Academic Sciences International Journal of Pharmacy and Pharmaceutical Sciences

ISSN- 0975-1491 Vol 4, Issue 2, 2012

Research Article

PHARMACOGNOSTIC AND ANTIOXIDANT STUDIES ON *CLERODENDRUM INERME* AND IDENTIFICATION OF URSOLIC ACID AS MARKER COMPOUND

[1]V. GAYATHRI DEVI*, [3]CHITHRA VIJAYAN, [1]ANITHA JOHN AND [2]K. GOPAKUMAR

[1]Assistant Research Officer (Chemistry), SRRI, Poojappura, Thiruvananthapuram - 695012, Kerala, India; [2]Assistant Director (S), SRRI, Poojappura, Thiruvananthapuram - 695012, Kerala, India [3]Research Scholar (Botany), Department of Botany, University of Kerala, Kariayavattom, Thiruvananthapuram - 695581, Kerala, India. *Email: vglatha@gmail.com

Received: 12 Sep 2011, Revised and Accepted: 23 Oct 2011

ABSTRACT

Clerodendrum inerme (Linn.) Gaertn (Family – Verbenaceae; English – Garden quinine, Hindi – Langai, Malayalam – Nirnochi, Tamil - Chankankuppi) is commonly used in Ayurveda and Siddha systems of medicine. It is a common shrub found in the Western Ghats of India near the sea coast. In the present paper, a detailed pharmacognostic study on the leaf of *Clerodendrum inerme* based on its macroscopic, organoleptic and physico-chemical properties were carried out to lay down the pharmacopoeial standards. High Performance Thin Layer Chromatographic (HPTLC) and Thin Layer Chromatographic (TLC) profiles were taken. The limit tests for arsenic & heavy metals were carried out and were found to be within the permissible limit. A preliminary phytochemical study was carried out and the plant material was found to possess polyphenols like phenolic acids, flavonoids etc., triterpenoids, steroids and sugars. A phenolic acid was isolated as marker compound and identified as Ursolic acid. A preliminary study on the antioxidant activity of the alcohol extract and the isolated Ursolic acid was carried out by evaluating the free radical scavenging activity by the method of DPPH radical scavenging assay. The extract gave very good radical scavenging activity comparable with that of Butylated Hydroxy Toluene BHT which was used as the standard.

Keywords: *Clerodendrum Inerme*, Chankankuppi, physico-chemical, DPPH radical scavenging assay, Antioxidant properties

INTRODUCTION

Clerodendrum inerme (Linn.) Gaertn (Family – Verbenaceae) is a straggling shrub occurring abundantly near the coastal regions of India and Ceylon. The plant is commonly known as Chankankuppi in Tamil, Nirnochi in Malayalam, Kundali in Sanskrit, Langai in Hindi and Garden quinine in English. The plant enters into the composition of many Siddha and Ayurvedic formulations. The plant is distributed throughout India near the sea[1].

The leaves of the plant are mucilaginous, bitter and fragrant and its medicinal properties resemble those of *Swertia chirayita*. Fresh and dry leaves possess alterative and febrifugal properties. It acts as a tonic, febrifuge and alterative and is used in buboes and rheumatism. Alcoholic extract of the leaves stimulated pregnant uterus, raised blood pressure and increased intestinal movements in rat. Aqueous extract of plant stimulated uterine motility at different stages of sex cycle in isolated rat uterus. Plant did not produce toxic effects with doses as high as 8 g/kg[2]. The medicinal properties of *Clerodendrum inerme* may be due to its antioxidant properties. The leaves are reported to contain Iridoid biglycosides, scutellarin-4-L-arabinoside, scutellarin, acacetin-7-glucoside, acacetin glucuronide, (24s) ethyl cholesta-5, 22, 25-trien-3β-ol and 7-O-glucuronides of apigenin, and clerodin. Scutellarin showed inhibition of phospholipase A2 activity and antifertility activity[2,3]. The ash of the leaves is rich in sodium chloride[1]. The objective of the present paper includes the pharmacognostic diagnosis, preliminary phytochemical study, estimation of total phenolics, and isolation of ursolic acid as marker compound and evaluation of antioxidant properties.

