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Full Length Research Paper

Studies on the genoprotective/antioxidant potential of methanol extract of *Anthocephalus cadamba* (Roxb.) Miq

Madhu Chandel[1], Satwinderjeet Kaur[1]* and Subodh Kumar[2]

[1]Department of Botanical and Environmental Sciences, Guru Nanak Dev University Amritsar-143005, Punjab, India.
[2]Department of Chemistry, Guru Nanak Dev University Amritsar-143005, Punjab, India.

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***Anthocephalus cadamba* (Roxb.) Miq. (Rubiaceae), an Ayurvedic medicinal plant is ethnomedicinally widely used in the treatment of fever, anaemia, uterine complaints, blood diseases, skin diseases, leprosy, dysentery, and for improvement of semen quality. The present work was carried out to evaluate the antioxidant potential of methanol extract from the bark of *A. cadamba.* The antioxidant activity was determined by *in vitro* assays viz. DPPH radical scavenging assay, ABTS radical cation decolorization assay and reducing power assay. The extract was evaluated for DNA protection activity in DNA protection assay using pBR322 plasmid DNA. The various antioxidant activities were compared to standard antioxidants such as butylated hydroxytoluene and ascorbic acid. The extract showed potent antioxidant activity in all the assays. The percentage inhibition of DPPH radical was 77.97±0.301% and for ABTS it was found to be 91.70±0.40% at a concentration of 200 µg/ml. The reducing power was observed to be 59.47±0.801% at the highest tested dose of 1000 µg/ml. The extract also showed good genoprotective potential comparable to gallic acid. In addition to the antioxidant activity of the extract, the total phenolic content and total flovonoid content were also measured.**

Key words: *Anthocephalus cadamba*, antioxidant activity, DPPH assay, ABTS assay, plasmid DNA protection assay, reducing power assay.

INTRODUCTION

The interest in medicinal plants has increased together with the number of investigations into their biological effects on human beings and animals (Gulcin et al., 2010; Veiga et al., 2005). Oxidative DNA damage is thought to be an important contributing factor in aging, as well as in the etiology of many human diseases, including atherosclerosis, diabetes, neurodegenerative diseases and even cancer (Marnett, 2000; Olinski et al., 2002). Oxidative damage can result when the critical balance between free radical generation and antioxidant defence is unfavourable (Lampe, 1999). Excessive reactive oxygen species (ROS), can oxidize critical target molecules. They can generate DNA damage by producing single strand breaks, double strand breaks, alkali labile sites, and oxidized purines and pyrimidines (Sankaranarayanan, 1991). Hydrogen peroxide (H_2O_2), a ROS may be involved in the formation of hydroxyl radicals which are highly reactive, destructive and results in direct DNA damage (Gulcin et al., 2010; Meneghini and Martins, 1993).

To protect molecules against toxic free radicals and other ROS, cells have developed antioxidant defences by endogenous enzymatic and/ non-enzymatic components that prevent radical formation, remove radicals before damage can occur, repair oxidative damage, eliminate damage molecules, and prevent mutations (Gordon, 1990).

Though an efficient antioxidant defence system is present in the cells, it may be overwhelmed under conditions of oxidative stress (Bansal et al., 2005). One of the potential uses of plant-derived compounds is as antimutagenic agents (Calomme et al., 1996; Hayder et al., 2008) and antioxidants (Yagi et al., 2002; Ben et al., 2007). Natural antioxidants contained in medicinal and

*Corresponding author. E-mail: sjkaur2011@gmail.com.

aromatic plants, fruits and vegetables may be useful in preventing the deleterious consequences of oxidative damage and are therefore considered as potential chemopreventive agents (Serbetci and Gulcin, 2010; Balaydin et al., 2010; Kitts, 1994; Verhagen et al., 1997; Kris-Etherton et al., 2002).

