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In vitro evaluation of antioxidant potential of *Artocarpus chama* Buch. fruits

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ABSTRACT

Phytochemicals possessing phenols and flavonoids are potential sources of antioxidants, which are useful to scavenge reactive oxygen species (ROS). The present study was undertaken to evaluate and compare the antioxidant potential of pet ether and methanol extracts of fruits of *Artocarpus chama* Buch., using DPPH (1,1-diphenyl-2-picrylhydrazyl) scavenging assay, cupric reducing antioxidant capacity, reducing power antioxidant capacity, determination of total phenol and flavonoid contents. Preliminary phytochemical study revealed the presence of alkaloid and flavonoid in both 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 pet ether and methanol extract was 27.64 µg/mL and 39.08 µg/mL, respectively, whereas IC_{50} value for the reference ascorbic acid was 12.70 µg/mL. Furthermore, both extracts showed similar cupric reducing power and reducing power capability. In addition, pet ether extract contains higher amount of phenols as compared with methanol extract, and possess similar flavonoid content expressed as Gallic acid and Quercetin, respectively. Based on these findings, it can be concluded that pet ether extract of the fruits of *A. chama* Buch possesses significant antioxidant potential comparing with methanol extract, which may be attributed to the high amount of phenols and flavonoids present in the extract.

INTRODUCTION

Research interest in phytoceuticals to inhibit chronic diseases and aging is gathering momentum. Reactive oxygen species such as hydroxyl ($OH^{\bullet}$) and peroxyl radicals ($ROO^{\bullet}$) and the superoxide anion ($O_2^{\bullet}$) are constantly produced as a result of metabolic reactions in living systems (Halliwell and Gutteridge, 1990). Furthermore, our body is bombarded daily with health hazards and irritants in the form of air pollutants, synthetic drugs, cigarette smoke and food additives which in turn increases free radical production (Shailja *et al.*, 2009). Living systems are protected from oxidative damage caused by these reactive species by enzymes such as superoxide dismutase and glutathione peroxidase and antioxidant compounds such as ascorbic acid, tocopherols, and carotenoids (Sies, 1997). However, when free-radical production exceeds the antioxidant capacity of the organism, these radical species attack lipids, proteins, and DNA, thus damaging structural integrity and function of cell membranes, enzymes, and genetic material. A growing body of evidence indicates that various pathological conditions, including cardiovascular disease, arthritis, and various cancers are associated, at least in part, with the damaging effects of uncontrolled free-radical production (Byers and Perry, 1992). Recent research on free radicals has confirmed that foods, rich in antioxidants play an essential role in the prevention of cardiovascular diseases and cancers (Kris-Etherton *et al.*, 2002, Serafini *et al.*, 2002) and neurodegenerative diseases, including Parkinson's and Alzheimer's diseases (Di Matteo *et al.*, 2002), as well as Inflammation and problems caused by cell and cutaneous aging (Ames *et al.*, 1993).

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Therefore, profound interest has been grown on plant materials to find promising compound having antioxidative potential. Recently, a number of natural compounds such as polyphenols and flavonoids have been reported as antioxidants, which entrap and deactivate damaging ROS and RNS (Pietta, 2000; Hossain *et al.*, 2006). Furthermore, they also possess anti-inflammatory, antiviral and anticancer properties (Di Pietro *et al.*, 2002; Barron *et al.*, 2002). *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. Triterpenoids were found in the bark of *A. chaplasha* Linn. (Mahato *et al.*, 1967). The juice of stem bark (5-10 mL, 3-4 times daily) is given orally in the treatment of diarrhea (Hemanta *et al.*, 2001). 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.* (Yong-Hong *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.* (Yong-Hong *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 (Yong-Hong *et al.*, 2007). As per as our literature survey could ascertain, in vitro antioxidant activities of the *A. chama* fruits have not previously been published. In this study, we examined the antioxidant activity of crude pet ether and methanol extracts, employing various in vitro assay systems, such as the DPPH (1,1-diphenyl-2-picrylhydrazyl) scavenging assay, cupric reducing antioxidant capacity, reducing power antioxidant capacity and determination of total phenolic 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 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 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.

Preparation of plant extract

Powdered dried fruits (100 g) were macerated with 70% pet ether and 70% methanol, 500 mL each, with occasional stirring at 25 ± 2°C for 3 days. The extracts were then filtered using a Buchner funnel and a sterilized cotton filter. The solvent was completely removed by rotary evaporator and 12.5 g pet ether and 10.8 g methanol extracts were obtained. These crude extracts were used for investigation of antioxidative 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, 2005.

