Academic Sciences International Journal of Pharmacy and Pharmaceutical Sciences

ISSN- 0975-1491 Vol 5, Issue 1, 2013

Research Article

EVALUATION OF ANTIOXIDANT AND CYTOTOXIC POTENTIAL OF *ARTOCARPUS CHAMA* BUCH. SEEDS USING IN VITRO MODELS

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Received: 05 Nov 2012, Revised and Accepted: 11 Dec 2012

ABSTRACT

Phytochemicals consisting of phenols and flavonoids possess antioxidant properties, which are useful to scavenge reactive oxygen species (ROS). The present study was conducted to evaluate antioxidant and cytotoxic potential of methanol extracts of seeds of *Artocarpus chama* Buch., using DPPH (1,1-diphenyl-2-picrylhydrazyl) scavenging assay, cupric reducing antioxidant capacity, reducing power antioxidant capacity, total antioxidant capacity, determination of total phenol and flavonoid contents and cytotoxic activity test using brine shrimp lethality bioassay. Preliminary phytochemical study revealed the presence of alkaloid and flavonoid in the extracts. The fraction showed significant antioxidant activities in the assay compared to the reference ascorbic acid in a dose dependent manner. In DPPH radical scavenging assay, the IC_{50} value of the crude methanol extract was 54.29 µg/mL, whereas IC_{50} value for the reference ascorbic acid was 14.56 µg/mL. Moreover, at 200 µg/mL extract concentration, profound total antioxidant activity (4373.58 mg/g equivalent to ascorbic acid) was observed. Furthermore, extract showed good cupric reducing power and reducing power capability. In addition, significant amount of phenols and flavonoids content were obtained from the extract. The extract also displayed strong cytotoxic potential with LC_{50} value of 10.41 µg/mL in brine shrimp lethality bioassay. Based on these findings, it can be concluded that significant antioxidant potential as well as cytotoxic potential of methanolic extract, might be due to the attributes of high amount of phenols and flavonoids present in the extract.

Keywords: *Artocarpus chama*, Antioxidant potential, DPPH, Total antioxidant, CUPRAC reducing, Cytotoxicity.

INTRODUCTION

Oxidative stress on human health has becoming increasingly an alarming concern in the modern era. According to the World Health Organization (WHO) around 80 % of the world population largely depends on traditional medicine for their primary health care needs, and most of this therapy involves the use of plant extracts and their active components[1]. Under stress, our body produces more reactive oxygen species (ROS) (e.g., superoxide anion radicals, hydroxyl radicals and hydrogen peroxide) than enzymatic antioxidants (e.g., superoxide dismutase (SOD), glutathione peroxidase (GPx), and catalase) and non-enzymatic antioxidants (e.g., ascorbic acid (vitamin C), α tocopherol (vitamin E), glutathione, carotenoids, and flavonoids)[2-4]. This imbalance leads to many diseases, such as brain dysfunction, cancer, heart diseases, age-related degenerative conditions, declination of the immune system, cancer, coronary arteriosclerosis, ageing processes, carcinogenesis, gastric ulcer and DNA damage [5-9]. Consequently, it is essential to develop and utilize effective natural antioxidants so that they can protect the human body from free radicals. Furthermore, epidemiological studies have shown that many of nature sourced antioxidant compounds possess anti-inflammatory, antiatherosclerotic, antitumor, anti-mutagenic, anticarcinogenic, antibacterial, or antiviral activities to a greater or lesser extent [10-13].

Artocarpus chama Buch. (synonym *A. chaplasha* Linn., locally known as 'Chamfol' in Bangladesh) is a tall deciduous tree of the Moraceae family, grows all over the South Asian region. It is generally used as timber for commercial purpose. The juice of stem bark (5-10 mL, 3-4 times daily) is given orally in the treatment of diarrhea[14]. Triterpenoids were reported from the bark of *A. chaplasha* Linn. [15]. However, *Artocarpus* species (Moraceae) provide a variety of prenylated flavonoids and a limited number of stilbenoids with interesting biological activities, such as cytotoxicity, antibacterial effects against cariogenic bacteria, and cyclooxygenase and tyrosinase inhibitory activities [16-20]. Five new isoprenylated flavones, artochamins A-E, along with eight known flavones, were isolated from the roots of *Artocarpus chama.* [21]. Furthermore, two new prenylated stilbenes, artochamins F and G, and their four novel derivatives, artochamins H–K, were isolated from the stems of *Artocarpus chama*. [22]. In addition, two new stilbenes with two isoprenoid groups, namely artostilbenes A and B were isolated from the stems of *Artocarpus chama* Buch [23]. As per as our literature survey could ascertain, in vitro antioxidant potentials of the *A. chama* seeds has not been reported yet. To evaluate the antioxidant potential of methanolic plant extract *A. chama* seeds, several assay strategies have been implemented in this study. These consists of various in vitro assay systems, such as the DPPH (1,1-diphenyl-2-picrylhydrazyl) scavenging assay, cupric reducing antioxidant capacity, reducing power antioxidant capacity, total antioxidant capacity and determination of total phenol and flavonoid content, in order to understand the usefulness of this plant as a functional food as well as in medicine.