MATERIALS AND METHODS

Plant material

The leaves of *Clerodendrum inerme* was collected from Mettur Botanical Garden. The plant material was washed in flowing water, dried in shade, cut, crushed and kept in airtight bottle for experimental purpose.

Reagents and Chemicals

All the biochemicals were purchased from Sigma Chemical Company, St. Louis, MO, USA and other chemicals and solvents were purchased from SRL Chemicals, India.

All the reagents used were of GPR grade.

Macroscopical analysis

The macroscopy of the plant was studied according to the method of Brain and Turner[4].

Organoleptic features

The organoleptic features such as colour, odour and taste were noted.

Physico-chemical parameters

The preliminary physico-chemical analysis such as determination of ash value, acid insoluble ash, extractable matter in water and alcohol, loss on drying at 105°C, pH value and volatile oil were carried out by standard methods[5].

Fluorescence study

The fluorescence characters of the petroleum ether, ethyl acetate, ethyl alcohol and water extracts of the plant material were detected in visible, short UV and long UV light[6].

Limit tests for arsenic & heavy metals

The limit test for Arsenic was determined in an Arsenic detection apparatus by comparing the yellow stain which is produced on the mercuric chloride paper if arsenic is present with the standard stains produced by operating in a similar manner with known quantities of dilute arsenic solutions. The test for heavy metals is designed to determine the content of metallic impurities that are coloured by sulphide ion under specified conditions. The limit for heavy metals is indicated in terms of the parts of lead per million parts of the substance by weight as determined by visual comparison of the colour produced by the substance with that of a control prepared from a standard lead solution[7].

Preliminary phytochemical study

In order to examine the presence of different natural products in the plant, characteristic phytochemical tests for sugar, starch, poly phenol, saponin, mucilage, steroid, alkaloid and flavonoid were performed using different extractives of the plant[8,9].

Preparation of plant extract for chromatographic studies

The extract for the study was prepared by soaking 4 g of the plant material in 40 ml of ethyl acetate and kept overnight. The solution was boiled for 10 min and filtered. The filtrate was concentrated and made up to 10 ml in a standard flask[10].

Development of thin layer chromatographic (TLC) profile

TLC profile of the ethyl acetate extract of the plant material was performed on silica gel 60 F_{254} pre-coated aluminium sheet. The plate was developed in Toluene: Ethyl acetate: Formic acid (9:1:0.5), air dried and visualized under UV light. The plate was then dipped in Vanillin-Sulphuric acid reagent and heated at 105ºC till the colour of the spots appeared. R_f values of the spots obtained in UV 254 nm, UV 366 nm and using vanillin-sulphuric acid as derivatising reagent were recorded.

Development of high performance thin layer chromatographic (HPTLC) profile

HPTLC is a micro analytical separation and determination method which has a wide application in herbal drug analysis. The plant extract was spotted in the form of bands with Camag microlitre syringe on a precoated silica gel plate with Camag Linomat V applicator. Mobile phase used was Toluene: Ethyl acetate: Formic acid (9:1:0.5). Linear ascending development was done in twin trough glass chamber saturated with mobile phase. The plate was scanned in UV 254 nm using Deuterium lamp. The plate was kept under UV 254 nm and 366 nm, and derivatised using vanillin-sulphuric acid reagent and photos were taken[10].

Isolation of Ursolic acid

The dried plant material (250 g) was cut into small pieces, crushed and extracted three times with 1000 ml hot 95 % ethanol under reflux. The combined extract was concentrated in vacuum. The aqueous concentrate was extracted successively with petroleum ether, chloroform and methanol at room temperature. After filtration, the solvent was evaporated under reduced pressure. The chloroform extract was subjected to column chromatography using silica gel G as stationary phase and chloroform-methanol in different ratios by increasing polarity as mobile phase. The fraction collected by elution using chloroform: methanol (20:1) gave a colourless solid. This was further purified by silica gel chromatography and again eluted with chloroform: methanol (20:1) to obtain ursolic acid. The identification of ursolic acid was carried out by determining the melting point, TLC and IR spectrum[11]. A TLC study was carried out to detect the ursolic acid isolated in the alcoholic plant extract as the marker compound.

