



Contents lists available at ScienceDirect

Asian Pacific Journal of Tropical Disease

journal homepage: www.elsevier.com/locate/apjtd

Document heading

Assessment of the antioxidant potential of *Pergularia daemia* (Forsk.) extract *in vitro* and *in vivo* experiments on hamster buccal pouch carcinogenesis

Kandhan Karthishwaran and Sankaran Mirunalini*

Department of Biochemistry and Biotechnology, Annamalai University, Annamalainagar, Tamil Nadu, India. 608002

ARTICLE INFO

ABSTRACT

Article history:

Received 15 March 2011

Received in revised form 27 April 2011

Accepted 28 May 2012

Available online 28 June 2012

Keywords:*P.daemia*

DMBA

Carcinogenesis

Reactive oxygen species

Antioxidants

Objective: The aim of the present study was to investigate the *in vitro* antioxidant assays and *in vivo* non enzymatic antioxidant potential of aerial parts of *Pergularia daemia* methanolic extract (PDME) on 7, 12-dimethylbenz(a)anthracene (DMBA) induced hamster buccal pouch (HBP) carcinogenesis. **Methods:** The plant extract was tested for their total phenol and flavonoid content and radical scavenging effect such as 2, 2'-azinobis- (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS*) radical decolorization assay, DPPH (2, 2-diphenyl, 2-picryl hydrazyl) radical scavenging, nitric oxide radical scavenging, reducing power assays. **Results:** According to IC₅₀ values obtained, PDME contains more powerful antioxidant compounds than gallic acid. The level of plasma vitamin C and the levels of Vitamin E and GSH in the plasma and erythrocyte lysate were decreased while the buccal tissue levels of vitamin E and GSH were increased in DMBA induced animals. Pretreatment with *P.daemia* (200 mg/kg bw) to DMBA induced animals significantly ($P < 0.05$) increased the levels of plasma and erythrocyte vitamin C, vitamin E and GSH where as the levels of vitamin E and GSH in the buccal tissue were increased compared to DMBA alone induced animals. **Conclusions:** The PDME exhibited strong antioxidant activity in free radical scavenging assays and hamster buccal pouch carcinogenesis.

1. Introduction

Carcinogenesis is the process by which cancer develops in a living organism. Oral squamous cell carcinoma can be induced in experimental animals by continued application of potent chemical carcinogens, such as DMBA and nitroquinoline-1-oxide etc. since 1950s, the Syrian golden hamsters are used in experimental studies on oral carcinogenesis. DMBA induced hamster buccal pouch carcinogenesis model has also histological, biochemical and molecular similarities to human oral cancer [1].

Reactive oxygen species (ROS) are a family of molecules

that are continuously generated, transformed and consumed in all living organisms as a consequence of aerobic life. The traditional view of these reactive oxygen metabolites is one of oxidative stress and damage that leads to decline of tissue and organ systems in aging and disease [2]. Recently attentions have been focused on the therapeutic potential of green foods and medicinal plants which is believed to reduce free radical induced tissue injury by trapping them [3]. Higher plants produce a variety of antioxidant compounds of which, polyphenols are assumed to be the most potent one [4]. Non enzymatic defence system included vitamin, Vitamin C and reduced glutathione (GSH). Vitamin C and E are non-enzymatic endogenous antioxidants also exist within normal cells and react with free radicals themselves which are less reactive than the radicals. The radical chain reactions by trapping peroxy and other reactive radicals [5].

The screening studies for antioxidant properties of medicinal and food plants have been performed increasingly for the last

*Corresponding author: Dr. S. Mirunalini Assistant Professor, Department of Biochemistry & Biotechnology, Annamalai University, Annamalainagar – 608 002, Tamil Nadu, India.

Tel No: +91 4144 239141

Fax: (91) 4144 238 145

E-mail: mirunasankar@gmail.com

few decades in hope of finding an efficient remedy for several present-day diseases including cancer [6]. In many developing countries, traditional medicine is one of the primary health care systems [7]. Herbs are widely exploited in the traditional medicine and their curative potentials are well documented [8]. Plants have been a rich source of medicines because they produce wide array of bioactive molecules, most of which probably evolved as chemical defense against predation or infection. The use of medicinal plants is increasing worldwide. Recent studies showed that a number of plant products including polyphenolic substances (e.g., flavonoids and tannins) and various plant or herb extracts exert potent antioxidant actions [9]. Plant and its products are rich sources of a phytochemicals and have been found to possess a variety of biological activities including antioxidant potential [10]. Natural antioxidants are in high demand for application as nutraceuticals, bio-pharmaceuticals, as well as food additive because of consumer preference.

P.daemia (Asclepiadaceae) is a well known Indian herbal drug. It is distributed all over India and has been used in many ways in medicine. The plant contains 2.4% of inorganic salts, a bitter resin and three bitter substances. It has a stimulant action on uterus, intestines, and other smooth muscles due to direct action on the smooth muscles. Aerial parts of this plant are reported to have various pharmacological activities like hepatoprotective [11], antifertility [12], anti-diabetic [13], wound healing [14], analgesic, antipyretic and anti-inflammatory [15] and Antifeedant activity [16]. Phytochemical screening of aerial parts of the herb showed positive test for alkaloids, flavonoids, hydrolysable tannins, phenols, sterols and terpenoids [17].

The objective of this study was to determine the antioxidant properties of *P.daemia* aerial parts using a set of in vitro antioxidant assays including scavenging of DPPH, nitric oxide and ABTS, as well as reducing power assay. The non enzymatic antioxidants such as Vitamin C, Vitamin E and GSH were also studied in vivo to reveal the antioxidant restoring capacity of *P.daemia*. The present study was to explore the antioxidant potential of aerial parts of *P.daemia*, a therapeutically important medicinal plant found in India and to make a comparison taking into account their ability to target multiple radical species and correlation of their antioxidant activity with total phenolic and flavonoid content were also explored.

