deoxysugar characteristic of cardenolides. A violet ring may appear below the brown ring, while in the acetic acid layer, a greenish ring may form just gradually throughout thin layer.

Test for Alkaloids (Wagner's test): One ml of the chloroform extract was mixed with 1 ml of Wagners reagent and the positive reaction results in a brown coloured precipitate.

Test for Steroids (Salkowski test): Five ml of chloroform extract was mixed concentrated H_2S0_4 (3 ml) and shaken. If the reaction is positive it gives red colouration on standing.

RESULTS AND DISCUSSIONS

Antioxidant activity

The antioxidant activity of methanol extracts of *Dracocephalum heterophyllum* Benth. was examined by comparing it with the activity of known antioxidants ascorbic acid and BHT with inhibition of DPPH radical. DPPH stable free radical method is an easy, rapid and sensitive way to survey the antioxidant activity of a specific compound or plant extracts (24). Phytochemicals recognized as possessing potent antioxidant activity are also strong scavengers of DPPH (25). Substances capable of donating electrons/hydrogen atoms are able to convert DPPH into their non-radical form 1,1-diphenyl-2-picrylhydrazine, a reaction which can be followed spectrophotometrically. Free radical scavenging activity

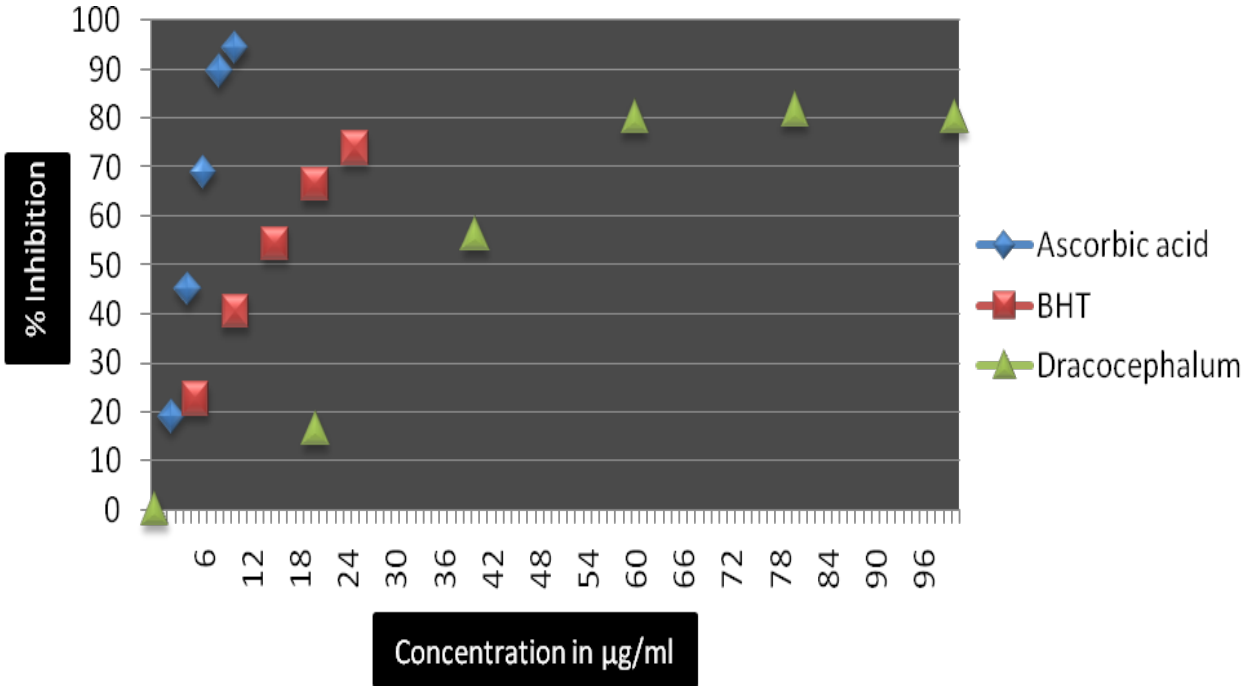

Figure 2 *DPPH free radical scavenging activity of ascorbic acid, BHT and methanolic plant extracts*

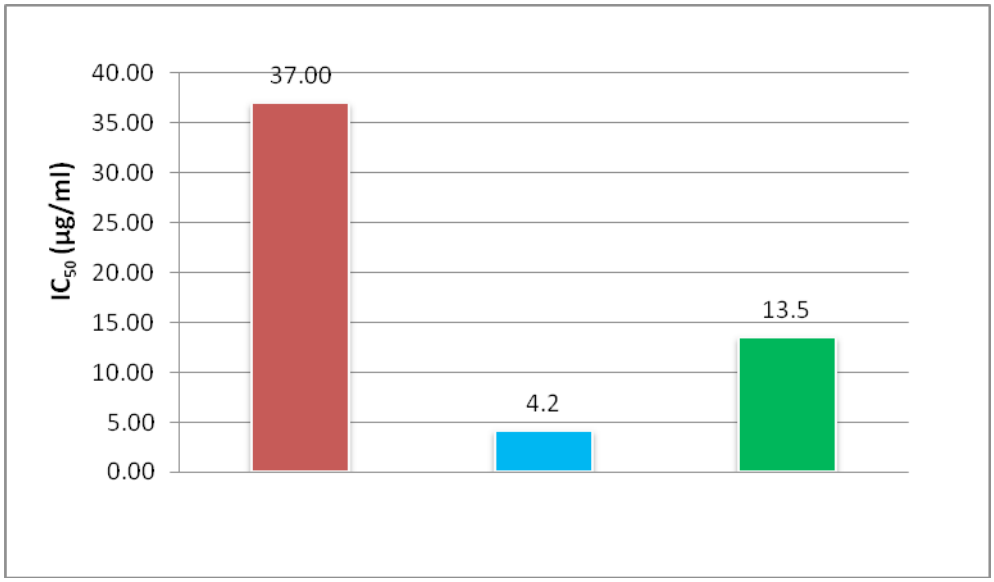

Figure 3 *IC $_{50}$(µg/ml) values of plant extract, AA and BHT for free radical scavenging activity by DPPH radical. Lower value indicates higher antioxidant activity*

of the extracts is concentration dependent and lower IC_{50} value reflects better protective action.