Determination of DPPH radical scavenging activity

The cut and crushed plant material (10 g) was extracted in hot ethyl alcohol. The extract was concentrated to dryness. The free radical scavenging activity of the extract was determined using the stable DPPH radical and the activity was compared using BHT as standard[12]. For quantitative analysis, different concentrations of the plant extract were prepared. 0.1mM solution of DPPH in ethyl alcohol was prepared and 1 ml of the solution was added to 3 ml of the plant extract in different concentrations. Thirty minutes later, the absorbance was measured at 517 nm. A lower absorbance of the reaction mixture indicates higher free radical scavenging activity. The capacity to scavenge the DPPH radical was calculated using the following equation:

$$\text{DPPH scavenging effect [\%]} = [\{ A_{control} - A_{sample} \} \div A_{control}] \times 100$$

Where $A_{control}$ is the absorbance of control reaction and A_{sample} is the absorbance of sample in the presence of the extract[13].

Estimation of the total phenolic content

1 gm of fresh tissue was finely chopped and put in boiling 80 % methanol and refluxed for 10 min. The extract was cooled and the tissue was homogenized with a pestle for a few minutes. The homogenate was filtered and centrifuged for 10 min at 10000 rpm. The supernatant was taken for the estimation of phenols[14]. The absorbance was recorded at 650 nm against the reagent blank. A standard graph of phenols was drawn with pyrocatechol by taking absorbance against the concentration and it was compared against the total phenolic content of the plant extract.

RESULTS AND DISCUSSION

The quality control parameters for the crude drugs as raw materials may be established with the help of several parameters based on macroscopic, organoleptic, microscopic, physico-chemical and phytochemical properties. The detailed microscopic properties are discussed by Dinesh Kumar *et al*[15]. The macroscopic, organoleptic, physico-chemical and preliminary phyochemical properties are discussed in this paper.

Clerodendrum inerme is an evergreen sprawling shrub 1-1.8 m tall. The stem is woody and smooth. The leaves (Fig 1) are ovate to elliptical, 5-10 cm long and 2-5 cm wide. The leaves have acute to acuminate tip, smooth and slightly shiny upper surface, pinnate venation, margins entire, leaves opposite and simple. Cyme or umbel is usually comprised of 3 flowers joined at a common base point. Corolla is white and fused with 5 lobes. There are 4 stamens, reddish to purple in colour and upwardly curved. Fruit is green in colour turning black, 1- 1.5 cm long and obovoid. The organoleptic features like colour, odour and taste were noted and found to have a greenish yellow colour, characteristic odour and bitter taste. The macroscopic features and organoleptic features were found to be in agreement with earlier reports[15,16].

Fig. 1: Leaves of *Clerodendrum inerme*

The physico-chemical parameters of the leaves of *Clerodendrum inerme* was determined and the values obtained are given in Table 1.

Table 1: Physico-chemical parameters

Sl. No.	Test	Result
1	Loss on drying at 105˚C (%)	14.55
2	Ash value (%)	11.63
3	Acid insoluble ash (%)	0.37
4	Extractable matter in water (%)	27.40
5	Extractable matter in alcohol (%)	16.15
6	pH of water extract	6.11
7	Volatile oil (%)	Nil

Details of observations recorded with respect to behaviour of different solvent extracts under visible and fluorescent light at 254 nm and 366 nm are given in Table 2.