2. Materials and methods

2.1. Chemicals

Dimethyl benza anthracene (DMBA), 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS^{•+}), 1,1-diphenyl-2-picryl hydrazyl (DPPH) radicals was purchased from sigma-aldrich, St Louis, MO, USA. All the other chemicals used were of analytical grade.

2.2. Plant Material

The fully mature aerial parts *Pergularia daemia* (Forsk.) were collected from Pudukkottai District, Tamil Nadu, India. The plant was identified by Dr. V. venkatesalu, Professor, Department of Botany, Annamalai University. A voucher specimen (ACC:196) was deposited in the herbarium of Department of Botany, Annamalai University.

2.3. Preparation of extracts

The flowering aerial parts of plants were dried in the shade and powdered so that all the material could be passed through a mesh not larger than 0.5 mm. powdered materials of each plant (1000 g) were soaked in 3 L of methanol (Merck Co., Germany) for 1 day, and the steps were repeated twice, followed by soxhlet apparatus by using methanol for 72 hrs. At the end of extraction, it was passed through Whatman filter paper No.1 (Whatman Ltd., England). The methanolic extract was concentrated to dryness under vacuum on rotary evaporator at 40 °C then reconstituted in minimum amount of DMSO and stored at 4 °C for further use.

2.4. Determination of total phenolic content

Total phenolic contents were determined by the Folin-Ciocalteu reagent method [18]. The extract samples (0.5 ml) were mixed with 2.5 ml of 0.2N Folin-Ciocalteu reagent for 5 min and 2.0 ml of 75 g/l sodium carbonate was then added. The absorbance of the reaction mixture was measured at 760 nm after 2 h of incubation at room temperature. The results were expressed as Gallic acid equivalents.

2.5. Total flavonoid content

Total flavonoids in the PDME were determined in the method of Jia et al., [19]. Different concentrations of PDME (100, 200, 300, 400, 500 µg/mL) were mixed with 0.075 mL of NaNO₂. After 6 min, 0.15 mL of aluminium chloride was added and allowed to stand for 5 min. After incubation, 0.5 mL NaOH was added and made upto 2.5mL with distilled water. The solution was well mixed and the absorbance was immediately measured at 510 nm using spectrophotometer. Quercetin at concentration ranging from (5–30 µg) was used as standard. The total flavonoid content in the extract was expressed as quercetin equivalents.

2.6. ABTS^{•+} radical cation decolorization assay

The generation of the ABTS^{•+} radical cation forms the basis of one of the spectrophotometric methods that has been applied for the measurement of the total antioxidant activity of solutions of pure substances [20]. ABTS^{•+} was dissolved in water at a concentration of 7mM. The stock solution was mixed

with 2.5 mM potassium persulphate (final concentration). The mixture was allowed to stand in the dark at room temperature for 12–16 h before use for incomplete oxidation of ABTS^{•+}. The radical was stable in this form for more than two days when stored in the dark at room temperature. The following reagents were added in the following order. The incubation mixture in a total volume of 5 mL contained 0.54 mL of ABTS^{•+}, 0.5 mL of phosphate buffer and varying concentrations of PDME (10, 20, 30, 40 and 50 μ g/ml). The blank contained water in place of PDME. The absorbance was read in spectrophotometer at 734 nm and compared with standard ascorbic acid at various concentrations.

$$\% \text{ Scavenging} = \frac{\text{Control OD} - \text{Test OD}}{\text{Control OD}} \times 100$$

2.7. DPPH radical scavenging activity

This method is based on the scavenging of DPPH through the addition of a radical species or an antioxidant that decolorizes the DPPH solution. Radical scavenging activity of *P.daemia* extract was measured according to the method of Williams et al., [21]. The reaction mixture containing 1 ml of 0.1 mM DPPH[•], various concentrations of extract (10, 20, 30, 40 and 50 μ g/ml) were made up to 3 ml with water. Then the tubes were incubated for 10 minutes. The blue colour chromophore is formed and the absorbance of this solution was measured at 517 nm. Gallic acid was used as standard for comparison. The percentage of free radical scavenging was calculated as per the formula mentioned above. A large decrease in the absorbance of the reaction mixture indicates significant free radical scavenging activity of the extract.

2.8. Reducing power assay

The reducing power of the samples is determined according to the method described by Oyaizu [22]. Substances, which have reduction potential, react with potassium ferricyanide to form potassium ferrocyanide, which then reacts with ferric chloride to form ferric–ferrous complex that has an absorption maximum at 700 nm. Varying volumes of (0.1–0.5 ml) of PDME containing 10, 20, 30, 40 and 50 μ g/ml were taken in the test tubes., mixed with a phosphate buffer (2.5 ml, 0.2 M, pH 6.6) and potassium ferricyanide (1%), and the mixture was incubated at 50 °C for 20 min. Next, 1.5 ml of trichloroacetic acid (10%) was added to the reaction mixture, which was then centrifuged at 3000 rpm for 10 min. The upper layer of the solution (0.5 ml) was mixed with distilled water (1 ml) and ferric chloride (0.5 ml, 1%), and the absorbance was measured at 700 nm. Gallic acid was used as standard for comparison.

2.9. Assay of nitric oxide–scavenging activity

The procedure is based on the principle that sodium nitroprusside in aqueous solution at physiological pH spontaneously generates nitric oxide which interacts with oxygen to produce nitrite ions that can be estimated using Griess reagent [23]. Scavengers of nitric oxide compete with oxygen, leading to reduced production of nitrite ions. For the experiment, sodium nitroprusside (10 mM), in phosphate–buffered saline, was mixed with different concentrations of each extracts dissolved in water and incubated at room temperature for 150 min. Following the incubation period, 0.5 ml of Griess reagent (1% sulfanilamide, 2% H₃PO₄ and 0.1% N–(1–naphthyl) ethylenediamine dihydrochloride) was added. The absorbance of the chromophore formed was read at 546 nm and Gallic acid was used as a standard.