Tests for antioxidant activity

DPPH free radical scavenging activity

The free radical scavenging activity of the extracts, 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.

Cupric reducing antioxidant capacity

The cupric reducing antioxidant activity of the pet ether and methanol extracts were 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.

Reducing power antioxidant capacity

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

Determination of total phenol content

The total phenolic content of plant extracts were determined using Folin–Ciocalteu reagent (Yu *et al.*, 2002). 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 both the extracts 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.

Statistical analysis

The results were expressed as means ± 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} was calculated using SigmaPlot 11 software.

RESULTS AND DISCUSSION

Preliminary phytochemical screening

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

$DPPH^-$ radical scavenging activity

In DPPH radical scavenging assay, as shown in Fig.1, both pet ether and 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 pet ether and methanol extract were 27.64 µg/mL and 39.08 µg/mL, respectively, while the IC_{50} value for the reference ascorbic acid was 12.70 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 $DPPH^-$ 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, 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 in comparing with methanol extract might be the resultant of containing 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.

Table. 1: Result of phytochemical screening of pet ether and methanol extracts of the fruits of *Artocarpus chama* Buch.

Extract	Carbohydrate	Glycoside	Glucoside	Alkaloid	Saponin	Steroid	Flavonoid	Tannin
ACFP	+	-	-	+	-	-	+	-
ACFM	+	-	-	+	-	-	+/-	-

ACFP: *Artocarpus chama* fruits pet ether; ACFM: *Artocarpus chama* fruits methanol, (+): Present; (-): Absent

Table. 2: Total phenol and total flavonoid contents of pet ether & methanol extracts of the fruits of *Artocarpus chama* Buch.

Extract	Total phenol (in mg/g, Gallic acid equivalents)	Total flavonoid (in mg/g, Quercetin equivalents)
ACFP	178.08 ± 2.05	24.95 ± 0.36
ACFM	41.12 ± 1.83	25.71 ± 0.59

Values are the average of triplicate experiments and represented as mean ± SD.

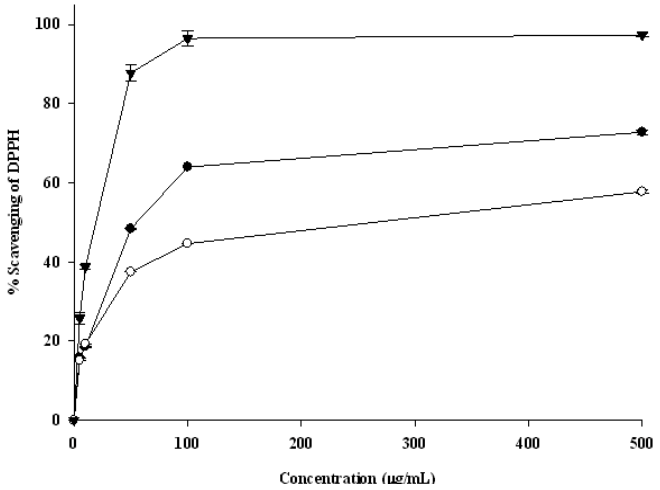

Fig. 1: DPPH radical scavenging activity of pet ether (●) and methanolic (○) extracts of the fruits of *Artocarpus chama* Buch along with the standard Ascorbic acid (▼). (Mean ± SD, n=3)

Cupric reducing antioxidant capacity (CUPRAC)

The reducing ability of a compound generally depends on the presence of reductants (Pin-Der *et al.*, 1999), which have been reported to exhibit antioxidative potential by breaking the free radical chain, donating a hydrogen atom (Gordon, 1990). 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:

$$n\text{-}Cu(Nc)_2^{2+} + n\text{-electron reductant (AO)} \leftrightarrow nCu(Nc)_2^{+} + n\text{-electron oxidized product} + n\,H^{+}$$