Table 2: Fluorescence behaviour of different extracts

Sl.No.	Extractives	Visible light	Short UV	Long UV
1	Petroleum ether	Greenish yellow	Yellow	Brown
2	Ethyl acetate	Greenish yellow	Light green	Greenish brown
3	Ethyl alcohol	Dark green	Brownish green	Brown
4	Distilled water	Light brown	Greenish brown	Dark brown

The limit test for arsenic was conducted and by matching the depth of colour with standard stain indicated that the proportion of arsenic was less than 1 ppm. The tests for heavy metals were also carried out and were also found to be within the permissible limit. This shows that the drug is not affected by environmental pollution and pesticides.

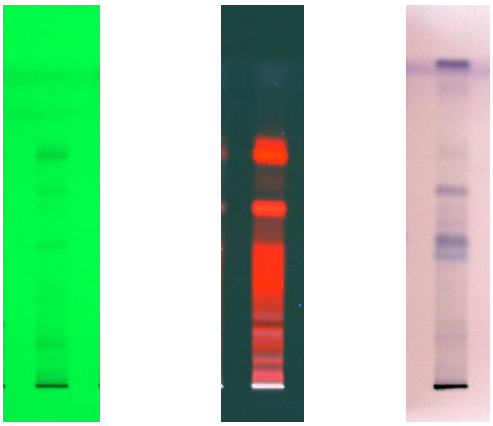
Phytochemical tests revealed the presence of sugar, poly phenol, mucilage, steroid and flavonoid and are recorded in the Table 3.

TLC of ethyl acetate extract of the plant material on a pre-coated Silica gel G 60 F_{254} plates using Toluene: Ethyl acetate: Formic acid (9:1:0.5) as mobile phase and when seen under UV_{254} nm showed 9 spots at R_f 0.11 (yellow), 0.15 (brown), 0.30 (light brown), 0.36 (light brown), 0.41 (light yellow), 0.45 (light brown), 0.56 (light brown), 0.64 (light brown), 0.69 (light brown); under UV_{366} nm, 7 fluorescent zones appeared at R_f 0.11 (orange), 0.18 (orange), 0.30 (light orange), 0.36 (orange), 0.41 (orange), 0.45 (light brown) and 0. 56 (light purple). On dipping the plate in vanillin- sulphuric acid reagent, 13 spots appeared at R_f 0.05, 0.11, 0.19, 0.23, 0.30, 0.40, 0.45, 0.49, 0.56, 0.64, 0.68, 0.71 and 0.74.

Table 3: Preliminary phytochemical tests

S No.	Natural products	Test performed	Inference
1	Sugar	Molisch'test	+ve
2	Starch	Iodine test	-ve
3	Poly phenol	Neutral $FeCl_3$ test	+ve
4	Saponin	Foaming in water	-ve
5	Mucilage	Swelling in water	+ve
6	Steroid	Liebermann's test	+ve
7	Alkaloid	Mayer's reagent test	-ve
8	Flavonoid	Shinoda test	+ve

The HPTLC of the ethyl acetate extract of the plant material was carried out. The plates were viewed under UV short, UV long and developed in vanillin-sulphuric acid reagent. HPTLC profile is a valuable parameter for identification of plant materials. HPTLC profile of *Clerodendrum inerme* is given in Figure 2.



(a) (b) (c)

Fig. 2: HPTLC profile of ethyl acetate extract of *Clerodendrum inerme*

(a) in UV short; (b) in UV long; (c) in vanillin-sulphuric acid

Solvent system: (Toluene: Ethyl acetate: Formic Acid (9;1;0.5)

Ursolic acid was isolated from the alcoholic plant extract as discussed earlier. The compound isolated is a colourless solid (mp. 287-288°C) reacted with bicarbonate showing it to be a carboxylic acid. A TLC study was performed on silica gel G 60 F_{254} plates using chloroform: methanol (30:1) as mobile phase and the R_f value was observed as 0.50. The identification of ursolic acid was performed by making direct comparison with commercially available authentic sample. The structure of ursolic acid isolated from the plant material was confirmed by determining mmp., co-TLC and co-IR. In the IR spectrum it showed absorption at 3430 cm^{-1}, 2625 cm^{-1}, 1703 cm^{-1} and 1045cm^{-1}. The absorption at 3430 cm^{-1} and 2625 cm^{-1} shows that there is at least one –OH group which is a part of carboxylic acid. The absorption at 1700 cm^{-1} shows the carbonyl group in a carboxylic acid group. The absorption at 1045 cm^{-1} indicated the presence of a secondary alcohol. The melting point and the TLC data (R_f =0.50) of the ursolic acid isolated is also in agreement with that of the standard ursolic acid[17]. The chemical structure of ursolic acid (3β-hydroxy-urs-12-en-28-oic acid) is shown in Figure 3. A TLC study of the isolated ursolic acid was carried out along with the alcoholic plant extract on silica gel G 60 F_{254} precoated aluminium sheet using Toluene: Ethyl acetate: Formic acid (9:1:0.5) as the mobile phase and is given in Figure 4.