MATERIALS AND METHODS

Chemicals

DPPH (1, 1-diphenyl, 2-picrylhydrazyl) was purchased from Sigma Chemical Co., USA, potassium fericyanide [$K_3Fe(CN)_6$] from Loba Chemie Pvt. Ltd., Mumbai, India, Ascorbic acid from SD Fine Chem. Ltd., Biosar, India, Vincristine sulphate from Jayson Pharmaceuticals Ltd, Bangladesh and neocaproin ($C_{14}H_{12}N_2$), ammonium molybdate, folin-ciocalteun phenol reagent, gallic acid ($C_7H_6O_5.H_2O$), quercetin were purchased from Merck, Germany.

Plant material

Artocarpus chama fruits were first collected from Jahangirnagar University, Savar, Dhaka, Bangladesh, in May 2009 while the fruits were matured but unripe and identified by the taxonomist of the National Herbarium of Bangladesh, Mirpur, Dhaka, Bangladesh. A voucher specimen of the plant has been deposited (Accession No.: 35650) in the herbarium for further reference. And seeds were separated carefully followed by drying.

Preparation of plant extract

Powdered dried seed (100 g) were macerated with and 70 % methanol, 500 mL, with occasional stirring at 25 ± 2°C for 3 days. The extract was then filtered using a Buchner funnel and a sterilized cotton filter. The solvent was completely removed by rotary evaporator and 11.2 g methanol extracts were obtained. These crude extracts were used for investigation of antioxidative and cytotoxic potential.

Preliminary phytochemical screening

The freshly prepared crude extracts were qualitatively tested for the presence of chemical constituents. Phytochemical screenings of the extracts were performed using the following reagents and chemicals: alkaloids with Dragendroff's reagents, flavonoids with the use of Mg and HCl; tannins with ferric chloride and potassium dichromate solutions and saponins with ability to produce stable foam and steroids with Libermann- Burchard reagent. Gum was tested using Molish reagent and concentrated sulfuric acid; reducing sugars with Benedict's reagent. These were identified by characteristic color changes using standard procedures by Ghani A. [24].

Tests for antioxidant activity:

DPPH radical scavenging activity

The free radical scavenging activity of the extract, based on the scavenging activity of the stable 1, 1-diphenyl-2-picrylhydrazyl (DPPH) free radical, was determined by the method described by Braca *et al* [25]. Plant extract (0.1 mL) was added to 3 mL of a 0.004 % ethanol solution of DPPH. Absorbance at 517 nm was determined after 30 min and the percentage inhibition activity was calculated from $[(A_o-A_1)/ A_o] \times 100$, where A_o is the absorbance of the control (DPPH solution) and A_1 is the absorbance of the extract/standard. The inhibition curves were prepared and IC_{50} values were calculated.

Determination of total antioxidant capacity

The antioxidant activity of the extract was evaluated by the phosphomolybdenum method according to the procedure describe by Prieto *et al.*[26]. A 0.3 mL extract was combined with 3 mL of reagent solution (0.6 M sulfuric acid, 28 mM sodium phosphate and 4 mM ammonium molybdate). The tubes containing the reaction solution were incubated at 95ºC for 90 min. Then the absorbance of the solution was measured at 695 nm using a spectrophotometer (UV-visible spectrophotometer, Shimadzu, 1601) against blank after cooling at room temperature. Methanol (0.3 mL) in the place of extract is used as the blank. The antioxidant activity is expressed as the number of equivalents of ascorbic acid.

