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Antioxidant Properties of Methanolic Extracts of *Argemone mexicana*

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ABSTRACT

Antioxidants activities from plant sources have attracted a wide range of interest across the world in recent times. This is due to growing concern for safe and alternative sources of antioxidants. The free radical scavenging activity using peroxidase assay, Ferric Reducing Ability of Plasma (FRAP), Lipid Peroxidation assay (LPO), ABTS (2,2'-azinobis (3-ethylbenzothiazoline-6-sulfonic acid) diammonium) and the estimation of total phenolics content using the Folin-Ciocalteu reagent and total flavonoid content using aluminium chloride method were carried out on the methanol extracts of different plant parts of *Argemone mexicana*. *Argemone mexicana* extract effectively scavenged free radicals at all different concentrations and showed its potent antioxidant activity. Further, these effects were in a dose dependent manner. The fruits exhibited remarkable ability to scavenge the species LPO (39.193±1.257 MDA/g DW), ABTS (68.23±3.02 mM L^{-1} g^{-1}) while flowers showed maximum peroxidase activity (0.513 mM min^{-1} g^{-1} DW) and FRAP (287±9.64). The total phenolic content was in the range 4.0-23.5 mg GAE/g DW while the total flavanoid content was in range 3.5-34.5 mg QE/g DW. The results suggest that methanol extract of the plant is very rich in antioxidant compounds and worthy of further investigations.

Key words: *Argemone mexicana,* free radical scavenging activity, polyphenols ABTS, FRAP

INTRODUCTION

Plants are important source of potentially useful structures for the development of new chemotherapeutic agents. The different plant parts used include root, stem, flower, fruit, twigs exudates and modified plant organs possess varied medicinal properties and they are traded in the market as the raw material for many herbal industries (Uniyal *et al*., 2006). Although, hundreds of plant species have been tested for antioxidant properties the vast majority of have not been adequately evaluated (Balandrin and Klocke, 1988). Terrestrial plants began producing antioxidants such as ascorbic acid, polyphenols and tocopherols as part of their adaptation. Development of angiosperm plants between 50 and 200 million years ago produced antioxidant pigments as chemical defenses against reactive oxygen species produced during photosynthesis (Venturi *et al*., 2000; Benzie, 2003).

Antioxidants are secondary constitute or metabolites found naturally in plants. An antioxidant can be defined in simple terms substances that delay or inhibits oxidative damage to a target

molecule. Oxygen is an element obligatory for life, living systems have evolved to survive in the presence of molecular oxygen and for most biological systems. Oxygen has double edged properties, being essential for life it can also aggravate the damage within the cell by oxidative events (Shinde *et al*., 2006). Reactive Oxygen Species (ROS) consist of superoxide radical anion ($O_2^{\bullet}$), hydroxyl radical ($^{\bullet}OH$), singlet Oxygen ($^1O_2$) and hydrogen peroxide (H_2O_2) are after generated as byproducts of biological reaction or from exogenous factors (Kikuzaki and Nakatani, 1993). Free radicals (superoxide, hydroxyl radicals and nitric oxide) and other reactive species (hydrogen peroxide, hypochloric acid and proxynitrite) produced during aerobic metabolism in the body (oxidation of carbohydrates, fats and protein) can cause oxidative damage of amino acids, lipids, protein and DNA (Halliwell, 1991; Gutteridge, 1995) are removed by antioxidative defence mechanism.

