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Strategies to evaluate antioxidant and cytotoxic activity of pet ether extract of *Artocarpus chama* Buch. seed

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ABSTRACT

Plant derived phytochemicals consisting of phenols and flavonoids possess antioxidant properties, eventually renders a lucrative tool to scavenge reactive oxygen species (ROS). In the current study various in vitro assay strategies were implemented to evaluate antioxidant and cytotoxic potential of pet ether extract 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 flavonoid and alkaloid in the extract. In DPPH radical scavenging assay, the fraction showed significant antioxidant activities in the assay compared to the reference ascorbic acid in a dose dependent manner.The IC_{50} value of the crude pet ether extract was 36.87 µg/mL, whereas IC_{50} value for the reference ascorbic acid was 14.56 µg/mL. Moreover, profound total antioxidant activity (3676.4 mg/g equivalent to ascorbic acid) was observed at 200 µg/mL extract concentration. 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 7.19 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 pet ether extract, might be due to the attributes of high amount of phenols and flavonoids present in the extract.

INTRODUCTION

Antioxidants are compounds that inhibit or delay the oxidation of other molecules by inhibiting the initiation or propagation of oxidizing chain reactions. There are two basic categories of antioxidants, namely, synthetic and natural. In general, synthetic antioxidants are compounds with phenolic structures of various degrees of alkyl substitution, whereas natural antioxidants can be phenolic compounds (tocopherols, flavonoids, and phenolic acids), nitrogen compounds (alkaloids, chlorophyll derivatives, amino acids, and amines), or carotenoids as well as ascorbic acid (Larson, 1988). The use of synthetic antioxidants such as butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT), however, has been limited because of their carcinogenicity (Branen, 1975; Ito *et al*, 1983). Thus, interest in natural antioxidants has increased considerably. Human bodies possess enzymatic and non-enzymatic antioxidative mechanisms for protection from oxidative damage, caused by the generation of reactive oxygen species. However, when the generation of the active oxygen-free radical is overgrown many degenerative 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 DNAdamage arise (Grzegorczyk *et al*, 2007; Kumaran and Joel, 2007; Shen *et al*, 2010; Kannan *et al*, 2010; Prakash *et al*, 2007).

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Thus, 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 (Halliwell, 1994; Mitscher *et al*, 1996; Owen *et al*, 2000; Sala *et al*, 2002). *Artocarpus chama* Buch. (synonym *A. chaplasha* Linn., local name 'Chamfol' in Bangladesh) is a tall deciduous tree of the Moraceae family, grows all over the South Asian region. The juice of stem bark (5-10 mL, 3-4 times daily) is given orally in the treatment of diarrhea (Hemanta *et al*, 2001). It is generally used as timber for commercial purpose. Triterpenoids were found in the bark of *A. chaplasha* Linn. (Mahato *et al*, 1967). 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 (Nomura *et al*, 1998; Soekamto *et al*, 2003; Su *et al*, 2002; Likhitwitayawuid and Sritularak, 2001; Likhitwitayawuid *et al*, 2000). Five new isoprenylated flavones, artochamins A-E, along with eight known flavones, were isolated from the roots of *Artocarpus chama* (Wang *et al*, 2004). 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* (Wang *et al*, 2006). In addition, two new stilbenes with two isoprenoid groups, namely artostilbenes A and B, were isolated from the stems of *Artocarpus chama* Buch (Wang *et al*, 2007). Our previous report on this plant including fruits showed significant antioxidant potential in methanol as well as pet ether extract (Taksim *et al*, 2012). Thus, it was of interest to evaluate the antioxidant potential of seeds of *A. chama*. Based on our literature survey in vitro antioxidant activities of the *A. chama* seeds have not been previously reported. To evaluate the antioxidant potential of pet ether plant extract of *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 phenolic and flavonoid content, and in vitro cytotoxicity study in order to reveal the effectiveness 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 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. Seeds were separated carefully from the fruit followed by drying.