Cupric reducing antioxidant capacity (CUPRAC)

The cupric reducing antioxidant activity of the methanol extracts were determined by the method described by Resat apak *et al* [27]. Different concentrations of the extract (5-200 µg) in 0.5 mL of distilled water were mixed with cupric chloride (1 mL, 0.01 M), Ammonium acetate buffer (1 mL, pH 7.0), neocaproin (1 mL, 0.0075 M) and finally distilled water (0.6 mL). The mixture was incubated for 1 hour at room temperature. Then the absorbance of the solution was measured at 450 nm against blank. Distilled water (0.5 mL) in the place of extract is used as the blank. The molar absorptivity of the CUPRAC method for each antioxidant was found from the slope of the calibration line concerned. Ascorbic acid was used as the standard solution.

Reducing power antioxidant capacity

The reducing power was determined according to the method previously described by Oyaizu [28]. Different concentrations of extracts (5-200 µg) in 1 mL of distilled water were mixed with phosphate buffer (2.5 mL, 0.2 M, pH 6.6) and potassium ferricyanide $[K_3Fe(CN)_6]$ (2.5 mL, 1 %). The mixture was incubated at 50°C for 20 min. An aliquot (2.5 mL) of trichloroacetic acid (10 %) was added to the mixture, which was then centrifuged at 3000 rpm for 10 min. The supernatant (2.5 mL) was mixed with distilled water (2.5 mL) and $FeCl_3$ (0.5 mL, 0.1 %) and the absorbance was measured at 700 nm. Increased absorbance of the reaction mixture indicated increased reducing power. Ascorbic acid was used as the reference.

Determination of total phenol content

The total phenolic content of plant extracts were determined using Folin–Ciocalteu reagent [29]. Plant extracts (100 µL) were mixed with 500 µL of the Folin–Ciocalteu reagent and 1.5 mL of 20 % sodium carbonate. The mixture was shaken thoroughly and made up to 10 mL using distilled water. The mixture was allowed to stand for 2 hour. Then the absorbance at 765 nm was determined. These data were used to estimate the phenolic contents using a standard curve obtained from various concentration of gallic acid.

Determination of total flavonoid content

The content of flavonoid compounds in the extract was determined by the method described by Chang *et al* [30]. 1.0 mL of extract was mixed with methanol (3 mL), aluminium chloride (0.2 mL, 10 %), potassium acetate (0.2 mL, 1 M) and distilled water (5.6 mL) and incubated the mixture for 30 min at room temperature. Then the absorbance was measured at 415 nm against blank. Methanol (1 mL) in the place of extract was used as the blank and quercetin was used as the standard solution. All determinations were carried out in triplicates. The amount of flavonoids in plant extracts in quercetin equivalents (QE) was calculated by the following formula: $X= (A \times m_0)/ (A_0 \times m)$, where X is the flavonoid content, mg/mg plant extract in QE, A is the absorption of plant extract solution, A_0 is the absorption of standard rutin solution, m is the weight of plant extract in mg and m_0 is the weight of quercetin in the solution in mg.

Cytotoxic activity test

Brine shrimp lethality bioassay was used for probable cytotoxic action [31,32]. The eggs of brine shrimp (*Artemia salina* Leach) were collected and hatched in a tank at a temperature around 37 °C with constant oxygen supply. Two days were allowed to hatch and mature the nauplii. Stock solution of the extract sample was prepared by dissolving required amount of extract in specific volume of pure dimethyl sulfoxide (DMSO). 10 living nauplii were taken to each of the vial containing different concentrations of test sample with pasteur pipette. Then specific volumes of sample were transferred from the stock solution to the vials to get final sample concentration. In the control vials same volumes of DMSO (as in the sample vials) were taken. Vincristine sulphate was used as the positive control. After 24 hour the vials were observed and the number of nauplii survived in each vial was counted. From this, the percentage of mortality of brine shrimp nauplii was calculated for each concentration of the extract.

Statistical analysis

The results were expressed as mean ± standard deviation (SD) from triplicate experiments, and evaluated with the analysis of student's t-test. Differences were considered significant at a level of $P<0.05$. IC_{50} and LC_{50} was calculated using SigmaPlot 11.0 software.

RESULTS AND DISCUSSION

Preliminary phytochemical screening

The preliminary phytochemical screening revealed that the extracts possess the presence of various bioactive components like carbohydrates, flavonoids and alkaloids (Table 1).

