PHYTOCHEMICAL STUDY AND DNA CLEAVAGE ACTIVITY OF CITRUS MAXIMA ETHANOLIC FRUIT EXTRACT

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ABSTRACT

Citrus maxima Merr is a tropical fruit from rutaceae family. Fruits of the plant had been utilized in folk medicine and showed various pharmacological properties. The present study include phytochemical screening, quantitation and identification of phenolics in fruit ethanolic extract of *C. maxima*. Furthermore, *C. maxima* ethanolic extract was evaluated against pathogenic *E.coli* palmid DNA by DNA cleavage assay. Phytochemical screening was carried out by colour reaction. The total phenolic (TPC) and total flavonoid content (TFC) were determined by Folin-Ciocalteu and aluminium trichloride colorimetric method, respectively. The identification of *C. maxima* phenolics present in ethanolic extract was done by HPLC equipped with Diode Array Detector. DNA cleavage activity was performed by gel electrophoresis method at various concentrations of the extract (0.125 µg/µl to 0.600 µg/µl). The preliminary phytochemical analyses, TPC and TFC results indicated *C. maxima* ethanolic extract is a good source of phenolics. 4-hydroxy-3-methoxy cinnamic acid and quercetin were identified as major phenolic acid and flavonoids in the ethanolic extract respectively. DNA cleavage activity exhibited ability of maximum bacterial DNA cleavage up to the linear form in a concentration dependent manner. Thus, overall results of the study suggests, *C. maxima* possess neutraceutical potential and may be exploited in future for therapeutic applications and anticancer drug discovey.

Keywords: phytochemical screening; phenolic acid; flavonoid; HPLC; DNA cleavage

INTRODUCTION

Citrus maxima Merr. (Rutaceae) commonly known as 'Pomelo' in Malaysia. It is widely cultivated in China, India, Indonesia, Japan, Malaysia, Taiwan and Thailand. The traditional knowledge and modern studies on *C. maxima* reported to have high nutritional and medicinal value (Arias & Ramon-Laca, 2005). The various parts of this plant known to be useful in epilepsy, chorea, coughing, hangovers, swelling, ulcers, dyspepsia and bacterial infections (Morton, 1987). The fruits of *C. maxima* are rich source of antioxidants mainly polyphenolic compounds such as flavonoids (Avinash *et al.*, 2012; Ghasemzadeh & Ghasemzadeh, 2011). Flavonoids of *C. maxima* exhibited anti-spasmolytic, anti-inflammatory, antioxidative and antimicrobial activities. Besides, flavonoids of *C. maxima* such as hesperidin, narirutin, naringin, neohesperidin, eriocitrin, neoeriocitrin, rutin, diosmin, neoponcirin and

nobiletin showed antitumor activity and anti cancer activity (Albach et al., 1996). Recently, various researchers have predicted the anti cancer potential of the plant by performing DNA cleaving activity of polyphenolic extracts from *Aloe vera*, *Averrhoa carambola* (star fruit), *Citrus maxima* (aqueous extract) and some brown algae (Gopalakrishnan and Vadivel, 2011; Khanam et al., 2014; Khanam et al., 2015; Naqvi et al., 2010). Thus, in continuation on our study to have deeper insight on *Citrus maxima* fruit, ethanolic extract was evaluated further for DNA cleaving potential against pathogenic *E. coli* DNA. The polyphenolic content of ethanolic extract was also analysed qualitatively and quantitatively, respectively.

MATERIALS AND METHODS

Plant Materials and Extraction

Fresh fruit of *Citrus maxima* Merr. of variety 'limau bali tambun' were purchased from local market of Jeli, Kelantan, Malaysia. Sample of uniform shape, size, colour and ripening stage were selected with no apparent physical and microbial damages. The fruits (100 g) were thoroughly washed, peeled manually and extracted with 200 ml of ethanol at 40 °C. The concentrated crude extract was stored in glass bottle at - 4°C for further study (Khanam 2014).

Phytochemical Screening

The extracts were analyzed for the presence of phenolic compounds, flavonoids, terpenoids, saponins, alkaloids, cardiac glycosides and chalcones following method of Raaman (2006).

