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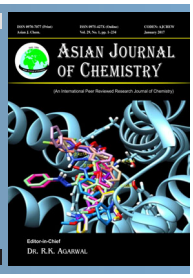

Isolation and Antioxidant Activity of Caffeic Acid from Roots of *Bryophyllum pinnatum* (Lam.) Kurz.

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Bryophyllum pinnatum (Lam.) Kurz. (Crassulaceae) is a perennial shrub, which possess bioactive phytoconstituents *viz*. terpenoids, phenolic compounds, flavonoid, anthraquinone, saponin and known to cure diabetes, cancer and act as anti-inflammatory, antiulcer, antihyperlipidemia. In the present study, isolation and antioxidant activity of caffeic acid from roots of *Bryophyllum pinnatum* (Lam.) Kurz. was carried out. Caffeic acid was characterized by TLC, FTIR and was elucidated by ^{1}H NMR and ^{13}C NMR technique. DPPH radical scavenging power of isolated caffeic acid showed IC_{50} value 66.58 ± 0.49 µg/mL in comparison to aqueous extract of root (139.52 ± 2.62 µg/mL).

Keywords: *Bryophyllum pinnatum* (Lam.) Kurz., Caffeic acid.

INTRODUCTION

India has a rich source of medicinal plants and a number of plant derived extracts are used against diseases in various systems of medicine such as Ayurveda, Unani and Siddha. Use of herbal medicines can be traced back as far as 2100 BC in ancient China (Xia dynasty) and India [1]. The use of medicinal plants in curing diseases is as old as man [2]. The World Health organization (WHO) has well recognized and drawn the attention of many countries to the ever increasing interest of the public in the use of medicinal plants and their products in the treatment of various ailments. These plants which are found in our environment enjoy wide acceptability by the population and serve as low-cost alternatives to orthodox medicine [3].

Secondary metabolites are organic molecules that are not involved in the normal growth and development of an organism. Absence of secondary metabolites does not result in immediate death however they play an important role in plant defense mechanism. Secondary metabolites are extremely diverse group of natural products synthesized by plants, fungi, bacteria, algae and animals. Most of the secondary metabolites, such as terpenes, phenolic and alkaloid compounds are classified based on their biosynthetic origin. Different classes of these compounds are often associated with narrow set of species within a phylogenetic group and constitute the bioactive compounds in several medicinal, aromatic, spice plants and/or functional foods [4].

Nature has been a potential source of therapeutic agents for thousands of years. An impressive number of modern drugs have been derived from natural sources. Over the last century, a number of top selling drugs have been developed from natural products. Anticancer drug vincristine from *Vinca rosea*, narcotic analgesic morphine from *Papaver somniferum*, antimalarial drug artemisinin from *Artemisia annua*, anticancer drug taxol from *Taxus brevifolia* and antibiotic penicillin from *Penicillium* sp. are few examples of drug developed from natural products. Recent studies are carried out in the identification and isolation of new therapeutic compounds of medicinal importance from higher plants for specific diseases [5,6].

Some bioactive compounds derived from plants include tannins, alkaloids, cardiac glycosides, flavonoids, sterols, triterpenes and anthraquinones, which play main role in nutrition, physiology and control of various diseases [7]. Identification and separation of phytoconstituents from different plants have been studied for it therapeutic use. One of such plants having different group of phytochemicals is *Bryophyllum pinnatum* (Lam.) Kurz. It possesses bioactive phytoconstituents *viz*. flavonoids (rutin, quercetin, kaemferol), terpenoids like α and β-amyrin. Cardenolides like bryophyllin and bufadienolide 1,3,5-orthoacetate [8]. However few works are available on isolation of phytoconstituents from *Bryophyllum pinnatum* (Lam.) Kurz.

In the present work, isolation of caffeic acid from root of *Bryophyllum pinnatum* (Lam.) Kurz. was carried out and its antioxidant activity using DPPH assay was evaluated.