The free radical scavenging activity of the extracts was investigated and results are given in Table 1 and Fig 2 and 3. BHT and AA were used as positive controls. The antioxidant activity of the extracts increased with an increasing amount of extract. The methanol extract was able to reduce the stable free radical DPPH to the yellow coloured diphennylpicrylhydrazine with an IC_{50} of 37 µg/ml, while AA was 4.2 µg/ml and the synthetic antioxidant BHT 13.5 µg/ml. The antioxidant activity of methanol extract may be attributed to high content of polar flavonoids extracted by methanol/water solution. The free radical scavenging activity of the methanolic extract was confirmed in the present investigation. It was observed that 40 µg/ml of the *Dracocephalum heterophyllum* extract was able to produce 54.18 % inhibition.

Phytochemical Screening

The preliminary phytochemical investigation revealed the presence of medicinally active phyto constituents. The phyto constituents of the medicinal plant *Dracocephalum heterophyllum* investigated using the aqueous and chloroform extracts are summarized in Table 2. The results indicated that the plant investigated were rich in tannins, flavonoids, saponins, steroids and alkaloids and these phytochemicals are known to exhibit medicinal as well as physiological activity (23). Polyphenols, phenolic compounds, flavonoids and terpenes are well known for their antioxidant activity. Phytochemicals such as saponins, terpenoids, flavonoids, tannins, steroids and alkaloids have anti-inflammatory effects (26–30). Glycosides, flavonoids, tannins and alkaloids have hypoglycemic activities (31,32). Tannins (commonly referred to as tannic acid) are water-soluble polyphenols that are present in many plants and inhibit the growth of many fungi, yeasts, bacteria, and viruses (33).Saponins are a special class of glycosides which have soapy characteristics and possess hypocholesterolemic and antidiabetic properties (34,35). The saponins and steroids are responsible for central nervous system activities. Steroids and triterpenoids showed analgesic properties (36). Steroids and phlobatannins were found to be of importance and interest in pharmacy due to their relationship with such compounds as sex hormones (37).

Table 1: Phytochemical constituents of Dracocephalum heterophyllum Benth.

		Extract(s)		
S.No.	Phytochemicals	Flower	Stem	Root
1	Tannins	–	–	+
2	Saponins	–	–	+
3	Terpenoids	–	–	–
4	Flavonoids	+	–	–
5	Alkaloids	+	+	–
6	Steroids	+	+	+

Table 2: Effect of methanol extracts from Dracocephalum heterophyllum Benth. and positive controls (BHT and AA) on the in vitro free radical DPPH scavenging

Sample	DPPH, IC_{50}(µg/ml)
Methanol extract	37
BHT	13.5
AA	4.2

CONCLUSIONS

Antioxidant properties of essential oils and various extracts have recently gained interest both research and the food industry, because of their possible role as natural additives to replace synthetic antioxidants. The preliminary phytochemical investigation indicates the presence of flavonoids in the plant. Polyphenols like flavonoids and tannins are the well known natural antioxidants. So, the antioxidant potential of the plant may be attributed to the presence of flavonoids. The separation and identification of flavonoids present in the roots can help researchers find new molecules which can be used as natural antioxidants. Identification of all the chemical constituents in the flower and leaf extracts responsible for antioxidant activity requires further investigation, although it is obvious that the constituents like tannins and flavanoids which are present in the extract may be responsible for such activity. Further studies will also be needed to evaluate the *in vivo* potential of the extract and its fractions in various animal models and the isolation and identification of the antioxidant principles.

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Quality Control Studies of *Ochrocarpus longifolius* Flower Buds

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Abstract

The flower buds of *Ochrocarpus longifolius* Benth. & Hook f. is the crude drug which has been used for the pharmacognostical standardization as per WHO guidelines-1998 and IHP-2002. The plant is a tree. Authentication and identification has been done taxonomically. The dried flower buds are light brown and round in shape. Oblate to suboblate pollen grains, lignified, spiral and elongated xylem vessels has been found. Stone cells, thick bordered tracheids and anther fractions have been observed in powder drug microscopy. Foreign matter (0.29%), loss on drying (13.16%), total ash (6.30%), acid insoluble ash (0.43%), water soluble ash (1.97%), alcohol soluble extractive (16.03%), water soluble extractive(12.57%), volatile oil (0.10%), foaming index 200 and swelling index 0.36 ml of the crude drug have been obtained. A fingerprint of fluorescence has been observed in fluorescence analysis. Preliminary phytochemical screening of methanolic extract of the crude drug has revealed the presence of glycosides, reducing sugars, phenolics, tannins, coumarins, sterols, flavanoids, saponins and volatile oil. Total phenolics (138.30 ± 4.58), total tannins (133.0 ± 1.52), total flavonoids (41 ± 1.28) and total flavonol (0.56 ± 0.04) content in mg/gm of plant extract have been estimated. Different amounts of chlorinated and phosphated pesticide were obtained in the crude drug. By establishing the quality parameters for *Ochrocarpus longifolius*, it can be a better used plant for further research and for trade and commerce.

Keywords: *Ochrocarpus longifolius,* Standardization, Quantitative standards, Fluorescence analysis, Pesticide residue

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INTRODUCTION

Ochrocarpus longifolius Benth. & Hook f. (*Syn Mammea longifolia*.) is a flowering plant belonging to the family Guttiferrea. In Sanskrit it is known as Nagakesara, in Hindi as 'Nagkesar' and in English as 'Sweet basil'. It is distributed mostly in the Western ghats of the Konkan, North Kanara, Malabar and Coimbatore. The tree is large with a cylindrical trunk, 12–18 m high. Fresh flowers of the tree are used for worship in temples and for personal adornment. Dried flowers keep their fragrance for a long time, a perfume, resembling that of violets, can be extracted from them. Flower buds contain a coloring matter which dyes silk red (1). This plant is valued as an avenue or compound tree and cultivated for its handsome foliage and sweet scented flowers. The flowers appear in the hot weather and fruits ripen during the rainy season. The flower buds of *O. longifolius* was reported to contain 0.50% to 1.5% volatile oil and 5–6% oleoresins. Thirty five chemical constituents of the oil were identified by gas chromatography (GC) and GC-mass spectrometry. Sesquiterpenes were the predominant constituents of the oil, and the major compounds were b-caryophyllene (28.25%), d-cadinene (14.22%), a-copaene (5.24%), linalool (3.46%), a-humulene (4.63%), and a-muurolene (3.35%) (1). Two phytoconstituents vitexin and meso-inositol have been isolated from the flowers of *O.longifolius* which was shown to have positive effect on treatment of leprosy(2). Surangin B a coumarin isolated from *Ochrocarpus longifolius* was shown to have antifungal and anthelmintic activity (3). Vitexin exhibited potent hypotensive, antiinflammatory, and antispasmodic (nonspecific) properties in laboratory animals (4).

