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BIOASSAY GUIDED FRACTIONATION AND QUANTIFICATION OF TOTAL PHENOLS FROM THE AQUEOUS ACETONE ROOT BARK EXTRACT OF *CLERODENDRUM INFORTUNATUM* L.

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ABSTRACT

Clerodendrum infortunatum L. is a traditional Indian medicinal plant used for the treatment of various diseases. Root bark of the plant is used to treat inflammation, abdominal pain, indigestion etc. Based on the traditional use root bark of the plant was selected for the present study. In our previous study on the screening of various extracts for the antioxidant activity, aqueous acetone extract was found to possess the greatest activity. Phytochemical evaluation of the extract showed the presence of polyphenolics. In the present study, purification of the aqueous acetone extract was carried out by bioassay guided fractionation and the total phenolic content was determined. The antioxidant activity of the fractions was carried out by DPPH radical scavenging assay and total antioxidant assay. The ethyl acetate fraction was identified as phenolic rich and it was found to possess significant radical scavenging activity and total antioxidant activity. Thus, the phenolic compounds may be responsible for the medicinal properties exhibited by the root bark of the plant. Further characterization of the fraction is necessary for the development of novel pharmaceutics.

KEY WORDS

Bioassay guided fractionation, Clerodendrum infortunatum, Polyphenolics, Total antioxidant activity

INTRODUCTION

Clerodendrum infortunatum L. is a traditionally used Indian medicinal plant belongs to the family Verbenaceae. The plant is commonly known as Bhant in Hindi and as Peruvelam in Malayalam. It is widely seen along the barren lands and plains of India. Leaves, bark, root, flower, stem and seed possess medicinal properties. Different parts of the plant have been used for the treatment of bowel complaints, anemia, malaria, inflammatory diseases, tumors, snake bite etc. Leaf juice of the plant is used to get rid of the vitiated conditions of cough, fever, removal of ascarides and scorpion sting. Root bark of the plant is used as an anthelmintic, and also against indigestion, abdominal pain and inflammatory diseases [1]. Phytochemical evaluation of the plant revealed the presence of phenolics, flavonoids, steroids, terpenoids, fixed oil and sugars [2]. Screening of various extracts of the root bark of plant revealed that aqueous acetone extract exhibited the greatest antioxidant activity. Phytochemical analysis of the aqueous acetone extract revealed the presence of polyphenolics [3].

The healing power exhibited by plants lies in the phytoconstituents present in them which inturn elicits specific pharmacological actions in the human body. These secondary metabolites include alkaloids, flavonoids, phenolics, steroids, tannins, saponins and coumarins. Of these, phenolics are the most diverse and the largest group of plant secondary metabolites present in the nature. Phenolics include simple phenols such as catechols to high molecular weight condensed tannins [4]. Phenolics have atleast one aromatic ring with one or more hydroxyl group attached. They usually occur in conjugation with sugars and organic acids [5].

The biochemical properties exhibited by phenolics may vary depending on the substituents attached to the hydroxyl group. They are involved in inhibition of the production of reactive oxygen species, inhibiton of leukocyte activation etc. [6]. The protective benefits of phenolics are well documented as they are reported to possess anticancer, anti-inflammatory, antimutagenic, antioxidant and antiviral properties and are used against numerous diseases. The activity exhibited by phenolics are mainly attributed to its antioxidant potential which inturn neutralize free radicals, quench singlet and triplet oxygen or decompose peroxides [7].

The aim of the present study was to partially purify the aqueous acetone extract from the root bark of *Clerodendrum infortunatum* by Bioassay guided screening and to identify the polyphenol rich fraction.

MATERIALS AND METHODS

Chemicals

Silica gel (230-400 mesh particle size), 1,1-diphenyl-2-picrylhydrazyl (DPPH), gallic acid and ascorbic acid were purchased from Sigma Aldrich, USA. Ammonium molybdate, Folin- Ciocalteu reagent, sodium phosphate were purchased from Sisco Research Laboratories, India. All other chemicals and solvents used were of analytical range.

The following analytical grade solvents; acetone, dichloromethane, petroleum ether, ethyl acetate, methanol and dimethyl sulfoxide (DMSO) were obtained from Merck, USA.

Plant material

The root bark of *Clerodendrum infortunatum* was collected from Idukki District, Kerala. It was identified and the voucher specimen (SBS BRL 21) was maintained at School of Biosciences, Mahatma Gandhi University, Kottayam, Kerala. The plant material was washed under running tap water, air dried and powdered and used for the extraction.