In addition to scavenging of ROS, chelation of metal ions (such as iron and copper, which initiate radical reactions) and inhibition of enzymes (responsible for free radical generation) (Edenharder and Grunhage, 2003), antioxidants can interfere with xenobiotic metabolizing enzymes, block activated mutagens/carcinogens, modulate DNA repair and even regulate gene expression (Brigelius-Flohe and Traber, 1999; Craig, 1999; Heo et al., 2001; Kris-Etherton et al., 2002; Nikolic et al., 2004). All these mechanisms may be important for the antimutagenic and anticarcinogenic properties of antioxidants (De Flora and Ferguson, 2005). In the last two decades, a wide range of evidence from epidemiological and laboratory studies have demonstrated that some plants eaten whole, or some of their active principles taken in isolation, have substantial protective effects against human carcinogenesis and mutagenesis (Surh and Ferguson, 2003). Several plant extracts have proved to contain a wide variety of antimutagenic substances (Verschaeve et al., 2004; Scassellati-Sforzolini et al., 1999; Khader et al., 2010; Wongwattanasathien et al., 2010; Kaur et al., 1998, 2000, 2001, 2009) and some can prevent cancer (Nishino, 1998; Saleem et al., 2005; Chang et al., 2009; Hu et al., 2010; Inayat-Hussain et al., 2010).

A. cadamba (Roxb.) Miq. (Rubiaceae) is known as wild cinchona and popular in India as “Kadamb”. Its bitter and pungent bark is used in ayurvedic medicine for uterine complaints, blood diseases, leprosy and dysentery. A decoction of the leaves is recommended as a gargle in cases of stomatitis (Kirtikar and Basu, 1935). Keeping in mind, the ayurvedic importance of *A. cadamba*, the present study was planned to evaluate the antioxidant potential of methanol extract of bark of this plant.

MATERIALS AND METHODS

Collection of plant material and extraction

The bark of *A. cadamba* was collected from the trees growing in the campus of Guru Nanak Dev University, Amritsar, Punjab, India. The plant was identified from the Herbarium of Department of Botanical and Environmental Sciences, G.N.D.U. Amritsar.

Bark was washed with running water to remove any dust impurities and dried at 40ºC. The material was finely powdered and percolated with 80% methanol to obtain the Methanol extract (MAC extract).

Chemicals

2, 2-Diphenyl-1-picrylhydrazyl (DPPH), Ferric chloride, L-Ascorbic acid were obtained from HiMedia Pvt. Limited. Mumbai, India. Potassium persulfate, ABTS [2,2-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt], Gallic acid, from Sigma (St. Louis, MO, USA). Plasmid pBR322 DNA was purchased from Genei Pvt. Ltd., Banglore. All other reagents used were of analytical grade.

Phytochemical analysis

Determination of total phenolic content

The total phenolic content of MAC extract was determined using Folin-Ciocalteu method (Yu et al., 2002) and gallic acid was used as standard. To 100 µl of MAC extract was added 900 µl of double distilled water followed by the addition of 500 µl of FC reagent. This was followed by the addition of 1.5 ml of 20% sodium carbonate. The mixture was shaken thoroughly and allowed to stand for 2 h. The volume of mixture was made up to 10 ml with distilled water and allowed to stand for 2 h. Finally absorbance was taken at 765 nm. The phenolic content was calculated as gallic acid (mg/g) equivalents on the basis of standard curve of gallic acid and described by equation $y = 0.004 x - 0.065$ ($R^2 = 0.997$) where y = absorbance and x = concentration.

Determination of total flavonoid content

The method given by Kim et al., (2003) was used for determination of total flavonoid content (TFC) employing rutin as a standard. Total flavonoid content of the MAC extract was determined using colorimetric method. To 1 ml of MAC extract, 4 ml of double distilled water was added followed by addition of 300 µl of $NaNO_2$ and 300 µl of $AlCl_3$. This mixture was incubated for 5 min. To this mixture 2 ml of NaOH was added and final volume was raised to 10 ml. Finally absorbance was taken at 510 nm. The flavonoid content was calculated as rutin (mg/g) equivalents.

Antioxidant activity

DPPH-radical scavenging assay

The DPPH radical scavenging assay is commonly employed in evaluating the ability of antioxidants to scavenge free radicals. The effect of antioxidants on DPPH is thought to be due to their hydrogen donating ability (Gulcin et al., 2010). It was carried out by the method of Blois (1958) with modifications. Different concentrations of extract of *A. cadamba* were dissolved in methanol and taken in test tubes in triplicates.

Then 2 ml of 0.1 mM methanol solution of DPPH (2,2-Diphenyl-1-picrylhydrazyl) was added to each of the test tubes and were shaken vigorously. After 30 min absorbance was taken at 517 nm using UV-VIS spectrophotometer. The control was prepared without the test compound. Ascorbic acid was used as standard. % Radical scavenging activity (%) = [Abs (control) – Abs (sample) /Abs (control)] ×100. where, Abs (control): Absorbance of DPPH radical + vehicle alone, Abs (sample): Absorbance of DPPH radical +MAC extract.