MATERIAL AND METHODS

Crude drugs – *Ochrocarpus longifolius* flower buds were collected from Bangalore, Karnataka, India. The taxonomical authentication and identification of the

crude drug was done by Prof. S. D. Dubey, Department of Dravyaguna, Faculty of Ayurveda, Institute of Medical Science, Banaras Hindu University, Varanasi. A herbarium (H.No. – 044) of the flower buds was prepared and deposited in the Pharmacognosy division of Department of Pharmaceutics, IT, BHU, Varanasi.

Chemicals and instruments – Digital microscope, Compound microscope, basic instruments and glass apparatus were used for the study. Chemicals were procured from Merck Limited, Worli, Mumbai, India.

Macroscopy and microscopy – Macroscopy of the flower buds (crude drug) was done as per WHO guidelines - 1998(5). The powder drug microscopy was done as per the method of Brain and Turner (1975b) (6) and Khandelwal (2007) (7).

Physico-chemical studies – Determination of quantitative standards - foreign matter, loss on drying, total ash, acid insoluble ash, water soluble ash, alcohol soluble extractive, water soluble extractive, foaming index, swelling index and volatile oil in the crude drug was done as per Indian Herbal Pharmacopoeia (IHP) (2002) (8). Fluorescence analysis was carried out according the method of Chase and Pratt (1949) (9) and Kokoski *et al*. (1958) (10).

Preliminary phytochemical screening – Preliminary phytochemical screening of methanolic extract of the crude drug was done as per Harborne (1998) (11) and Khandelwal (2007) (7).

Quantitative estimation of the phytoconstituents – Estimation of total phenolic and tannin content was done as per the method of Hagerman A *et al*, 2000. Estimation of total flavonoid and flavonol content was carried out as per the method of Kumaran, A., *et al,* 2006.

Determination of pesticide residues - Determination of chlorinated and phosphated pesticide residues in the crude drug was done according to WHO guidelines – 1998(5).

RESULTS AND DISCUSSION

Macroscopic characters – The flower buds (Figure 1) when dried were light brown, small and round in shape. They were numerously arranged in short fascicles on tubercles. Buds were globose in shape, orange-red in colour, pedicles 1.1–2 cm long and thickened upwards; bracts numerous. Calyx was seen to be bursting out into two valves and was reflexed during flowering. Petals were 4 in number, ovate to oblong, acute. Stamens many and were found sterile in the female flowers. Styles were short, stout; stigma broad and peltate.

Powdered drug microscopy – The powdered flower buds of dried *O. longifolius* appeared brownish in color with a slight characteristics taste and odor. Various powder drug characteristics observed were pollen grains, xylem vessels, stone cell, tracheids and anther fraction. Numerous pollen grains were observed. The pollens were oblate to somewhat suboblate in shape having an average size of 40 × 54 µm. Lignified, spiral shaped and elongated xylem vessels were found. Numerous more or less round stone cells were viewed which stained pink with phloroglucinol

Table 1: Quantitative standard values of *O. longifolius* flower buds

Parameters	Obtained values in percentage
Foreign matter	0.29
Loss on drying	13.16
Total ash	6.30
Acid insoluble ash	0.43
Water soluble ash	1.97
Alcohol soluble extractive	16.03
Water soluble extractive	12.57
Volatile oil	0.10