2.10. Experimental animals and diet.

The whole experiment was carried out according to the guidelines of the Committee for the Purpose of Control and Supervision of Experiments on Animals, New Delhi, India and approved by the Animal Ethical Committee of Annamalai University (Proposal no.647: Dated 25.09.2009). The study was conducted on 24 golden Syrian hamsters (*Mesocricetus auratus*) weighing 90–120g, obtained from the Central Animal House, Department of Experimental Medicine, Rajah Muthiah Institute of Health Sciences, Annamalai University, Tamil Nadu, India. They were housed in polypropylene cages (47 x 34 x 20 cm), (six hamsters /cage) lined with husk, renewed every 24 h under a 12:12 h light dark cycle at around 22°C and had free access to tap water and food. The animals were fed on a standard pelleted diet (Pranav Agro Industries Limited, Maharashtra, India).

2.11. Experimental Design

The animals were divided into four groups of six animals each. Group I: Untreated control, animals were painted with paraffin alone; Groups II: Animals were given the extract of *P.daemia* at a concentration 200 mg/kg bw dissolved in 0.5% DMSO orally in alternative days for a period of fourteen weeks using intragastric tube; Groups III and IV: The left buccal pouches of animals in group 3 and 4 were painted with 0.5% DMBA three times a week. In addition with, group 4 received the intragastric administration of *P.daemia* methanolic extract at a concentration of 200 mg/kg bw on days alternate to DMBA application.

All the experiment was terminated at the end of 14 weeks and all animals were anesthetized with ketamine hydrochloride (24 mg/kg bw) and sacrificed by cervical decapitation. Blood was collected in two different tubes, i.e., one with anticoagulant for the separation of plasma and other without anticoagulant for the serum. Plasma and serum were separated by centrifugation for various biochemical estimations. The buccal tissues

were excised immediately and rinsed in ice-chilled normal physiological saline. Known weights of the tissues were homogenized in 5.0 ml of 0.1 M Tris hydrochloric acetic acid buffer (pH 7.4). The homogenates were centrifuged and the supernatant was used for the estimation of various biochemical parameters

2.12. Biochemical estimations

Biochemical studies were conducted on circulation of control and experimental hamsters in each group. A known amount of enzyme preparation was allowed to react with H₂O₂ in the presence of GSH for a specified time period. Then the remaining GSH content was measured. Reduced glutathione was estimated by the method of Ellman [24]. This method was based on the development of yellow colour when 5,5'-dithio-bis-(2-nitrobenzoic acid) (DTNB) was added to compounds containing sulphhydryl groups. Vitamin E level in the plasma and erythrocyte membranes were determined colorimetrically by the method of Desai [25]. Vitamin E present in the lipid residue forms a pink colored complex with bathophenanthroline-phosphoric acid reagent, which was measured at 536 nm. Tissue vitamin E was measured by the fluorimetric method of Palan et al., [26]. The level of plasma vitamin C was determined by the method of Omaye et al.,[27].

2.13. Statistical analysis

Statistical analysis was performed using one way analysis of variance followed by Duncan's Multiple Range Test using Statistical Package for the Social Science software (SPSS) package version 12.00. Results were expressed as mean±standard deviation for six animals in each group. *P* values <0.05 were considered statistically significant.

3. Results

3.1. The content of phenol and flavonoid

The content of phenolic compounds (mg/g) in methanolic extract was found 10.2 mg/g plant extract and expressed in gallic acid equivalents (GAE). The flavonoid content were found to be 4.65 respectively mg/g plant extract in quercetin equivalent.

3.2. ABTS free radical scavenging activity

The graph (Fig. 1) indicates the ABTS radical cation scavenging potential of the methanol extracts of the *P.daemia* at each concentration, in the range of 10–50 μ g/ml. The mean values across the concentration range indicate that the methanol extracts of *P.daemia* was highly potent in neutralizing ABTS cation radicals and was comparable with that of GAE. The scavenging capacity of the PDME was 54% at 20 μ g and 58

at 40 μ g GAE. The IC₅₀ value indicated that the ABTS free radical scavenging activity with a 50% inhibition (IC₅₀) at a concentration of 19.72 μ g/ml of PDME was nearly higher than GAE ((14.55 μ g/ml).

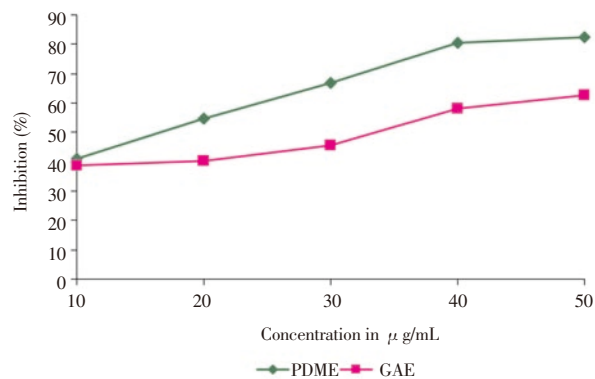


Figure 1. ABTS free radical scavenging activity of the methanolic extracts of *Pergularia daemia* in comparison with Gallic acid. Values are the average of triplicate experiments and represented as mean±standard deviation.

