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Industrially Feasible Method for the Extraction, Purification and Quantification of Mangostin (Alpha-Mangostin) from *G. mangostana* Fruit Hulls

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ABSTRACT

Garcinia mangostana fruit hulls extract having major bioactive substance α-mangostins and good amount of polyphenols with very strong anti-oxidant and good anti-diabetic activities. The present study was designed for the extraction and quantification of α-mangostin from the fruit hull of *Garcinia mangostana* L. which was extracted with methanol (>99.0% pure) to get Crude extract which is dried further in Rotoevoparator under vacuum to get yellow colored powder and Yield of extract was estimated (3.9 ± 1.8 %). Alpha-mangostin (36.1 ± 0.7% w/w) and Polyphenols (64.0 ± 3.3 mg/g Tannic acid) content was estimated by HPLC and Spectrophotometrically by Folin-ciocalteu method respectively. Crude extract was again subjected to purification by Liquid-Liquid partitioning using organic solvents, followed by separation, Concentration to remove the solvents, Crystallization and dried the crystallized phytocompound in Rotoevoparator under vacuum to obtain fine, shiny yellowish colored powder. Yield of purified fraction was estimated (2.0 ± 0.7 % w/w). Alpha-mangostin (66.0 ± 0.82% w/w) and Polyphenols (38.6.0 ± 3.3 mg/g Tannic acid) content was estimated by HPLC and Spectrophotometric method respectively. Results obtained from the present study indicate one of the easy and feasible processing methods for the purification of Alpha-Mangostin. Due to the simplicity of the existing method, it can be adopted by the Herbal Industries for commercial scale manufacturing of pure alpha-mangostin.

INTRODUCTION

Garcinia mangostana L, commonly known as "Mangosteen" is one of the most widely recognized tropical evergreen tree, belonging to the family *Guttiferae,* its origin is in Southeast Asia It can now be found in Northern Australia, Brazil, Central America, Hawaii, Southern India, Indonesia, Malaysia, Thailand and other tropical countries. The edible fruit is deep reddish, purple when ripe. In Asia, it is known as the "Queen of Fruits" due to its pleasant flavor. *Garcinia mangostana* L. (Guttiferae), a fruit from topical tree containing phytochemicals called Xanthones have demonstrated interesting biological activities[1,2]. Other biological activities of the constituents of the fruit hulls have demonstrated antibacterial,[3-5] antifungal,[6] and antitumor-promotion[7]. *In vitro* studies have been conducted to examine the antioxidant and antidiabetic properties of the extracts or particular xanthones obtained from the fruit hulls of this plant.

Our study as explained in this paper focuses on the easy and industrial feasible method of purification of alpha-mangostin from fruit hulls of Garcinia mangostana, Identification and quantification by TLC, UV-Vis Spectrophotometer and HPLC. Purity of the purified alpha-mangostin was confirmed with working standard (standardized against pure alpha-mangostin from chromadex) of Alpha-mangostin which is received as complimentary sample from Phytoteck extracts Pvt Ltd,(1564-1566, Nilgiri's Supermarket Cross Road, St. Thomas Town PO, Kammanahalli, Bangalore 560084) by TLC, UV-Vis spectrophotometer and HPLC.

We hereby tried to get purified Alpha-mangostin and to report a rapid and highly efficient extraction, purification and quantification procedure for the Alpha-mangostin. We also examined the Total polyphenols content in the purified Alpha-mangostin fraction which is needed for the synergetic effect with Alpha-mangostin.

Compared to the previous reports on this fruit hull extracts, the present procedure for the purification and quantification is much more efficient and rapid and resulted in higher content of Alpha-mangostin which is the main component (as per the literatures) responsible for the various useful pharmacological activities.

MATERIALS AND METHODS

Materials:

Methanol, Acetone, Benzene, chloroform, Toluene and TLC, HPTLC plates were purchased from Merck, Frankfurter, Strabe, 25064293, Darmstadt, Germany. Working standard of Alpha-mangostin (90.0% purity) was obtained as complimentary sample from Phytoteck extracts Pvt. Ltd., Bangalore, India (With certificate of analysis). Anisaldehyde reagent, Sulphuric acid and Folin-ciacalteu reagent, unless and otherwise mentioned all the other chemicals were purchased from Ranbaxy, SRL Chemicals and Qualigens, India.