ABTS radical scavenging assay

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 (Talaz et al., 2009). The spectrophotometric analysis of

$ABTS^{\cdot+}$ scavenging activity will be determined according to the protocol given by Re et al., 1999. ABTS radical cation was produced by reacting ABTS stock solution (7 mM) and potassium persulfate (2.45 mM) and allowing the mixture to stand in the dark at room temperature for 12 to 16 h before use. The ABTS radical cation solution was diluted with ethanol to an absorbance of 0.70 (±0.02) at 734 nm. 300 µl of MAC extract/standard compound was added to the diluted ABTS radical cation solution and absorbance reading was taken up to 5 min.

Reducing power assay

Reducing potential of MAC extract was determined using the method of Oyaizu (1986). Different concentrations of the extract of *A. cadamba* were dissolved in methanol and taken in test tubes in triplicates. To the test tubes 2.5 ml of phosphate buffer (pH 6.6, 0.2 M) and 2.5 ml of 1% Potassium ferricyanide solution was added. These contents were mixed well and were incubated at 50˚C for 20 min. After incubation, 2.5 ml of 10% TCA was added and were kept for centrifugation at 3000 rpm for 10 min. After centrifugation, 2.5 ml of supernatant was taken and mixed with double distilled water (2.5 ml) and ferric chloride (0.5 ml, 0.1%). The O.D (absorbance) was measured spectrophotometrically at 700 nm. Increase in absorbance of reaction mixture was interpreted as increase in reducing ability of the extract and the results were compared with ascorbic acid. The percentage of reduction of the sample as compared to standard (ascorbic acid) was calculated using the formula:

Percentage (%) of reduction power = [1-(1-As/Ac)×100]
As = absorbance of standard at maximum concentration tested
Ac = absorbance of sample.

Plasmid DNA protection assay

DNA nicking experiment was performed according to the protocol of Lee et al. (2002). Plasmid DNA (5 µg) was incubated with Fenton's reagent (30 mM H_2O_2, 50 mM ascorbic acid and 80 mM $FeCl_3$) containing extract and finally the volume of the mixture was raised up to 20 µl. The mixture was then incubated for 30 min at 37°C followed by addition of loading dye. Electrophoresis was carried out in TAE buffer (40 mM Tris base, 16 mM acetic acid 1mM EDTA, pH 8.0) and DNA was analyzed followed by ethidium bromide staining. The gallic acid was used as the standard reference compound.

RESULTS

Total phenolic and flavonoid content

The total phenolic content of the methanol extract (100 µg/ml) of *Anthocephalus cadamba* was found to be 280.7 mg gallic acid equivalent/g of extract, by reference to standard curve ($y = 0.004x-0.059$, $r^2 = 0.998$). The flavonoid content was found to be 424 mg rutin equivalents/g of the extract, by reference to standard curve ($y = 0.001x-0.001$, $r^2 = 0.998$).

DPPH radical scavenging activity

The method is based on the reduction of methanolic DPPH solution because of the presence of antioxidant substances having hydrogen donating groups (RH) such as phenolics and flavonoids compounds due to the formation of non radical DPPH form (Paixao et al., 2007). The antioxidant activity of MAC extract from bark of *A. Cadamba* was 77.97±0.301% at the highest tested dose of 200 µg/ml. The quality of antioxidants in the extract was determined by the IC_{50} value, low IC_{50} value indicates strong antioxidant activity in a sample. IC_{50} (concentration providing 50% inhibition) was calculated graphically using a calibration curve by plotting the extract concentration vs the corresponding scavenging effect. The IC_{50} value of MAC extract was found to be 97.43 µg/ml. The potential of ascorbic acid to scavenge DPPH radical became almost stable after 60 µg/ml and there was no increase in scavenging activity from the concentration 60 µg/ml (93.27±0.046%) to 200 µg/ml (94.11±0.028%) (Figure 1).

