

Evaluation of antioxidant potential and HPLC based identification of phenolics in *Polygonum amplexicaule* extract and its fractions

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Abstract: There is a growing interest for the plant-based medicines in pharmaceutical industry. Plant derived Antioxidants have gained huge importance regarding their medicinal value. The present study was designed to establish pharmaceutical value of *Polygonum amplexicaule* for their antioxidant activity using shoot, leaf and rhizome crude methanolic extract along with their n-butanolic, ethanolic, ethyl acetate and aqueous fractions. DPPH assay was used to assess antioxidants, which shows the maximum activity by crude methanolic extract of leaves (CMEL) having IC₅₀ 1.03 µg/mL where all other fractions showed IC₅₀ in a range of 1.03-58.2 µg/mL. The DNA plasmid protection assay showed that 10 ppm and 100 ppm concentrations of crude methanolic extracts (rhizome and leaf), aqueous fractions (shoot and leaf extract), n-butanolic fractions (shoot and leaf extract) and ethanolic fraction (rhizome extract) have DNA protection properties. TLC and HPLC based Identification of different antioxidants present in shoot, leaf and rhizome crude extracts and their fractions showed the presence of gallic acid, quercetin, catechin, caffeic acid, rutin, myricetin and kaempferol. This study suggested that this plant have high content of antioxidants, which needs to be investigated further for their medicinal and/ cosmaceutical applications.

Keywords: *P. amplexicaule*, antioxidants, bioassays, TLC, HPLC.

INTRODUCTION

Antioxidants are compounds with the ability to neutralize the action of free radicals in normal body cells. Naturally two types of defense system, against free radicals, exist in human body including: (i) enzymatic and (ii) non-enzymatic. Catalase, Superoxide dismutase (SOD), Glutathione Reductase, Thioredoxin and Glutathione Peroxidase are the enzymatic antioxidant system whereas non-enzymatic antioxidant systems includes vitamins like Vitamin E, vitamin C and phytochemicals like flavonoid, carotenoids, polyphenols and alpha lipoic acid etc. (Devasagayam *et al.*, 2004).

Non-enzymatic antioxidants are broadly categorized into two vast assemblies, viz., endogenous and exogenous antioxidants. The exogenous antioxidants are generally of dietary origin and are vitamins like Vitamin C and Vitamin E, carotenoids, quinones and polyphenols. They usually work as scavengers and metal chelators (Krinsky, 2002; Vaya and Aviram, 2001). As far as endogenous antioxidants are concerned they are divided into two categories protein and non-protein endogenous antioxidants. Among those, which are protein in nature are metal binding proteins in blood plasma. They are thought to regulate the fabrication of free radicals, which are activated or promoted by metal catalysis. Examples include albumin, ceruloplasmin and copper ion sequesters. Oxidation process is also hampered by non

proteinaceous endogenously localized antioxidants by foraging activity and is lipoic and uric acids, bilirubin, ubiquinone, and glutathione (Ivanova and Ivanov, 2000; Shahidi, 1997 and Halliwell and Gutteridge, 1990).

The genus *Polygonum* (*Polygonaceae*) includes about 150 species, producing secondary metabolites like flavonoids like quercetin, avicularin, plantagin and taxifolin etc (Isobe *et al.*, 1980; 1981). This Genus is widely distributed around the world, mostly in temperate regions. High polyphenolic content of genus *Polygonum* might have associated with certain alluring biological activities (Gong *et al.*, 2002).

The *Polygonum amplexicaule* has common name “masloon” and is widely distributed in the North Pakistan with flowering season June to September. *P. amplexicaule* is a member of the genus *Polygonum*, it is being speculated that like the other member of genus antioxidants content is high. The motivational and inspirational thought for the related study came from the traditional use of *P. amplexicaule* for its medicinal attributes for treatment of flue, fever joint pain and gastrointestinal disorders (Qureshi *et al.*, 2007).

It has also been reported that the respective specie is found active in curing heart problems, dysentery, menstruation issues and ulcer (Matin *et al.*, 2001). In a study it was reported that plant is very high in antioxidants and contains two novel antioxidant viz, kellaone and amplexicine (Tantry *et al.*, 2012).

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The present study, which was based upon the antioxidants assays, will significantly contribute towards the development of antioxidants based medicines for the cure of patho-physiological disorders. The objectives of the present study were to determine the antioxidant activity from different parts of *P. amplexicaule* through bioassay guided fractionation. Second objective was to determine the phenolics through HPLC.