CAMAG TLC system, CAMAG TLC III scanner, stationary phase used was silica gel G60F254 and 10x5 cm TLC plate. Standard of Alpha-mangostin >90.0% purity (ChromaDex Inc, #10005, Muirlands Blvd, Irvine, CA- 92618, USA) was obtained as complimentary from Phytoteck extracts Pvt. Ltd., (1564-1566, Nilgiri's Supermarket Cross Road, St.Thomas Town PO, Kammanahalli, Bangalore, India).

Plant material, Extraction and purification of Alpha-mangostin:

Plant Material: Mangosteen fruits were purchased from local markets in Bangalore, India during August, 2009 and authenticated by Prof. B.K Manjunath, Department of Biotechnology, Oxford College of Engineering, Bangalore, Karnataka, India. The voucher specimen (KU/AB/KSV/3012) was deposited in the University herbaria at Post Graduate Department of Studies and Research in Botany, Shankaraghatta, Karnataka for future reference.

Extraction: Fresh fruits obtained from the local market, Bangalore were washed with water and hand processed to remove hulls and inner layer of white colored flesh and seeds. The fruits were cleaned thoroughly and the edible part was removed. The fruit hull was cut into small pieces and shade dried for 15-20 days. Shade dried (2.1 kg) mangosteen fruit hulls were made into powder for the extraction. Dried powder was checked for the Alpha-mangostin content by HPLC before taking for the extraction.

2.0 Kg of the coarsely grinded RM was extracted with fresh 8.0 Ltrs of methanol for about 2 hrs under reflux condition, filtered the extract and two more similar extractions were done with 6.0 liters of fresh methanol. All the extracts were combined, concentrated and dried the combined liquid extract in Buchii rotoevoparator at temperature < 70°C under Vacuum (500 mm Hg) to afford 78.0 Gms of Crude extract (E1). The crude extract obtained was washed with 1:1 volume of DM (Demineralized) water (i.e., 78.0 ml) by constant stirring and allowed for the setting for 2 to 3 hrs. The mixture was filtered through whatman No.1 filter paper to separate water and settled crude extract. The filtrate was discarded and extract obtained was dried on the filter paper under vacuum (500 mm Hg) to afford 72.0 Gms of Water washed crude extract (E2).

Purification: Purification of the Alpha-mangostin was performed as shown below and the starting material used for the purification was water washed crude extract (E2). 70.0 Gms of E2 was dissolved in 70.0 ml of Benzene (1:1 volume), heated at 50°C to dissolve and filtered into a separate beaker. Filtrate collected was concentrated in a buchii vacuum rotoevoparator at temperature <70°C under vacuum (500 mmHg) to get dark brown colored thick paste. Transferred the hot thick paste obtained immediately into separate beaker and left for 2-3 hrs for cooling to get hard cake like extract. Dissolved this hard thick cake like substance in 50 ml Toluene at 55°C with constant Stirring and filtered into a separate beaker. Filtrate collected was concentrated in a buchii vacuum rotoevoparator at temperature <70°C under vacuum (500 mmHg) to get dark brown colored thick paste. Transferred the hot thick paste obtained immediately into another separate beaker and left for 2-3 hrs at room temperature to get hard cake like material. Obtained hard extract material was next dissolved in mixture of Benzene, toluene and water (30ml, 30 ml and 10 ml respectively) at 50°C under stirring for 30 min. Cooled the mixture for 5 to 6 hrs at room temperature resulted in the yellow colored shiny amorphous powder which are separated clearly from the rest of solvent mix. Separate the yellow colored shiny amorphous powder from the solvent mix by filtration through whatman No.1 filter paper. Powder obtained by filtration is treated with hot water followed by separation by filtration by Whatman filter paper again. Now the separated yellow colored amorphous powder was dried very carefully under vacuum (500 mm Hg) at < 50°C to get dried, light yellow colored shiny powder. This purified material was again dried at low temperature (40-45°C) to lower the moisture content, grinded in mortar and pestle to get uniform size powder (Fraction E3) (34.0 Gms).

Presence of purified Alpha-mangostin in the above fraction was confirmed by TLC. Purity of the alpha-mangostin was checked by HPLC with the Working standard obtained from Phytoteck. (Qualified against Alpha-mangostin from Chromadex Inc).