ABTS radical scavenging activity

The MAC extract exhibited effective ABTS radical cation scavenging activity. It showed a concentration dependent scavenging of ABTS radicals with IC_{50} being 77.82 µg/ml compared to that of BHT (42.75 µg/ml). The radical scavenging of the extract at the highest tested dose of 200 µg/ml (91.70 %) was close to that of BHT at the same concentration (95.75%) (Figure 2).

Reducing power assay

The IC_{50} value of the extract was found to be 829.73 µg/ml. Ascorbic acid was used as the standard compound. The reducing capacity of the extract increased with an increase in concentration (Figure 3). The reducing properties are generally associated with the presence of reductones (Duh, 1998), which have been shown to exert antioxidant action by breaking the free radical chain by donating hydrogen atom (Gordon, 1990). Reductones are also reported to react with certain precursors of peroxide, thus preventing peroxide formation (Gulcin, 2009, 2010).

Plasmid DNA protection assay

DNA protective activity was assessed by measuring the degree of protection on DNA scission that was induced by the attack of hydroxyl radicals, which was shown by the agarose gel electrophoresis pattern. It is clear from the results that extract scavenged the hydroxyl radicals and protected the pBR322 plasmid DNA (Figure 4).

DISCUSSION

In this study, MAC extract exhibited a concentration

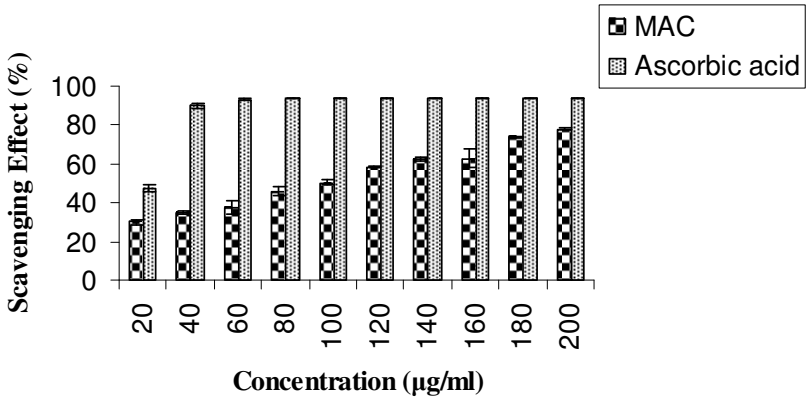

Figure 1. DPPH radical scavenging activity of Methanol extract (MAC) from bark of *Anthocephalus cadamba.*

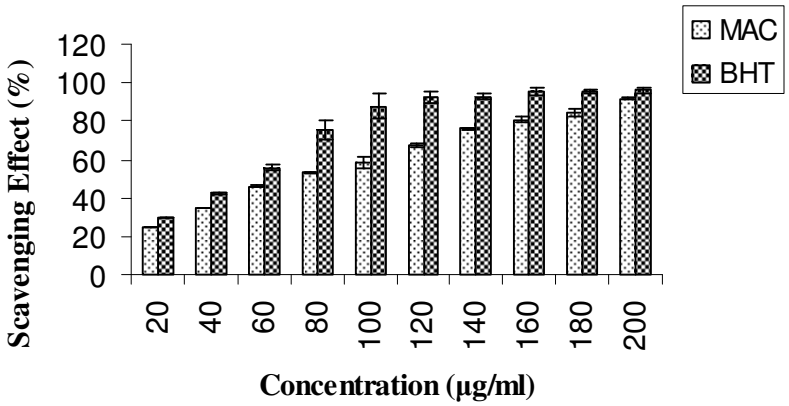

Figure 2. ABTS radical cations Scavenging activity of Methanol extract (MAC) from bark of *Anthocephalus cadamba*.

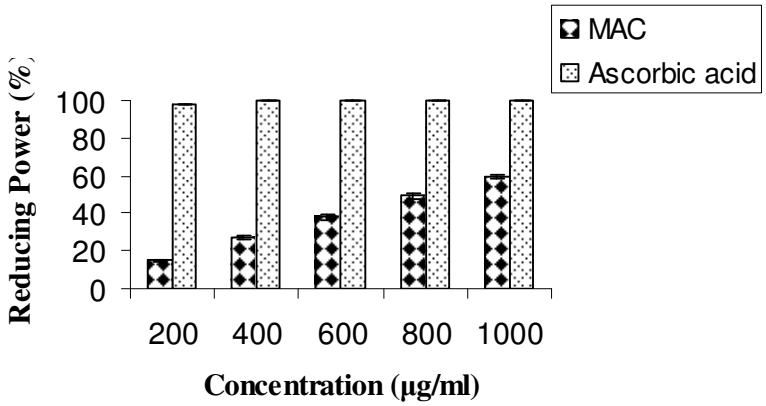

Figure 3. Reducing potential of the Methanol extract from bark of *Anthocephalus cadamba.*