Table 1: Result of phytochemical screening of methanolic extracts of *A. chama* seeds

Phytochemicals	Results
Carbohydrate	+
Glucoside	-
Alkaloid	+
Saponin	-
Steroid	-
Flavonoid	+
Tannin	-

(+): present, (-): absent.

DPPH radical scavenging activity

In DPPH radical scavenging assay, as shown in Fig.1, methanol extract exhibited a concentration-dependent antiradical activity by inhibiting $DPPH^-$ radical. Ascorbic acid, which is a well known antioxidant, showed higher degree of free radical-scavenging activity than that of the plant extract at each concentration points. The IC_{50} value of the crude methanol extract was 54.29 ± 1.98 µg/mL, while the IC_{50} value for the reference ascorbic acid was 14.56

± 0.24 µg/mL. The DPPH antioxidant assay is based on the ability of 1, 1-diphenyl-2-picryl-hydrazyl (DPPH), a stable free radical, to decolorize in the presence of antioxidants [33]. The method is based on the reduction of ethanolic $DPPH^-$ solution in the presence of a hydrogen donating antioxidant, due to the formation of the non-radical form DPPH-H by reaction. The extracts were able to reduce DPPH radical (visible deep purple color) to the yellow-coloured diphenylpicrylhydrazine. It has been found that cysteine, glutathione, ascorbic acid, α-tocopherol, polyhydroxy aromatic compounds (e.g. hydroquinone, pyrogallol, gallic acid), and aromatic amines (e.g. p-phenylene diamine, p-aminophenol), reduce and decolorise 1,1-diphenyl-2-picrylhydrazyl by their hydrogen donating ability [34]. Therefore, one of the possible mechanism of the methanol extract's better antioxidant capacity might be attributed to good amount of phenolic compounds, which shows antioxidant activity due to their redox properties, play an important role in absorbing and neutralizing free radicals, quenching single and triple oxygen or decomposing peroxide.

