Available online on www.ijppr.com
International Journal of Pharmacognosy and Phytochemical Research 2019; 11(3);129-134
doi: 10.25258/phyto.11.3.6
ISSN: 0975-4873

Research Article

Antioxidant Activitiy Guided Isolation of Chemical Constituents from Whole Plant of *Canscora Perfoliata*

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Received: 13th Mar, 19; Revised 30th Apr, 19; Accepted 10th May, 19; Available Online:25th Jun, 19

ABSTRACT
Canscora perfoliata is an important ethno medicine plant belonging to the family *Gentianaceae*. The plant is reported to have many important pharmacological activities even though the reports on the chemical constituents are few. The present study was designed for the bio assay guided identification of chemical constituents from most active extract of *C. perfoliata*. Various in vitro antioxidant assays like ABTS assay, NO quenching assay, Ferric reducing assay and DPPH scavenging assay were carried out on different extracts. Column chromatographic isolation for major chemical constituents was conducted on the most active extract. Hydroalchol extract showed predominant results in various antioxidant tests performed among the extracts. Column chromatographic isolation led to the identification of three compounds including a hydroxyl cinnamic acid, an alkaloid and a xanthanoid glycoside from the hydroalcohol extract. The identified compounds are the first report from this plant to the best of our knowledge. The plant extracts and identified compounds were active antioxidants and can be used a potentially as a bioactive source of natural antioxidants for contributing beneficial health effects.

Keywords: *Canscora perfoliata*, hydroalcohol, antioxidant activity.

INTRODUCTION

Canscora perfoliata is an important medicinal plant belonging to *Gentiananceae* family. *C. perfoliata* is distributed mainly in *Western Ghats*, India. *C. grandiflora* is the official synonym of this plant. The plant is being used by tribal people for various medicinal purposes. Various pharmacological activities of this plant were reported. The ethanolic extract of the plant showed significant hepatoprotective activity in CCl_4 induced hepatotoxic rats[1]. Ethanolic extract showed hypoglycemic and hypolipidemic activity in alloxan induced diabetic rats. In acute toxicity study, ethanolic extract of the whole plant was non-toxic up to 2000 mg/kg in rats[2]. It also showed antihyperlipidemic activity in Triton X-100 induced hyperlipidemia in male Wistar albino rats[3,4], immunomodulatory activity in swiss albino mice and significant anti-inflammatory activity in carrageean induced paw edema in wistar albino rats[5]. Yet now the reports on the main chemical constituents responsible for the medicinal activities of this plant were not available. So a detailed study was initiated for the identification of major chemical constituents. Various extracts of *C. perfoliata* were analysed for the different in vitro antioxidant assays including ABTS, NO quenching, ferric reducing and DPPH scavenging. The most antioxidant active extract was screened for the identification of active constituents using various chromatographic methods. The identified compounds were characterized using various spectrometric techniques like proton, carbon nuclear magnetic resonance and mass spectroscopy. The identification of chemical constituents in the plant extract will be useful for future pharmacological studies and also for checking the quality control of the plant.

MATERIALS AND METHODS

Collection of Plant material

Fresh material of whole plant was collected from Palakkad district Kerala. The materials were authenticated by Plant Systematic and Genetic Resources division, Centre for Medicinal Plants Research, Arya Vaidya Sala, and Kottakkal. A voucher specimen (9949) was deposited in CMPR herbarium.

Preparation of extract

10 gram each of the shade dried and powdered material was extracted with ethyl acetate, methanol, and methanol water (50:50) for 5 hours in soxhlet method. Filtered, solvents were removed by rotary evaporator under reduced pressure. 1 mg of all extracts was made up to 10 ml in respective solvents and these extracts were screened for various antioxidant activities and quantitative estimations.

Chemicals

Chemicals used in the study are quercetin, gallic acid, ascorbic acid, 2,2'azinobis (3-ethylbenzothiazoline-6-sulphonic acid), diphenyl picryl hydryzyl, Greiss reagent, ferric chloride, potassium persulfate, Folin-Ciocalteu reagent were procured from Sigma Chemicals Co.