3.3. DPPH radical scavenging activity

The graph revealed for DPPH radical-scavenging activity of the methanol extracts of the *P.daemia* (Fig. 2) elucidating the mean values across the concentration range, indicates that the methanol extracts of *P.daemia* are more potent in scavenging the DPPH radicals generated in vitro, when compared to the standard GAE. The methanolic extract of PDME exhibited a significant dose dependent inhibition of DPPH activity, with a 50% inhibition (IC₅₀) at a concentration of 33.36 μ g/ml. The IC₅₀ value of the extract was found to be close to that of the standard; GAE (34.12 μ g/ml). Compared to GAE the extract exhibited a similar curve of antioxidant activity. This result demonstrated that PDME extract has inhibitory activity against the DPPH radical.

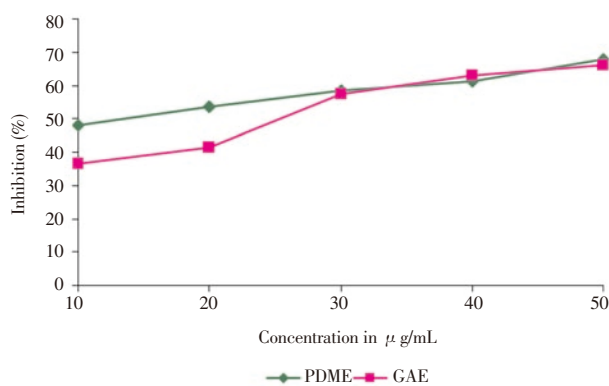


Figure 2. DPPH radical scavenging activity of the methanolic extracts of *Pergularia daemia* in comparison with Gallic acid. Values are the average of triplicate experiments and represented as mean±standard deviation.

3.4. Reducing power

Fig. 3 shows the reductive capabilities of the plant extract at each concentration, in the range of 10 to 50 μ g/ml compared to GAE. The reducing power of extract of PDME was very

potent and the reducing power of the extract was increased with quantity of sample. The plant extract could reduce the most Fe^{3+} ions, which had a lesser reductive activity than the standard of GAE. The methanolic extract of PDME exhibited a significant dose dependent inhibition of reducing power activity.

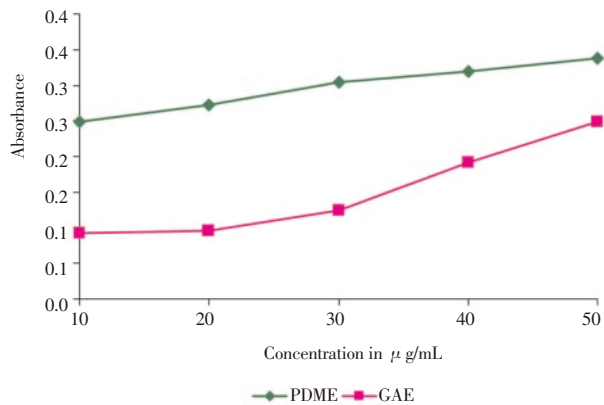


Figure 3. Reducing power of methanolic extracts of *Pergularia daemia* as compared to Gallic acid. Values are the average of triplicate experiments and represented as mean±standard deviation.

3.5. Nitric oxide radical inhibition

The scavenging of nitric oxide by plant extract was increased in a dose dependent manner as illustrated in fig. 4. The extract of the aerial parts showed moderately good nitric oxide scavenging activity of 58% at 10 µg/ml and 55 at 20 µg GAE. At concentration of 32.37 mg/ml of extract 50% of nitric oxide generated by incubation was scavenged, while IC_{50} value of standard GAE was found 34.19 mg/ml. The aerial parts extract showed higher nitric oxide radical scavenging activity.

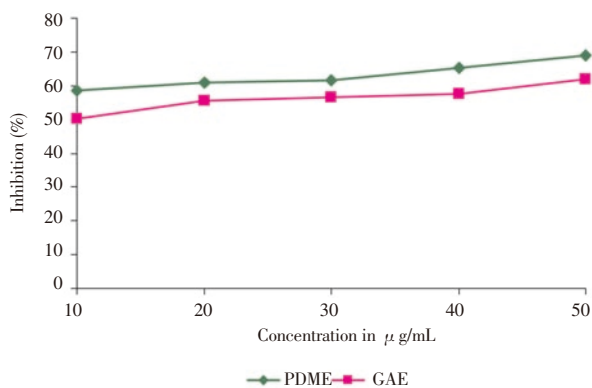


Figure 4. Scavenging effect of the methanolic extracts of *Pergularia daemia* and standard Gallic acid on Nitric oxide radical. Values are the average of triplicate experiments and represented as mean±standard deviation.

3.6. In vivo experiments—hamster buccal pouch model

Fig. 5 shows the levels of GSH in plasma, erythrocyte and buccal tissue of normal and experimental animals. Animals induced with DMBA showed significant ($P<0.05$) decrease in the levels of plasma and erythrocyte vitamin C, vitamin E and GSH

while the buccal tissue showed significant ($P<0.05$) increase in the levels of vitamin E and GSH compared to normal control animals. Animals treated with PDME alone showed no significant difference in non-enzymatic antioxidant status as compared to control animals. Oral pretreatment with PDME (200mg/kg) to DMBA painted animals significantly ($P<0.05$) increased the levels of plasma and erythrocyte where as the levels of GSH in the buccal tissue were increased compared to DMBA alone treated animals.