Preparation of polyphenolic fraction

100 g of powdered root bark was defatted with petroleum ether and extracted with aqueous acetone (70% acetone) in a mechanical shaker for 72 hours at room temperature. The extract was filtered and concentrated in a rotary evaporator and the residue was lyophilised (Lark Penguin Classic Plus, Chennai). A column (50 x 1000 mm) was filled with silica gel (230-400) and the lyophilized aqueous acetone residue was layered on top of the silica gel equilibrated with n-hexane. It was then eluted with petroleum ether, dichloro methane, chloroform, ethyl acetate and methanol at a flow rate of 1 mL/min. 75 fractions were collected and pooled based on the thin layer chromatography and UV spectral analysis at 280 nm.

Determination of total phenolic content

The total phenolic content of the fractions were determined by the modified method of Singleton and Rossi [8]. To 100 µl of the standard gallic acid and different fractions of the aqueous acetone extract of *Clerodendrum infortunatum*, 500 µl of Folin-Ciocalteu reagent and 1ml of distilled water were added. It was kept at room temperature for 5 minutes and then added 1.5 ml of 20% sodium carbonate. The resulting mixture was incubated in the dark for 2 hrs at room temperature and the absorbance value was read at 760 nm. The total phenolic content was expressed as mg gallic acid equivalents (GAE)/ g plant extract.

Free Radical Scavenging Assay

The free radical scavenging potential of the fractions was determined by using DPPH by the modified method of Blois [10]. 2 ml reaction mixture containing 1 ml methanolic solution of DPPH and 1 ml of standard ascorbic acid or fractions at various concentrations was incubated in dark at 37ºC for 30 minutes. After incubation the absorbance was read at 517 nm in a UV-Vis spectrophotometer (Hitachi U-5100). Radical scavenging activity was expressed as the inhibition percentage and can be calculated using the formula

$$\% \text{ inhibition of DPPH radical} = \frac{\text{Abs control-Abs sample}}{\text{Abs control}} \times 100$$

Extract concentration providing 50% inhibition (IC_{50}) was calculated from the graph by plotting inhibition percentage against extract concentration.

Total antioxidant activity

The total antioxidant activity of the fractions was determined by the phosphomolybdenum method described by Prieto et al [9]. 0.1ml of the fractions (100µg) was combined with 1 ml of reagent solution (0.6M sulphuric acid, 28mM sodium phosphate and 4mM ammonium molybdate) and incubated in a boiling water bath at 95°C for 90 minutes. After cooling, the absorbance was measured at 695 nm against a blank in a UV-Vis spectrophotometer (Hitachi U-5100). 0.1 ml of ascorbic acid (5-30 µg) serves as the standard. The antioxidant activity was expressed as mg ascorbic acid equivalents per gram of sample on a dry weight basis.

Statistical analysis

The statistical analysis was carried out in GraphPad prism 5.03. All the results are presented as mean ± standard deviation and significant difference among the groups are compared at $p<0.05$ level by one way Analysis of Variance (ANOVA) followed by Tukey's post hoc analysis.

RESULTS AND DISCUSSION

Total phenolic content

The lyophilized aqueous acetone residue is a dark brown coloured powder. In the column chromatography of the aqueous acetone residue, total 75 fractions were obtained. The fractions obtained were pooled based on the UV spectral analysis and TLC into 15 fractions. Polyphenolic content of the pooled fractions (n=15) were determined by Folin-ciocalteu method and the ethyl acetate fraction (F12) possess the greatest content (264.3 ± 7.11 mg GAE/g plant tissue). The total phenolic content of different fractions obtained was presented in figure 1. Polyphenolics are widely distributed in the plant kingdom and they include simple phenylpropanoids and flavonoids to high molecular weight tannins and lignans that exhibit a variety of biological properties such as antibacterial, antioxidant, anticancer properties. Human studies related to the health benefits of polyphenols such as resveratrol also proved ensuring results [11].