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Table 1: Results of quantitative assays and antioxidant activities of *C. perfoliate* mg- Milligram, Eq-Equivalent, µg-Microgram, IC_{50}-Inhibition Concentration At 50 Percentage.

Extract	Phenolics content (mg eq gallic acid)	Flavonoids Content (mg eq quercetin)	Ferric reducing assay (100µg)	Nitric oxide assay (IC_{50})	ABTS assay (IC_{50})	DPPH assay (IC_{50})
Ethyl acetate	73±0.5	31±0.5	0.15±0.03	403 ±1.1	113±0.7	19.5±0.01
Methanol	191±0.3	71±0.3	0.51±0.05	351 ±1.5	115±1	23±0.05
Hydro alcohol	193±0.7	75±0.5	0.53±0.07	345 ±1.3	193±1.1	131±0.03
Ascorbic acid			0.57±0.01	343±1.3	103±0.3	15±0.01
Quercetin						5.1±0.01

(Bangalore, India). Methanol, formic acid and acetonitrile (LC/MS grade) were obtained from Burdick & Jackson, USA All other chemicals employed were of standard analytical grade from Merck India.

Total Phenolics assay

The total phenolics content were determined by using the Folin-Ciocalteu assay[6]. An aliquot of extracts or standard solution of Gallic acid with varying concentrations was added to volumetric flask, containing distilled water. Reagent blank was prepared using distilled water. Folin-Ciocalteu phenol reagent was added to the mixture and shaken. After 5 minutes Na_2CO_3 solution was added to the mixture. The volume was then made up to the mark. After incubation for 90 minutes at room temperature, the absorbance against the reagent blank was determined at 550 nm with an UV-Visible spectrophotometer. Total phenolics content was expressed as mg Gallic acid Equivalents (GAE)

Total Flavonoid Assay

Total flavonoid content was measured by the aluminium chloride colorimetric assay[7]. An aliquot of extracts or standard solutions of quercetin with varying concentrations was added to volumetric flask containing distilled water. To the flask was added $NaNO_2$, after five minutes $AlCl_3$ was added. After five minutes, NaOH was added and the volume was made up to 10 ml with distilled water. The solution was mixed and absorbance was measured against the blank at 510 nm. The total flavonoid content was expressed as mg quercetin equivalents (QE)

Free radical-scavenging activity on ABTS

ABTS assay was based on the slightly modified method[8]. ABTS radical cation (ABTS+) was produced by reacting 7 mM ABTS solution with 2.45 mM potassium persulfate aqueous solution and allowing the mixture to stand in the dark at room temperature for 12-16 h before use. The ABTS+ solution was diluted with ethanol to an absorbance of 0.70 ± 0.02 at 732 nm. After addition of extracts to 3.9 ml of diluted ABTS+ solution, absorbance was measured at exactly 6 min. Ascorbic acid was used as the standard for study.

Nitric oxide quenching activity

Nitric oxide scavenging activity was measured spectrophotometrically by using the Greiss reagent, with which nitrate reacts to give a stable product was by slightly modified method[9,10,11]. Nitric oxide radicals were generated from sodium nitroprusside. 1ml of 10mM sodium nitroprusside and 1.5 ml of 0.2M, pH7.4phosphate buffer saline was added to the various concentrations like 10, 25, 50, 75 and 100 µg/ml of the plant extracts and incubated for 150 min at 25^0C. After incubation, 1ml of the reaction mixture was treated with 1ml of Griess reagent (1% sulfanilamide, 2% H_3PO_4 and 0.1% napthalenediamine dihydrochloride). The absorbance was measured at 546 nm. Ascorbic acid was used as standard.