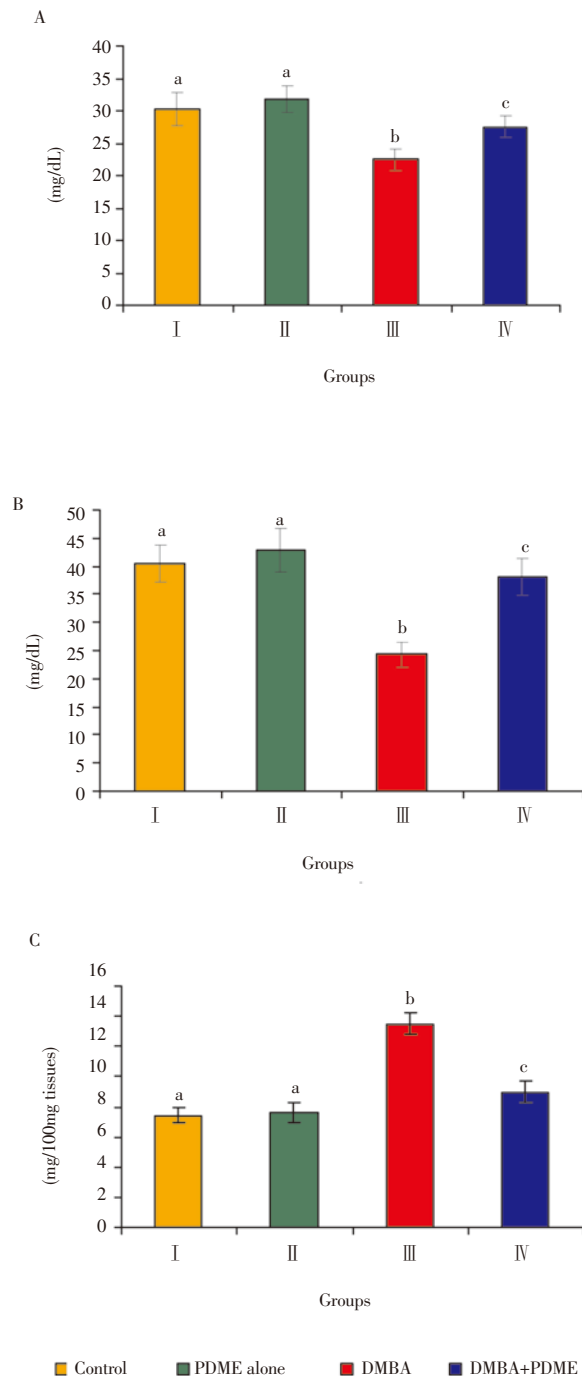


Figure 5. The levels of GSH in (A) Plasma, (B) erythrocytes and (C) Buccal tissue of control and experimental animals in each group. Group I: control; Group II: PDME alone (200 mg/kg bw); Group III: DMBA; Group IV: PDME (200 mg/kg bw) + DMBA. Each column is mean±SD for six animals in each group; columns that have different letters (a, b, c) differ significantly with each other ($P<0.05$; Duncan's multiple range test).

Fig. 6 depicts the levels of plasma vitamin C in normal and DMBA painted animals. Animals induced with DMBA exhibited significant ($P<0.05$) decreased in the levels of plasma vitamin-C compared with normal control animals. Animals treated with PDME alone (Groups II–IV) showed no significant difference in non-enzymatic antioxidant status as compared to control animals. Where as oral pretreatment with PDME (200mg/kg) to DMBA painted animals significantly ($P<0.05$) increased in the level of plasma in Vitamin C compared to DMBA alone induced animals

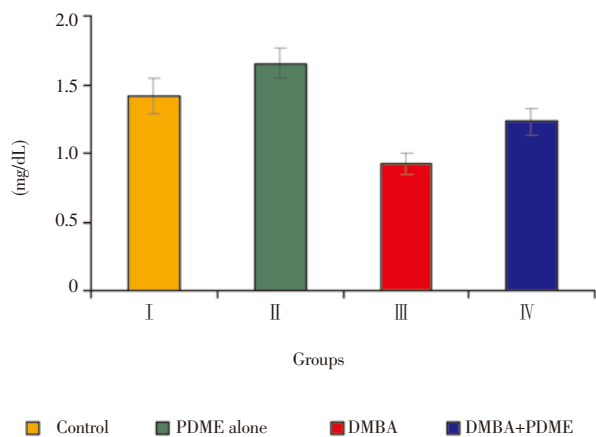


Figure 6. The levels of Vitamin C in Plasma of Control and experimental animals in each group of experimental design. Group I: control; Group II: PDME alone (200 mg /kg bw); Group III: DMBA; Group IV: PDME (200 mg/ kg bw) + DMBA. Each column is mean±SD for six animals in each group; columns that have different letters (a, b, c) differ significantly with each other ($P<0.05$; Duncan's multiple range test).

Fig. 7 depicts the levels of vitamin-E in plasma, erythrocyte lysate and buccal tissues of normal and DMBA painted animals. Animals induced with DMBA exhibited significant ($P<0.05$) decrease in the levels of Vitamin E in circulation, whereas increased in buccal tissue compared with normal control animals. Animals treated with PDME alone showed no significant difference in non-enzymatic antioxidant status as compared to control animals. Oral pretreatment with PDME (200mg/kg) to DMBA painted animals significantly ($P<0.05$) increased the level of circulation in Vitamin E, decreased in buccal tissue compared to DMBA alone induced animals.