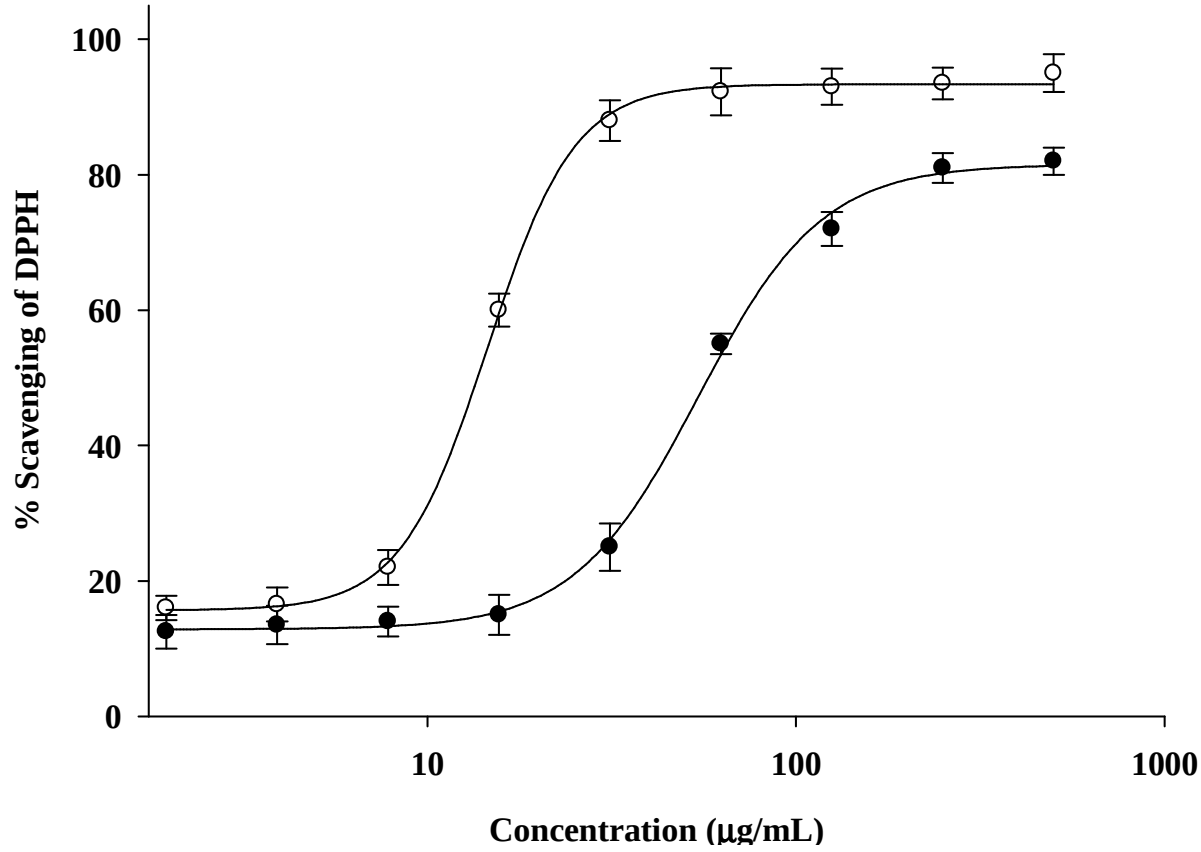

Fig. 1: DPPH radical scavenging activity of methanolic (●) extracts of *A. chama* seeds along with standard Ascorbic acid (○) (Mean ± SD, n=3). Concentrations are displayed on logarithmic scales.

Determination of total antioxidant capacity

The total antioxidant capacity of the methanolic extract of the *A. chama* seeds are given in Table 2. Significant amount of total antioxidant activity was obtained from the extract (4373.58 mg/g equivalents of ascorbic acid). The phosphomolybdenum method was based on the reduction of Mo(VI) to Mo(V) by the antioxidant compound and the formation of a green phosphate/Mo(V) complex with a maximal absorption at 695 nm. The assay is successfully used to quantify vitamin E in seeds and, being simple and independent of other antioxidant measurements commonly employed, it was decided to extend its application to plant extracts [26]. Moreover, it is a quantitative one, since the antioxidant activity is expressed as the number of equivalents of ascorbic acid.

Cupric reducing antioxidant capacity (CUPRAC)

The reducing ability of a compound generally depends on the presence of reductants [35], which have been reported to exhibit antioxidative potential by breaking the free radical chain, donating a hydrogen atom [36]. The CUPRAC method of reducing antioxidant capacity assay uses bis (2, 9-dimethyl-1, 10 phenanthroline: neocuproine) Cu (II) chelate cation as the chromogenic oxidant, which is reduced in the presence of n-electron reductant antioxidants to the cuprous neocuproine chelate [Cu(I)–Nc] showing maximum light absorption at 450 nm. Color development in the CUPRAC method is based on the following reaction:

n $Cu(Nc)_2^{2+}$ + n-electron reductant (AO) ↔ n$Cu(Nc)_2^{+}$ + n-electron oxidized product + n H^+

Where, the electrons required for the formation of the Cu (I)–Nc chromophore are donated by the tested antioxidants. In this reaction, the reactive Ar-OH groups of polyphenolic antioxidants are oxidized to the corresponding quinones (Ar=O) (ascorbic acid is oxidized to dehydroascorbic acid) and Cu (II)-Nc is reduced to the highly colored Cu (I)-Nc chelate [37,38]. As observed from Fig. 2, at concentration level of 200 µg/mL, the reducing capacity of methanol extract and ascorbic acid is 0.324 and 0.744, respectively. According to changed concentration trend, we concluded that the reducing power of extracts were lower than that of ascorbic acid. The probable mechanism of Cupric reducing power of extracts, would be the resultant of having a good number of polyphenolics and flavonoids, as the reactive hydroxyl groups of polyphenolics, oligomeric flavonoids, and is oxidized with the CUPRAC reagent to the corresponding quinines [27].