Reducing power assay

The reducing power of the extracts was determined according to previous method with slight modification[12]. Different concentrations of plant extracts (25, 50, 250 and 500µg/ml) prepared in methanol were mixed with phosphate buffer (2.5 ml, 0.2M, pH 6.6) and potassium ferricyanide $[K_3Fe(CN)_6]$ (2.5ml, 1%). The mixture was incubated at 50°C for 20 min and 2.5ml of trichloro acetic acid (10%) was added to the mixture, which was then centrifuged at 3000 rpm for 10 min. The upper layer of the solution (2.5 ml) was mixed with distilled water (2.5 ml) and FeCl3 (0.5 ml. 0.1%) and the absorbance was measured at 700nm. Increased absorbance of the reaction mixture indicated increased reducing power. Ascorbic acid was used as standard.

DPPH radical scavenging assay

The antioxidant activity of different extracts was determined by DPPH radical scavenging assay[13]. The analysis was done with minor modifications. 1 ml of 0.004% DPPH in methanol was mixed with equal volume of extract and standard solution. Each extracts was tested in triplicate at five concentrations. The mixture was slightly shaken and kept in dark for 30 minutes. The absorbance at 517 nm was measured by using UV/Visible spectrophotometer. Quercetin and ascorbic acid were used as the standards for the test. Where A_1 is the absorbance of DPPH and A_0 is absorbance of extract. Results were expressed in terms of inhibition concentration.

Statistical analysis

Data were given as mean ± standard deviation (SD) of three values. Statistical analyses of the all tests were performed using a one-way analysis of variance. The IC_{50} values for all activities were calculated by linear-regression analysis method. Results were calculated by employing the statistical software (COSTAT, Monterey, U.S.A)

Preparation of extract for isolation chemical constituents

Powdered whole plant material (1 kg) was extracted with hydroalcohol (methanol: water, 50:50) by soxhlet technique for 24 hrs. Extract was filtered and solvent was

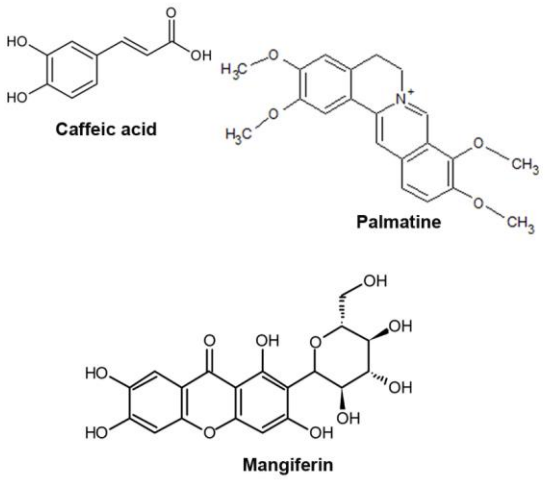

Figure 1: Structures of isolated compounds.

removed by rotary evaporator method under reduced pressure.

Column chromatography

The extract was adsorbed on activated silica gel 100-200 mesh size. Column of 1000 mm height and 50 mm diameter was packed with silica gel 100-200 mesh with hexane as solvent in wet packing method. The sample was loaded to the column and eluted with the solvents such as ethyl acetate and methanol. Eluting solvents started from ethyl acetate 100% to ethyl acetate with methanol in increasing polarity order 10%, 30%, 50% and methanol 100%. The fractions obtained from the column were analyzed using thin layer chromatography to identify the similar fractions. The similar fractions were clubbed together and further purification methods were carried out for isolation. A total of 30 fractions of 100 ml were collected from column. Various fractions were selected and pooled on the basis of thin layer chromatography analysis are 3-10, 13-15 and 23-30. The combined fractions were concentrated to eliminate the solvent and finally under Rota vapor to remove the traces of solvent in extract.

Characterization of compounds

NMR spectra were recorded on a Bruker DRX 500 NMR instrument operating at 400 MHz for ^{1}H and 400 MHz for ^{13}C at room temperature. A region from 0 to 15 ppm for ^{1}H and 0 to 200 ppm for ^{13}C was employed. Signals were referred to as the internal standard tetramethylsilane (TMS). About 10 mg of the sample were used and $CDCl_3$ and DMSO were used for recording the spectra.