MATERIALS AND METHODS

Sample preparation and fractionation

The plant *P. amplexicaule* was obtained from Murree hills and a voucher specimen after identification was deposited in ISL Herbarium Islamabad, Pakistan. Rhizome and aerial parts (shoots and leaves) of the plants were washed, dried, ground and dissolved in 80% methanol in 1:3 proportions to obtain crude methanolic extract (CME). From each crude extract, aqueous (Aq), Ethanolic (Eth), Ethyl acetate (EAc) and n-Butanolic (NB) fractions were made according to their polarity index.

Antioxidant activity (DPPH assay)

To determine antioxidant activity in various fractions (NB, EAc, Eth and Aq) of all three crude extracts i.e leaf, shoot and rhizome, the free radical scavenging activity was calculated by using DPPH assay. The assay was executed in accordance to the method illustrated by Kulisic *et al.* (2004) modified by Obeid *et al.* (2005). About 2800 µl of DPPH solution and 200 µl of sample solutions (1000 ppm, 100 ppm, 10 ppm, 1 ppm, 0.1ppm, 0.01) made in methanol were incubated for one hour at room temperature. OD was taken at 517 nm by spectrophotometer (Hitachi). A mixture of 200 L of methanol and 2800 L of DPPH solution was taken as negative control, Methanol (82 %) as blank and ascorbic acid as positive control. The formula given below was used to calculate percentage inhibition and graphical method was used to calculate IC₅₀ values.

Scavenging effect (%) = [(Ac-As)/Ac] x100

Whereas “As” is the Absorbance of test sample and “Ac” is Absorbance of negative control

DNA plasmid protection assay

For DNA protection assay pBR322 (Fermentas) plasmid was obtained by transformation in *E. coli* DH5α. Eppendorf tube was used to make reaction mixture with final volume of 15 µL comprising of; about 0.5 µg plasmid, 3 µL of 50 mM phosphate buffer (pH 7.4), 3 µL of 2 mM FeSO₄, 5 µL of each samples. As control untreated supercoiled DNA was either treated with 30 % H₂O₂ or 2 mM FeSO₄ or both. After 1 hr incubation at 37 °C, agarose gel electrophoresis was conducted. Ethidium bromide was used to stain DNA bands, which might be supercoiled, linear, and open circular depending upon the conditions and were analyzed qualitatively with the help

of scanning by Doc-IT computer program (VWR). The results hence obtained were evaluated for antioxidant or prooxidant effects on DNA, which were obvious by the increase or loss percentage of supercoiled monomer, as compared to the control value.

Thin Layer Chromatography (Tlc)

Extract which was having the maximum antioxidant activity was detected through TLC. The plate used was of alumina. An aliquot of the respective solution (1 mg/ mL, 3 mL) was directly spotted onto the TLC plates. TLC plates were developed in the chamber with n-hexane, toluene, ethyl acetate and formic acid (2:5:2.5:0.5) as developing reagents and the solvent was allowed to run until it reached 1 cm from the top of plates. The developed TLC plates were removed from the TLC chamber, and were air-dried for at least 30 min. After drying, spraying was done with a 2.54 mM DPPH solution in methanol solution. Formation of yellow bands on purple background was an indication of DPPH scavenging activity. Each TLC plate was also monitored under UV light at 254 and 366 nm (Gu *et al.*, 2009).

High-pressure liquid chromatography (HPLC)

For the qualitative and quantitative identification of antioxidants, extract with high antioxidant activity was analyzed through HPLC. For the separation of antioxidants chromatographic conditions for HPLC includes the use of reversed phase C18 column with UV-VIS diode assay and a binary solvent system which includes two solvents, solvent A and solvent B. Solvent A consisted of acidified water like aqueous acids. Solvent B was acetonitrile. The separation time was set to 1 hour at a flow rate of 1.0-1.5 mL/min and automated results were analyzed (Apati *et al.*, 2003).

Results

This study was appraised to evaluate the antioxidants activities in *P. amplexicaule* by DPPH radical scavenging assay and DNA plasmid protection assay and determination of active fractions by using HPLC.

DPPH radical Scavenging Assay

The antioxidant activity of crude methanolic extract of rhizome, shoot and leaf of *Polygonum amplexicaule* was determined. The Tables 1-3 indicate the IC₅₀ value for the crude methanolic extract and fractions of leaf, shoot and rhizome of *P. amplexicaule*. According to the results; the IC₅₀ value of *P. amplexicaule* leaf extract is 1.03 µg/mL that is analogous to ascorbic acid that is 0.49 µg/mL. Among the different fractions of the leaf extracts studied antioxidant activity of leaf CME and EAc was highest with an IC₅₀ of 1.03 and 3.1µg/mL of DPPH respectively. Among different IC₅₀ values Ethylacetate fraction (EtAc) is most active and n-Butanol fraction (NB) of leaf is least active.