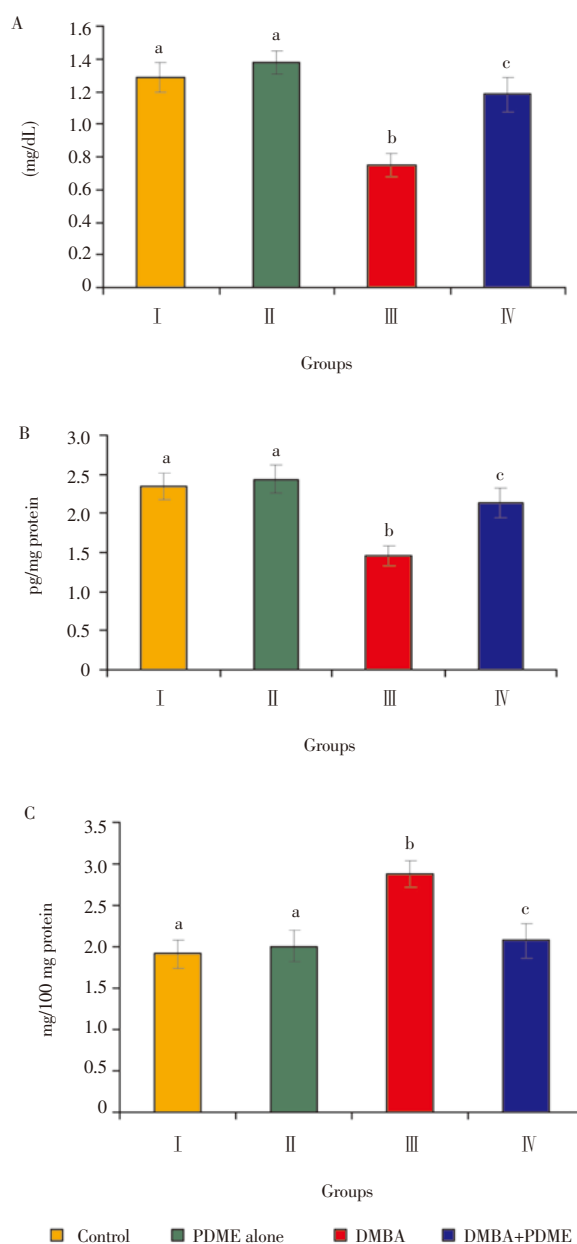


Figure 7. The levels of Vitamin E in (A) Plasma, (B) erythrocyte lysate and (C) Buccal tissue of Control and experimental animals in each group of experimental design. Group I: control; Group II: PDME alone (200 mg /kg bw); Group III: DMBA; Group IV: PDME (200 mg/kg bw) + DMBA. Each column is mean±SD for six animals in each group; columns that have different letters (a, b, c) differ significantly with each other ($P<0.05$; Duncan's multiple range test).

4. Discussion

The antioxidant activity of PDME is probably due to its polyphenolic contents. It is well known that phenolic compounds are constituents of many plants, and they have attracted a great deal of public and scientific interest because of their health promoting effects as antioxidants. Studies have shown that increasing levels of flavonoids in the diet could reduce the incidence of certain human diseases [28]. Numerous plant constituents have proven to show free radical scavenging or antioxidants activity [29]. The antioxidant activity of PDME

could be attributed to its flavonoidal content. Flavonoids act as scavengers of various oxidizing species i.e. super oxide anion ($O_2^{\cdot-}$), hydroxyl radical or peroxy radicals, they also act as quenchers of singlet oxygen [30].

DPPH \cdot and ABTS \cdot^{+} have been widely used to test the free radical scavenging ability of various antioxidants commonly found in plant extracts [31]. The antioxidant activity of PDME was also determined in the DPPH radical assay, which primarily evaluates proton radical-scavenging ability [32]. In the present study, PDME showed a concentration dependent scavenging of DPPH and ABTS radical, which may be attribute to its hydrogen-donating ability.

ABTS \cdot^{+} radicals and unlike the reaction with DPPH \cdot radical which involve H atom transfer the reactions with ABTS \cdot^{+} radicals involve electron transfer process. The kinetics of DPPH \cdot and ABTS \cdot^{+} radical involve electron transfer process. The kinetics of DPPH and ABTS \cdot^{+} radical scavenging efficiency of the extract analyzed by stopped-flow kinetic spectrometry showed the presence of two active components in the extract (kaempferol and Quercetin glycosides), evidently responsible for the antioxidant activity.

The methanolic extract of *P.daemia* possesses reducing power property. The reducing power property means that several compounds derived from *P.daemia* are electron donors, and therefore it can reduce the oxidized intermediates of lipid peroxidation process, so that they can act as primary and secondary antioxidants [33]. In reducing power assay, the yellow colour of the test solution changes to various shades of green and blue depending upon the reducing power of *P.daemia* extract. The presence of reductants (i.e. antioxidants) in the herbal extracts causes the reduction of the Fe $^{3+}$ /ferricyanide complex to the ferrous form. Therefore, the Fe $^{2+}$ can be monitored by measuring the formation of Perl's Prussian blue at 700 nm [34].

The aerial parts of PDME showed moderate nitric oxide-scavenging capacity. In addition to ROS, nitric oxide is also implicated in inflammation, cancer and other pathological conditions [35]. Consequently, this plant has potentials for counteract the effect of NO formation and it may be got considerable interest in preventing the ill effects of excessive NO generation in the human body. Furthermore, NO scavenging activity may also help to arrest the chain of reactions initiated by excess generation of NO that are detrimental to human health.

The Syrian golden hamster buccal pouch carcinogenesis model closely mimics events in the development of precancerous lesions and epidermoid carcinomas of the oral cavity in humans using topically and chronically applied DMBA. DMBA is a member of polycyclic aromatic hydrocarbons that are present in the environment as products of incomplete combustion of complex hydrocarbons. Being an indirect carcinogen, DMBA, after metabolic activation, has been suggested to induce cancer through oxidatively mediated genotoxicity by incorporating diolepoxide and other ROS into DNA [36].

The non-enzymic antioxidant systems are the second line of defence against free radical damage. GSH is an important non-protein cellular thiol conjugates with GPx which plays a regulatory role in cell proliferation [37]. GSH dependent enzymes are involved in scavenging the electrophilic moieties involved in the cancer initiation [38]. We also observed decreased activity of GSH in tumor-bearing animals. GSH serves as a marker for evaluation of oxidative stress and it acts

as an antioxidant at both intracellular and extracellular levels [39]. Ascorbic acid is a good scavenger of free radicals and it protects cellular membranes thereby preventing degenerative disease like cancer [40]. Decreased levels of water-soluble antioxidant in cancer-bearing animals may be due to the utilization of antioxidants to scavenge free radicals. Vitamin C undergoes a synergistic interaction with tocopheroxyl radical in the regeneration of α -tocopherol [41]. Vitamin E is a potent oxygen radical scavenger that protects cell membranes from oxidative damage initiated by carcinogens [42]. The free radical scavenging capacity of vitamin E is due to the localization of unpaired electron on its conjugated double bond. Whereas treated animals showed PDME increased the GSH levels, which clearly suggest their antioxidant property. The redox cycle of GSH has a vital role in the cellular response to bioreduction/oxidation of various classes of toxic free radicals in disease processes and hence, has an important physiological role in cancer.