EXPERIMENTAL

The plants of *Bryophyllum pinnatum* (Lam.) Kurz. were collected from Kalyan, India. The plant was identified and authenticated from Blatter Herbarium, St. Xavier's College, Mumbai. The shade dried material of root was separately cut into small pieces and powdered using mixer grinder. The powdered material was stored separately in labeled air tight bottles.

IR spectra were recorded (KBr discs) on a FT-IR spectrophotometer (ν_{max}, cm^{-1}). ^1H NMR and ^{13}C NMR spectra were recorded on a Bruker (400 MHz) and (75 MHz) instrument in CD_3OD with TMS as an internal standard (chemical shifts in δ, ppm). TLC was performed with silica gel GF_{254}. All solvents used were of analytical reagent grade.

Extraction and isolation: Root powder of *Bryophyllum pinnatum* (Lam.) Kurz. (600 g) was macerated with 95 % ethanol for 12 h and the resulting extract was concentrated under reduced pressure. Aqueous suspension of this ethanolic extract of root was further partitioned with petroleum ether, followed by ethyl acetate and *n*-butanol. These fractions were concentrated to obtain respective extracts.

From the above fractions, ethyl acetate fraction was subjected to column chromatography on silica gel with various proportions of petroleum ether and acetone (100:0-0:100) as the eluting solvent. Out of 25 fractions (R1-R25) obtained, prominent bands of fraction R6-R10 were combined and subjected to column chromatography, using various proportions of chloroform: methanol (1:1 to 0:1) as an eluting solvent. The 10 fractions (25 mL) collected from column chromatography were subjected to TLC analysis. Out of 10 fractions (E1-E10), fractions E5-E8 with single band were further combined on TLC. This isolated compound (ER1) was subjected to ^1H and ^{13}C NMR data for identification and characterization.

in vitro antioxidant activity by DPPH method

Extraction method: Root (50 g) of *Bryophyllum pinnatum* (Lam.) Kurz. was macerated separately in 100 mL of distilled water for 24 h in mechanical shaker at 120 rpm. The contents were filtered through Whatman filter paper No. 1 and residues were further macerated thrice using same procedure.The filtrates obtained at each step were combined and evaporated on water bath (60 ± 2 °C).

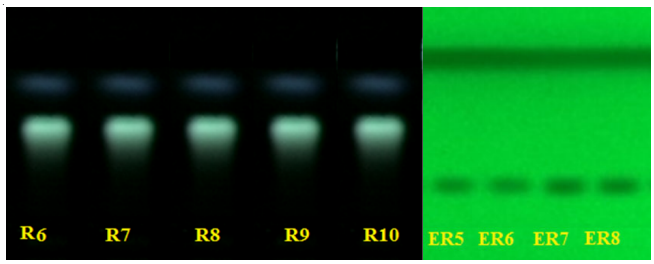
Antioxidant activity (DPPH free radical scavenging activity) determination: The antioxidant activity of the plant extracts was examined on the basis of the scavenging effect on the stable DPPH free radical activity [9]. Ethanolic solution of DPPH (0.05 mM) (300 µL) was added to 40 µL of root extract solution and caffeic acid with different concentrations (0.02-2 mg/mL) separately. DPPH solution was freshly prepared and kept in the dark at 4 °C. Ethanol (96 %, 2.7 mL) was added and the mixture was shaken vigorously. The mixture was left to stand for 5min and absorbance was measured spectrophotometrically at 517 nm. Ethanol was used to set the absorbance zero. A blank sample containing the same amount of ethanol and DPPH was also prepared. All determinations were performed in triplicate. The radical scavenging activities of the tested samples, expressed as percentage of inhibition were calculated according to the following equation [10].

$$\text{Inhibition of DPPH activity (\%)} = \frac{(AB - AA)}{AB} \times 100$$

where AA and AB are the absorbance values of the test and of the blank sample, respectively. A percent inhibition *versus* concentration curve was plotted and the concentration of sample required for 50 % inhibition was determined and represented as IC_{50} value for each of the test solutions.