Determination of Total Phenolic Content (TPC)

The total phenolic content of the extract was determined spectrophotometrically by using Folin-Ciocalteu (FC) reagent described by Singleton et al. (1999) with slight modifications. Briefly methanolic solution of extract (1 mg/ml) was added into 2.5 ml of 10% FC reagent, previously diluted with distilled water. After 8 min, 2.5 ml of 7.5% sodium hydrogen carbonate ($NaHCO_3$) was added. The mixture was covered with aluminium foil, vortexed for 10 s and allowed to stand for 1 hour in dark at 45 °C. The absorbance was measured at 765 nm, using UV-vis spectrophotometer. A calibration curve was prepared with gallic acid as standard and results were expressed as mg gallic acid equivalents (GAE)/100 g sample in fresh weight (fw) basis.

Determination of Total Flavonoidic Content (TFC)

The total flavonoidic content of the extracts were determined spectrophotometrically using aluminium trichloride colorimetric method with slight modification (Marinova et al., 2005). Briefly, methanolic solution of the extract (1 mg/ml) was mixed with 0.3 ml of 5% (w/v) sodium nitrite ($NaNO_2$) solution. After 5 min, 0.3 ml of 10% aluminium trichloride ($AlCl_3$) solution was added to the mixture. Then, after 6 min, 2 ml of 1 M sodium hydroxide (NaOH) solution was added followed by the volume of reaction mixture was made up to 10 ml with distilled water and vortexed for 10s. The absorbance was measured at 510 nm after pink colour developed using UV-vis spectrophotometer. A calibration curve was prepared with quercetin as standard and results were expressed as mg quercetin equivalents (QE)/100 g sample in fresh weight (fw) basis.

HPLC Analyses of Extract

HPLC analyses of the fruit extracts and standards were performed on Shimadzu HPLC System equipped with Diode Array Detector (DAD). About 5µl of analyte solution was injected into HPLC valve using Puradisc (Sterile and Endotoxin Free 0.2 µm PES Filter Media) and Terumo Syringe (5cc/ml) at room temperature. Phenolic compounds were separated on a Thermo Scientific Hypersil Gold reverse phase (RP-18) column, 250 mm x 4.6 mm (Merk) packed with C_{18} stationary phase with particle size of 5µ. The binary mobile phase consisted of a solvent A (water: acetic acid; 99:1; v/v) and solvent B (acetonitrile) was used. The phenolic acids and flavonoids were detected by UV detector (200 to 500 nm).

Plasmid DNA Extraction from *E. coli*

Plasmid DNA was isolated from *E.coli* culture by conventional method reported elsewhere (Yadav et al., 2011). The *E.coli* solution was mixed in a test tube containing detergent. The solution was heated on hot water bath at 55-60 °C for 10 minutes and about two drops of contact lens cleaning solution were mixed gently. Later, plasmid DNA was precipitated by adding cold absolute ethanol into the test tube. The solution was left stand for 2-3 minutes and the DNA precipitate was spool on to a glass rod from alcohol layer. The DNA was air dried and suspended in 300 µl TAE buffer (10 mM Tris, pH 7.5, 1 mM EDTA).

Gel Electrophoresis

The DNA cleavage of supercoiled *E.coli* plasmid DNA was carried out using agarose gel electropohoresis, according to the method followed by Khanam et al. (2015). About 1.0 g of powdered agarose was mixed in 100 mL of 1x TAE buffer (working) and heated to dissolve agarose completely. The ethidium bromide (1.0 µg/ml) was added to the above solution and mixed well. The molten agrose was cooled to 60 ^{0}C before pouring into cassette and clamped immediately with comb to form sample wells. After setting of gel, the comb was removed and the gel was mounted into electrophoretic tank filled with working buffer. The sample at various concentrations (0.125 µg/µl to 0.600 µg/µl) along with plasmid DNA (15 µl) and loading dye were filled into the wells (40 µl) of the submerged gel using a micropipette. The plasmid DNA without extract is taken as control. The gel electrophoresis was carried out at 60 V and finally, gel was photographed under UV transilluminator.

Statistical Analyses

All data were expressed as means ± standard deviations (SD) of triplicate measurements. ANOVA was used to determine the significant differences (P<0.01) between the means.