Preparation of plant extract

Powdered dried seed (100 g) were macerated with 500 mL pet ether by 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 9.25 g pet ether extract was obtained. These crude extract was subjected to antioxidative and cytotoxic potential assay.

Preliminary phytochemical screening

The freshly prepared crude extract was qualitatively tested for the presence of chemical constituents. Phytochemical screenings of the extract was 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 , 2005.

Tests for antioxidant activity

DPPH free 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*, 2001. 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*,1999. 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 to ascorbic acid.

Reducing power antioxidant capacity

The reducing power was determined according to the method previously described by Oyaizu, 1986. Different concentrations of extract (25 – 500 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.

Cupric reducing antioxidant capacity

The cupric reducing antioxidant activity of the pet ether extract was determined by the method described by Resat *et al*, 2004. 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.

Determination of total phenol content

The total phenolic content of plant extract was determined using Folin–Ciocalteu reagent (Yu *et al*, 2002). Plant extract (100 µL) was 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 flavonoids compounds in the extract was determined by the method described by Chang *et al*, 2002. 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: $X= (A \times m0)/ (A_0 \times m)$, 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 m0 is the weight of Quercetin in the solution in mg.

Cytotoxic activity test

Brine shrimp lethality bioassay was used for probable cytotoxic action (Meyer *et al*, 1982; McLaughlin *et al*, 1991). 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.

Table. 1: Result of phytochemical screening of pet ether extract of the seeds of *Artocarpus chama* Buch.

Extract	Carbohydrate	Glycoside	Glucoside	Alkaloid	Saponin	Steroid	Flavonoid	Tannin
ACSP	+	-	-	+	-	-	++	-

ACSP: *Artocarpus chama* seeds pet ether; (+): Present; (-): Absent

Table. 2: Total antioxidant capacity, total phenol and total flavonoid contents of pet ether extracts of the seeds of *Artocarpus chama* Buch.

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)
ACSP	3676.4 ± 15.03	135.01±6.19	41.29±1.08

ACSP: *Artocarpus chama* seeds pet ether. Values are the average of triplicate experiments and represented as mean ± SD.

RESULTS AND DISCUSSION

Preliminary phytochemical screening

Preliminary phytochemical screening studies, as is observed in Table.1, the extract possess the presence of various bioactive components like carbohydrates, flavonoids and alkaloids (Table 1).

DPPH radical scavenging activity

As can be seen from Fig.1, in the DPPH radical scavenging assay pet ether extract exhibited a concentration-dependent antiradical activity by inhibiting $DPPH^{\cdot}$ 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 pet ether extract was 36.87 µg/mL, whereas the IC_{50} value for the reference ascorbic acid was 14.56 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 (Kumarasamy *et al*, 2007). The method is based on the reduction of ethanolic $DPPH^{\cdot}$ solution in the presence of a hydrogen donating antioxidant, due to the formation of the non-radical form DPPH-H by reaction. The extract was able to reduce DPPH radical (visible deep purple color) to the yellow-coloured diphenylpicrylhydrazine. It has been found that cysteine, glutathione, ascorbic acid, 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 (Blois, 1958). Therefore, one of the possible mechanism of the pet ether extract's better antioxidant capacity might be the resultant of containing good amount of phenolic compounds, which shows antioxidant activity due to their redox properties, plays an important role in absorbing and neutralizing free radicals, quenching single and triple oxygen or decomposing peroxide.

