ISSN 0974 – 5211

Journal of Natural Products

Volume 2 (2009)

www.JournalofNaturalProducts.com

Research Paper

Screening of xanthone from mangosteen (*Garcinia mangostana* L.) peels and their effect on cytochrome c reductase and phosphomolybdenum activity

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(Received 05 December 2008; Revised 15 January 2009; Accepted 20 January 2009)

ABSTRACT

The aim of this study was to separate and determine the xanthones from mangosteen (*Garcinia mangostana*) pericarp. The xanthones in the acetone, ethyl acetate and hexane extract, was investigated by means of high performance liquid chromatography–electrospray ionisation/mass spectrometry (HPLC–ESI/MS) technique. Chromatography of the xanthones on a RP-C-18 HPLC column followed by MS detection using ESI negative mode showed the presence of seven major known xathones: 3-isomangostin, 8-desoxygartanin, gartanin, α-mangostin, garcinone E (tentatively identified), 9-hydroxycalabaxanthone and β- mangostin. The antioxidative properties of mangosteen pericarp extracts which is the mixture of these xanthones obtained by different solvents were investigated for reactive oxygen species scavenging, like cytochrome c assay, molybdenum activity and lipid peroxidation inhibition assay. Of the solvent extracts acetone and ethyl acetate extracts reduced oxidized cytochrome c to an OD of 0.455 and 0.321 at 400 µg; each of the extract reduced Mo (1V) to Mo (V) at 100 µg to 7967 ± 47.1 and 6803 ± 42.1 µmole/g of ascorbic acid equivalent. The IC_{50} value for lipid peroxidation was 9.2 ±3.8 and 10.0 ± 1.7 µg/ml which was higher than standard BHA respectively. Hexane extract showed less antioxidant activity in the above test because of the poor solubility of the xanthones. The results indicate that xanthones show good antioxidant property and a method was validated to identify them by HPLC-MS method.

Keywords: Xanthones, HPLC, ESI/MS, Antioxidant activity.

INTRODUCTION

Mangosteen (*Garcinia mangostana* L.) is a tropical fruit available in Southeast Asia. It is used as traditional medicine to treat skin infections, wounds, and diarrhea (Mahabusarakam, et al., 1987). Mangosteen has become recently popular as an alternative medicinal product. There are over 50 natural xanthones (Pedraza, et al., 2008) reported in mangosteen. Xanthones are secondary metabolites commonly

occurring in a few higher plant families, and in fungi and lichens. Xanthones and tannins assure astringency to discourage infestation by insects, fungi, plant viruses, bacteria and animal predation while the fruit is immature (Akao, et al., 2008). Their taxonomic importance in such families and their pharmacological properties has aroused great interest not only for the chemosystematic investigation but also from pharmacological point of view like antimalarial (Mahabusarakam, et al., 2006; Laphookhieo, et al., 2006), antibacterial (Chomnawang, et al., 2005; Rassameemasung, et al., 2007), antifungal and antiviral properties (Gopalakrishnan, et al., 1997; Vlietinck, et al., 1998), anti-inflammatory and antiallergy (Deschamps, et al., 2007; Chen, et al., 2008), antioxidant properties (Chin, et al., 2008; Haruenkit, et al., 2007) and antitumoral properties (Nakagawa, et al., 2007; Suksamran, et al., 2006).

Xanthones are commonly separated by chromatography on silica gel, using an appropriate solvent mixture by HPLC. Reverse phase- high performance liquid chromatography (RP-HPLC), with diode array detector (DAD) is widely applied for the analysis of these compounds due to its high sensitivity and easy operation. The structures of xanthones are derived mainly by IR, mass spectral data and NMR data (Peres, et al., 2000).

Xanthones like any other class of phenolic compounds cannot be produced by the human body. Nutritional and therapeutic role of dietary phenolic antioxidants is essential for the development of functional foods which requires knowledge about chemical composition of foods. Phenolic compounds widely distributed in plants, spices, vegetables, fruits, grains, pulses and other seeds are an important group of natural antioxidants with possible beneficial effects on human health. They can participate in protection against the harmful action of reactive oxygen species, mainly oxygen free radicals. Free radicals are produced in higher amounts in a lot of pathological conditions and are involved in the development of the most common chronic degenerative diseases, such as cardiovascular disease and cancer. Hence our objective was to screen and analyze the chemical composition of *G. mangostana* extracted by SOXTEC (Foss Tecator, Sweden) apparatus using acetone, hexane and ethyl acetate as extraction solvent and examine their effect on cytochrome c reductase, phosphomolybdenum activity and lipid peroxidation. The chemical compositions of the above extracts were characterized by HPLC / ESI/MS.