dependent antiradical activity resulting from reduction of $DPPH^{\bullet}$, $ABTS^{+\bullet}$ radicals to their non-radical forms. The results are similar to those reported by Gulcin et al. (2009), where they evaluated the antioxidant activities of secoiridoids from root bark of *Chionanthus virginicus* using $DPPH^{\bullet}$, $ABTS^{+\bullet}$ superoxide scavenging, reducing

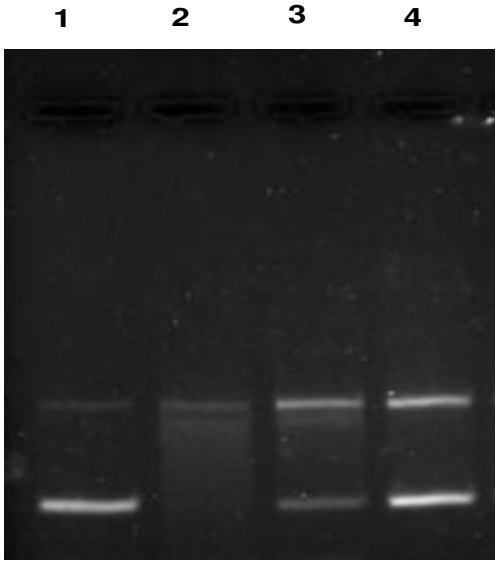

Figure 4. Effect of methanol extract of bark of *A. cadamba* in DNA protection assay. Lane 1: Negative Control (DW+DNA), Lane 2: Fenton's reagent (FR) + DNA, Lane 3: FR+DNA+gallic acid (200 µg/ml), Lane 4: FR+DNA+methanol extract (MAC) (200 µg/ml).

activity, hydrogen-peroxide scavenging and ferrous metal chelating activity assays and observed potent antioxidant activity of secoiridoids in all the assays. In an another study Gulcin (2009) studied the antioxidant activity and antioxidant mechanism of L-adrenaline using various *in vitro* assays and found L-adrenaline as potent scavenger of free radicals. $DPPH^{\bullet}$, $ABTS^{+\bullet}$ radicals accept an electron from compounds (antioxidants) to become stable non-radical forms. DPPH assay is considered as a valid assay to evaluate radical scavenging activity of antioxidants, since the radical compound is stable and does not have to be generated as in other radical scavenging assays. From Figure 1, we observe that a dose response relationship is found in the DPPH radical scavenging activity. The involvement of free radicals, especially their increased production, appears to be feature of most human diseases, including cardiovascular disease and cancer (Deighton et al., 2000). It has been found that cysteine, glutathione, ascorbic acid, tocopherol, flavonoids, tannins and aromatic amines reduce and decolourise DPPH by their hydrogen donating ability (Blois, 1958; Yokozawa et al., 1998).