RESULTS AND DISCUSSION

Characterization and elucidation of ER1 (ethyl acetate fraction of root) from root of *Bryophyllum pinnatum* (Lam.) Kurz.: Ethyl acetate fraction from root, when eluted with different proportions of petroleum ether and acetone gave 5 fractions (R6-R10). R6 to R10 fractions were combined and were subjected to column chromatography using chloroform: methanol (8: 2 v/v) as eluting solvents. From above separation, E5-E8 fractions were obtained which showed single band when subjected to TLC using chloroform:methanol (8: 2). Fractions E5-E8 (Plate 1-2) was combined and light yellow colour crystals were obtained which was labeled as ER1.



ER1 was soluble in methanol. It gave positive result for 5 % ethanolic ferric chloride test performed for the presence of phenol group. It was identified as caffeic acid based on ^1H NMR, ^{13}C NMR and FTIR data.

^1H NMR (400 MHz, CD_3OD, δ ppm): 6.32 (d, 1H, *J* = 16.1 Hz, H-2), 6.93 (d, 1H, *J* = 7.5 Hz, H-5'), 7.029 (d, 1H, *J* = 1.8 Hz, H-2'), 6.98 (dd, 1H, *J* = 8.1, 2.0 Hz, H-6'), 7.46 (d, 1H, *J* = 15 Hz, H-3) (Figs. 1 and 2).

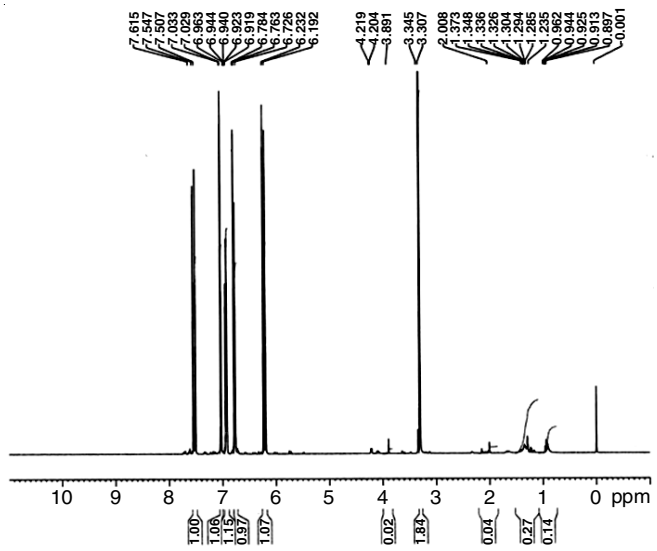


Fig. 1. ^1H NMR data of ER1 isolated from root of *Bryophyllum pinnatum* (Lam.) Kurz.

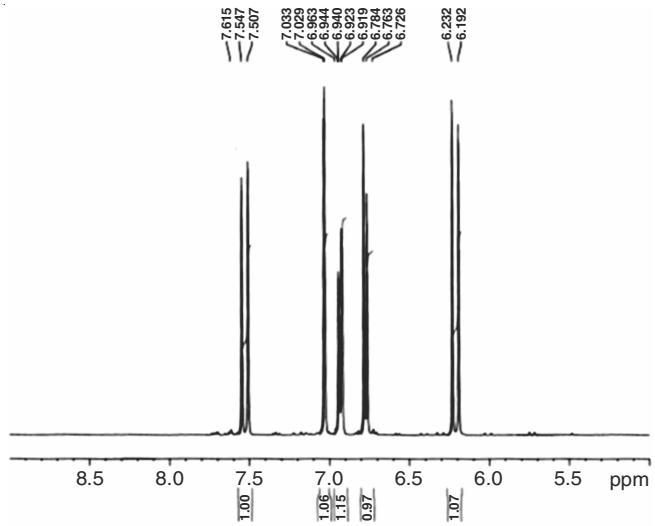


Fig. 2. ^{1}H NMR data for ER1 isolated from root of *Bryophyllum pinnatum* (Lam.) Kurz.