MATERIALS AND METHODS

***Chemicals*:** Acetic acid, methanol, acetone and water were of HPLC grade, butylated hydroxyanisole (BHA), L-ascorbic acid, ferrous sulfate, tris buffer, thiobarbituric acid (TBA), trichloroacetic acid (TCA), ammonium molybdate were from Merck (Mumbai, India) linoleic acid was from Sigma (MO,USA), cytochrome c was purchased from Himedia (Mumbai, India). All the other chemicals used were of analytical grade.

***Preparation of extracts*:** The fresh pericarp of mangosteen was tray dried at 40-52 °C and ground into powder using hammer mill equipment. The fine powder (5g) was extracted using SOXTEC apparatus with various solvents. The solvent systems used were ethyl acetate (EtOAc), hexane (HX) and acetone (Ace). Extractions were carried out for 2 h that include initial boiling for 30 min. After filtering the extract through Whatman No. 1 paper, each of the filtrates was concentrated using rota evaporator at 40 °C, the weight of the each extract was noted and the final volume was made up to

25 ml in a volumetric flask. The extracts were kept in airtight amber bottles after flushing with nitrogen gas for 30s and stored in freezer at -20 °C until further analysis.

Determination of total phenol content: Total phenol was determined by Folin-Ciocalteu reagent and was expressed as milligrams of gallic acid equivalents (GAE) per gram dry of the extract (Kujala, et al., 2000).

HPLC/ESI-MS analysis*:* The separation of xanthones compounds was performed using Shimadzu – LC 10A (Japan) with UV detector system. The column was RP-C 18, 150 X 4.6 mm, SS, Exsil ODS 5 µM particle size operated at a temperature of 40 °C. The mobile phase consisted of 0.1 % (v/v) acetic acid in water (eluent A) and 95% (v/v) methanol (eluent B). The gradient program was as follows: 65 to 90% B over 0–40 min at the flow rate of 1.0 ml/min. The injection volume for all samples was 10 µl. Ultraviolet (UV) spectra were recorded at 254 nm. For the MS, a mass spectrum (CFTRI, Mysore) was acquired using Ultima ESI-Q- TOF. Drying gas: N_2, drying gas temperature was 325 °C. For negative ESI analysis, the parameters were, capillary voltage: -3.00 KV; Cone: 100, source temperature: 120 °C, desolvation temperature: 300 °C, cone gas flow 50 l/hr, desolvation gas: 500 l/hr. The mass range was from 200 to 900 m/z, scan sped 1000 amu/sec. Data acquisition and processing was done with the software Masslynx 4.0 and held at that composition for another 5 min. Negative ion mass spectra of the column elute was recorded in the range m/z 50-1000. The flow rate was adjusted to 0.8 ml/min. The injection volume was 10 µl. The data was collected and compared with mass spectra and data from Chem draw software, Cambridge, USA.

Cytochrome c reduction: The cytochrome c reducing capacity of the mangosteen extract was determined according to the method of Suter and Richter, (2000). Unaltered cytochrome c due to its active heme group, which contains a ferrous ion has a characteristic spectrum with λ max of 550 nm, when subjected to oxidation by oxygen saturated phosphate buffer the peak at 550 nm will diminish quantitatively. In the assay, cytochrome c (15 M) was subjected to oxidation by oxygen saturated phosphate buffer (0.1 mM, pH 7), extracts (400 µg)/BHA (800 µM) was added and incubated at ambient temperature for 30 min. Absorbance at 550 nm was measured spectrophotometrically. Appropriate controls were maintained.

Determination of total antioxidant capacity by phosphomolybdenum method**:** The assay is based on the reduction of molybdate (VI) to Mo (V) by the extract (Miyke, et al., 1997). The tubes containing extract and reagent (0.6M sulfuric acid, 28 mM sodium phosphate and 4 mM ammonium molybdate) were incubated at 95 °C for 90 min. After the mixture had cooled to room temperature, the absorbance of each solution was measured at 695 nm against a blank. The mixture prepared without sample or standard served as a control.

Thiobarbituric acid assay: Lipid peroxidation was induced by ferrous sulfate–ascorbic acid solution according to the procedure of Shimazaki, et al., (1984) an aliquot of extract 5-100µg was incubated with ferrous sulfate and ascorbic acid (10:100 µmole) in a final volume of 0.5 ml of Tris buffered saline (10 mM Tris, pH 7.4, 150 mM NaCl), the reaction mixture was incubated at 37 °C for 1 h. The reaction mixtures was treated with 1% TBA and incubated in a hot water bath for 15 min. BHA was used as positive controls while the negative control was without any antioxidant or extract. The color developed was measured spectrophotometrically at 535 nm.