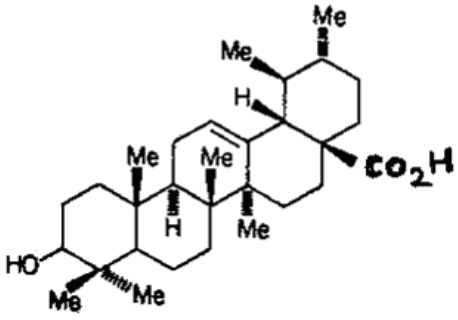


Fig. 3: chemical structure of ursolic acid

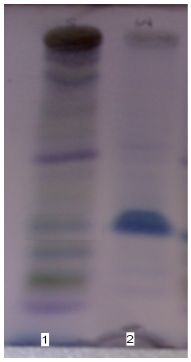


Fig. 4: Identification of ursolic acid as marker compound by TLC

1. Alcohol extract of Clerodendrum inerme; 2. Isolated ursolic acid

Detection: vanillin-sulphuric acid

DPPH assay is one of the most widely used methods for screening antioxidant activity of plant extracts[12]. DPPH is known to extract labile hydrogen. In the DPPH assay, the antioxidants were able to reduce the stable radical DPPH to yellow coloured diphenyl-picryl hydrazine, which is a non-radical form[18]. The antioxidant activity assay is based on the activity of DPPH, a stable free radical, to decolourize in the presence of antioxidants. In DPPH radical scavenging assay, a lower absorbance of the reaction mixture indicates higher free radical scavenging activity. The results of DPPH-scavenging activity (Fig. 5) of the plant extract suggests that the plant material contain a free radical scavenging system which could exert a beneficial action against pathological alterations caused by the generated free radical. The activity was increased by increasing concentration of the sample extract.

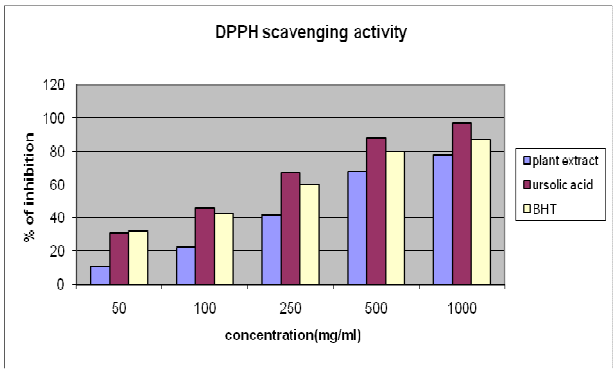


Fig 5: DPPH radical scavenging activity of alcoholic plant extract, ursolic acid and BHT as standard

The total phenolic content was also estimated. Total phenolic content present in the alcoholic extract of the leaves was determined and was found to be 4.8 µg pyrocatechol/ 100 µg plant extract.

CONCLUSION

TLC and HPTLC profiles developed along with the physico- chemical parameters can be conveniently used as a tool for the pharmacognostic identification of the leaves of *Clerodendrum inerme*. Ursolic acid isolated from the plant material may be used as a marker compound. The antioxidant activity of the plant extract may be due to the presence of polyphenols which are reported as strong antioxidants.