RESULTS AND DISCUSSION

Phytochemical Screening

The phytochemical screening of ethanolic extract was performed by color reaction tests. The extract exhibited the presence of phenolic acids, flavonoids, terpenoids and cardiac glycosides (Table 1).

Table 1. Phytochemical screening of *C. maxima* ethanolic fruit extract.

Phytochemicals	Tests	Ethanolic Extract
Phenolic acids	Ferric chloride test	+
Flavonoids	Alkaline reagent test	+
Chalcones	Ammonium hydroxide test	-
Terpenoids	Salkowski's test	+
Saponins	Froth test	-
Alkaloids	Wagner's test	-
Cardiac glycosides	Keller-Killani's test	+

Total Phenolic Content (TPC)

Among various secondary metabolites of plant, phenolics have one or more hydroxyl groups directly associated with the aromatic ring. On the basis of the number of phenol units, phenolics are broadly classified as simple phenols (phenolic acids) and polyphenols (flavonoids). Phenolic compounds are responsible for unique taste, flavour, colour and health-promoting properties of vegetables and fruits (Khanam et al., 2015). According to Pichaiyongvongdee and Hanruenkit (2009) polyphenols are the major antioxidant among citrus fruits. In the current study Folin-Ciocalteau method was employed to determine TPC, based on the principle of reduction of phosphomolybdic acid by phenols in the presence of aqueous alkali. The results were expressed as mg gallic acid equivalent (GAE) as this compound represents the simplest form of a phenolic compound. From the calibration curve; straight line equation was obtained as $y = 0.0099x - 0.0478$ ($R^2 = 0.9954$). The TPC of *C. maxima* extract was ranged from 33.08 to 33.11 mg GAE/g of extract. The average TPC was obtained as 31.96 ± 1.34 mg GAE/g of extract on dw basis (Table 2).

Table 2. TPC and TFC of *C. maxima* ethanolic fruit extract.

Sample	TPC (mg GAE/g dw)	TPC (mg GAE/g dw)	TFC (mg QE/g dw)	TFC (mg QE/g dw)
Sample 1	30.08	31.96 ± 1.34*	16.0	19.33 ± 3.12*
Sample 2	33.11		18.5	
Sample 3	32.71		23.5	

Results are presented as the mean of triplicate measurements (n = 3 ± SD). * Values are significantly different from each other ($P < 0.01$).

Total Flavonoidic Content (TFC)

Flavonoid comprise of a large group of polyphenolic compound, having benzo-γ-pyrone structure with basic skeleton of C_{15} (C_6-C_3-C_6). Flavonoids are plant pigments synthesized by phenylalanine pathway and generally display excellent colours in flowering plants. Recent interest in flavonoids attributed to numerous pharmacological activities and potential health promoting properties arising due to antioxidant activities (Yao et al., 2004; Ghasemzadeh and Ghasemzadeh, 2011). It is believed that flavonoid protect cells from degradation, stress, toxins, assist transport across membranes, attract or repell pollinators or pests, responsible for rich aroma and taste (Bala et al., 2010). In the present study almunium chloride method was used, based on the reaction of almunium ion with flavonoids to form red chelates in alkaline medium. TFC results were expressed as mg quercetin equivalent (QE)/g of extract on dry weight (dw) basis. From the calibration curve straight line equation was obtained as $y = 0.0004x - 0.0024$ ($R^2 = 0.964$). The TFC ranged from 16.00 to 23.50 mg

QE/g of *C. maxima* extract. The average TFC was obtained as 19.33 ± 3.12 mg QE/g of extract on dw basis (Table 2).

HPLC Analysis

The HPLC analyses (Table 3) of *C. maxima* ethanolic fruit extract showed the presence of caffeic acid, gallic acid, 4-hydroxycinnamic acid, 4-hydroxy-3-methoxycinnamic and vanillic acid (Figure 3, Table 3). Flavonoids such as naringenin, kaempferol, luteolin, quercetin and myricetin were detected in ethanolic extract. Chlorogenic acid and *trans*-cinnamic acid were absent among the tested phenolic acids. The flavonoid apigenin was not found in the fruit extract of *C. maxima*. The highest amount of flavonoid was identified as quercetin (38.734 %) followed by kaempferol, luteolin, naringenin and myricetin. The major phenolic acid in *C. maxima* was identified as gallic acid (14.230 %) followed by 4-hydroxy-3-methoxycinnamic, 4-hydroxycinnamic acid, vanillic acid and caffeic acid.