There is a balance between generation of ROS and antioxidant system in organism. In pathological condition, ROS are over produced and result in lipid peroxidation and oxidative stress (Dharmistitha *et al*., 2010). It has been established that oxidative stress is among the major causative factors in the induction of many chronic and degenerative diseases including atherosclerosis, ischemic heart disease, ageing, diabetes mellitus, cancer, immuno suppression, neuro degenerative diseases and others (Branen, 1975; Buyukokuroglu *et al*., 2001; Gulcin *et al*., 2005; Gyamfi *et al*., 1999; Devasagayam *et al*., 2004; Duan *et al*., 2006). As a result of this, much attention has been focused on the use of antioxidants, especially natural antioxidants to inhibit lipid peroxidation and to protect from damage due to free radicals Phytoconstituents present in medicinal plant are important source of antioxidant and capable to terminate the free radical chain reaction (Cody *et al*., 1986; Liu and Ng, 2000; Oluwaseun and Ganiyu, 2008). The importance of the antioxidant constituents of plant materials in the maintenance of health and protection from aging related disease has intrigued scientist for a long time. It has been mentioned that antioxidant activity of plants might be due to their phenolic compounds (Duh *et al*., 1999).

Argemone mexicana Linn. (Papaveracae), also known as mexican prickly poppy (Khare, 2007) is a well known weed in the agricultural and waste lands. The seed are used as emetic, demulcent, laxative (Nadkarani, 1982) and antidote in snake poisoning. It has also been investigated for anticatalepsy activity, antihistamic activity (Facchini, 2001), hepatoprotective activity (Adam *et al*., 2011), antimicrobial activity (Willcox *et al*., 2007), antiallergic activity, antistress, larvicidal activity, antidiabetic activity (Rout *et al*., 2011), antioxidant, anti-inflammatory activity (Srivastava and Srivastava, 1998) and wound healing activity (Dash and Murthy, 2011). It is also used in the treatment of jaundice, leprosy, piles, dysentery and warts (Magora and Cole, 2001).

In this study, we presented the results of the antioxidant activities of methanol extracts of the different plant parts (leaves, stems, fruits, flowers and roots) of *A. mexicana*. The findings from this study may add to the overall value of the medicinal potential of the herbs.

MATERIAL AND METHODS

Plant material: The different plant parts (leaves, stem fruits, flowers and roots) of *A. mexicana* were collected in the month of November-December from the Jaipur-Delhi highway. It was washed with tap water, air dried at room temperature to constant weights and ground to fine powder. The plant was botanically identified and authenticated in the Department of Botany, University of Rajasthan, Jaipur, Rajasthan, India and a voucher specimen (No. RUBL20874) was deposited at the herbarium of botany department of the University of Rajasthan.

Chemicals: Chemical like 2,2_-azinobis-3-ethylbenzothiazoline-6-sulfonic acid (ABTS), ascorbic acid, quercetin, gallic acid, potassium persulphate was obtained from Sigma (St. Louis, Missouri, USA). Folin-Ciocalteus's phenol reagent, sodium carbonate, hydrogen peroxide, pyrogallol, sodium dihydrogen phosphate, dihydrogen hydrogen phosphate were from Merck Chemical Supplies (Damstadt, Germany). All the chemicals used were of analytical grade.

Total phenolic and flavonoidal content

Plant extraction: Two gram each of the dry material was extracted with 25 mL of methanol at room temperature for 48 h, filtered through Whatman No. 1 filter study, stored and used for quantification.

Total phenolic content: Total phenolic compound contents were determined by the Folin-Ciocalteau method (Mcdonald *et al*., 2001; Ebrahimzadeh *et al*., 2008a, b; Nabavi *et al*., 2008). The extract samples (0.5 mL; 1; 10 diluted) were mixed with Folin Ciocalteu reagent (5 mL, 1:10 diluted with distilled water) for 5 min and aqueous Na_2CO_3 (4 mL, 1 M) were then added. The mixture was allowed to stand for 15 min and the phenols were determined by colorimetric method at 765 nm. The standard curve was prepared using the standard solution of Gallic acid in methanol in the range 20-200 μg mL^{-1} (R^2 = 0.987). Total phenol values are expressed in terms of Gallic acid equivalent (mg g^{-1} of dry mass)which is a common reference compound. Total phenolic content can be calculated from the equation:

$$T = \frac{CV}{M}$$

Where:
T = Total phenolic concentration
C = Concentration of gallic acid from caliberation curve (μg mL^{-1})
V = Volume of extract (mL)
M = Wt. of methanol plant extract