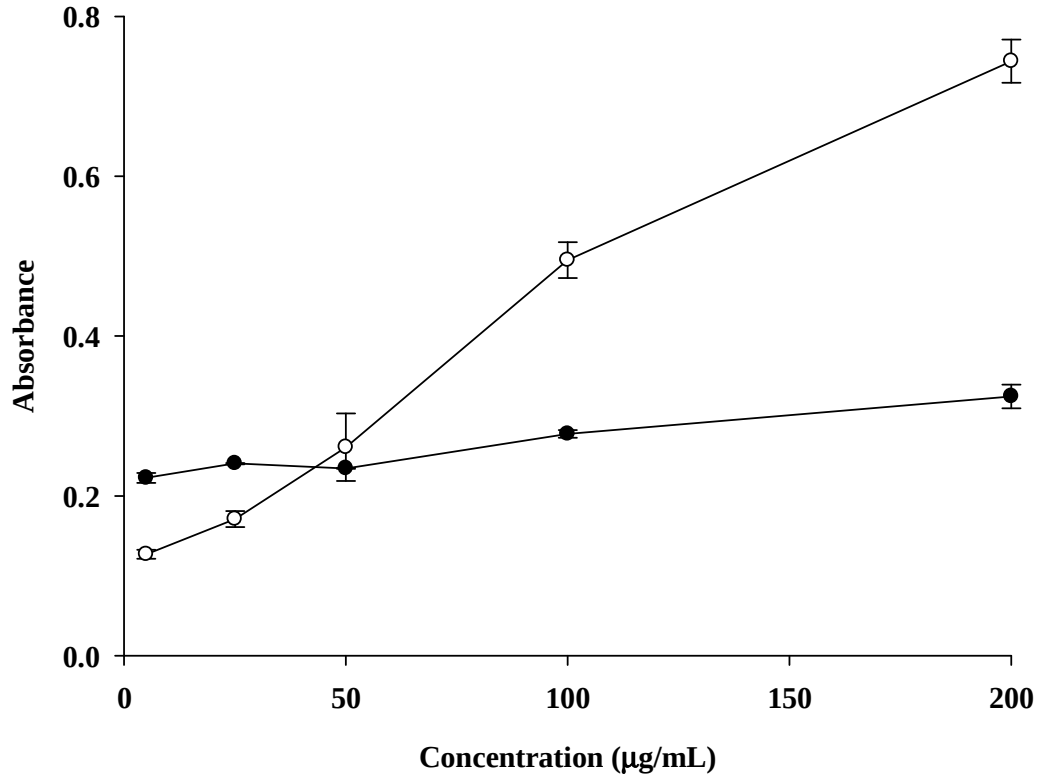

Fig. 2: Cupric reducing power of methanolic (●) extracts *A. chama* seeds along with standard Ascorbic acid (○) (Mean ± SD, n=3).