Table 3. Phenolic acids and flavonoids of *C. maxima* ethanolic fruit extract.

Standard	Retention time R_t	Area (%)
Caffeic acid	2.116	0.041
Chlorogenic acid	nd	-
Gallic acid	1.98	14.230
4-Hydroxycinnamic acid	9.300	0.242
4-Hydroxy-3-methoxycinnamic	5.288	1.036
Trans-cinnamic acid	nd	-
Vanillic acid	5.760	0.147
Apigenin	nd	-
Kaempferol	5.001	0.976
Luteolin	6.707	0.944
Myricetin	9.529	0.218
Naringenin	8.270	0.460
Quercetin	0.752	38.734

nd= not detected

DNA Cleavage Activity

In the present study, DNA cleavage activity was assessed by treating double stranded supercoiled plasmid DNA (Form I) isolated from *E. coli* with *C. maxima* ethanolic fruit extract at various concentrations (0.125 µg/µl to 0.600 µg/µl). It is reported that compounds or extracts may have potential to cleave DNA band from Form I (native plasmid DNA) to Form II (single-stranded, nicked circular plasmid DNA) or from Form II to Form III (linear plasmid DNA) (Khanam et al., 2015). The cleavage activity has been evaluated by observing the intensity or removal of bands with increasing concentration. In *C. maxima* a notable cleavage activity has been observed after introduction of ethanolic extract (Fig.1). The gel electrophoresis of extract exhibited the dose dependent appearance of Form II and Form III with simultaneous disappearance of Form I (Khanam et al., 2014). With the increase in concentration, there was almost complete removal of Form I, suggesting no circular form of DNA sustained in the presence of the extract at 0.600 µg/µl concentration (Fig. 1, lane 6). The further cleavage of nicked form was indicated by its reduced band width as compared to Form III with increase in concentration of the extract (Arianingrum, 2009). The broadening of Form III band seems to begin merely at 0.125 µg/µl of ethanolic extract treatment (Fig. 1, lane 2), indicating impeccable DNA

cleavage potential of the extract.

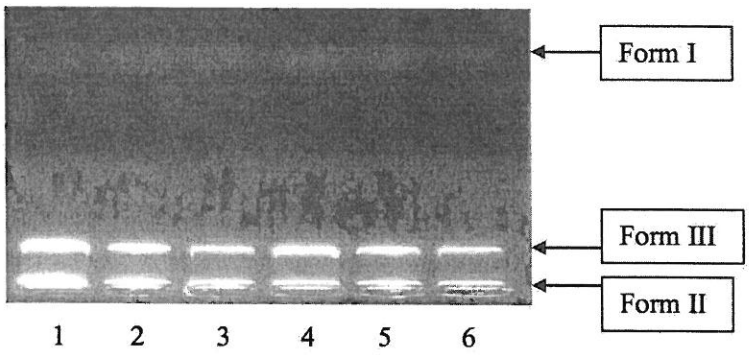

Figure 1. DNA cleavage activity of *C. maxima* ethanolic fruit extract. Lane. 1: DNA (control), Lane 2: DNA + 5 µl extract, Lane 3: DNA + 10 µl extract, Lane 4: DNA + 15 µl extract, Lane 5: DNA + 20 µl extract, Lane 6: DNA + 25 µl extract.

CONCLUSIONS

Quantitative and qualitative analyses of *C. maxima* ethanolic fruit extract showed it to be a good source of phenolics and flavonoids, indicating high antioxidant potential. As there is a growing interest in polyphenolics due to its antioxidant property, *C. maxima* may have future in food and cosmetics industry. Also, the ethanolic extract exhibited an efficient DNA cleavage activity against pathogenic *E. coli* plasmid DNA to Form III. Thus, the present study provides preliminary evidence with regard to neutraceutical value of *C. maxima* and is worthwhile to be incorporated in daily diet for beneficial polyphenolic supplementation. Furthermore, extensive study is a prerequisite to exploit DNA cleaving properties of *C. maxima* for therapeutic applications such as antimicrobial and anticancer.