5. Conclusion

In conclusion, the results obtained that *P. daemia* was proved to be the richest in phenolic compounds, and the largest share of flavonoids was found in aerial parts of methanolic extract. In experiments evaluating the efficacy and safety of the aerial parts of *P.daemia* extract showed high potency of the extract (200mg/kg bw) in the experimental in vivo system. We consider that this methanolic extract deserves more intensive study, bioavailability and possible protection against anticancer activity to understand their real potential as nutraceuticals. This study suggested that the PDME possess antioxidant activity, which might be helpful in preventing or slowing the progress of various types of cancer.

Conflict of interest statement

We declare that we have no conflict of interest.

Acknowledgements

The authors wish to thank the Annamalai University and University Research fellowship for support (AURS/H5/2010).

References

- [1] Miyata M, Furukawa M, Takahasi K, Gonzalez FJ, Yamazoe Y. Mechanism of 7,12-dimethylbenz(a)anthracene induced immunotoxicity: role of metabolic activation at the target organ. *Jap J Pharmacol* 2001; **86**: 302–309.
- [2] Bryan CD, Christopher JC. Chemistry and biology of reactive oxygen species in signaling or stress responses. *Nature Chemical Biology* 2011; **7**: 504–511.
- [3] Dudonne S, Vitrac X, Coutiere P, Woillez M, Merillon JM. Comparative study of antioxidant properties and total phenolic content of 30 plant extracts of industrial interest using DPPH, ABTS, FRAP, SOD, and ORAC assays. *Journal of Agriculture and Food Chemistry* 2009; **57**, 1768–1774.
- [4] Guangrog H, Jiaxin J, Dehui D. Antioxidative and antibacterial activity of the methanol extract of *Artemisia anomala* S. Moore. *African*