RESULTS AND DISCUSSIONS

Phenolics and flavonoid content

The total phenolics content of ethyl acetate, methanol and hydro alcohol extracts was estimated by Folin-Ciocalteu assay and expressed as milligram equivalent of gallic acid. Hydro alcoholic extract showed the highest phenolic content among the extracts (193 MgEG) followed by methanolic and ethyl acetate extracts. Total flavonoid content of ethyl acetate, methanol and hydro alcohol extracts were estimated by aluminium chloride colorimetric assay and expressed as milligram equivalent of quercetin. Like phenolics, hydro alcohol extract showed maximum flavonoid content (73 MgEQ) then methanolic extract and least shown by ethyl acetate extract (Table 1). The results revels the rich percentage of medicinally active phenolics and flavonoids type of compounds in hydro alcohol extract. Phenolics and flavonoids are active group of phytocompounds with well known medicinal and therapeutic activities. The phenolic and flavonoid percentage in *C. perfoliata* is comparable with recognized highly active medicinal plants.

ABTS activity

ABTS radical is a widely using compound to measure the antioxidant activity of plant extracts. The activity of sample was calculated from the decolorization of ABTS·+, which was measured spectrophotometrically at 734 nm. A high inhibition value indicates that the mechanism of antioxidant action of extracts was as a hydrogen donor is efficient and it could terminate the oxidation process by converting free radicals to the stable forms[8]. The results were expressed as percentage of inhibition concentration. Hydro alcohol extract exhibited the higher antioxidant activity by giving the lowest IC_{50} value 113 µg/ml. Methanolic extract also showed a comparable activity with IC_{50} value 115 µg/ml. The least activity was shown by ethyl acetate IC_{50} value 193 µg/ml. Hydroalcohol and methanol extracts showed inhibition activity comparable to the standard ascorbic acid (Table 1). From the results it is clear that the nature of extracting solvent significantly influencing the antioxidant activity in ABTS assay.

Nitric oxide activity

Nitric oxide is an essential bioregulatory molecule required for several physiological processes like neural signal transmission, immune response, cardiovascular dilation and blood pressure. However, elevated NO radical results in several pathological conditions including cancer. The efficiency of the plant extract to consume the nitric oxide radical is an effective technique for measuring the antioxidant activity[9,10,11]. All extracts used for study showed the activity to counteract the formation of nitric oxide dose-dependent way. Hydro alcohol extract showed the higher activity by giving the lowest IC_{50} value 345 µg/mL, followed by methanol extract 351 µg/mL. Ethyl acetate extract showed the lowest activity 403 µg/mL. Ascorbic acid was used as the standard for the study and values are given in table (Table 1).

Reducing Power

In reducing power assay Fe^{3+} was transformed to Fe^{2+} in the presence of plant extracts and the reference compound to measure the reductive capability. Ascorbic acid was used as the standard for study[12]. In the present study reducing power of the extracts increased with increasing concentrations. The concentration of extracts used are 100, 500, 1000µg/ml and the values showed 0.53, 0.65 & 0.73 with hydro alcohol extract, 0.51, 0.63 & 0.71 with methanol extract, 0.15, 0.31 & 0.35 with ethyl acetate extract, and 0.57, 0.63 & 0.65 with ascorbic acid respectively. The result shows the higher reducing activity of Hydro alcoholic extract among the tested extracts.

DPPH activity

DPPH radical is one of the free radicals widely used for analyzing the radical scavenging activity of the plant extract as it is a direct and reliable method for determining the activity[13]. It is expressed as IC_{50} value in µg per ml. Lower the IC_{50} value higher will be the antioxidant activity of the extract. The radical scavenging assay follows the same manner of phenolics as the highest activity was shown by hydro alcohol extract with IC_{50} value (19.5 µg/ml). Methanolic extract also showed an excellent activity IC_{50} value (23 µg/ml). Ethyl acetate extract showed low activity compared to the other extracts IC_{50} value (131 µg/ml). Detailed results are given in Table 1. The results showed that hydro alcohol extract is endowed with powerful antioxidant active compounds higher than that of ascorbic acid and comparable with quercetin. In addition, methanol extract also exhibited a strong scavenging activity comparable to that of hydro alcohol extract, whereas ethyl acetate extract was less active.