ACKNOWLEDGEMENT

The authors are thankful to the Director General, Central Council for Research in and Siddha, New Delhi for providing necessary facilities to carry out this work. The authors are also thankful to the Assistant Director-in-charge, SRRI, Poojappura, Thiruvananthapuram and Dr. G. M. Nair, Professor & Head, Dept. of Biotechnology, University of Kerala, Thiruvananthapuram for giving facilities, help and support.

REFERENCES

1. Anonymous, *The Wealth of India*, Raw materials Publication and Information Directorate, CSIR, Vol. II, Delhi, India, 1, 116-118, 1985.
2. Ram P. Rastogi and Mehrrotra B.N., *Compendium of Indian Medicinal Plants*. CDRI, Lucknow and NISC, New Delhi, India, 2, 194, .1999.
3. Ganapathy and Rao, *Indian J. Pharm. Sci*., 47, 167, 1985.
4. Brain K.R., Turner T.D., *The practical evaluation of phytopharmaceuticals*, Wright-Sentechnica, Bristol., 4-9, 1975.
5. Anonymous, *Quality control Methods of Medicinal Plant Materials*, World Health Organization (WHO), Geneva, 28-34, 45-46, 1998.
6. Chase C.R. and Pratt R., *Fluorescence of powdered vegetable drugs with particular reference to development of system of identification*, J. Am. Phar.Assoc. (Sci.ed.), 38, 324-331, 1949.
7. Anonymous, Workshop documents, *Quality Assurance of Ayurveda, Siddha, Unani and Homeopathic Drugs*, June 10-11, Dept. of Ayush, Govt. of India, New Delhi 2008.
8. Arther I. Vogel, *Vogel's Text Book of Practical Organic Chemistry*, Longman Group Limited London, 4th edition, 1978.
9. Raman N., *Phytochemical Techniques*, New India Publishing Agency, New Delhi, 2006.
10. Wagner H. and Bladt S. *Plant drug analysis - A thin layer Chromatography Atlas*, Springer - Verlage, Berlin, 1-2, (1996).
11. Hideaki Yamaguchi, Toshiro Nostika, Yumi Kidachi, Hironori Umetsu, Masahiko Hayashi, Kanki Komiyama, Shinji Funayama and Kazuo Ryoyama, *Isolation of Ursolic acid from Apple peels and its specific efficiency as a potential antitumour agent*, Journal of Health Science, 54(6), 654-660, 2008.
12. Nanjo F., Goto K., Seto R., Suzuski M., Sakai M. & Hara Y., *Scavenging effect of tea catechins and their derivatives on 1,1-diphenyl-2-picryl hydrazyl radical*, Free Rad. Biol. Med., 21, 895, 1996.
13. Gulcin I., Beydemir S., Sat I.G., Kufrevioglu O.I., *Evaluation of antioxidant activity of cornelian cherry (Cornus mas L.)*, Acta Aliment Hung 34, 193-202, 2005.
14. Mayr U., Treutter D., Santos- Buelga C., Bauer H. & Feucht W., *Developmental changes in the phenol concentrations of 'golden delicious' apple fruits and leaves*, Phytochemistry, 38, 1151, 1995.
15. Dinesh Kumar, Pravin V. Buge , Zulfikar Ali Bhat , Jeevan . Dhumal , Yogesh . katare , Santosh . Bhujbal, *Macroscopical and microscopical evaluation of leaves of Clerodendrum inerme Gaertn.*, Int. J. Biol. Med. Res. 2(1), 404-408, 2011.
16. Turner R.J.Jr. and Wasson E., *Botanica*. Mynah, USA, 1997.
17. He X., and Liu R.H., *Triterpenoids isolated from apple peels have potent antiproliferative activity and may be partially responsible for apple's anticancer activity*, J. Agric. Food Chem., 55, 4366-4370, 2007.
18. Gulcin I., Mshviladze V., Gepdiremen A., & Elias R., *Anti-oxidant activity of triterpenoid glycoside isolated from the berries of Hedera colchica- Hederagenin*. Phytotherapy Research. 20, 130, 2006.