- Journal of Biotechnology* 2008; **7**: 1335–1338.
- [5] Bose KSC, Vyas P, Singh M. Plasma non-enzymatic antioxidants–vitamin C, E, b–carotenes, reduced glutathione levels and total antioxidant activity in oral sub mucous fibrosis. *Eur Rev Med Pharmacol Sci* 2012; **16**: 530–532.
- [6] Halliwell B. Are polyphenols antioxidants or pro-oxidants? What do we learn from cell culture and in vivo studies?. *Arch Biochem Biophys* 2008; **476**: 107–112.
- [7] E Rossi, MD Stefano, S Baccetti, F Firenzuoli, L Roti, M Da Frè, MP Viña, V Stambolovich. Complementary and traditional medicine in tuscan (italy): its integration in the public health system and international cooperation experiences. *Universitas Forum* 2011, **2**: 1–9.
- [8] Dubey NK, Kumar R, Tripathi P. “Global promotion of herbal medicines: India’s opportunity”. *Cur Sci* 2004; **86**: 37–41.
- [9] Sheng MS, Tay SY, Jung CP. Antioxidant activities of citrus herbal product extracts. *Food Chemistry* 2008 ; **111**: 892–896.
- [10] Zhimin Xu. Important antioxidant phytochemicals in agricultural food products. Analysis of Antioxidant–Rich Phytochemicals. Zhimin Xu and Luke R. *Howard (Eds.)* 2012; 1–24.
- [11] Bhaskar VH, Balakrishnan N. Protective effects of Pergularia daemia roots against paracetamol and carbon tetrachloride–induced hepatotoxicity in rats. *Pharmaceutical Biology* 2010; **48**: 1265–1272.
- [12] Golam S, Gafur MA, Shah ABM, Khurshid AAHM, Helal UBM, Parvez H, Abdul M, Omar FKM, Chowdhury AKA. Antifertility Activity of Pergularia daemia. *The Sci* 2001; **1**: 22–24.
- [13] Wahi AK, Ravi J, Hemalatha S, Singh PN Anti diabetic activity of Daemia extens. *J Nat Remed* 2002; **2**: 80–83.
- [14] Omale James, Ebiloma Godwin Unekwojoand Agbaji Ann Ojochenemi. Assessment of Biological Activities: A Comparison of Pergularia daemia and Jatropa curcas Leaf Extracts. *British Biotechnology Journal* 2011; **1**: 85–100.
- [15] Bhaskar V.H., 2Balakrishnan N. Analgesic, anti–inflammatory and antipyretic activities of Pergularia daemia and Carissa carandas. *Daemia. DARU* 2009; **17**: 168–174.
- [16] Pavunraj M, Muthu C, Ignacimuthu S, Janarthanam S, Duraipandian V, Raja N, Vimalraj S. Antifeedant activity of a novel 6–(4,7–hydroxy–heptyl) quinone® from the leaves of the milkweed Pergularia daemia on the cotton bollworm *Helicoverpa armigera* (Hub.) and the tobacco armyworm *Spodoptera litura* (Fab.). *Phytoparasitica* 2011; **39**:145–150.
- [17] Meena TI, Jolly CI, Rinku M, sheela D. Preliminary phytochemical analysis and antibacterial activity of Pergularia daemia (Forsk) Chiov. *Journal of Pharmacy Research* 2011; **4**:662–663.
- [18] Singleton, VL, Rossi JA. Colorimetry of total phenolics with phosphomolybdic–phosphotungstic acid reagents. *Am J Enol Vitic* 1965; **16**:144– 158.
- [19] Jia Z, Tang M, Wu J. The determination of flavonoids content in mulberry and scavenging effect on superoxide radicals. *Food Chem* 1999; **64**, 555–599.
- [20] Wolfenden BS, Willson RL. Radical–cations as reference chromogens in kinetic studies of one– electron transfer reactions; pulse radiolysis studies of 2,2’–azinobis–(3–ethylbenzothiazoline–6–sulfonate). *J Chem Soc Perkin Trans* 1982; **2**: 805–812.
- [21] Williams BW, Cuverlier ME, Berset C. Use of free radical method to evaluate antioxidant activity. *Food Sci Technol* 1995; **28**: 25–30.
- [22] Oyaizu M. Studies on products of browning reaction prepared from glucoseamine. *Japan J Nutri* 1986; **44**: 307–315.
- [23] Nehir ELS, Karakaya S. Radical scavenging and iron chelating activities of some greens used as traditional dishes in Mediterranean diet. *Inter J Food Sci Nutri* 2004; **55**(1): 67 –74.
- [24] Ellman GL. Tissue sulphhydryl groups. *Achiev Biochem Biophys* 1959; **82**: 70–77.
- [25] Desai FD. Vitamin E analysis methods for animal tissues. In: Feischer S, Packer L, eds. *Methods Enzymol*, vol.105. *Academic Press, New York* 1984; 138–145.
- [26] Palan PR, Mikhail BS, Basin J, Romney SL. Plasma Levels of antioxidant beta–carotene and tocopherol in uterine cervix dysplasia and cancer. *Nutri Can* 1973; **15**:13–20.
- [27] Omaye ST, Turubull TD, Sauberlich HE. Selected method for the determination of ascorbic acid in animal cells, tissues and fluids. In: Mc Cormic D, Band Wright DL, eds. *Methods Enzymol*, vol.62. *Academic Press, New York* 1979; 3– 11.
- [28] Dehpour AA, Ebrahimzadeh MA, Nabavi SF, Nabavi SM. Antioxidant activity of methanol extract of *Ferula assafetida* and its essential oil composition. *Grasas Aceites* 2009; **60**: 405– 412.
- [29] Chang CL, Lin CS, Lai GH. Phytochemical Characteristics, Free Radical Scavenging Activities, and Neuroprotection of Five Medicinal Plant Extracts. *Evidence–Based Complementary and Alternative Medicine* 2012; **2012**:1–8.
- [30] Demira F, Uzuna FG, Durakb D, Kalende Y. Subacute chlorpyrifos–induced oxidative stress in rat erythrocytes and the protective effects of catechin and quercetin. *Pesticide Biochemistry and Physiology* 2011; **99**: 77–81.
- [31] Alnajjar ZAA, Abdulla MA., Ali HM, Alshawsh MA and Hadi AHA. Acute Toxicity Evaluation, Antibacterial, Antioxidant and Immunomodulatory Effects of *Melastoma malabathricum*. *Molecules* 2012; **17**: 3547–3559.
- [32] Sanda V–K, Biljana B, Maja BŠ, Antun A, Tamás K and József P. Antioxidant Activities and Polyphenolic Contents of Three Selected Micromeria Species from Croatia. *Molecules* 2011; **16**: 1454–1470.
- [33] Mudoit T, Deka DC and Devi R. In vitro antioxidant activity of *Garcinia pedunculata*, an indigenous fruit of North Eastern (NE) region of India. *International Journal of PharmTech Research* 2012; **4**: 334–342.
- [34] Chung Y–C, Chang C–T, Chao W–W, Lin C–F, Chou, S–T. Antioxidative activity and safety of the 50% ethanolic extract from red bean fermented by *Bacillus subtilis* IMR–NK1. *J Agri Food Chem* 2002; **50**: 2454–2458.
- [35] Ebrahimzadeh MA, Bahramian F. Antioxidant activity of *Crataegus pentagyna* subsp. *subsp. bursifolia* Fruits extracts used in traditional medicine in Iran. *Pak J Biol Sci* 2009; **12**: 413–419.
- [36] Li N, Chen X, Liao J, Yang G, Wang S, Josephson Y, Han C, Chen J , Huang M–T and Yang CS. Inhibition of 7,12–dimethylbenz[a]anthracene (DMBA)–induced oral carcinogenesis in hamsters by tea and curcumin. *Carcinogenesis* 2002; **23**: 1307–1313.
- [37] Bhuvaneshwari V, Velmurugan B, Nagini S. Lycopene modulates circulatory antioxidants during hamster buccal pouch carcinogenesis. *Nutri Res* 2001; **21**: 1447–53.
- [38] Rosa MS, Felipe L and Juan CM. Radical Decisions in Cancer: Redox Control of Cell Growth and Death. *Cancers* 2012; **4**: 442–474.
- [39] Comporti M. Three models of free–radicals induced cell injury. *Chem Biol Interact* 1989; **72**: 1–56.
- [40] Vijayavel K, Gopalakrishnan S, Thilagam H, Balasubramanian MP. Dietary ascorbic acid and α –tocopherol mitigates oxidative stress induced by copper in the thorn fish *Terapon jarbua*. *Sci Tot Environ* 2006; **372**: 157–63.
- [41] Raymond FB, Joani MC, Mark JM, Lori MA, William OWJ, James MM, Kristina E H and Ford FE. A Combined Deficiency of Vitamins E and C Causes Severe Central Nervous System Damage in Guinea Pigs. *J Nutr* 2006; **136**: 1576–1581.
- [42] Schindler R, Mentlein, R. Flavonoids and vitamin E reduce the release of the angiogenic peptide vascular endothelial growth factor from human tumor cells. *J Nutri* 2006; **136**: 1477–82.