The shoot extract and its fractions are not very active as compare to leaf extract. Here the highest activity was observed by CME of shoot with an IC_{50} value of 1.90 $\mu\text{g/mL}$ and nearest to it was shown by EAc fraction of shoot which is 13.4 $\mu\text{g/mL}$. The NB fraction of shoot and Aq fraction of shoot have also shown significant activity having IC_{50} value 33 $\mu\text{g/mL}$ and 39 $\mu\text{g/mL}$ respectively. Least active was Eth fraction of shoot with an IC_{50} 58.2 $\mu\text{g/mL}$. Ethanolic fraction (Eth) of rhizome extract of *P. amplexicaule* has shown the maximum activity and its IC_{50} value was found to be 3.1 $\mu\text{g/mL}$, whereas NB fraction of Rhizome and EAc fraction of Rhizome were found to be 4.2 and 5.4 $\mu\text{g/mL}$ respectively.

DNA plasmid protection assay

DNA protective activity of *P. amplexicaule* for all three samples (and their fractions) was assessed using pBR322 plasmid. Three concentrations (1000 ppm and 100 ppm and 10 ppm) of all extracts along with their fractions were used. The experiments were conducted in dark to avoid photo excitation of samples. The results of the assay for CME of leaf have as is obvious in table 4 that only 1000 ppm has not shown any DNA protection potential whereas rest of the concentrations 100 and 10 ppm has shown plasmid protection potential. Shoot extract and its fractions have shown very good potential as far as the results of Plasmid protection assay is concerned as is shown in table 5. Among all the experimented concentrations 1000, 100 and 10 ppm of CME of shoot only 100 ppm has shown Plasmid protection. Rhizome and its fractions were also evaluated for DNA protection potential of the relevant antioxidants present in it as is shown in table 6. It was observed that both 10 and 100 ppm has got DNA protection effects except 1000 ppm in the case of CME of rhizome.

Isolation of Antioxidants by Thin Layer Chromatography (TLC)

The TLC bio-autography-guided strategy was used for the qualitative analysis of the antioxidant compounds from plant extracts of *P. amplexicaule* since very less was known about the antioxidant potential of the plant under consideration.

Comparing all three samples shoot EAc, leaf EAc and rhizome EAc give two most obvious and intense bands whose R_f value was calculated to be 0.40 and 0.30 respectively. All other samples have got R_f value in a range of 0.18-0.5.

Identification of Antioxidants by HPLC

The extract and fractions, which gave the maximum antioxidant activity, were further subjected to HPLC. The extracts and fractions, which were analyzed included ethyl acetate fractions of rhizome, leaf and shoot extracts along with crude methanolic extracts of leaf and rhizome. The total content of flavonoids and phenolics along with carotenoids and polyphenols set the criteria to assess the

antioxidant content of any medicinal plant (Larson, 1997). Promising results were obtained as far as flavonoids and phenolics are concerned. Catechin and caffeic acid were found in all the fractions tested, whereas rutin was found in EAc fraction of shoot, CME fraction of leaf and CME fraction of Rhizome. Gallic acid was found in all the fractions except EAc fraction of shoot. Only EAc fraction of shoot and CME of leaf have found to have myricetin. Quercetin was found in CME of leaf only whereas Kaempferol was found in EAc fraction of leaf and CME of Rhizome. The HPLC analysis of *Polygonum multiflorum* has also been reported for presence of emodin and quercetin as antioxidants in stem and root (Lin et al., 2010).

DISCUSSION

Plants are considered potent for the production of biologically active phytochemicals. These phytochemicals show certain biological activities in vitro depending upon the nature of the plant product. Antioxidants are the compounds capable of inhibiting oxidation of other compounds. Inside the plant they are found playing vital roles as accessory pigments and pollinators and thwart oxidative harms of tissues from reactive oxygen species to DNA, enzymes, RNA and proteins. They are anti-inflammatory as well. These biological activities of antioxidants are widely exploited in combating diseases and benefit of mankind in various ways. Antioxidants are highly concerned with Alzheimer's Diseases and Parkinson's disease. Rheumatic and pulmonary disorders are also found in association with the level of antioxidants in the body and there is a much more to be explored yet.

P. amplexicaule is well known for its phytochemicals and been traditionally used as folk medicine since centuries. In order to establish its pharmaceutical value as source of antioxidants, certain bioassays were employed using crude methanolic extracts (CME) of shoot, leaf and rhizome and their various fractions (NB, EAc, Eth and Aq). The extracts and fractions with significant activities were also analyzed for active phenolics and flavonoids by chromatographic techniques like TLC and HPLC.