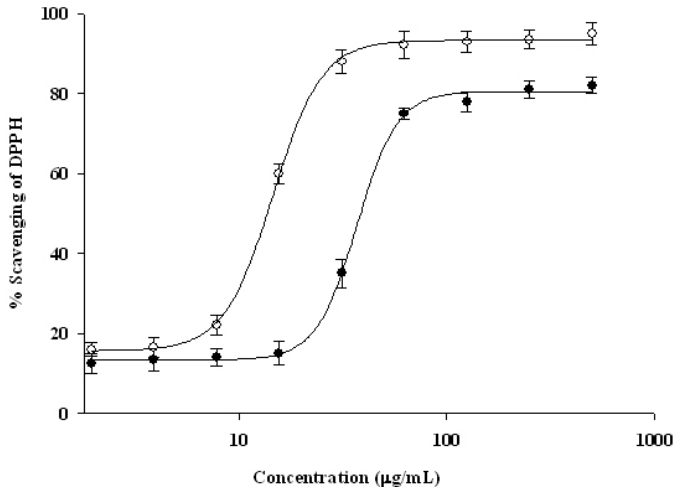

Fig. 1: DPPH radical scavenging activity of pet ether (●) extract of the seeds of *Artocarpus chama* Buch along with the standard Ascorbic acid (○). (Mean ± SD, n=3). Concentrations are displayed on logarithmic scales.

Determination of total antioxidant capacity

The total antioxidant capacity of the pet ether extract of the *A. chama* seeds is given in Table 2. Significant amount of total antioxidant activity was obtained from the extract (3676.4 mg/g equivalents of ascorbic acid). The phosphomolybdenum method is 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 (Prieto *et al*, 1999). Moreover, it is a quantitative one, since the antioxidant activity is expressed as the number of equivalents to ascorbic acid.

Reducing power antioxidant capacity

Fig. 2 shows the reducing power capabilities of the plant extract compared to ascorbic acid. The extract displayed good reducing power which was found to rise with increasing concentrations of the extract. At 200 µg/mL concentration level, the absorbance of standard ascorbic acid and pet ether extract was 1.01 and 0.67, 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* extract 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 (Gordon, 1990).

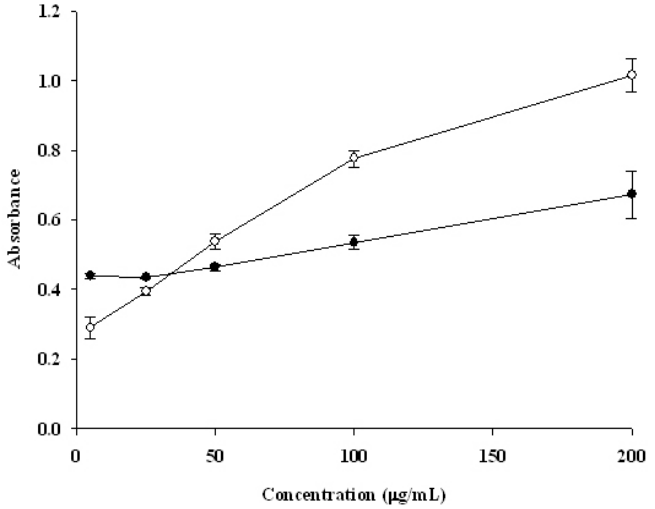

Fig. 2: Reducing power of pet ether (●) extract of the seeds of *Artocarpus chama* Buch. along with the standard Ascorbic acid (○). (Mean ± SD, n=3).

Cupric reducing antioxidant capacity (CUPRAC)

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. Colour 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 (Resat *et al*, 2008; Reşat *et al*, 2007). As observed from Fig. 3, at concentration level of 200 µg/mL, the reducing capacity of pet ether extract and ascorbic acid is 0.362 and 0.744, respectively. According to changed concentration trend, we concluded that the reducing power of extract was lower than that of ascorbic acid. The probable mechanism of Cupric reducing power of extract, 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 (Resat *et al*, 2004).