Identification of Alpha-mangostin:

a) By TLC:

Preparation of standard Alpha-mangostin solution: The standard solution was prepared by dissolving 10 mg of working standard in 10 ml methanol solution. The working standard of 100 µg/ml was prepared from standard solution by diluting with methanol.

Preparation of Fraction E3 solution: The sample solution was prepared by dissolving 10 mg of Fraction E3 in 10 ml methanol solution. The sample solution of appx 100 µg/ml was prepared by diluting further with methanol.

Mobile phase for the Separation: Chloroform: Acetone: Benzene (4:3:3 v/v/v).

TLC Condition: 10 cm × 5 cm TLC silica gel G60F254 plate was used for the analysis. TLC plate was cleaned by pre-development with methanol, followed by drying in an oven 105°C for 5 min. Sample and standard were applied manually to the layer as bands. TLC plates were developed in a CAMAG twin trough glass chamber (20 x 10 cm) by ascending method and detection was done by spraying the spray reagent Anisaldehyde sulphuric acid (ANS) and heating the plate at 105°C.

b) By HPTLC:

Preparation of standard Alpha-mangostin solution: The standard solution was prepared by dissolving 10 mg of working standard in 10 ml methanol solution. The working standard of 100 µg/ml was prepared from standard solution by diluting with methanol.

Preparation of Fraction E3 solution: The sample solution was prepared by dissolving 10 mg of Fraction E3 in 10 ml methanol solution. The sample solution of appx 100 µg/ml was prepared by diluting further with methanol.

Documentation report:

Instrument name	:	CAMAG Linomat 5 "Linomat5_140435" S/N 140435 (1.00.12)
Stationary phase	:	Silica gel 60 F 254
Manufacturer of Stationary phase	:	Merck KGaA
Plate size (X x Y)	:	20 X 10 cm
Calibration mode	:	Single level
Evaluation mode	:	Peak Height
Mobile solvents	:	Chloroform: Acetone: Benzene
Solvent ratio (%v/v)	:	4:3:3
Spray reagent used	:	Anisaldehyde Sulphuric acid
Linomat 5 application parameters		
Spray gas	:	Inert gas
Sample solvent type	:	Methanol
Dosage speed	:	150 nl/s
Predosage volume	:	0.2 ul
Sequence		
Syringe size	:	100 ul
Number of tracks	:	8
Application position Y	:	8.0 mm
Band length	:	6.0 mm
Solvent front position	:	83.0 mm

d) Determination of Molecular weight of Phytocompound present in Faction E3:

Aim: To determination of Molecular weight of Phytocompounds present in the Purified Fraction E3						
Sample preparation	**Sample dilution:** ~100mg of Purified Fraction E3 is dissolved in 10ml of Methanol Note: all the extraction and concentrations were done at room temperature					
LC conditions	**Solvent –A**	:	0.1% TFA in water			
	Solvent –B	:	0.1% TFA in Methanol			
	Gradient	:	**Time**	**TFA (%)**	**Methanol (%)**	
			0.0	95.0	5.0	
			0.01	95.0	5.0	
			10.0	10.0	90.0	
			15.0	5.0	95.0	
			25.0	5.0	95.0	
			30.0	10.0	90.0	
			35.0	95.0	5.0	
			40.0	95.0	5.0	
	Flow rate	:	0.8ml/min			
	Injection vol	:	20µl			
	Column	:	C18, LCMS col Thermo			
MS conditions	**Source type**	:	APCI			
	Vaporizer temp	:	350° C			
	Capillary temp	:	220° C			
	Sheath gas	:	(15)Nitrogen			
	Auxiliary Gas	:	(5)Nitrogen			
	Discharge Current	:	5K µA			
	Capillary volt	:	35.00V			
	Tube Lens	:	124.83 v			
Scan details	Detection of the peak is done in SRM mode and pseudo SRM mode					
	STD		**SRM conditions**		**Pseudo SRM conditions**	
	Alpha-mangostin					
Observations						

e) HPLC Quantification of Alpha-Mangostin in partially purified Fraction E3:

Time	Solvent A (%)	Solvent B (%)
0.0	95.0	5.0
0.01	95.0	5.0
10.0	10.0	90.0
15.0	5.0	95.0
25.0	5.0	95.0
30.0	10.0	90.0
35.0	95.0	5.0
40.0	95.0	5.0
45.0	Stop	

Active/Marker compounds are separated from the raw material and extracted by reverse phase chromatography. Separated compounds are identified with their retention times. The chromatographic profile of the sample obtained by this method is compared with the profile obtained using reference sample. The marker compounds are identified by comparing the retention time and quantified with the corresponding peak areas.