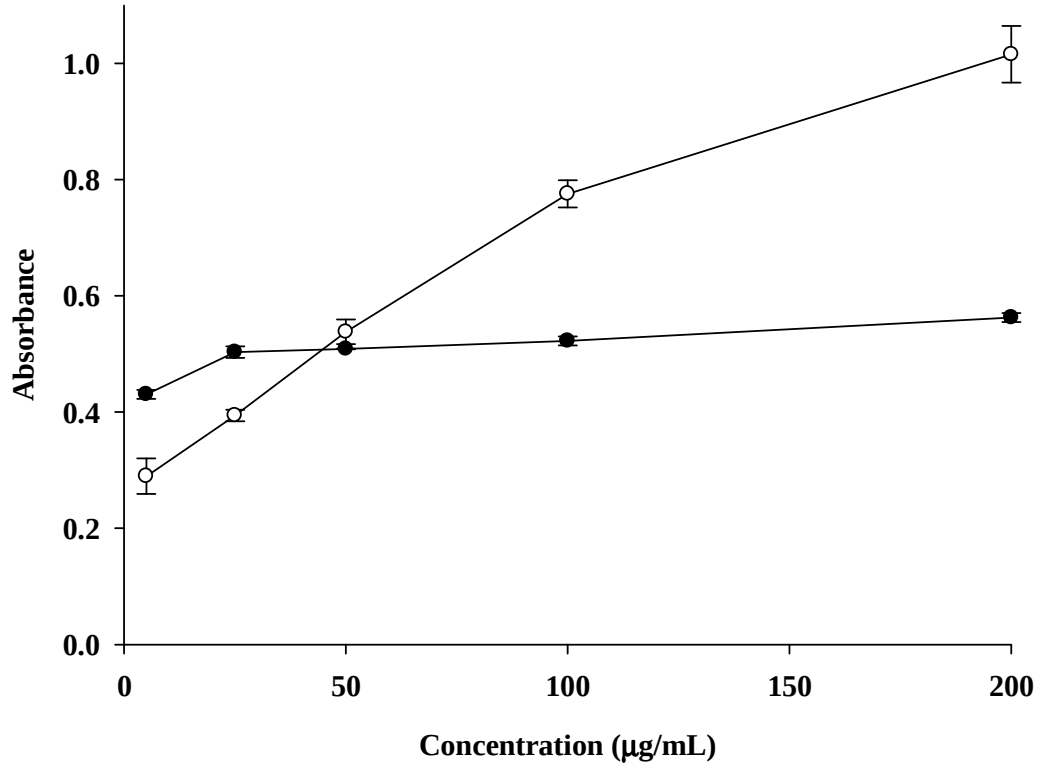

Fig. 3: Reducing power of methanolic (●) extracts of *A. chama* seeds along with standard Ascorbic acid (○) (Mean ± SD, n=3).

Reducing power antioxidant capacity

Fig. 3 shows the reducing power capabilities of the plant extracts in comparison with ascorbic acid. The extract displayed good reducing power which was found to rise with increasing concentrations of the extracts. At 200 µg/mL concentration level, the absorbance of standard ascorbic acid and methanol extract was 1.01 and 0.56, respectively. In reducing power assays, the presence of antioxidants in the seeds can reduce the oxidized form of iron (Fe^{3+}) to its reduced form (Fe^{2+}) by donating an electron. Thus, it can be assumed that the presence of reductants (i.e. antioxidants) in *A. chama* extracts causes the reduction of the Fe^{3+}/ferricyanide complex to the ferrous form. Therefore, the Fe^{2+} complex can be monitored by measuring the formation of perl's prussian blue at 700 nm. A higher absorbance indicates greater reducing power ability [36].

Determination of total phenol content

Several reports have conclusively shown close relationship between total phenolic content and antioxidative activity of the fruits and vegetables. Phenolic compounds, as natural antioxidants exhibit therapeutic potential in multiple diseases including cardiovascular disease, aging and cancer [39]. Moreover, the antioxidant activity of phenolic compounds is 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 [40]. However, as can be seen from Table 2, the methanolic extract of the seeds of *A. chama* Buch. was found to contain higher amount of phenolics, 61.04 mg/g gallic acid equivalent(GAE) using Folin-Ciocalteau method. As the exact chemical nature of the Folin-Ciocalteu reagent is not known, but it is believed to contain heteropolyphosphotunstates molybdates. Sequences of reversible 1 or 2 electron reduction reactions lead to blue species, possibly $PMoW_{11}O_{40}$ [29]. Furthermore, stilbenes are naturally occuring polyphenolic compounds which have been found in many families of higherplants, such as Vitaceae, Gnetaceae, Polygonaceae, Liliaceae, Moraceae and Cyperaceae [41,42]. Since stems of *A. chama* contains stilbenes, one other possible mechanism of antioxidant activity might be the presence of stilbenes in *A. chama* seeds, thus further extensive studies following structural elucidation is required to evaluate the various pharmacological properties other than antioxidant properties of stilbenes.

Determination of total flavonoid content

Flavonoids, the main class of polyphenols in plants, are known to be antioxidants and free radical scavengers having the basic structure of diphenylpyrans. The antioxidative activities of flavonoids are multifaceted. Flavonoids possess phenolic hydrogens responsible for the radical scavenging activity. It has been reported that the *O*-dihydroxyl (catechol) structure in the B ring is the obvious radical target site for all flavonoids. The additional presence of both 3 and 5-hydroxyl groups is responsible for maximal radical scavenging potentials and strongest radical absorption [43]. Flavonoids can exhibit their antioxidant activity in several ways: (i) Radical scavenging activity toward either reactive species (e.g. reactive oxygen species: ROS) such as $OH^{\cdot}$, $O2^{\cdot-}$, $O^{\cdot}{}_2$, or toward lipid peroxidizing radicals such as $R^{\cdot}$, $RO^{\cdot}$, and $ROO^{\cdot}$. Radical scavenging action generally proceeds via hydrogen atom transfer or electron donation; (ii) prevention of the transition metal-catalyzed production of reactive species (i.e. via Fentontype reactions) through metal chelation; (iii) interaction with other antioxidants (such as cooperative actions), localization, and mobility of the antioxidant at the microenvironment [44]. However, total flavonoid content of *A. chama.* seeds methanolic extract is shown in Table 2. The results were exhibited as quercetin equivalent of flavonoids per gm of extracts of the sample. The total flavonoid content of methanolic extract was found to be 33.71 mg/ quercetin equivalent. These results suggested that the antioxidant activities of *A. chama* might be due to its flavonoid content since *A. chama* roots contains a variety of prenylated flavonoids e.g. isoprenylated flavones, flavones [21].