RESULTS

The yield and phenolic content of the extracts are presented in Table 1, the maximum and minimum yields were obtained in ethyl acetate and hexane extracts respectively. The phenolic content was analysed in all the extracts using Folin-Ciocalteu method. Ethyl acetate (269 mg/g) showed maximum phenolic content followed by acetone extract (205 mg/g) and hexane extract (135 mg/g) in turn of gallic acid equivalent.

Table-1: Extraction yield and total phenols in different extracts of mangosteen pericarp

Extract	Yield *(%db)	Polyphenol content (mg GAE/g)
Ethyl acetate	15.4±0.02	269.9±0.02
Hexane	07.7±0.09	135.9±0.03
Acetone	12.0±0.07	205.2±0.02

*Dry weight basis of original sample of pericarp.
Values are the mean ± std deviation (n=3).

The HPLC-ESI-MS method used is based on concepts used in previous papers (Xiuhong Ji, et al., 2007), in a single 35 min chromatographic run. Ethyl acetate, acetone and hexane extracts of mangosteen peel were analysed by HPLC. The relative retention time (RRT) is presented in Table-2. The compounds were identified by comparison with literature and mass spectral data. The chromatograms of the various extracts showed that the compounds are isolated in accordance with the polarity of the solvents used. The ESI permits the observation of ($[M - H]^-$) molecules, fragment ions. The characteristic data and contents of xanthones detected in extracts by mass spectra are presented in Table-3. In the negative ion mode xanthones produced a deprotonated $[M-H]^-$ molecule. As can be seen, seven xanthones 3-isomangostin-m/z 427 (1), 8-desoxygartanin-m/z 379 (2), gartanin-m/z 395 (3), α-mangostin-m/z 409 (4), garcinone E (tentatively identified)-m/z 463 (5), 9-hydroxycalabaxanthone –m/z 407 (6) and β-mangostin - m/z 423 (7) were detected with α mangosteen being the predominant compound in all the extracts.

Table-2: Relative retention time (RRT) of the extracts.

Compound	Hexane extract RRT (min)	Acetone extract RRT (min)	Ethylacetate extract RRT (min)
3-Isomangostin	0.58	0.56	0.58
8-Desoxygartanin	0.73	0.72	0.72
Gartanin	0.79	0.78	0.77
α-Mangostin	1.00	1.00	1.00
Garcinone E (tentatively identified)	1.14	1.12	1.13
9-Hydroxycalabaxanthone	1.47	1.46	1.46
β-Mangostin	1.54	1.54	1.53

Table-3: Mass spectra of compound with ESI-MS method.

Compound	Negative mass spectra (m/z)
3-Isomangostin	427.24 $[M+H_2O–H]^-$
8-Desoxygartanin	379.22 $[M–H]^-$
Gartanin	395.15 $[M–H]^-$
α-Mangostin	409.17 $[M–H]^-$
Garcinone E (tentatively identified)	463.37 $[M–H]^-$
9-Hydroxycalabaxanthone	407.20 $[M–H]^-$
β-Mangostin	423.22 $[M–H]^-$

Cytochrome c, located on the inner mitochondrial membrane is used as a marker for this membrane and is the major electron transport protein of the respiratory chain, was used as a model protein to investigate the direct reductive capacity of the extracts as one of the reaction mechanisms of antioxidant activity. The extracts significantly reduced the oxidised cytochrome c (Figure 1); up to 0.455 in acetone extract which was almost same as standard BHA, followed by ethyl acetate extract in a time dependent manner at 30 min. Hexane extract showed the lowest activity of 0.214.

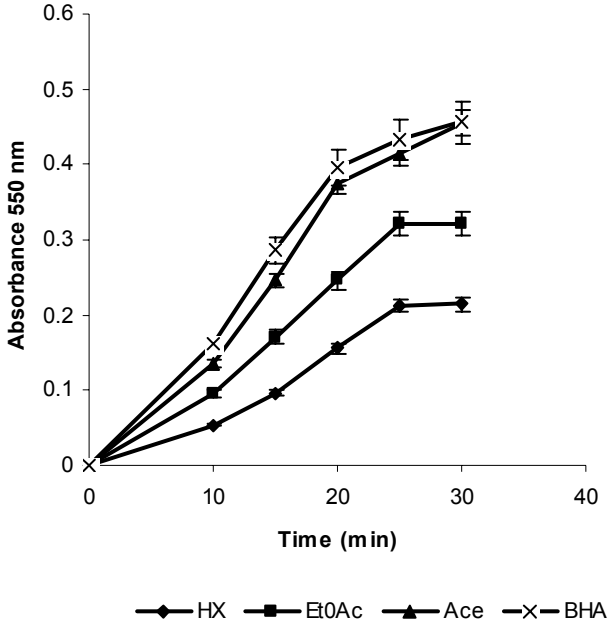

Figure-1: Cytorchrome c reduction capacity of the extracts
HX-Hexane, EtOAc-Ethyl acetate, Ace-Acetone, BHA-Butyl hydroxy anisole