^{13}C NMR (75 MHz, CD_3OD, δ ppm): 171.51 (COOH), 116.32 (C-2), 145.49 (C-3), 129.24 (C-1'), 115.22 (C-2'), 146.91 (C-3'), 147.65 (C-4'), 117.58 (C-5'), 123.22 (C-6') (Fig. 3).

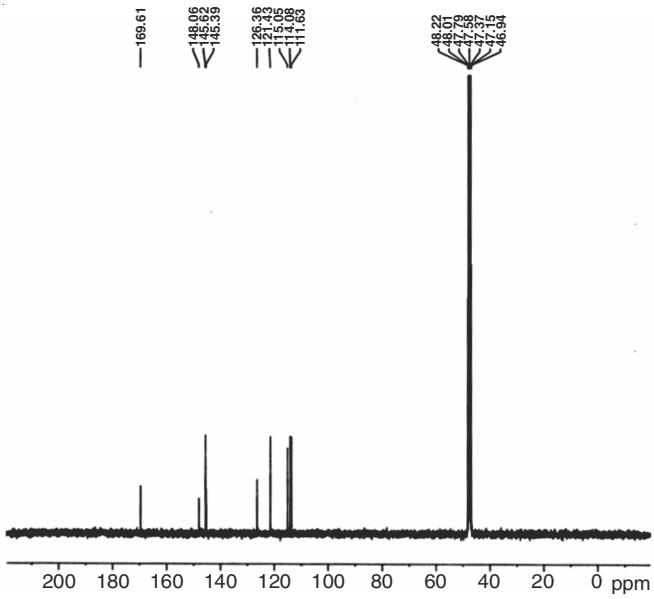


Fig. 3. ^{13}C NMR data of ER1 isolated from root of *Bryophyllum pinnatum* (Lam.) Kurz.

FTIR (ν, cm^{-1}): 3610, 3285, 2990, 1717, 1576, 1454 (Fig. 4). The FTIR spectrum of ER1 exhibited broad absorption band at 3350-2600 cm^{-1} for OH group of carboxylic acid, an absorption band at 1680 for –C=O of carboxylic acid and an absorption band near 1610 cm^{-1} for –C=C- stretching.

The ^{1}H NMR spectrum of ER1 displayed two *ortho*-coupled doublet (J = 8.0 Hz) each for 1H, at δ 6.807 and 7.029 and broad singlet for 1H, at δ 7.101 in the aromatic region thus indicated the presence of a tri-substituted aromatic ring in the molecule. The chemical shifts of these signals indicated the presence of catechol moiety in the molecule, which was confirmed by ^{13}C NMR chemical shifts of the hydrogen carrying carbon atoms at δ 114.86 (C-2), 115.75 (C-5) and

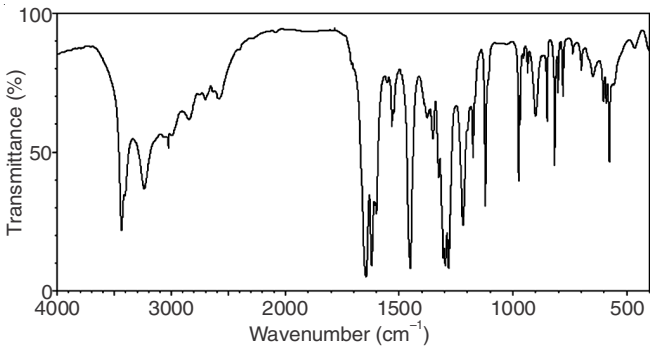


Fig. 4. FTIR data for ER1 isolated from root of *Bryophyllum pinnatum* (Lam.) Kurz.