Chromatographic system:

(1) Shimadzu integrated High-Performance Liquid Chromatography system LC/2010 comprising of system controller unit, degassing unit, low-pressure gradient unit, 4 solvents pump unit, mixer, Autosampler, column oven, UV-Vis detector and class VP Ver. 6.0 workstation is used for analysis.

(2) High-Performance Liquid Chromatography system equipped with LC10A pump, SPD-M 10AVP Photo Diode Array Detector in combination with Class VP software wherever PDA analysis is carried out.

Method:

Gradient reversed phase (RP), HPLC procedure is adopted.

- *Preparation of Standard solution:* 100 mg of working standard is dissolved in 10 ml of Methanol. Filter the preparation through 0.45 Micron syringe filter before injection.
- *Preparation of Sample (Fraction E3):* 100 mg of Sample (Fraction E3) is dissolved in 10 ml of Methanol. Filter the preparation through 0.45 Micron syringe filter before injection.
- *Mobile phase: Solvent A:* 0.1% TFA in water

Solvent B: 0.1% TFA in Methanol

- *Gradient Conditions:*

- ***Column:*** ODS, C-18. Phenomenex: Type: Luna 5µ C18 (2), Size: 250 x 4.60mm 5µ micron.

- ***Chromatographic conditions:***

- Detector: SPD- 10 AVP Photodiode array detector or Uv-Vis. detector.
- Flow rate: 1.0 ml /min
- **Wavelength:** 242nm for monitoring.
- **Injection value:** 20 µl

Procedure:

Set the instrument as per the chromatographic conditions described. Inject 20 µl of standard solution, record chromatogram. Repeat the injections twice and calculate the RSD (Relative Standard Deviation). RSD does not exceed by 2%. Injected 20µl of sample solution, and record the chromatogram.

The chromatographic profile of the sample obtained by this method is compared with the profile obtained using crude drug reference sample. The marker compounds are identified by comparing the Retention time and quantified with the corresponding peak areas.

All separated compounds in the chromatogram were identified using their retention times in comparison with the retention time of the reference standard or by the relative retention time.

Calculate their percentage of Alpha-mangostin in Fraction E3 using the peak area.

$$\frac{\text{Area of sample}}{\text{Area of Std.}} \times \frac{\text{Wt. of std. (mg)}}{\text{Wt. of sample (mg)}} \times \frac{\text{Dilution}}{\text{Std. Dilution}} \times \frac{100}{100} \times \text{Purity} = \%\text{w/w}$$

f) Estimation of Total Polyphenolic Content in the Fraction E3 (Partially purified Alpha-mangostin):

Total Polyphenolics content was determined by Folin-Ciocalteu method[8-15]**.** In brief, 200 µL of test sample was added to a test tube that containing 2.6 ml of distilled water. After vortexing the tube, 200 µL of Folin-Ciocalteu's phenol reagent was added. The tubes were vortexed and after 6 min, 2 ml of 7 % Na_2CO_3 was added. The tubes were vortexed again and allowed to stand for 90 min at room temperature and the absorbance of reaction mixture was measured against a blank at 750 nm using a spectrophotometer (Shimadzu UV-1609). A calibration curve was constructed, using Tannic acid as standard. The total phenolics content of extract was expressed in terms of milligrams of Tannic acid (mg GAE) per gram of dry extract.