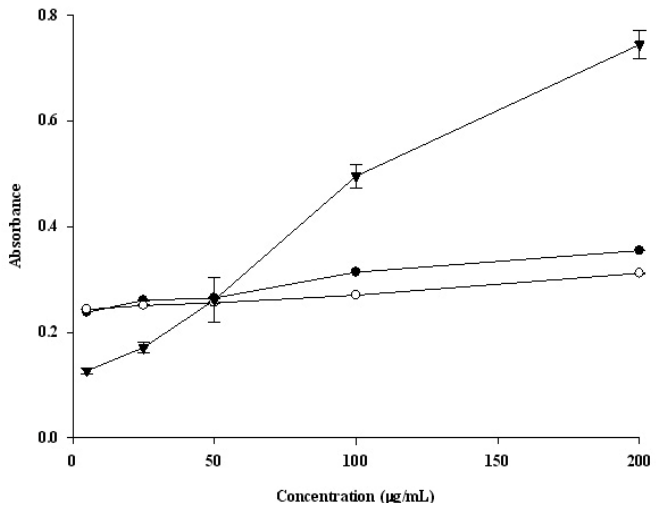

Fig. 2: Cupric reducing power of pet ether (●) and methanolic (○) extracts of the fruits of *Artocarpus chama* Buch. along with the standard Ascorbic acid (▼). (Mean ± SD, n=3)

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. 2, at concentration level of 200 µg/mL, the reducing capacity of pet ether, methanol extract and ascorbic acid is 0.3115, 0.3545 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, is oxidized with the CUPRAC reagent to the corresponding quinines (Resat *et al.*, 2004).

Reducing power antioxidant capacity

Fig. 3 shows the reducing power capabilities of the plant extracts compared to ascorbic acid. Both extracts 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, pet ether extract and methanol extract was 1.01, 0.52 and 0.60, respectively. Both the plant extracts showed almost similar reducing power capacity. In reducing power assays, the presence of antioxidants in the fruits 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 (Gordon, 1990).

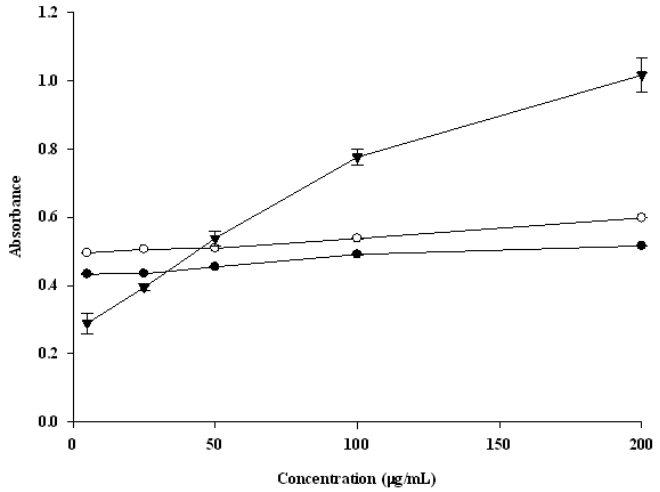

Fig. 3: Reducing power of pet ether (●) and methanolic (○) extracts of the fruits of *Artocarpus chama* Buch. along with the standard Ascorbic acid (▼). (Mean ± SD, n=3).

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). It has been reported that phenolic compounds with *ortho*- and *para*-dihydroxylation or a hydroxy and a methoxy group are more effective than simple phenolics (Frankel *et al.*, 1995). 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, the pet ether extract of the fruits of *A. chama* Buch. was found to contain large amount of phenolics, 178.08 ± 2.05 mg/g Gallic acid equivalent(GAE) while methanolic extract contain moderate amount, 41.12 ± 1.83 mg/g GAE using Folin-Ciocalteau method. The results were represented in Table 2. 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* fruits, 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. Most flavonoids possess the ability to scavenge free radicals by acting as hydrogen as well as electron donors. Some flavonoids can also act as antioxidant by direct reaction with radicals to form less reactive products, and some species possess a capacity to chelate transition elements. Some flavonoids have been found to possess not only anti-inflammatory, antibacterial, antiviral, antiallergic, and antitumor activities but also been reported to possess antioxidant, antiradical properties as well as inhibit the activities of an array of enzymes such as xanthine oxidase (Pietta *et al.*, 2000). 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 .OH, $O_2^{\cdot-}$, O_2, or toward lipid peroxidizing radicals such as $R^{\cdot}$, RO., and ROO. 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*. fruits extracts 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 and methanol extracts were found to be 24.95 ± 0.36 and 25.71 ± 0.59 mg/ quercetin equivalent, respectively. 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 (Yong-Hong *et al.*, 2004).

CONCLUSION

The study clearly indicates that the pet ether and methanol extract 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. 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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