121.35 (C-6). The ^{1}H NMR spectrum also displayed two doublets (J = 15.0 Hz), each for 1H, at δ 7.511 (H-7) and 6.351 (H-8). The large value of coupling constant indicated the presence of *trans*-di-substituted ethylene moiety in the molecule. The ^{1}H NMR and ^{13}C chemical shifts of olefinic protons and carbons were δ 141.41 (C-7) and 127.50 (C-8).

The ^{13}C NMR spectrum of ER1 exhibited presence of nine carbon atoms in the molecule. The ^{13}C chemical shifts of a carbon at δ 174.65 indicated the presence of carboxylic functional group in the molecule. The up-field chemical shifts of one of the ethylenic carbon (C-8) and proton (H-1) indicated that the carboxylic group is located at C-8 position. The ^{13}C chemical shifts of carbon atoms at δ 145.21 (C-3) and 148.35 (C-4) indicated that the hydroxyl group are attached at C-3 and C-4 positions. The position of ethylene function was determined by chemical shift of C-1 carbon at δ 125.42 and the downfield chemical shifts of C-7 carbon and H-7 proton of ethylene moiety. Thus on the basis of these spectral data, compound ER1 was characterized as caffeic acid (Fig. 5).

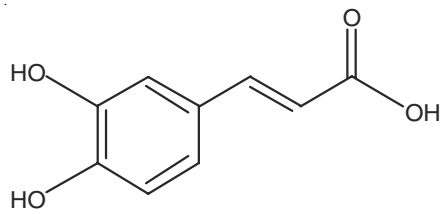


Fig. 5. Structure of caffeic acid isolated from *Bryophyllum pinnatum* (Lam.) Kurz.

***in vitro* Antioxidant activity by DPPH method:** Free radicals are involved in many disorders like neuro degenerative diseases, cancer and AIDS. Antioxidants due to their scavenging activity are useful for the management of those diseases. DPPH stable free radical method is a sensitive way to determine the antioxidant activity of plant extracts [11,12]. The DPPH radical is scavenged by antioxidants through the donation of electrons forming the reduced DPPH. The colour changes from purple to yellow after reduction and the accompanying decrease in absorbance can be quantified at wavelength 517 nm.

In DPPH assay conducted on caffeic acid has the lowest IC_{50} value 66.58 ± 0.49 µg/mL in comparison with aqueous extract of root (139.52 ± 2.62 µg/mL) of *Bryophyllum pinnatum* (Lam.) Kurz. The positive control glutathione (GSH) had an IC_{50} value of 71.77 ± 2.09 µg/mL. The above IC_{50} values showed that caffeic acid demonstrated even higher radical

scavenging activities than the positive control in the DPPH assay.

Phytochemicals, generally have a wide range of pharmacological activities or actions [13]. Most of these phytochemical constituents are potent bioactive compounds found in medicinal plant parts, which are the precursors for the synthesis of useful drugs [7]. The synthesis of bioactive compounds is chemically difficult, because of their complex structure and high cost [14]. All plant parts synthesize some chemicals in themselves, which metabolize their physiological activities. These phytochemicals are used to cure the disease in herbal and homeopathic medicine. Caffeic acid (a poly-phenolic compound) was isolated from root of *Bryophyllum pinnatum* (Lam.) Kurz. using column chromatography. It was identified by using TLC and FTIR and characterized by ^{1}H NMR and ^{13}C NMR. Caffeic acidis known to possess antioxidant, anticancer and antiobesity activity.

Conclusion

Natural products are of great significance to man, fulfilling the roles of medicines, stimulants, perfumes, spices, antimicrobial agents, hallucinogens and as components of industrial products. Phytoconstituents have been known to possess various health benefits *viz*. antimicrobial, anti-inflammatory, cancer preventive, antidiabetic and antihypertensive effects. The present study can be an important path of information for selection of active constituents in pharmacological studies as secondary metabolites are responsible for various biological activities.

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