Total flavonoidal content: Total flavonoid content was determined by using aluminium chloride colorimetric method ($AlCl_3$) according to the known method (Dewanto *et al*., 2002; Sakanaka *et al*., 2005) with slight modifications using quercetin as standard. About 1 mL of test material was added to 10 mL volumetric flask containing 4 mL of water. To above mixture, 0.3 mL of 5% $NaNO_2$ was added. After 5 min, 0.3 mL of 10% $AlCl_3$ was added. After 6 min, 2 mL of 1M NaOH was added and the total volume was made up to 10 mL with distilled water. Then the solutions were mixed well and absorbance was measured against blank at 510 nm. The standard curve was prepared using the standard solution of Quercetin in methanol in the range 0.5-5.0 mg mL^{-1} (R^2 = 0.991). Total flavonoidal content of the extracts was expressed in milligram of quercetin equivalents per gram of dry weight. Total flavonoidal content can be calculated from the equation:

$$T = \frac{CV}{M}$$

Where:
T = Total flavonoidal concentration
C = Concentration of quercetin from caliberation curve (mg mL^{-1})
V = Volume of extract (mL)
M = Wt. of methanol plant extract

Ascorbic acid content

Extraction and quantification: Each of the fresh experimental material was homogenized thoroughly with 10 mL of acetate buffer (ph 4.8) and centrifuged (1200 rpm, 20 min). The supernatants were separately collected, out of which 1 mL was measured to other test tube; From the stock solution of ascorbic acid (10 mg/100 mL in 4% TCA), varied concentrations (0.01-0.09 mg mL^{-1}) were also prepared in different test tube, 4 mL of 4% trichloroacetic acid (TCA) was then added to all test tubes, left overnight and later, centrifuged (Roe and Kuether, 1943). To the supernatant of each sample, 1 mL of the colour reagent (prepared by mixing 90 mL of 2.2% 2, 4 dinitrophenylhydrazine in 10 N H_2SO_4, 5 mL of 5% thiourea and 5 mL of 0.6% $CuSO_4$ solution) was added and incubated at 57°C for 45 min. Later, on cooling 7 mL of 65% was H_2SO_4 added to each mixture, brought to the room temperature and the ODs were measured at 540 nm in spectrophotometer against a blank. A regression curve was computed between the optical density and the concentration of standard ascorbic acidwhich followed beer's law.

Determination of antioxidant activity

Reducing ability (FRAP assay): The determination of the total antioxidant activity (FRAP assay) in the extract is a modified method of Benzie and Strain (1996). The stock solutions included 300 mM acetate buffer (0.3 M acetic acid and 0.3 M sodium acetate), pH 3.6, 10 mM TPTZ (2, 4, 6-tripyridyl-*s*-triazine) solution in 40 mM HCl and 20 mM ferric chloride ($FeCl_3.6H_2O$) solution. The fresh working solution was prepared by mixing 25 mL acetate buffer, 2.5 mL TPTZ and 2.5 mL $FeCl_3.6H_2O$. The temperature of the solution was raised to 37°C before use. Plant extracts (100 µL each of methanolic ethyl acetate and hexane) were allowed to react with 2900 µL of the FRAP solution for 30 min in the dark condition. Readings of the coloured product (ferrous tripyridyltriazine complex) were taken at 593 nm. The standard curve was linear between 100 and 1000 µM $FeSO_4$. Results are expressed in mM Fe (II) g^{-1} dry mass.

Peroxidase assay: The method of assay measures the oxidation of pyrogallol to purpurogallin by peroxidase when catalyzed by peroxidise at 420 nm and at 20°C. Plant sample (200 mg) was homogenized with 10 mL of phosphate buffer and refrigerated centrifuged at 10000 rpm for 20 min. The clear supernatant was taken as the enzyme extract. The activity was assayed after the method of Chance and Maehley (1955) with following modifications. 2.4 mL of phosphate buffer, 0.3 mL pyrogallol (50 µM) and 0.2 mL of H_2O_2 (30%) were added. The amount of purpurogallin formed was determined by taking the absorbence at 420 nm immediately after adding 0.1 mL enzyme extract. The extinction coefficient of 2.8 mM cm^{-1} was used in calculating the enzyme activity that was expressed in terms of millimole per minute per gram dry weight.