DPPH assay was used to assess antioxidants. The IC_{50} value of crude methanolic extracts (CME) of shoot, leaf and rhizome samples along with their fractions were determined. The maximum activity was shown by CME of leaf having IC_{50} 1.03 $\mu\text{g/mL}$, where as all other fractions show a positive result for the presence of antioxidants with varying degrees of IC_{50} ranging from 1.03-58.2 $\mu\text{g/mL}$. The maximum potent fraction was EAc for shoot, leaf and rhizome extracts with IC_{50} 3.1, 13.4 and 5.4 $\mu\text{g/mL}$ respectively.

Comparing all the three samples along with their fractions, the CME of leaf has shown maximum activity with IC_{50} 1.03 $\mu\text{g/mL}$, as compared to the CME of shoot

and CME of rhizome with IC_{50} values of 1.90 and 3.5 $\mu\text{g/mL}$ respectively. Among butanolic fractions rhizome NB has been proven most active with IC_{50} value of 4.2 $\mu\text{g/mL}$ while rest of both NB fraction of shoot and NB fraction of leaf have got 33 $\mu\text{g/mL}$ and 51 $\mu\text{g/mL}$ IC_{50} values. It has been observed that plant extracts contains a significant amount of antioxidants and this biological activity is found associated with the presence of polyphenols counting tannins and flavonoids (Shahidi *et al.*, 1992; Pietta 1998).

Highes antioxidant activities were shown by the EAc fraction of rhizome and EAc fraction of leaf with IC_{50} value of 3.1 $\mu\text{g/mL}$ while EAc fraction of shoot has 13.4 $\mu\text{g/mL}$ IC_{50} value. Aqueous fractions of all the three samples have shown IC_{50} values 39 $\mu\text{g/mL}$, 39 $\mu\text{g/mL}$ and 41.2 $\mu\text{g/mL}$ respectively. All These results were comparable to Ascorbic acid, which was taken as standard whose IC_{50} value was found out to be 0.49 $\mu\text{g/mL}$. In another study hydroxy-methyl-furfural was found as an effective antioxidant from *P. multiflorum* Thunb. but after steam processing its IC_{50} value was found to be 1.6 $\mu\text{g/mL}$ (Liu *et al.*, 2011). In another study phenyl propanoid esters of sucrose namely hidropiperosides were isolated form *P. hydropiper* L. from its methanolic extract and their IC_{50} was calculated as 23.4 and 26.7 $\mu\text{g/mL}$, respectively (Kiem *et al.*, 2008).

Antioxidants from the plant under study were also analyzed for its DNA plasmid protection capabilities when the DNA was exposed to the ROS along with the three different concentrations of samples 1000, 100 and 10 ppm in order to assess DNA damage and protective effects of the sample under study. Very encouraging results were obtained through Agarose gel electrophoresis. Maximum protection was observed in the case of 10 ppm and 100 ppm concentrations of crude methanolic extract of rhizome and leaf extract, aqueous fractions of shoot and leaf extract and n-butanolic fractions of shoot and leaf extract along with ethanolic fraction of rhizome extract. Higher concentrations i-e 1000ppm has pro-oxidant activities mainly but only a few of them show DNA protection ability, which was aqueous fractions of leaf and shoot extract.

For isolation of different antioxidants present in shoot, leaf and rhizome crude extracts and their fractions was assessed by the help of autobiography TLC. In which the presence of antioxidants in the sample was evaluated in VIS for the formation of yellow spots on purple background. The entire set of fractions gave positive results in HPLC profile for the highly potent fractions has shown very promising results regarding the presence of antioxidants in rhizome and aerial parts of plant. Ethyl acetate fractions of rhizome, shoot and leave were subjected HPLC along with CME fraction of rhizome and CME of leaf. Results indicated the presence of gallic acid

in EAc fraction of leaf, EAc fraction of rhizome, CME fraction of leaf and CME fraction of rhizome. Whereas Catechin and Caffeic acid was found in all the fractions. Rutin was found EAc fraction of shoot, CME fraction of rhizome and CME fraction of leaf. Myricetin was found in EAc fraction of shoot and CME of leaf. CME of leaf was the only fraction which has shown the presence of quercetin and Kaempferol was found in EAc fraction of leaf and CME fraction of rhizome respectively.

It is suggested that highly potent fraction should be used for the pharmaceutical and cosmetic formulations and plant should be worked out for the enhanced production of antioxidants *in vitro* in order to save the highly valuable medicinal plant from being extinct from local flora.

CONCLUSION

The *P. amplexicaule* extracts has shown significant antioxidant and DNA protection potential. The active fractions contains phenolics and flavonoids in the plant leave, shoots and rhizome which includes gallic acid, quercetin, catechin, caffeic acid, rutin, myricetin and Kaempferol with already established antioxidant activities.

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