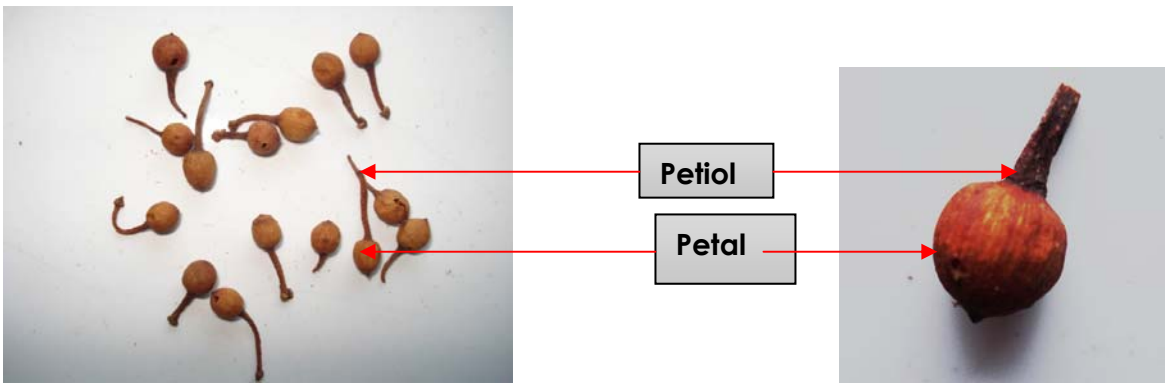

Figure 1: Flower buds *Ochrocarpus longifolius*

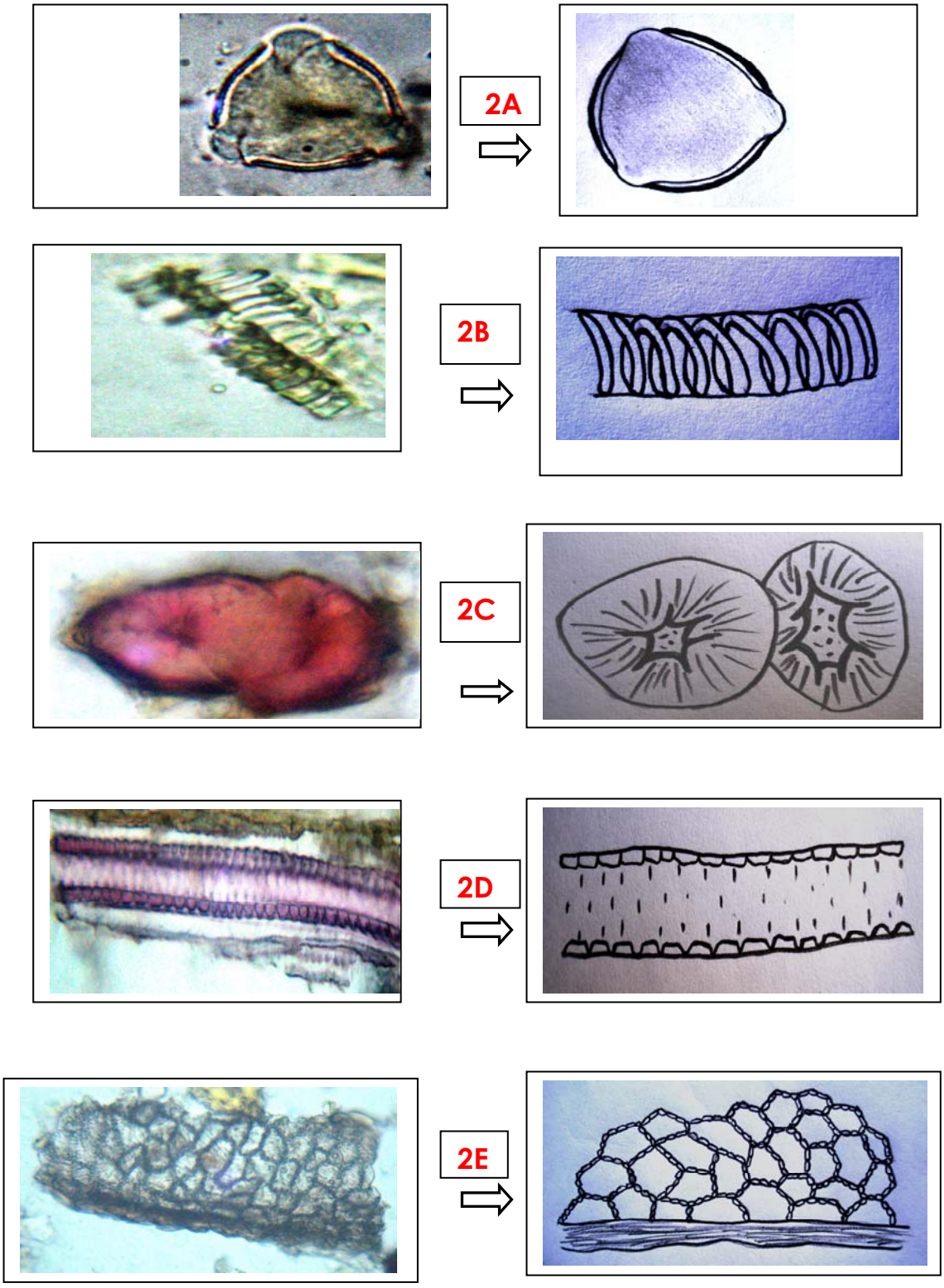

Figure 2: Powdered drug characters of *Ochrocarpus longifolius*
A: Pollen Grain, B: Xylem vessel, C: Stone Cell, D: Tracheid, E: Anther Fraction

Table 2: Fluorescence powder drug analysis of *O. longifolius* flower buds

Sl.no	Powder + Reagent	Fluorescence in daylight	Fluorescence under UV(365nm)
1.	Powder as such	Saddle Brown (139,69,19) (8B4513)	NF
2.	Powder + 1N NaOH in methanol	Yellow (255,255,0) (FFFF00)	Yellow (255,255,0) (FFFF00)
3.	Powder + 1N NaoH in water	Dark red (139,0,0) (8B0000)	NF
4.	Powder + 1N HCl in methanol	Saddle brown (139,69,19) (8B4513)	Tan (210,180,140) (D2B48C)
5.	Powder + 1N HCl in water	Tan (210,180,140) (D2B48C)	Pale green (152,251,152) (98FB98)
6.	Powder + 1N HNO_3 in methanol	Brown (165,42,42) (A52A2A)	NF
7.	Powder + 1N HNO_3 in water	Light yellow (255,255,224) (FFFFE0)	NF
8.	Powder + Iodine (5%)	Golden rod (218,165,32) (DAA520)	Pale green (152,251,152) (98FB98)
9.	Powder + $FeCl_3$ (5%)	Dark olive green (85,107,47)	NF
10.	Powder + KOH (50%)	Brown (165,42,42) (A52A2A)	NF
11.	Powder + Ammonia (25%)	Saddle brown (139,69,19) (8B4513)	Green yellow (173,255,47) (ADFF2F)
12.	Powder + Picric acid (saturated)	Yellow (255,255,0) (FFFF00)	NF
13.	Powder + Acetic acid	Golden rod (218,165,32) (DAA520)	Antiquewhite (250,235,215) (FAEBD7)

and HCl (1:1). Thick bordered tracheids in large numbers were seen which also stained pink with phloroglucinol and HCl (1:1). Few fractions of anthers were also seen. The characteristics are shown in Figure 2A–2E.