RESULTS AND DISCUSSION

Identification of Alpha-mangostin by TLC:

Several mobile phase mixtures were tried to separate the spot of Alpha-mangostin. TLC Chromatogram with good resolution in **Figure no. 1** was attained in the solvent system Chloroform: Acetone: Benzene (4:3:3 v/v/v). Spray reagent used for the derivatization was Anisaldehyde sulphuric acid (ANS). Identification of Alpha-mangostin in partially purified extract was confirmed against standard Alpha-mangostin at Rf value 0.59 ± 0.06 **(Table 1).**

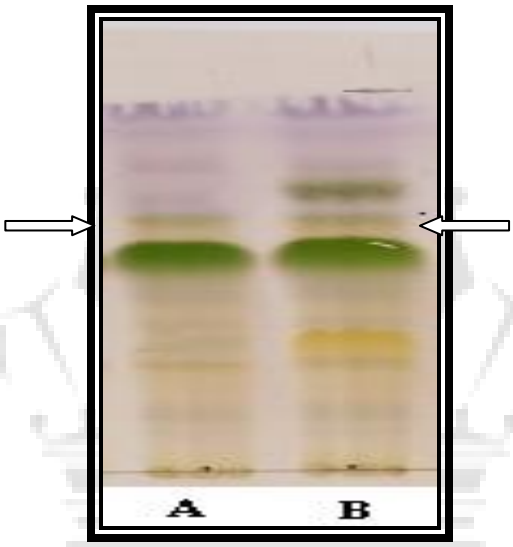


Figure: 1: TLC Fingerprint profile of partially Purified Alpha-mangostin (B) and standard (A)

Table 1: Rf Values of Alpha-mangostin (Major greenish band) - Standard Vs Partially purified.

	Presence of Alpha-mangostin	Color of the Spot	Rf Values	No of major spots
Standards Alpha-mangostin	+++	Dark green colored	0.59 0.63	1
Partially purified Alpha-mangostin	+++	Dark green colored	0.36 0.59 0.63 0.66	3

+++ indicates the presence very prominent

Identification of Alpha-mangostin by HPTLC:

HPTLC Chromatogram with clear resolution of all the phytochemical compounds in Fraction E3 was obtained in the solvent system Chloroform: Acetone: Benzene (4:3:4 v/v/v). Chromatograms observed at various wavelengths (Under UV and Normal light). TLC plate developed in a CAMAG twin trough glass chamber (20 x 10 cm) by ascending method and detection was done by spraying the spray reagent Anisaldehyde sulphuric acid (ANS) and heating the plate at 105°C (Figure No.2). Identification of Alpha-mangostin in partially purified extract was confirmed against standard Alpha-mangostin at Rf value 0.64 ± 0.05 (Table 2).

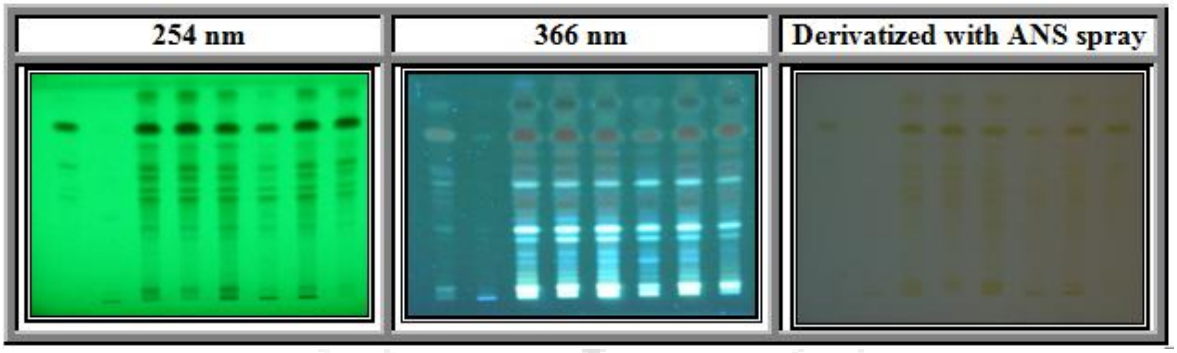


Figure 2: HPTLC fingerprint profile of Fraction E3 (From Track no; 3-8) and Standard Alpha-mangostin (Track no;1). Track no-2 is blank.