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REFERENCES

Albach, R. F., Juarez, A. T., & Lime, B. J.(1996). Time of naringin production in grapefruit. *Journal of the American Society Horticultural Science*, 94, 605-609.

Arianingrum, R. (2009). In vitro activity of supercoiled double stranded DNA cleavage by proteins extracted from sweet potato (*Ipomea Batatas* L.) peel, *NU Science Journal*, 6, 73-79.

Arias, B. A., & Ramon-Laca, L. (2005). Pharmacological properties of citrus and their ancient and medieval uses in the Mediterranean region. *Journal of Ethnopharmacol.*, 97, 89-95.

Avinash, P., Swapneel, K., Anita, P. (2012). A comprehensive review of an important medicinal plant: *Averrhoa carambola* L. *Pharmacognosy Communications*,2(2), 26-39.

Bala, A., Kar, B., Haldar, P. K., Mazumder, U. K., & Bera S. (2010). Evaluation of anticancer activity of *Cleome gynandra* on Ehrlich's ascites carcinoma treated mice. *Journal of Ethnopharmacology.*, 129(1), 131–134.

Ghazemzadeh, A., & Ghazemzadeh, N. (2011). Flavonoids and phenolic acids: Role and biochemical activity in plants and human. *Journal of Medicinal Plants Research*, 5(31), 6697-6700.

Gopalakrishnan, S., & Vadivel, E. (2011). DNA cleavage studies of the ethanolic extracts of the bark of Bohinia tomentosa L. and the whole plant of Mussaenda frondosa L., *Int. Res. J. Pharm.*, 2,

153-154.

Khanam, Z., Ching, C. H., Zakaria, N. H. B. M., Sam, K. H., & Bhat, I. U. H. (2014). Phytochemical analyses and DNA cleavage activity of *Citrus maxima* fruit. International Conference on Chemistry and Environmental Sciences Research 2014 (ICCESR 2014), 17-18 Sept, 2014, Pulau Pinang, Malaysia.

Khanam, Z., Sam, K. H., Zakaria, N. H. B. M., Ching, C. H., & Bhat, I. U. H. (2015). Determination of polyphenolic content, HPLC analyses and DNA cleavage activity of Malaysian *Averrhoa carambola* L. fruit extracts, nal of King Saud University-Science 27, 331–337

Marinova, D., Ribarova, F., & Atanassova, M. (2005). Total phenolics and total flavonoids in Bulgarian fruits and vegetables. *Journal of the University Chemical Technology and Metallurgy*, 40, 255-260.

Morton, J. F. (1987). *Fruits of warm climates*, Winterville, North Carolina, Creative Resources Systems Inc.

Naqvi, S., Ullah, M. F., & Hadi, S. M. (2010). DNA degradation by aqueous extract of *Aloe vera* in the presence of copper ions., *Indian J. Biochem. Biophys.*, 47, 161-165.

Pichaiyongvongdee, S., & Haruenkit, R. (2009). Investigation of limonoids, flavanones, total polyphenol content and antioxidant activity in seven thai pummelo cultivars. *Kasetsart J. (Nat. Sci.)*, 43, 458-466.

Raaman, N. (2006). *Phytochemical techniques*. New Delhi, New India Publishing Agency.

Singleton, V. L., Orthofer, R., & Lamuela-Raventos, R.M. (1999). Analysis of total phenols and other oxidation substrates and antioxidant by means of Folin-Ciocalteu reagent. *Methods in Enzymology*, 299, 152-178.

Yadav, P., Yadav, A., Garg, V., Gupta, T. K., Goswami, S. L., & De S. (2011). A novel method of plasmid isolation using laundary detergent, *Indian Journal of Experimental Biology*, 49, 558-560.

Yao, L.H., Jiang, Y. M., Shi, J., Tomas Barberan, F. A., Datta, N., Singanusong, R., & Chen, S. S. (2004). Flavonoids in food and their health benefits, *Plant Foods for Human Nutrition*, 59, 113-122.