Physico-chemical studies – The obtained values of foreign matter, loss on drying, total ash, acid insoluble ash, water soluble ash, alcohol soluble extractive, water soluble extractive and volatile oil are in *Table 1*. The foaming index was 200 and swelling index was 0.36 ml. The percentage of foreign matter suggests that it is almost free from any other plant part or any other matter other than the crude drug. Loss on drying shows that there is a good amount of moisture in the crude drug. The total ash value of 6.30% includes both "physiological ash", which is derived from the plant tissue itself, and "non-physiological" ash, which is the residue of the extraneous matter (e.g. sand and soil) adhering to the plant surface. Acid insoluble ash value of 0.43% measures the amount

Table 3: Preliminary phytochemical screening of methanolic extract of *O. longifolius* flower buds

Alkaloids	Glycosides	Reducing Sugars	Tannins & Phenolics	Coumarins	Sterols	Flavanoids	Saponins	Proteins	Mucilage	Volatile oil
–	+	++	++	+	+	++	++	–	–	+

(–): Absent, (+): Present in small quantity, (++): Present in good quantity

Table 4: Quantitative estimation of phytoconstituents of methanolic extract of *O. longifolius* flower buds

Sl. No.	Phytoconstituent class	Total content mg/gm of plant extract
1	Total phenolics	138.30 ± 4.58 (in gallic acid equivalent)
2	Total tannins	133.0 ± 1.52 (in tannic acid equivalent)
3	Total flavonoids	41 ± 1.28 (in rutin equivalent)
4	Total flavonol	0.56 ± 0.04 (in rutin equivalent)

of silica present, especially as sand and siliceous earth. Water soluble ash is used to detect the presence of material exhausted by water. The water soluble ash is subject to a much greater reduction than is the total ash and is therefore used as an important indication of the presence of exhausted material substituted for the genuine article (12). There was negligible amount of volatile oil present in the crude drug.

The result of fluorescence analysis is presented in *Table 2*. From the results obtained it was monitored that florescence dominate mostly when the powder drug was made to interact with the reagent mixtures of 1 N NaOH in methanol, 25% ammonia and 5% iodine. So from this it can be concluded that the phytoconstituents of the crude drug seems to be more soluble in the basic medium as compared to that of acid medium. The identification of the colors was done using the color index (13). This showed that certain phytoconstituents that had the capability to produce fluorescence seemed to get expelled out from the power drug and reacted with the reagent under a suitable medium of light to produce different colors. Fluorescence analysis provides limited information regarding the identification of the crude drugs.

Preliminary phytochemical screening – The result of preliminary phytochemical screening of methanolic extract of the crude drug is presented in *Table 3*. It is a qualitative test. This reveals the various phytoconstituent classes present in the crude drug.

Quantitative estimation of the phytoconstituents class – The result of total phenolics, tannin, flavonoid and flavonol content is presented in *Table 4*.

Determination of pesticide residues – >Chlorinated pesticide obtained in first and second elute from column were 0.081 and 0.034 mg/kg of the crude drug respectively. Phosphated pesticide obtained in first, second and third elute from column were 0.012, 0.095 and 0.004 mg/kg of the crude drug respectively. Care should be taken in cultivated variety to limit the pesticide residue. IHP-2002 does not prescribe any limits for the pesticide residues. Certain official books like Quality control methods for medicinal plant materials, WHO, Geneva has provided some information about the limits of pesticides in which the phosphated pesticide having the content in between 0.05-0.1 mg/kg of the plant material is considered to be of no contamination. So from this we can conclude that *O. longifolius* flower buds was shown to have no contamination with phosphate pesticides.

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Comparative Phytochemical and Antibacterial Studies on the bark of *Alstonia scholaris* R.Br. and *Alstonia macrophylla* Wall. ex G.Don

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ABSTRACT

Comparative phytochemical and antibacterial activities of bark of *Alstonia scholaris* and *Alstonia macrophylla* were investigated. The successive different solvent extracts showed the presence of alkaloids, phenolics, saponins and tannins were found in both the species. The antibacterial activities of bark of *A. scholaris* and *A. macrophylla* in successive different solvent were tested against gram +ve and gram –ve organisms. The chloroform extracts of *A. macrophylla* showed broader spectrum of antibacterial activity when compared with *A. scholaris*. However. *Alstonia scholaris* is widely used medicinal plant.

Keywords: Comparative, bark, *Alstonia* species, phytochemical, antibacterial

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INTRODUCTION

Green plants represent a reservoir of effective chemotherapeutics and can provide valuable source of natural antimicrobials (1, 2). Plant species which have one or more of its organs containing substances that can be used for therapeutic purpose are called medicinal plants (3).Plants have been used as medicinal agents from the earliest day of mans existence (4, 5) and has made it necessary to study them in details in order to discriminate the kinds employed for different purposes (6). In particular, the antimicrobial activities of plant extracts have formed the basis of many applications, including raw and processed food formation, pharmaceuticals, alternative medicine and natural therapies (7).

Now a day, infectious diseases continue to be the major concern for health institutions, pharmaceutical companies and Governments all over the world, especially with the current increasing trends of multidrug resistance among emerging –reemerging bacterial pathogens to the available modern drug or antibiotics (8, 9). It is therefore very necessary that the search for newer antibiotic serves to be a continued process. Plants are the cheapest and safer alternative source of antimicrobials (10, 11). Therefore, with increasing acceptance of traditional medicine as an alternative form of health care, the screening of plants for active compounds has become very important.

Alstonia scholaris R. Br. (Apocynaceae), popularly known as "Saptaparni" or "Devil's tree", and *A. macrophylla*, are used in the traditional systems of medicine. However, the former one is widely recognized medicinal plant.

The bark of *A. scholaris* is bitter, tonic, astringent, expectorant, alterative, anthelmintic, emmenagogue and galactogogue. It has proved valuable in fever, chronic diarrhea and in advanced stages of dysentery (12–15). Whereas the bark of *Alstonia macrophylla* is used for the same purpose as that of *Alstonia scholaris* (16). Decoction of the stem bark is effective in stomach ache, skin diseases and urinary infections (17). Moreover, phytochemistry on these two species studied earlier (18–21). The genus *Alstonia* has been the subject of antimicrobial activities (22, 23). This work, however, is designed to evaluate the comparative account of antibacterial activity of related species.

MATERIAL AND METHODS

COLLECTION OF PLANT MATERIALS

The fresh barks of *A. scholaris* R. Br. were collected in the month of October 2005 from forest area of Aurangabad

district (M.S.) and that of *Alsotia macrophylla* from the Botanical Garden of Dr. Babasaheb Ambedkar Marathwada University, Aurangabad. The plant species were identified with the help of Flora of Marathwada (24) and voucher specimens have been deposited at the Botany department of the university. Plant samples were washed, shade dried at room temperature for 15 days.