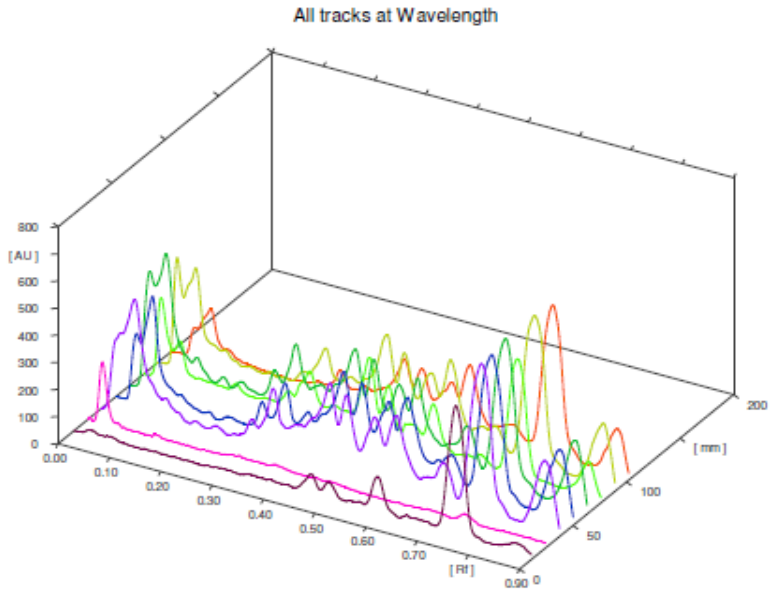


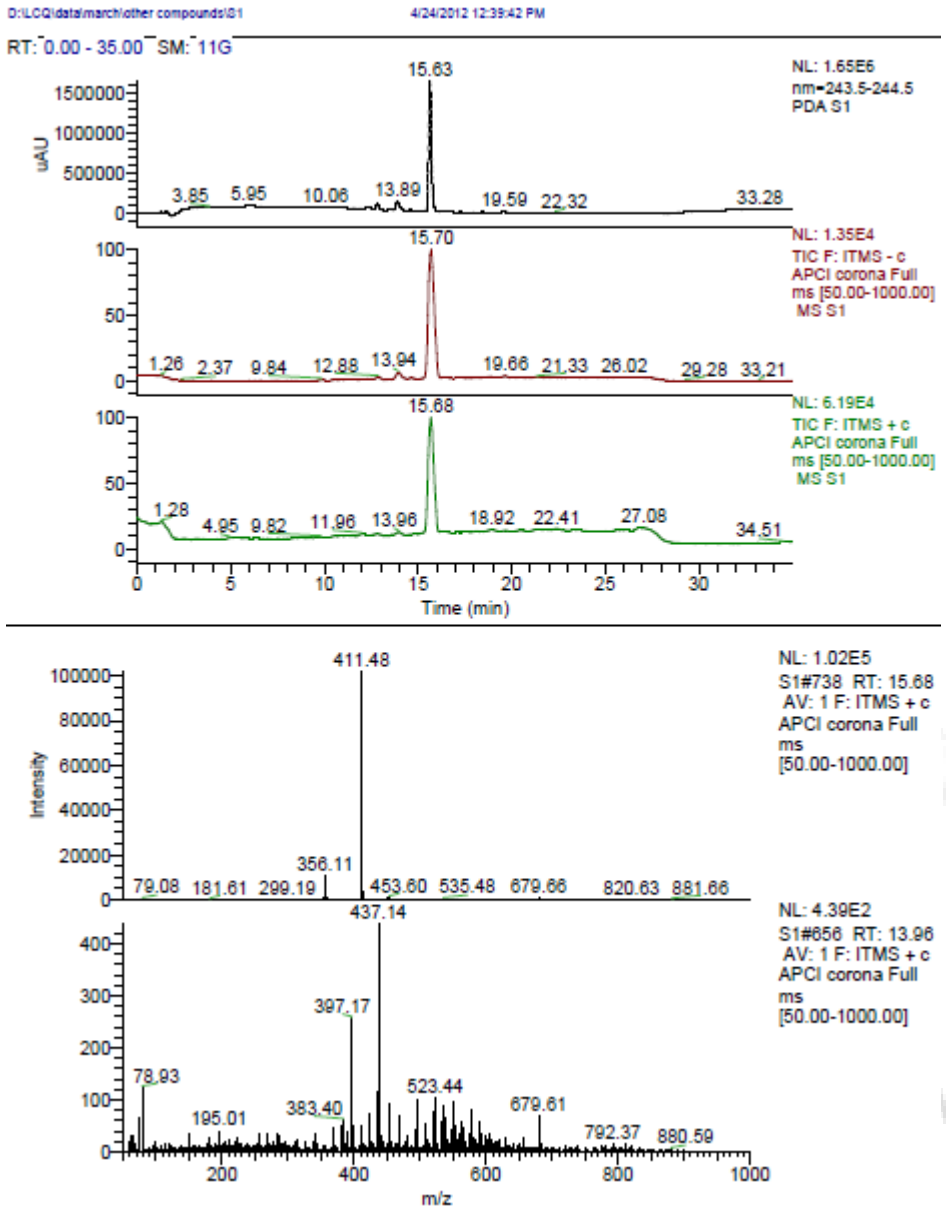
Figure No.3: All tracks at different wavelength