Table 2: Total antioxidant capacity, total phenol and total flavonoid contents of methanolic extracts of *A. chama* seeds

Extract	Total antioxidant capacity equivalent to ascorbic acid mg/g plant extract	Total phenol (in mg/g, gallic acid equivalents)	Total flavonoid (in mg/g, quercetin equivalents)
ACSM	4373.58 ± 11.53	61.04 ± 2.15	33.71 ± 0.27

ACSM: *Artocarpus chama* seeds methanol. Values are the average of triplicate experiments and represented as mean ± SD.

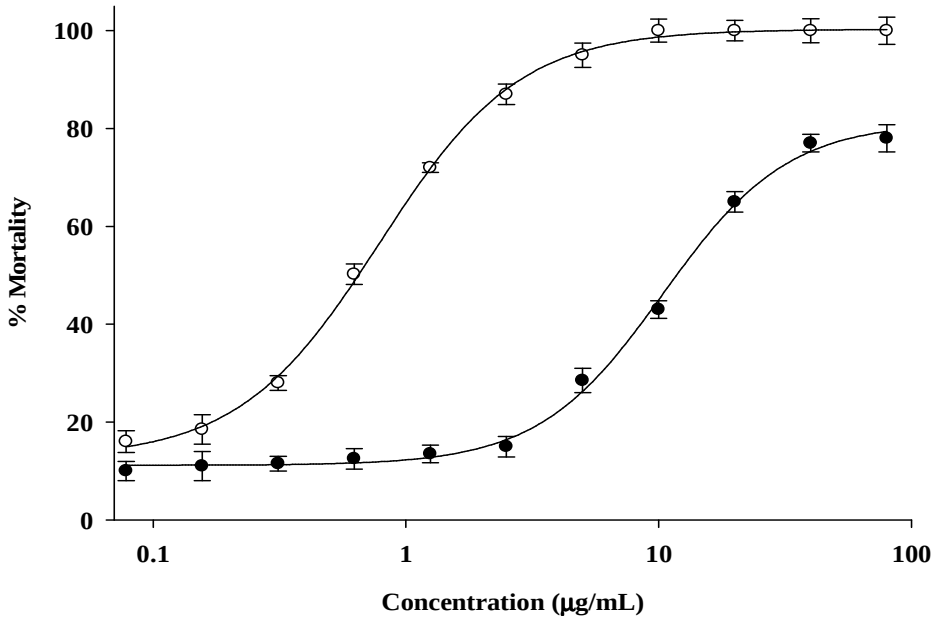

Fig. 4: Cytotoxic potential of methanolic (●) extracts *A. chama* seeds along with standard vincristine sulphate (○) (Mean ± SD, n=3). Concentrations are displayed on logarithmic scales.

Cytotoxic activity test

The cytotoxicity of the crude extracts to brine shrimp was determined on *A. salina* [31]. Fig. 4 shows the results of the brine shrimp lethality testing after 24 hours of exposure to the samples and the positive control, vincristine sulphate. The LC_{50}, obtained from the semi log plot were found to be 0.78 ± 0.03 µg/mL and 10.41 ± 0.60 µg/mL for vincristine sulphate (positive control) and DMSO soluble fraction of methenolic crude extract, respectively. The results of this study indicate the presence of potent bioactive principles in this crude extract which might be very useful as antiproliferative, antitumor, pesticidal and other bioactive agents.

CONCLUSION

The study clearly indicates that the methanolic extracts have the significant amount of antioxidants. This might be rationale behind the using of this plant extract as folk medicine. Since the chemical composition and structures of active extract components are important factors governing the efficacy of natural antioxidants, the extract of *Artocarpus chama* Buch. seeds need their characterization. Therefore, further research is necessary for elucidating the active principles e.g. phenolic compounds and also in vivo studies are needed for understanding their mechanism of action as an antioxidant.

ACKNOWLEDGEMENT

We would like to thank Mohammad Ehsanul Haque Mazumder, Discipline of biomedical science, School of medical sciences, University of Sydney, Australia, for his valuable information, suggestions and critical reading of the manuscript.

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