PREPARATION OF EXTRACTS AND PHYTOCHEMICAL SCREENING

The dried plant material was pulverized into fine powder using a grinder (mixer). About 50 gm of powdered material was extracted in soxhlet extraction apparatus successively with 250 ml of each of the following solvents. Petroleum ether, chloroform, acetone and methanol (25). The extracts obtained with each solvent were filtered through Whatman filter paper No. 1 and the respected solvents were evaporated (at 40°C) with the help of heating mantle. The sticky greenish-brown substances were obtained and stored in refrigerator and were suspended in dimethyl sulphoxide (DMSO) for prior to use (26).

Some of the extracts of each solvent were used for the qualitative phytochemical screening for the identification of the various classes of active chemical constituents, using standard prescribed methods (27–30). The positive tests were noted as weak (+), moderate (++), strong (+++) and absent (–).

TEST CULTURE

The test bacteria used for the screening antimicrobial activity were *Staphylococcus aureus, Bacillus subtilis, Micrococcus luteus* MTCC 106 *Escherichia coli, Salmonella typhi, Pseudomonas aeruginosa* MTCC 2488, *Klebsiella planticola* and *Bacillus megaterium*. The cultures were obtained from Microbial Type culture Collection (MTCC), IMTEC, Chandigarh, India. Cultures were maintained as nutrient agar slants in screw-capped bottles and stored at 4°C. All cultures were checked for viability and purity by regular plating. Test cultures were prepared by transferring a loop full of bacteria from stock culture nutrient broth and incubated at 37±1°C for 24 hours.

SCREENING FOR ANTIBACTERIAL PROPERTIES

The antibacterial activities of the successive bark extracts of petroleum ether, chloroform, acetone and methanol of both the plant species were tested by Agar well diffusion method (31). The culture plates were prepared by pouring 20 ml of sterile nutrient agar. 1ml inoculum suspension was spread uniformly over the agar medium using sterile glass rod to get uniform distribution of bacteria. A sterile cork borer (6 mm) was used to make wells in each plate for extracts. These plates were labeled and 100µl of each plant extracts (at concentration of 100mg/ml) was added aseptically into the well. Then the plates were incubated for 24 h at 37°C during which the activity was evidenced by the presence of zone of inhibition surrounding the well. Ampicillin (40 µg/ml) was used as standard antibiotics. Each test was repeated three times and the antibacterial activity was expressed as the mean of diameter of the inhibition zones (mm) produced by the plant extracts when compared to the controls.

RESULTS AND DISCUSSION

The results of the phytochemical screening of the bark of both species of *Alstonia* are shown in Table 1. It indicates the presence of alkaloids, phenolics, saponins and tannins. The presence of steroids and terpenoids are indicated only in *Alstonia scholaris* while cardiac glycosides and flavonoids are found alone in *A. macrophylla*. However there were complete absence of anthraquinones and leucoanthocyanins in both species.

The antibacterial activities of *A. scholaris* and *A. macrophylla in* petroleum ether, chloroform, acetone and methanol extracts against bacteria examined in the current study and their potency were qualitatively assessed by the presence or absence of inhibition zones and zone diameter (Table 2). The results showed that the extracts of *A. scholaris* mediated some degree of activity against bacteria. Except, *S. aureus* and *S. typhi* other strains like *M. luteus, B.subtilis, B. megaterium, K. planticola E. coli* and *P. aeruginosa* are inhibited by acetone extracts only, while other extracts such as petroleum ether, chloroform and methanol showed negative inhibition at test concentration.

The antibacterial activity of *A. macrophylla* extracts were the most prominent in activity against all bacteria tested at test concentration (Table 2). Except petroleum ether extracts, others such as chloroform, acetone and methanol showed broader spectrum of activity. However, chloroform extracts of *A. macrophylla* exhibited significant antibacterial activities against all the bacteria tested but, particularly, to *B. subtilis, S.aureus* and *E. coli* with a diameter same or greater than standard antibiotic ampicillin.

Based on these results, it is possible to conclude that of two species of *Alstonia*. *A. macrophylla* exhibited broader range of antibacterial activity to varying degrees, as it is a less known medicinal plant in the Indian literature. Particularly, the chloroform extracts of *A. macrophylla* showed significant antibacterial activities and could be used as antimicrobial agents in new drugs for therapy.

Table -1: Phytochemical constituents of bark extracts of *A.scholaris* and *A. macrophylla*

	Alstonia scholaris				*Alstonia macrophylla*			
Phytochemicals	**A**	**B**	**C**	**D**	**A**	**B**	**C**	**D**
Alkaloids	—	+++	—	+++	—	+++	—	+++
Anthraquinones	—	—	—	—	—	—	—	—
Cardiac glycosides	—	—	—	—	—	—	++	+++
Coumarins	—	—	—	—	—	—	—	—
Flavonoids	—	—	—	—	—	++	++	+++
Leucoanthocyanins	—	—	—	—	—	—	—	—
Simple phenolics	—	—	+	++	—	++	++	+++
Steroids	—	—	—	++	—	—	—	—
Saponins	—	—	+	++	—	++	++	+++
Tannins	—	—	+	++	—	+	++	+++
Terpenoids	—	++	++	++	—	—	—	—

Note:- A - Pet ether, B - Chloroform, C - Acetone, D - Methanol, (+) - weak, (++) - moderate, (+++) - strong and (–) - absent.

Table -2: Antibacterial activity of bark extracts of *A. scholaris* and *A. macrophylla*

		Inhibition zone in diameters (mm / sensitive strains)									
		A. scholaris				***A. macrophylla***					
Organisms	**Gram stain + / –**	**A**	**B**	**C**	**D**	**A**	**B**	**C**	**D**	**DMSO**	**Ampicillin**
Staphylococcus aureus	+	—	—	—	—	—	24	16	15	—	23
Bacillus subtilis	+	—	—	11	—	—	32	16	18	—	21
Bacillus megaterium	+	—	—	11	—	—	10	15	15	—	25
Micrococcus luteus	+	—	—	13	—	—	27	15	17	—	30
Escherichia coli	–	—	—	10	—	—	18	15	17	—	17
Salmonella typhi	–	—	—	—	—	—	11	13	10	—	19
Pseudomonas aeruginosa	–	—	—	10	—	—	—	14	14	—	16
Klebsiella planticola	–	—	—	11	—	—	22	18	15	—	21

Note:- A - Petroleum ether, B - Chloroform, C - Acetone, D - Methanol, — no inhibition, Figures are diameter of zone of inhibition.