TABLE TITLE MISSING

	No of major peaks	Rf Values	Alpha-mangostin
Standard Alpha-mangostin solution	1	0.64	+++
Sample (Fraction E3) solution	3	0.64	+++
		0.89	
		0.39	

Determination of Molecular weight of Fraction E3 by LC-MS:

Fraction E3 (purified Alpha-mangostin) obtained from the purification of crude mangostin extract is subjected for the molecular weight determination by LC-MS. Procedure followed was explained in the experiment section. LC chromatograms and Mass spectroscopy chromatograms (Figure No.4) clearly gave us the molecular weight of Alpha-mangostin (the main phytocompound isolated) as 410.48 (411.48-H^+).



LC Chromatogram: indicating different phytocompounds in the Fraction E3

MS/MS Spectra :Indicating Molecular weight of separated phytocompounds in Fraction E3

Figure 4: LC and Mass chromatograms of the separated phytocompound in the Fraction E3

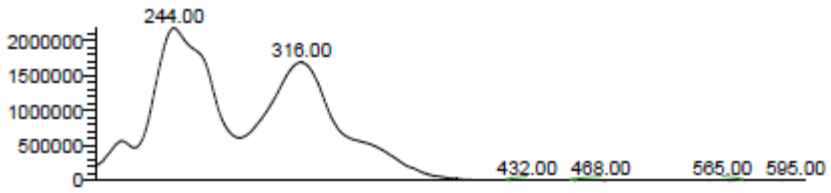


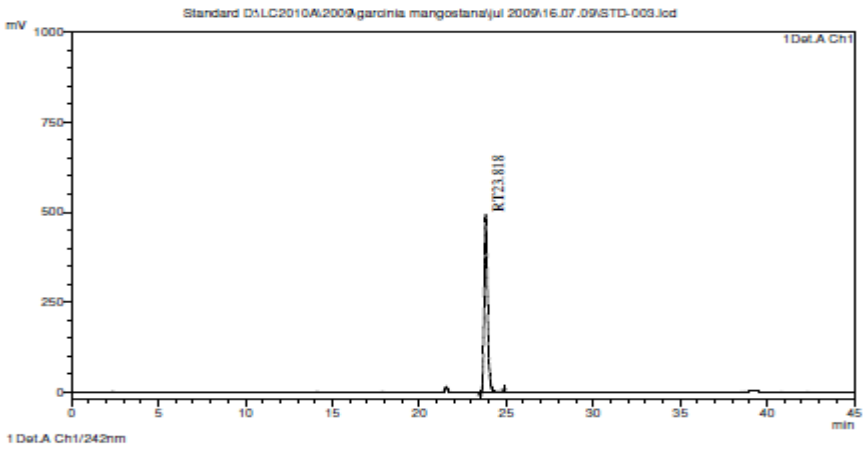
Figure 5: UV pattern of separated phytocompound (Fraction E3)

UV absorption study of the Fraction E3 at 1mg per ml concentration dissolved in methanol shows us the strong absorption at 244 nm and 316 nm; characteristic absorption pattern of Alpha- mangostin (Figure No.5).

Purity checking /Quantification of Alpha-mangostin in the partially purified Fraction E3:

Quantification of Alpha-mangostin in the purified extract was done by HPLC. Major peak corresponds to Alpha-mangostin at Retention time 23.8 min (Figure No. 6) was identified and calculated as 66.0 ± 0.82 % W/W. Result of this study indicated that there is a direct increase of 83.3 ± 3.6% of Total purity from the methanol extract (36.1 ± 0.7% w/w) [13,16].