Antioxidant activity of hydro alcohol extract could in part be attributed to the presence of active phenolic compounds or flavonoid compounds in the extract. The various antioxidant activities are in correlation with their phenolics and flavonoid contents. The plant extracts showed a good percentage of phenolics and flavonoids and these compounds exhibited prominent biological properties in various previous studies. Various investigations have established a relationship between the structure of different phenolics and flavonoids and their relative efficiencies as antioxidants. The capacity of phenolics and flavonoids to act as antioxidants *in-vitro* studies has been the subject of several studies in the past years and role in antioxidant activities has been recognized.

Isolation of compounds

The highest phenolic, flavonoid contents and predominant results in antioxidant activities was put on view that hydro alcoholic extract is a rich source of active chemical constituents. Column chromatography isolation was done for the identification of chemical compounds from hydroalcohol extract. Compound 1 was obtained from fractions 3-10 and was a phenolic compound on preliminary test with ferric chloride and was recrystallised in methanol. Compound 2 was obtained from fractions 13-15 and was recrysatllied in methanol. The compound was an alkaloid on preliminary test. Compound 3 was obtained fractions 23-30 as a yellow powder and recrysalised in methanol.

Compound 1

3,4-Dihydroxycinnamic acid - ^{1}H NMR (DMSO, 400 MHz) δ: 7.41(1 H, d, H-7), 7.03 (1 H, d, H-2), 6.94 (1 H, d, H-6), 6.75 (1 H, d, H-5), 6.21(1 H, d, H-8). ^{13}C-NMR (DMSO, 400 MHz) δ 125.6 (C-1), 114.2 (C-2), 144.7 (C-3), 148.0 (C-4), 115.7 (C-5), 121.4 (C-6), 145.3(C-7), 114.9 (C-8), 168.1 (C-9). On LC MS Q TOF analysis on negative mode it showed a M-H molecular mass ion at 179.1543.

Compound 2

Palmatine - ^{1}H NMR (DMSO, 400 MHz) δ: 9.81 (1 H, d, H-7), 8.87, 8.14 (d), 7.95(d), 7.73, 7.02, 6.10, 4.87 (t), 4.03 (d), 3.15 (t). ^{13}C-NMR (DMSO, 400 MHz) δ: 150.45, 149.89, 147.68, 145.43, 143.66, 137.49, 133.03, 130.68, 126.76, 123.50, 121.39, 120.43, 120.18, 108.42, 105.41, 102.06, 68.81, 61.90, 57.05, 55.17, 26.29. On LC MS Q TOF analysis on positive mode it showed a M+H molecular mass ion at 352.4131

Compound 3

C-2-β-D-glucopyranosyl-1,3,6,7-tetrahydroxyxanthone - ^{1}H NMR (DMSO, 400 MHz) - 13. 75 (s, HO-1), 10.64(s, HO-6), 10.60(s, HO-3), 8.30(s, HO-7), 7. 38(s, H-8), 6.86(s, H-5), 6.37(s, H-4), 4.57(d, H-1). Peaks of glucose moiety are 4.97 (s), 4.61(d), 4.03(t), 3.6(d), 3.3 (s), 3.14 (m). ^{13}C NMR (DMSO, 400 MHz) – carbons on xanthone ring are 162.5(1), 108.3(2), 164.5(3), 94.0(4), 156.9(4a), 103.3(5), 154.7(6), 144.4(7), 112.4(8), 108.7(8a), 179.8(9), 102.0(9a), 151.4(10a). Six carbons on glycoside moiety are C-1 73.8, C-2 71.3, C-3 79.7, C-4 70.9, C-5 82.3, C-6 62.2. On LC MS Q TOF analysis on negative mode it showed a M-H molecular mass ion at 421.0881. Structures of all isolated compounds were given below.