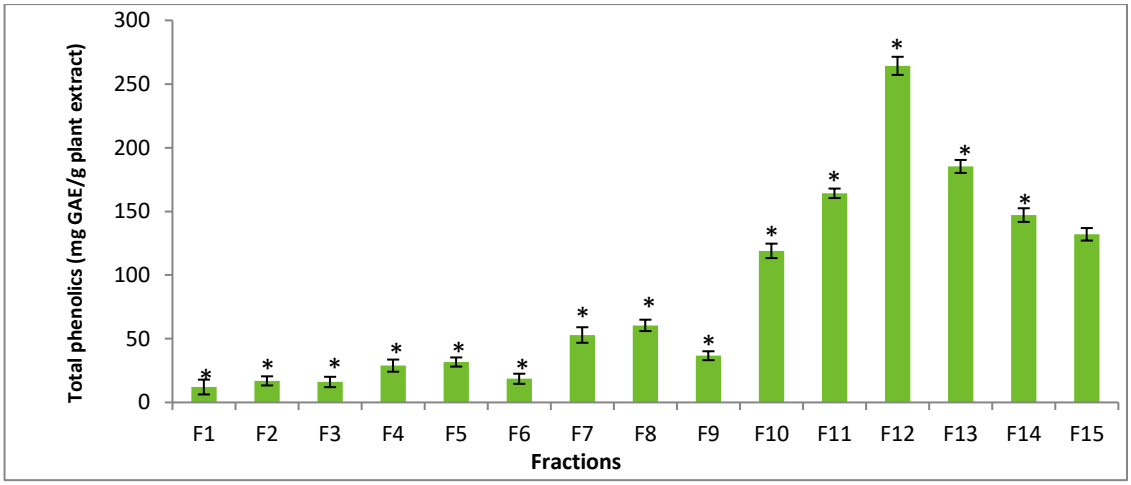

Fig. 1 Total phenolic content of the various fractions obtained from the aqueous acetone root bark extract of *Clerodendrum infortunatum*

F1- F15 are pooled fractions obtained during fractionation. All the values are mean ± SD (n=6).'*' indicates p <0.05 when compared with F12

Antioxidant activity

1,1-diphenyl-2-picrylhydrazyl (DPPH) is a stable free radical with an absorption maximum at 517 nm and DPPH radical scavenging assay is one of the most widely used method for antioxidant screening of plant extracts [12]. DPPH is reduced to a yellow coloured solution in presence of an antioxidant. Thus it is used as a substrate to assess the antioxidant potential of plant extracts. The percentage inhibition of radical formation increases with increase in concentration of the fractions as evident from the decrease in absorbance. The IC_{50} value of the fractions was given in fig. 2. IC_{50} value is the concentration required to scavenge 50% of the free radical's present. The ethyl acetate fraction (F12) showed an IC_{50} value of 225.2 ± 2.17µg/ml which was equivalent to that of the standard antioxidant ascorbic acid (262.5±3.68µg/ml). The hydrogen donating ability of the fraction is assessed as a measure of its antioxidant potential in DPPH radical scavenging assay [13].

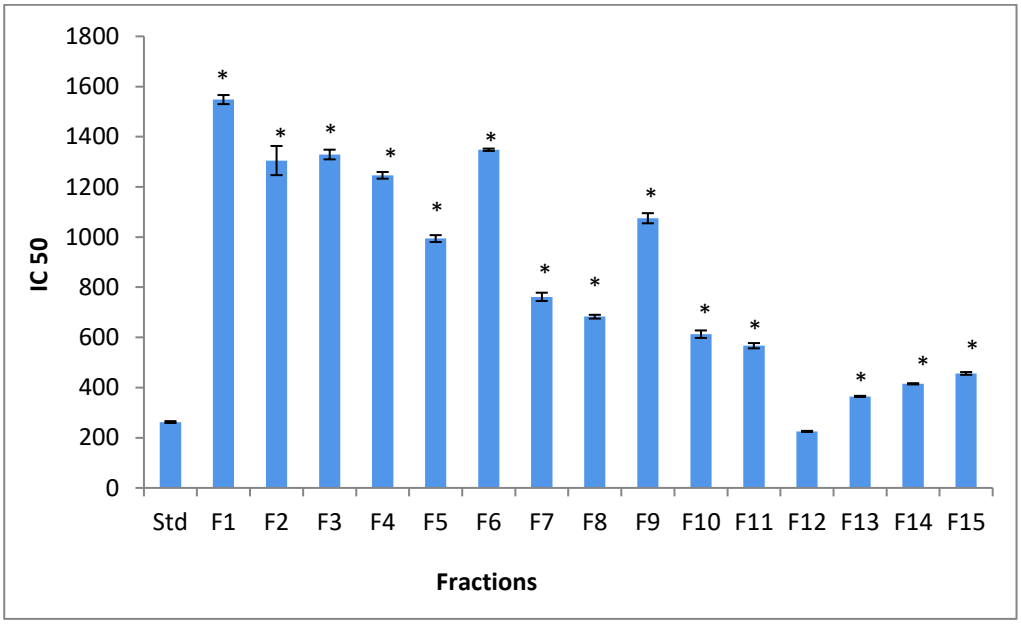

Fig. 2 DPPH radical scavenging activity of the various fractions obtained from the aqueous acetone root bark extract of *Clerodendrum infortunatum*