Phytoconstituents of the *Anthocephalus cadamba* extract are probably involved in their antiradical activity. Generation of ABTS radical cation forms the basis of one of the spectro-photometric methods for the measurement of radical scavenging activity. *A. cadamba* reduced ABTS radical cations to non-radical forms in a concentration dependent manner. Therefore, the plant has significant antioxidant capacity in terms of scavenging both $DPPH^{\bullet}$ and $ABTS^{+\bullet}$ radicals. Mariod et al. (2009) studied the antioxidant activity of methanol extract and polyphenol rich fractions of *Nigella sativa* seedcake and observed potent free radical scavenging activity on $DPPH^{\bullet}$. The antioxidant activity of aqueous and methanol extracts of the stem bark of *Dalbergia sissoo* was measured using DPPH assay and the IC_{50} value of aqueous extract was found to be 12.23±1.11 µg/ml while that of methanol extract was 23.63±1.65 µg/ml which were comparable to gallic acid (12.14±1.05 µg/ml) (Roy et al., 2011). Loo et al. (2008) studied the $ABTS^{+\bullet}$ scavenging activity of three com-pounds isolated from pyroligneous acid of *Rhizophora apiculata* and the scavenging activity was found to be 90.82±6.33% in 3-methoxycatechol, 89.39±6.86% in catechol and 83.48±4.71% in syringol. The MAC extract was studied for its reduction potential in reducing power assay. The reducing power of the MAC extract of *A. cadamba* increases with increase in concentration. The substances with reduction potential, react with potassium ferricyanide ($Fe[(CN)_6]_3$) to form potassium ferrocyanide ($Fe[(CN)_6]_2$), which then reacts with ferric chloride to form ferric ferrous complex ($Fe_4[Fe(CN^-)_6]_3$) that has an absorption maxima at 700 nm. An increase in the absorbance of reaction mixture would indicate an increase in reducing potential due to an increase in the formation of the complex. The reducing capacity of extract may serve as a significant indicator of the potential antioxidant activity. Visavadiya et al. (2009) found the greater reductive activity of ethanolic extract of seeds of *Sesamum indicum* than that of standard of α-tocopherol. Rohman et al. (2010) found the direct correlation between the phenolic compounds and reducing potential of extracts of *Pandanus canoideus*.

Moein et al. (2008) showed that the reducing power of the *Salvia mirzayanii* ethyl acetate fraction was less than all subfractions that may be due to the fact that during fractionation process an increase in the antioxidant activity occurred. DNA nicking assay is a sensitive assay for the detection of potent oxidants such as hydroxyl radicals generated via Fenton type reaction which interacts with DNA and cause single-strand breaks of supercoiled DNA into the open circular state or convert supercoiled DNA to linear form by double strand breaks. These forms can be separated from each other and detected by monitoring their migration in agarose under the influence of an electric field. It is clear from Figure 4 that the MAC extract of *A. cadamba* has the ability to protect the pBR322 plasmid DNA from the damage caused by hydroxyl radicals generated in Fenton's reaction. The protective effect of MAC can be explained by its ability to scavenge ROS or it may directly interact with DNA thereby protecting DNA from free-radical induced strand breaks. Kalpana et al. (2008) studied the antioxidant potential of aminothiazole derivatve and its protective effects on H_2O_2-induced oxidative damage on PBR322 DNA and RBC cellular membrane and attributed the significant capacity of the molecule to its ability to

scavenge various radicals. Thind et al. (2008) assessed the extracts of leaves of *Tabernaemontana divaricata* for their ability to scavenge hydroxyl radicals in plasmid nicking assay with pBR322 and observed that all the extracts inhibited the unwinding of supercoiled DNA. Manikandan et al. (2009) evaluated the *Azadirachta indica* leaf fractions for *in vitro* antioxidant potential and protective effects against H_2O_2-induced oxidative damage to pBR322 DNA and red blood cells and suggested that the antioxidant and protective effects of neem leaf fractions can be attribute to their ability to inhibit various free radicals. Total phenolic content in MAC extract was found to be 280.7 mg GAE/g of the extract and the flavonoid content was 424 mg RE/g of the extract. Many of the phenolics have been shown to possess high antioxidant activity. These polyphenols have attracted a great deal of public and scientific interest because of their health promoting effects as antioxidants (Rice-Evans et al. 1997). Phenolic compounds may contribute to the overall antioxidant activity of plants mainly due to their redox properties, which can play an important role in adsorbing and neutralising free radicals, quenching singlet and triplet oxygen, or decomposing peroxides (Osawa, 1994). Liu et al. (2008) evaluated the antioxidant activity of two types of buckwheat sprouts viz. *Fagopyrum tataricum* and *Fagopyrum esculentum* and observed that rutin was the major flavonoid in both the plant extracts and that it played an important role in the antioxidant capacity of buckwheat sprouts. Phenol, flavonoid contents and antioxidant activity of Iranian medicinal plants was determined by Pourmorad et al. (2006) and concluded that high content of phenols and flavonoids in *Mellilotus officinalis* could be responsible for its high radical scavenging activity. Thus, from The results obtained it is observed that MAC extract which is rich in phenolic/flavonoid compounds showed significant antioxidant potential in terms of scavenging free radicals and protection of pBR322 plasmid DNA. The present work indicated that *A. cadamba* harbours phytoconstituents with potent genoprotective/antioxidant potential which need to be isolated.

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