Lipid Peroxidation Assay (LPO): LPO activity was calculated using the protocol of Heath and Packer (1968). The 0.5 g of dry material was homogenized with 10 mL of 0.1% (w/v) Trichloroacetic acid (TCA). The homogenate was centrifuged for 5 min (15000 g, 4°C). Supernatant was collected

and 1 mL of supernatant was mixed with 4 mL of 0.5% (w/v) TBA (Thiobarbituric acid) in 20% (w/v) TCA and then incubated in water bath at 95°C for 30 min. Reaction was quickly ended by incubating on an ice bath. In case the solution is not clear, centrifuge at 10000 g for 10 min and the absorbance was measured at 532 and 600 nm. The OD_{600} values were subtracted from MDA-TBA complex values at 532 nm and MDA concentration was calculated using the Lambert-Beer law with an extinction coefficient èM = 155/mM/cm. Results were presented as µmol MDA/g.

ABTS radical scavenging assay: To determine ABTS radical scavenging assay, the method of Re *et al.* (1999) was adopted. The stock solutions included 0.002 M ABTS solution and 0.07 M potassium persulphate solution. The working solution was then prepared by mixing the 25 mL of ABTS stock and 0.1 mL of potassium persulphate stock and allowing them to react for 12 h at room temperature in the dark. The solution was then diluted by mixing ABTS solution with ethanol to obtain an absorbance of 0.706±0.001 units at 734 nm using the spectrophotometer. Fresh ABTS solution was prepared for each assay. Plant extracts (1 mL) at varying concentration were allowed to react with 3 mL of the ABTS solution and the absorbance was taken at 734 nm after 6 min using the spectrophotometer. The ABTS scavenging capacity of the extract was compared with that of BHT and percentage inhibition calculated as:

$$\text{Inhibition (\%)} = \frac{\text{Abs}_{\text{sample}}}{\text{Abs}_{\text{control}}} \times 100$$

where, $Abs_{control}$ is the absorbance of ABTS radical + methanol and Abs_{sample} is the absorbance of ABTS radical + sample extract/standard.

Statistical analysis: Experimental results are expressed as Means±Standard Deviation (SD). All measurements were replicated three times. The IC_{50} values were also calculated by linear regression analysis. Experiments results were further analyzed for Pearson correlation coefficient (r) between total phenolic, flavanoid and different antioxidant assay systems using the Microsoft Excel 2007 software and two way analysis of variance (ANOVA) was applied to investigate the differences among means by using software. The values were considered to be significantly different at $p<0.05$.

RESULTS AND DISCUSSION

Total phenolics, flavonoids, ascorbic acid: Results obtained in the present study revealed that the level of phenolic compounds in methanol extracts of *A. mexicana* were considerable (Table 1). Highest amount of TPC, TFC and ascorbic acid was recorded in flowers (23.5 mg GAE/g DW and

Table 1: Total phenolic, flavonoidal and ascorbic acid content in different parts of *A. mexicana*

Plant parts	Total phenolic content (mg GAE/g DW)	Total flavonoidal content (mg QE/g DW)	Ascorbic acid content(mg g^{-1} DW)
Leaves	4.5±0.035	32.5±3.28	0.503±0.023
Stems	13.5±2.312	6.25±0.335	0.3±0.01
Fruits	10.5±0.730	9.375±0.36	0.225±0.01
Flowers	23.5±1.010	34.50±0.221	1.6±0.036
Roots	4.0±0.426	3.5±0.35	0.1±0.002

Table 2: Total antioxidant (FRAP) activity of methanol, ethyl acetate and hexane extracts of different plant parts of *A. mexicana*