The antioxidant capacity of the mangosteen pericarp extracts was measured by phosphomolybdenum method, which is based on the reduction of Mo (IV) to Mo (V)

by the sample analyte and the subsequent formation of green phosphate/Mo (V) compounds with a maximum absorption at 695 nm. The antioxidant capacity of fruit extracts was found to decrease in the order acetone > ethyl acetate > hexane (Table 4). All the extracts showed increase in antioxidant capacity with increase in dose. The extracts were expressed as equivalents of ascorbic acid (µmol/g of extract).

Table-4: Antioxidant capacity of mangosteen peel extracts as ascorbic acid equivalents (µmol/g of extract).

Concentration (µg/ml)	Ethyl acetate extract	Hexane extract	Acetone extract
25	4137 ± 26.2	1486 ± 32.0	4912 ± 28.6
50	5526 ± 33.5	1680 ± 38.8	6302 ± 35.2
100	6803 ± 42.1	3199 ± 45.0	7967 ± 47.1

- Values are the mean ± std. deviation (n=3).

Thiobarbituric acid reactive substances are produced as by-products of lipid peroxidation induced by the ferrous sulfate:ascorbate system. Increasing concentrations of the extracts were tested for antioxidant activity in the linolenic acid medium as lipid phase model system. The extracts exhibited a dose-dependent inhibition of $FeSO_4$ and ascorbate induced lipid peroxidation. Ethyl acetate and acetone extract showed the maximum IC_{50} value of 9.2±3.8 and 10.0±1.7 µg/ml and the lowest being hexane which had an activity of 26.4±3.2 µg/ml. The antioxidant activity of the extracts was compared with standard antioxidants, such as BHA which was observed to have an IC_{50} of 17.3±1.9 µg/ml. The results suggest that the magnitude of the antioxidant potential of the acetone and ethyl acetate extract is high at low concentration compared to known standard antioxidants.

DISCUSSION

In majority of the cases xanthones occur as substituted compounds hence the adsorption capacity are generally different therefore, their separation in reversed phase separation is not possible using isocratic eluent systems. In order to overcome this difficulty and to increase the separation capacity of liquid chromatographic method gradient elution in HPLC are the methods of preference for the analysis. In the present study we could identity seven xanthones 3-isomangostin, 8-desoxygartanin, gartanin, α-mangostin, garcinone E (tentatively identified), 9-hydroxycalabaxanthone and β- mangostin. The compounds were confirmed by ESI-MS and spectral data (Walker, 2007; Xiuhong Ji, 2007).

To verify the radical scavenging ability of the extracts we carried out the following assay mentioned in methodology. The cytochrome c reductase results indicate that the extract is a potent reducing agent for the active heme group of cytochrome c. Absorbance increases with increase in reduction of oxidized cytochrome c. Similar studies have reported that the aqueous extract of herbal remedy PADMA 28, derived from traditional Tibetan medicine, contains reducing compounds that reduce ferric cytochrome c to ferrocytochrome c (Suter and Richter, 2000). Similarly in phosphomolybdenum assay we observed the effective antioxidant activity of the extract. Lipid peroxidation is a key process in many pathological events and is one of the reactions induced by oxidative stress. The rearrangement of the double bonds in unsaturated lipids and the destruction of membrane lipids to produce breakdown products such as malondialdehyde, is known to be mutagenic and carcinogenic (Miyake and Shibamoto, 1997) hence the extract were tested for their

inhibition of lipid peroxidation. From the results we could imply the extracts to be effective against lipid peroxidation activity. The antiradical activity of xanthones is principally based on the redox properties of their hydroxy groups and structural relationship between different parts of their chemical structure. The low antioxidant activity in hexane extract can be attributed to fact that the compounds are polar in nature and are not completely extracted in hexane whereas acetone and ethyl acetate extracts are able to percolate through the matrix thus enhancing their solubility. Also there may be variation in the quality and quantity of xanthones and other bioactive compounds present in different extracts as observed in total phenol content.

CONCLUSION

The present work reports the chemical nature of xanthones present in the mangosteen pericarp powder by HPLC–MS. The high antioxidant activity of the extract at low concentrations makes mangosteen a potential source of antioxidants.

Acknowledgements: The authors thank the Director, CFTRI, Mysore, for his support in this research. The first author Miss. Zarena A.S thanks CSIR, India for the grant of Senior Research Fellowship.

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