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Pharmacognostic Screening of *Dendrophthoe falcata*

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Abstract

Preliminary pharmacognostical screening of crude drug as well as pet ether, chloroform, ethanol and water extracts were done on *Dendrophthoe falcata* (Loranthaceae). *Dendrophthoe Falcata* showed the presence of alkaloids, flavonoids, glycosides, reducing sugar, saponin, terpenoids, tannins and steroids. Morphological and anatomical study has been done with the aim to aid pharmacognostic and taxonomic species using light microscopy. WHO recommended physicochemical determination and authentic phytochemical procedures. Physicochemical, morphological and histological parameters evaluated in this article helps to standardize and authenticate the species *Dendrophthoe falcata* and possibly help to differentiate from its other species.

Keywords: *Dendrophthoe falcata*, Loranthaceae, Pharmacognosy, Standardization

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INTRODUCTION

Number of crude drugs used today having urolithiasis activity. *Dendrophthoe falcata* (Loranthaceae) commonly known as 'Vanda' in Marathi is dried as well as fresh stem parasitic on Magnifera indica. It is an evergreen shrub with bark smooth grey, leaves opposite unequal, thick 1.6–25.4 cm long, flowers single, orange-red or scarlet softly pubescent, berries soft ovoid-oblong, 1.3 cm diameter and indigenous to India, Sri lanka, Thailand, Indo-china and Australia. The aerial parts are used in wounds, menstrual troubles, asthma, psychic disorders, pulmonary tuberculosis, consumption and mania by the tribal of India. Leaf paste is used in skin diseases (1, 2). Its paste is applied on boils, setting dislocated bones and extracting pus. The plant has been scientifically proved to have antilithiatic, diuretic, cytotoxic and immunomodulatory activities (3, 4). Phytochemical investigation reveals the presence of β-sitosterol, β-amyrin, olionelic acid, and terpenoids in these extracts.

MATERIAL AND METHODS

Plant material- Whole plant of *Dendrophthoe falcata* was collected from Shirur (Pune) region in the month of May-June and identified by botanical survey of India, Pune; voucher specimen has been deposited in library. Plant material was shade dried and powdered and passed through sieve no. 40 and stored in well closed vessels.

Chemicals and instruments- Binocular microscope, Photomicroscope, glass slides, cover slips, watch glass and common glassware were the basic apparatus and instruments used for the study. Solvents viz petroleum ether, chloroform, ethanol (95%), HCl, chloral hydrate and sodium hydroxide were used.

Macroscopic and microscopic analysis- The macroscopy and microscopy of different parts like leaf, stem and root were studied. For the microscopical studies transverse sections were prepared and stained. Micropowder analysis was done according to reported method (5).

Physico-chemical analysis- Physicochemical analysis i.e. percentage of ash values, extractive values, determination of volatile oil, loss on drying (LOD), swelling index, bitterness index, determination of arsenic and heavy metals, foaming index, powder fineness and TLC were performed according to the official methods prescribed and the WHO guidelines on the quality control methods for medicinal plant materials. Fluorescence analysis was carried according to the method described by Latha et. al. (6).

"Table I: Preliminary phytochemical screening f the entire plant powder of D. falcata"

Test	Petroleum Ether	Chloroform	Ethanol	Water
Alkaloids	–	–	–	–
Carbohydrates	–	–	–	+
Phytosterols	+	–	–	–
Terpenes	–	–	–	–
Fixed oil and fats	+	–	–	–
Saponins	–	–	+	+
Phenolic compounds and tannins	–	–	+	+
Flavonoids	–	–	–	–
Gums and mucilage	–	–	–	+

+ present, – absent

Preliminary phytochemical screening- Preliminary phytochemical screening was carried out by using standard procedures (7, 8, 9).

Extraction- The powdered material was extracted in succession with petroleum ether (40–60°), chloroform, ethanol and water using soxhlet apparatus. The solvent was removed under reduced pressure. The extracts were concentrated under vacuum at 40–60° yield a residue (%w/w), which was stored in desicator at room temperature.

RESULTS AND DISCUSSION:

Macroscopic characters- of the plant: A stem parasite with smooth grey bark. Leaves usually opposite, thick, variable in shape from ovate to linear oblong, mid rib prominent, secondary nerves obscure, flowers orange red or scarlet in short spreading stout, axiliary racemes, calyx present, often as a rim flowers bisexual. Fruits ovoid or oblong, berries crowned by cup shaped calyx.

Microscopic characters

Transverse section of leaf- The leaf has prominent midrib and even lamina. Midrib is shallowly convex on the adaxial side and broadly semicircular on the abaxial side. It shows prominent cuticle as well as radial wall. Leaf is dorsiventral and embedded in nature. The ground tissue has dilated circular compact parenchyma cell most of them having filled with dense tannin mass. It contains collateral type of vascular bundle.

Transverse section of stem- The stem is circular in cross section view. The epidermis consists of single layer of papillate cells with cuticles. The cortex is wide homocellular and parenchymatous. The cells have tannin body. The cortical zone is about 12 layer and 400 mm wide. The stele consists of wide hollow cylindrical discrete vascular segments which are separated from each other by parenchymatous medullary rays. Vascular bundles are radial type. Pith is wide and parenchymatous. There are small clusters of sclerides scatter in the pith.

"Table II: Ash value of entire plant powder of D. falcata"

Parameters	Values % (w/w)
Total ash	5.5
Acid insoluble ash	0.5
Water soluble ash	0.6

"Table III: Extractive values of the entire plant powder of D. falcata"

Parameters	Values % (w/w)
Water soluble extractive	7.0
Ethanol soluble extractive	11.4
Pet. Ether soluble extractive	5.7

Preliminary phytochemical screening - Preliminary phytochemical screening revealed the presence of carbohydrates, saponins, Phytosterols, fixed oils and fats, Phenolic compounds, gums and mucilage (Table I).