The first compound 3,4-Dihydroxycinnamic acid (caffeic acid) is a derivative of hydroxycinnamic acid. It is found as the major phenolic acid in *C. perfoliata* extract. It has reported anti-inflammatory, anti-cancer, antiviral, antioxidant, anti-ischemia reperfusion, anti-thrombosis, anti-hypertension, anti-fibrosis, anti-tumor and neuroprotective properties[14]. Studies demonstrated that it can improve memory and interfered with the cholinergic signaling. As a natural and promising compound it can be considered potentially therapeutic in disorders that involve the cholinergic system[15]. It showed a good inhibition of acetycholonesterase activity[16]. Its treatment alters the extracellular adenine nucleotide hydrolysis in platelets and lymphocytes[17]. It has the potency to reduce oxidative stress, learning and memory deficit[18]. Palmatine also known as proptoberberine is an alkaloid naturally produced by many of plants. This natural product has been used for centuries in health care. It has a wide range of pharmacological applications in medicine[19]. C-2-β-D-glucopyranosyl-1,3,6,7-tetrahydroxyxanthone (mangiferin) a xanthanoid phenolic compound formed from xanthone. Mangiferin is an important xanthanoid and it is a glucoside of norathyriol. Mangiferin is a heat-stable molecule and a natural pharmacologically active phytochemical that has various biological activities. The mangiferin was very effective against many lifestyle related disorders[20]. It is a promising anti-cancer agent and the mechanism of its activity was well described[21] and other activities were reported[22]. Mangiferin showed many nutraceutical activities beneficial for human health[23]. It showed many other pharmacological activities and protective activities also[24]. Mangiferin showed good results in various free radical scavenging assays and a proved antioxidant[25]. The compounds such as 3,4-Dihydroxycinnamic acid and mangiferin were highly active antioxidants from previous reports and from our study it is clear that mangiferin is the most abundant phenolics compound in extract. So these compounds might be behind the improved antioxidant activity of this plant. Since 3,4-Dihydroxycinnamic acid and mangiferin are efficient anticancer compounds more pharmacological

studies might identify the plant as a promising natural drug.

CONCLUSION

Various antioxidant assasys including DPPH, ABTS, Nitric oxide and Ferric reducing also quantitative estimations for pharmacological important chemical groups were done in different extracts such as hydro alcohol, methanol and ethyl acetate of *C. perfoliata*. Hydro alcohol showed the predominant results in various assays among the extracts and it showed the presence of active constituents in extract. The detailed chromatographic studies on hydroalcohol extract leads to the isolation of three compounds such as 3,4-Dihydroxycinnamic acid, Palmatine and C-2- glucopyranosyl-1,3,6,7-tetrahydroxyxanthone. The studies proved that the plant is an important source of C-2- glucopyranosyl-1,3,6,7-tetrahydroxyxanthone since it obtained in high quantity. Also the compounds such as 3,4-Dihydroxycinnamic acid and C-2- glucopyranosyl-1,3,6,7-tetrahydroxyxanthone are efficient antioxidants and anticancer agents. These compounds might be responsible for the high antioxidant activity of the extract. The study reveals the medicinally beneficial activities of the extract and also the compounds responsible for the important activities. The plant extracts and identified compounds were active antioxidants and can be used a potentially as a bioactive source of natural antioxidants for contributing beneficial health effects. Detailed pharmacological studies must require for proving the medicinal efficiency of the extract.

ACKNOWLEDGEMENTS

The authors are thankful Dr. Prabhu kumar to his help in identification of the plant material. Thanks are also due to Indira Balachandran Director, CMPR for valuable contribution. Sincere thanks to Bharathiar University Coimbatore for providing the facility for the successful completion of this work.

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