Plant part	Methanol extract	Ethyl acetate extract	Hexane extract
Leaves	249.19±1.32	51.80±3.16	22.33±1.38
Stems	112.67±2.08	38.33±1.53	19±4.58
Fruits	197.66±7.78	40.33±2.89	30.39±2.08
Flowers	287±9.64	69.43±5.03	18.16±1.15
Roots	30.68±1.15	18.39±2.52	13.66±3.51

Table 3: ABTS activity (IC_{50}) in different plant parts of *A. mexicana* (mmole/min/g DW)

Plant part	Methanol extract
Leaves	262.18±21.88
Stems	517.82±7.08
Fruits	68.96±3.48
Flowers	553.75±39.10
Roots	120.85±11.80

34.5 mg QE/g DW). Polyphenolic compounds are known to have antioxidant activity and it is likely that the activity of the extracts is due to these compounds (Tepe *et al*., 2006). This activity is believed to be mainly due to their redox propertieswhich play an important role in quenching singlet and triplet oxygen, or decomposing peroxides and adsorbing and neutralizing free radicals (Zheng and Wang, 2001). In fact, many medicinal plants contain large amount of phytochemicals. Many of these phytochemical possess significant antioxidant capacities that are associated with lower occurrence of human diseases (Djeridane *et al*., 2006).

Reducing ability (FRAP): The reducing ability of the extracts was in the range of 12-290 μM Fe (II)/g (Table 2). The antioxidant potentials of the methanol, ethyl acetate and hexane extracts of different plant parts of *A. mexicana* were estimated from their ability to reduce TPRZ-Fe (III) complex to TPTZ-Fe (II). Ferric Reducing Antioxidant Power (FRAP) method is based on the reduction of a ferroin analogue, the Fe^{3+} complex of tripyridyltriazine $Fe(TPTZ)^{3+}$ to the intensely blue-coloured Fe^{2+} complex $Fe(TPTZ)^{2+}$ by antioxidants in acidic medium. The FRAP values for the methanol extracts were significantly higher than that of ethyl acetate and hexane fractions. The ferric reducing/antioxidant power (FRAP assay) is widely used in the evaluation of the antioxidant component in dietary polyphenols (Luximon-Ramma *et al*., 2002). Antioxidant activity increased proportionally to the polyphenol content. According to recent reports, a highly positive relationship between total phenols and antioxidant activity appears to be the trend in many plant species (Sharma *et al*., 2013; Bhardwaj *et al*., 2014).

ABTS radical scavenging activity: The methanol extracts were fast and effective scavengers of the ABTS radical. Proton radical scavenging is an important attribute of antioxidants. ABTS, a protonated radical, has characteristic absorbance maxima at 734 nm which decreases with the scavenging of the proton radicals (Mathew and Abraham, 2006). Fruits were most effective in scavenging the ABTS radical (IC_{50} = 68.96±3.48 mmole/min/gDW) (Table 3). Factors like stereo selectivity of the radicals or the solubility of the extract in different testing systems have been reported to affect the capacity of extracts to react and quench different radicals (Yu *et al*., 2002). This further showed the capability of the extracts to scavenge different free radicals in different systems, indicating that they may be useful therapeutic agents for treating radical-related pathological damage.

Table 4: Peroxidase activity in different plant parts of *A. mexicana* (mmole/min/g DW)

Plant part	Methanol extract
Leaves	0.295±0.007
Stems	0.102±0. 004
Fruits	0.143±0.011
Flowers	0.326±0.046
Roots	0.096±0.015

Table 5: Lipid peroxidation assay (LPO) activity in different plant parts of *A. mexicana* (mmole/min/g DW)

Plant part	Methanol extract
Leaves	28.15±1.45
Stems	3.89±0.30
Fruits	39.19±1.26
Flowers	24.12±1.43
Roots	10.126±0.49

Peroxidase assay: Peroxidase are widely distributed in nature and are found in plants, micro-organisms and animals, where they catalyze the reduction of hydrogen peroxide (H_2O_2) to water, rendering it harmless. The H_2O_2 is a common and product of oxidative metabolism and being a strong oxidizing agent, could prove toxic if allowed to accumulate. Thus, peroxidases serve to rid plant cells of excess H_2O_2 under normal and stress conditions. In present investigation, flowers showed maximum peroxidise activity (0.326±0.045 mmole/mim/g DW) and decreases in the order leaves, fruits, stems and roots (Table 4).