Physico-chemical studies- Ash values of drug gives an idea about inorganic material present in drug. The percentage of total ash, acid insoluble ash and water soluble ash were carried out (Table II). Extractive values are primarily useful for the determination of exhausted or adulterated drug. Water soluble, alcohol soluble and ether soluble extractive values was carried out (Table III).

The other Physico-chemical parameters such as swelling index, foaming index and moisture content was carried out according to WHO guidelines (Table IV).

"Table IV: Swelling index, foaming index and moisture content of D. falcata"

Parameters	Values
Swelling index	1 ml
Foaming index	111.11
Moisture content (LOD)	8.7 %

The results of fluorescence analysis of entire plant powdered drug and different plant extracts under UV and visible light are tabulated in Table V and Table VI respectively. Behavior analysis of entire plant powder drug is also carried out with various reagents (Table VII).

"Table V: Fluorescence analysis of the entire plant powder of Dendrophthoe falcata"

Reagents	*Dendrophthoe falcata* (Visible Light)	*Dendrophthoe falcata* (UV Light)
Powder as such	Brown	Brown
Powder + 1N NaOH	Grayish Green	Light green
Powder + 1N NaOH in ethanol	Grayish Brown	Light green
Powder + Ethanol	Dark Brown	Green
Powder + 50% HNO_3	Brown	Orange green
Powder + HCL	Dark Brown	Dark green
Powder + H_2SO_4	Black	Black
Powder + Picric acid solution	Yellowish brown	Yellowish green
Powder + Glacial acetic acid	Reddish Brown	Green
Powder + Concentrated HNO_3	Red	Green

"Table VI: Analysis of different solvent extracts of D. falcata under UV and visible light"

	Observation	
Extracts	**D.F. extract Visible light**	**D.F. extract UV light**
Pet ether	Green	No Change
Chloroform	Light Green	No Change
Ethanol	Chocolate Brown	Light Green
Water	Dark Brown	Light Green

"Table VII: Behavior analysis of powder of entire plant of D. falcata with various reagents"

S.No.	Procedure	*Dendrophthoe falcata*
1	Powder as such	Brown
2	Powder + Picric acid	Yellowish green
3	Powder + HNO_3	Faint brown
4	Powder + HCI	Faint brown
5	Powder + H_2SO_4	Faint brown
6	Powder + glacial acetic acid	Faint brown
7	Powder + Ferric chloride (5% aqueous solution)	Dark brown
8	Powder + NaOH (5N aqueous solution)	Dark brown
9	Powder + I_2 (Aqueous solution)	Dark red

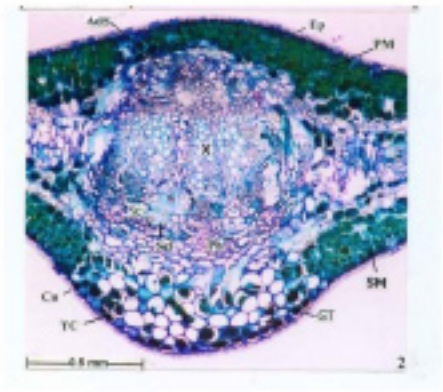

Fig. IA

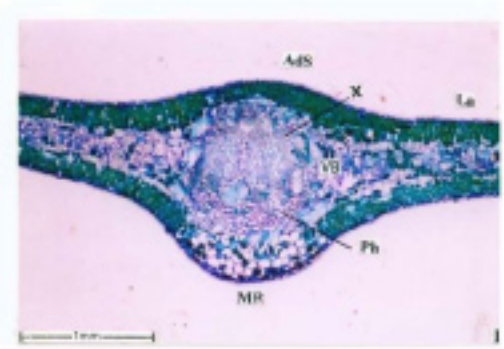

Fig. IB

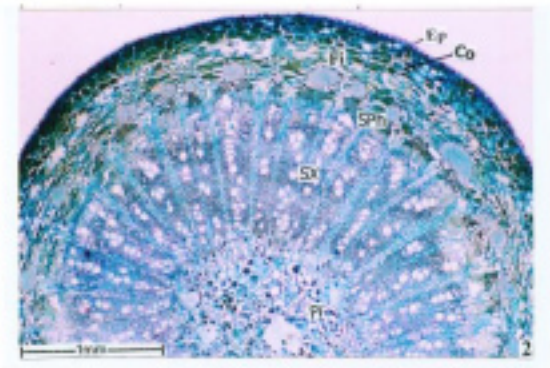

Fig. IC

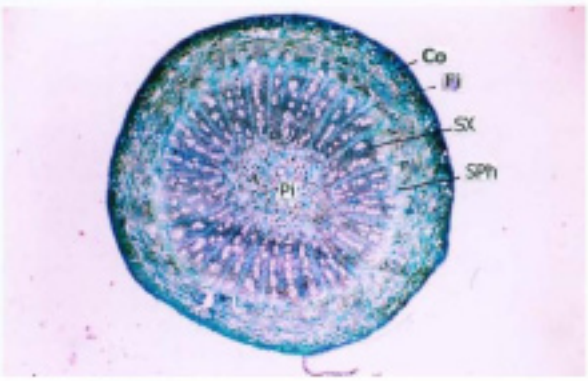

Fig. ID

Histology of leaf and stem

Fig. IA and Fig IB: T.S. of leaf through midrib with lamina (Ads-Adaxial cell, EP-Epidermis, PM-Palisade mesophyll, X-Xylem, Ph-Phloem, MR-Midrib, Scl-Scleride, Cu-Cuticle, TC-Taniferous cell, GT-Ground tissue, SM-Spongy mesophyll, La-Lamina, VB-Vascular bundle)

Fig. IC and Fig ID: T.S. of stem (Ep-Epidermis, Co-Cortex, SPh- Secondary phloem, SX-Secondary xylem, Pi-Pith, Fi-Fiber)

CONCLUSION

The present study on pharmacognostical evaluation of *Dendrophthoe falcata* will provide useful information for its identification. Macro, micro and physicochemical standards discussed here can be considered as identifying parameters to substantiate and authenticate the drug.

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