F1- F15 are pooled fraction obtained during fractionation. All the values are mean ± SD (n=6). '*' indicates p <0.05 when compared with F12

The total antioxidant activity of the fraction was determined by phosphomolybdenum method. It is a routinely used method to evaluate the total antioxidant capacity of plant extracts and is based on the reduction of Mo(VI) to Mo(V) which leads to the formation of green phosphate / Mo(V) complex at acidic pH [9]. Figure 3 shows the total antioxidant capacity of the various fractions obtained from the aqueous acetone root bark extract. The ethyl acetate fraction exhibited the greatest antioxidant activity (257.5±1.91 mg ascorbic acid equivalents/g dry extracts) compared to other fractions.

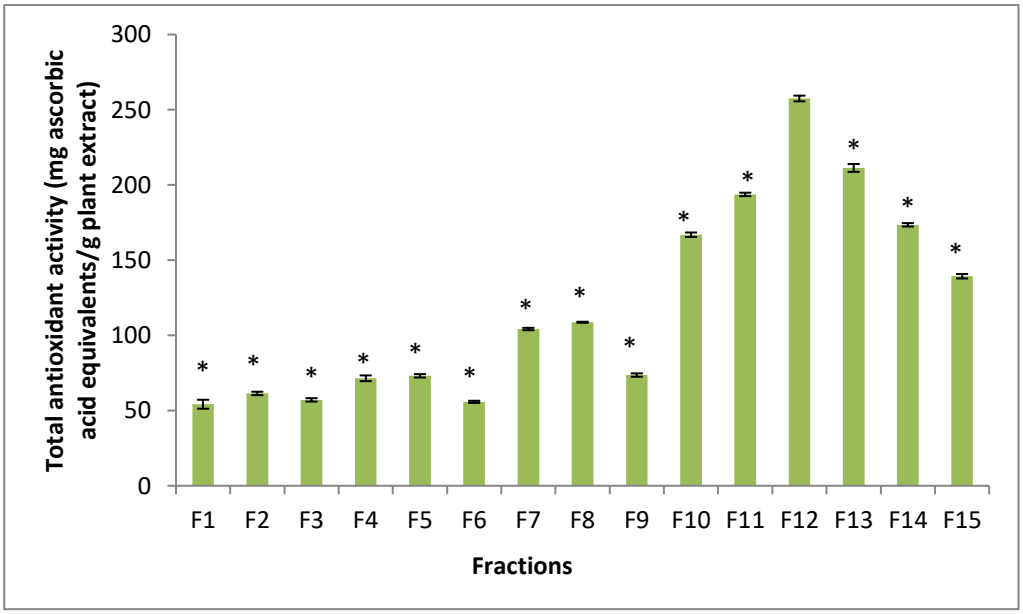

Fig. 3 Total antioxidant activity of the various fractions obtained from the aqueous acetone root bark extract of *Clerodendrum infortunatum*

F1- F15 are pooled fraction obtained during fractionation. All the values are mean ± SD (n=6). '*' indicates p <0.05 when compared with F12

There has been an emerging interest towards the protective role of traditional medicinal plants and other natural products in the treatment of cancer, neurodegenerative diseases, ageing and a growing

concern regarding the health effects of synthetic antioxidants as well. Antioxidants are free radical scavengers that delay or inhibit the process of oxidation and thereby protect the biological system from the toxic effects of reactive oxygen and nitrogen species and free radicals. Phenolic compounds constitute the main class of natural antioxidants due to their redox property [14]. The present study showed that the ethyl acetate fraction from the aqueous acetone root bark extract was phenolic rich and also exhibited greater antioxidant activity, which may contribute to the pharmaceutical potential of the plant.

CONCLUSION

The present study revealed that the phenolic rich ethyl acetate fraction possesses potent radical scavenging activity and total antioxidant capacity. Thus, it can form a preventive drug for the management of oxidative stress related disorders. Polyphenolics isolated from the root bark of the plant may be responsible for the medicinal properties attributed by the plant. Characterization of the fraction may further be carried out so that it can form a promising future drug.

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