Lipid peroxidation assay (LPO): Lipid Peroxidation (LPO) is oxidative deterioration of polyunsaturated lipids and it involves Reactive Oxygen Species (ROS) and transition metal ions. It is a molecular mechanism of cell injury leading to the generation of peroxides and lipid hydroperoxideswhich can decompose to yield a wide range of cytotoxic products, most of which are aldehydes such as malondialdehyde (MDA) and 4 hydroxynonenal. The LPO is a highly destructive process that affects cellular organelles and causes them to lose biochemical function and/or structural integritywhich may lead to irreparable damage or cell death. In present investigation, fruits have showed maximum activity (39.19±1.26 mmole/min/g DW) (Table 5).

Correlations: Pearson correlation coefficient was positively high if $0.65 \leq, r \geq 0.97$ (Thaipong *et al.*, 2006). The correlation coefficients of TPC, TFC and different antioxidant activities are depicted in Table 6. The highest positive correlation between TPC and peroxidise activity was observed in leaves (r = 0.904) but there was poor correlation between TFC and peroxidise activity. Fruits have showed highest and positive correlation between TPC and LPO activity (r = 0.797) and TPC and ABTS scavenging activity (r = 0.985) and TFC and LPO activity (r = 0.974). The highest negative correlation was observed between TFC and LPO activity in flowers (r = -0.975). The fruits had positively high correlation between phenolic content and ABTS and LPO activity. This data indicated that higher total phenolic content in fruits would have given higher scavenging activity in both LPO and ABTS activity. There was negatively high correlation between TFC and LPO activity in flowers (r = -0.975) and TPC and ABTS activity in leaves (r = -0.844). It illustrated that higher content of phenols and flavonoids in them would give lower ABTS and LPO activity. Further

Table 6: Pearson's correlation coefficient of total phenolic and total flavonoid of methanolic extracts of different plant parts of *A. mexicana* and ABTS, LPO and peroxidase antioxidant activity

Activity	Total phenol	Total flavanoid
Peroxidase		
Leaves	0.904	0.130
Stems	-0.366	0.006
Fruits	-0.342	-0.500
Flowers	-0.877	0.026
Roots	0.293	0.275
LPO		
Leaves	0.602	-0.950
Stems	-0.643	0.340
Fruits	0.797	0.974
Flowers	0.691	-0.975
Roots	-0.930	-0.916
ABTS		
Leaves	-0.844	0.770
Stems	0.334	0.023
Fruits	0.985	0.763
Flowers	-0.710	-0.250
Roots	0.230	0.265

the negative correlation between TPC, TFC and different antioxidant activity assay models suggested that it could be related to other antioxidant compounds contained in the plants (Sharma *et al*., 2013).

CONCLUSION

Determination of the natural antioxidant compounds of plant extracts will help to develop new drug candidates for antioxidant therapy. In the present study, the total phenolic, flavonoidal, ascorbic acid content and antioxidant activity of methanolic extracts of different plant parts was measured by different assay models in *A. mexicana*. The study reveals the potential of methanolic extracts of plant to scavenge the harmful free radicals. The methanol extracts of plant exhibited different levels of antioxidant activity in all models studied. Well proved potent antioxidant activity of *A. mexicana* in the present study, strongly emphasize that it can be used as an accessible source of natural antioxidants with potential to provide protection against free radicals induced damage to biomolecules. Further investigation of individual compounds with their *in vivo* antioxidant activities and different antioxidant mechanisms is needed.

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