A. Alpha-Mangostin working Standard Chromatogram



Detector A Ch1 242nm

Peak #	Ret. Time	Name	Area	Area %
1	23.818	RT23.818	6465001	100.000
Total			6465001	100.000

B. Alpha-Mangostin from Purified Fraction E3

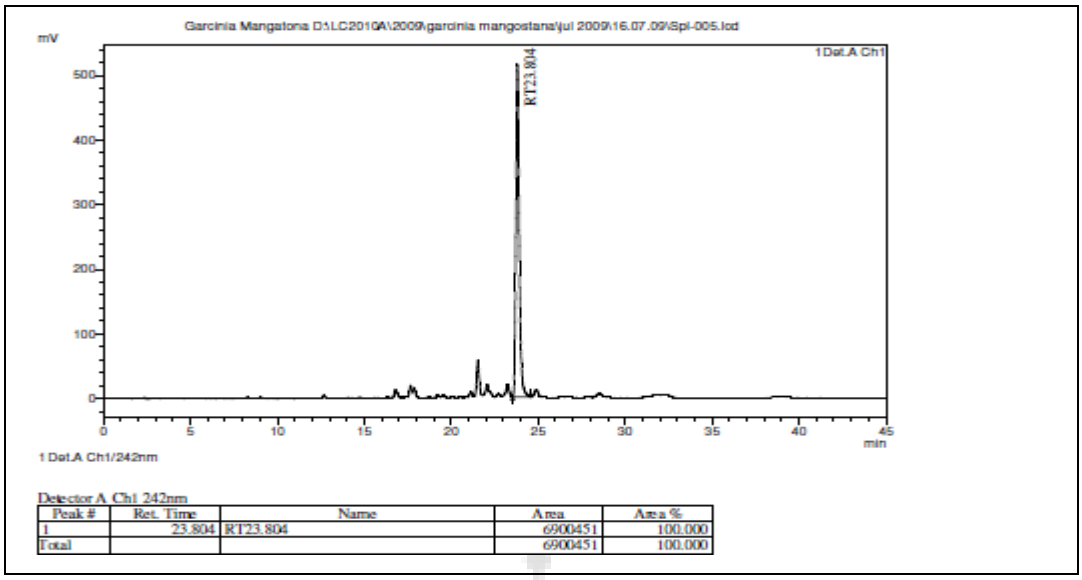


Figure 6: HPLC chromatograms of Alpha-mangostin Working standard (A) and Fraction E3 (B)

Total Polyphenolic content

The antioxidant activity of phenolic compounds is mainly due to their redox properties, which can play an important role in absorbing and neutralizing free radicals, quenching singlet and triplet oxygen or decomposing peroxides[16]. Folin-Ciocalteu phenol reagent was used to obtain crude estimation of the amount of phenolic compounds present in the extract. The total polyphenolic content in the partially purified extract was found to be 38.6.0 ± 3.3 mg/g of dry extract (in Tannic acid equivalents).

CONCLUSION

As an outcome, the present study reveals the easy and industrial feasible procedure for the Partial purification of Alpha-mangostin (Fraction E3) to >65.0% which is very much simple and rapid. Partially purified mangosteen extract (Alpha-mangostin) had potent antioxidant activity that could be attributed to the presence of high concentrations of xanthones (Alpha-mangostin) and Phenolic compounds. Characterization studies (Both qualitative and quantitative) which are performed with the different techniques for the Purified Fraction E3 supports us purified phytocompound is Alpha-mangostin. Current study reveals us Alpha-mangostin (single

compound or with other related mangostins) may be the responsible compound for different pharmacological activities.

Present study provides complete purification method for the Alpha-mangostin. Its Isolation techniques followed by the support for the qualitative and quantitative estimation of the isolated phytocompound i.e., Alpha-mangostin (Confirmed by the Identification tests TLC, HPTLC and UV pattern, Molecular weight determination by Liquid chromatography and LC-MS and Quantification by HPLC).

Alpha-mangostin as powerful antioxidant and Antiinflammatory agent which can be a suitable molecule to develop drugs for the particular disease related to free radical damage such as Cardiovascular, Arthritis, Atherosclerosis and inflammation etc. Potent Inhibition of Alpha-Glucosidase activity by Alpha-mangostin also supported some earlier reports where xanthones from *Garcinia mangostana* fruit hulls showed very good antidiabetic activity and could be used for the development of drug for the management of Type II diabetes and its associated diseases.

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