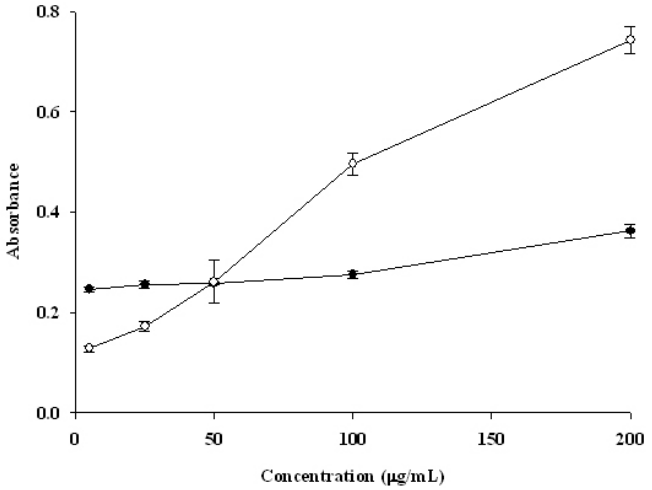

Fig. 3: Cupric reducing power of pet ether (●) extract of the seeds of *Artocarpus chama* Buch. along with the standard Ascorbic acid (○). (Mean ± SD, n=3)

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 (Vinson *et al*, 1998). 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 (Uritani *et al*, 1994). However, as can be seen from Table 2, the pet ether extract of the seeds of *A. chama* Buch. was found to contain higher amount of phenolics, 135.01 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 $PMoW_{11}O_{40}$ (Yu *et al*, 2002).

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 (Teguo and Fauconneau, 1998; Li and Wang, 2001). 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 (Feng *et al*, 1998). 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 $^{\cdot}OH$, $O2.^{-}$, $^{\cdot}O_2$, 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 (Bombardelli *et al*, 1993). However, total flavonoid content of *A. chama*. seeds pet ether 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 pet ether extract was found to be 41.29 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 (Wang *et al*, 2004).

Cytotoxic activity test

The cytotoxicity of the crude extract to brine shrimp was determined on *A. salina* (Meyer *et al*, 1982). 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 µg/mL and 7.19 µg/mL for Vincristine sulphate (positive control) and DMSO soluble fraction of pet ther 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. Moreover, a recent report describes that prenylated isoflavoniods can cause in vitro cell death to several cancer cell including MCF-7 (Phi *et al*, 2012). Thus, the significant cytotoxic effect of *A. chama* seed extract might be attributed to the presence of flavonoid related compounds.

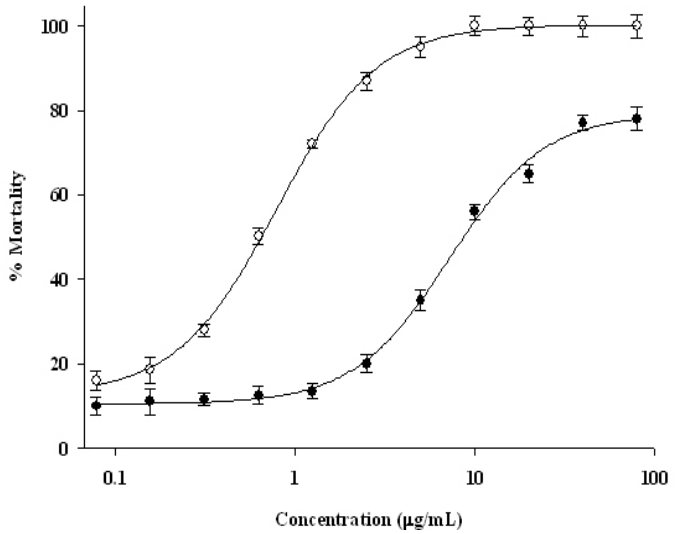

Fig. 4: Cytotoxic potential of pet ether (●) extract of the seeds of *Artocarpus chama* Buch along with the standard Vincristine sulphate (○). (Mean ± SD, n=3). Concentrations are displayed on logarithmic scales.

CONCLUSION

The study clearly indicates that the pet ether extract have the significant amount of antioxidants. This might be rationale behind the using of this plant extract as folk medicine. Our previous findings showed fruits of *A. chama* as a potent antioxidant source, likewise seeds of *A. chama* also possess significant antioxidant activity. 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. seed needs 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.

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