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Evaluation of Alpha-Mangostin, Isolated and Purified from the Crude Extract of *Garcinia mangostana* for the Anti-Diabetic, Anti-Inflammatory and Antioxidant Activity

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ABSTRACT

Garcinia mangostana Linn belongs to the family Guttiferae is used as an anti-oxidant, anti-diabetic and anti-inflammatory drug in Southeast Asia. Fruit hulls extract having major bioactive substances alpha and gamma-mangostins, good amount of polyphenols with very strong anti-oxidant and good anti-diabetic activities. Objective of the present study was to provide an in-vitro evidence for the potential inhibition activity of the α-magostin rich fraction on the *α-glucosidase* enzyme, DPPH free radical scavenging and α-amylase. In the present research work, α-magostin rich fraction was evaluated for the anti-diabetic activity, Antioxidant and anti-inflammatory activity. Partially purified α-magostins from the fruit hulls crude extract was confirmed by both qualitative and quantitative test like TLC and HPLC (Detailed purification techniques, characterization, qualitative and Quantitative analysis of the Alpha-mangostin from the crude solvent extract was explained in our earlier publication). IC_{50} value for the Inhibition of *Alpha-glucosidase* and *Alpha-amylase enzyme* for the evaluation Anti-diabetic activity, Scavenging of free radicals by DPPH for the evaluation of Antioxidant activity and Anti-inflammatory activity measured by using Carrageenan induced rat paw edema was found to be 45.2 ug/ml and 30.58 ug/ml (measured against Standard Acarbose, IC_{50}= 11.6 ug/ml and 5.4 ug/ml), 7.4 ug/ml (measured against Standard Ascorbic acid, IC_{50}= 4.5 ug/ml) and showed same potency as that of standard drug (Sulindac)for the inhibition of inflammation on carrageenan induced paw edema in mice (at the dose of 20 mg/kg body weight) respectively. Estimation of Total polyphenolic content was performed by Folin-ciocalteu method and it was found to be 52.2 ± 2.5 mg/g.

INTRODUCTION

Chronic metabolic disorder diabetes mellitus characterized by the hyperglycemia, carbohydrate, fat and protein metabolism disturbances. It causes malfunctioning of insulin action. Approximately 366 million people are suffering from diabetes as per the IDF (International Diabetic Federation) and it may double within another 15 years. Type 2 is more prevalent than Type 1 and more than 90% of the total diabetic patient's suffering from it. Regulation of plasma glucose level is vital for preventing the Type II diabetes. Recent therapeutic approach for the diabetic therapy is found to be development of the drugs for the inhibition of the carbohydrate hydrolyzing enzymes such as *alpha-glucosidase and alpha-amylase.* These enzyme inhibitors control diabetes by reducing the absorption of glucose (one such example is Acarbose, leading inhibitor of carbohydrate metabolic enzymes in GI tract but it is associated with so many side effects like diarrhea and other intestinal disturbances). Therefore screening of *Alpha-glucosidase and alpha-amylase* inhibitors in medicinal plants has received much attention.

There are many reports available regarding the phytochemicals responsible for the inhibition of carbohydrate digestive enzymes which can able to manage diabetes. Hence the main objective of the present study was to investigate in-vitro, *α-glucosidase and α-amylase.*

Garcinia mangostana L. (Clusiaceae), 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, 4] antifungal [5,6] 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.

Use of DPPH for a radical scavenging measuring method is described by Yen and Duh (1994), Yordanov and Christova (1997), Masuda et al. (1999), Anderson and Padhye (2004), and Iwashima et al. (2005). DPPH is a stable free radical in a methanolic solution. In its oxidized form, the DPPH radical has an absorbance maximum centered at about 520 nm (Molyneux, 2004). The DPPH method is described as a simple, rapid and convenient method independent of sample polarity for screening of many samples for radical scavenging activity (Koleva et al. 2001)[8].therefore DPPH free radical scavenging activity was used to evaluate the antioxidant activity of partially purified Alpha-mangostin.

Acute inflammation is a complex process that can be induced by a variety of means. Anti-inflammatory agents exert their effects through a spectrum of different modes of action [9,10]. In the screening of new anti-inflammatory compounds, carrageenan-induced edema in the hind paw as an acute inflammation mode is widely employed. Therefore, the carrageenan-induced mice paw edema model was also used to evaluate the anti-inflammatory effects of partially purified Alpha- mangostin in this study.

MATERIALS AND METHODS

Materials:

Working standard of Alpha-mangostin (60.0% purity) was obtained as complimentary sample from Phytoteck extracts Pvt. Ltd., Bangalore, India (With certificate of analysis). Anisaldehyde reagent, Sulphuric acid, Folin-ciacalteu reagent, Sodium carbonate, Gallic acid, 2,2-diphenyl- 1-picrylhydrazyl (DPPH), Ascorbic acid, p-nitrophenyl-Alpha-D-glucopyranoside (DNPG), *Alpha-Glucosidase*, Acarbose, Carrageenan and sulindac from Sigma Chemical Co. (St. Louis, MO). 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).

Methanol, Acetone, Benzene, chloroform, Toluene, TLC plates were purchased from Merck, Frankfurter, Strabe, 25064293, Darmstadt, Germany.

Animals

Animals used for the study were Male Wister rats weighing 110 to 160gms, maintained at standard environmental conditions; standard diet and clean water were provided to the rats. Experiments were planned after the approval of Institution Animal Ethical Committee (IAEC), SJM College of pharmacy, Chitradurga, Karnataka, 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 kgs) 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 Liters 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. Combined all the extracts, concentrated and dried the combined liquid extract in Buchii rotoevoparator at temperature $< 70^0$C under Vacuum (500 mm Hg) to afford 78.0 Gms of Crude extract (E1). Washed the crude extract obtained, with 1:1 volume of DM (Demineralized) water (i.e., 78.0 ml) by constant stirring and allowed for the settling for 2 to 3 hrs. Filtered the mixture through Whatman No.1 filter paper to separated water and settled crude extract. Discard the filtrate and dried the extract obtained 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 50^0C to dissolve and filtered into a separate beaker. Filtrate collected was concentrated in a buchii vacuum rotoevoparator at temperature $<70^0$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 55^0C with constant stirring and filtered into a separate beaker. Filtrate collected was concentrated in a buchii vacuum rotoevoparator at temperature $<70^0$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 50^0C 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 is 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 < 50^0C to get dried, light yellow colored shiny powder. This purified material was again dried at low temperature (40-45^0C) to lower the moisture content, grinded in mortar and pestle to get uniform size powder (Alpha-mangostin Rich Fraction) (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).

METHOD AND PROTOCOLS:

1.0. Identification of the Alpha-mangostin and other Phytocompounds by HPTLC:

Isolated fraction containing Alpha-mangostin is qualitatively analyzed with working standard by HPTLC. Detailed method is mentioned in the Table No.1.

Table.1:

HPTLC METHOD		
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
INSTRUMENT CONDITION		
Spray gas	:	Inert gas
Sample solvent type	:	Methanol
Dosage speed	:	150 nl/s
Predosage volume	:	0.2 ul
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
WORKING STANDARD AND SAMPLE PRPERATION:		
Standard Alpha-mangostin		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.
Sample preparation		The sample solution was prepared by dissolving 10 mg of Alpha-mangostin rich Fraction in 10 ml methanol solution. The sample solution of appx 100 µg/ml was prepared by diluting further with methanol.

2.0. Quantification of Alpha-mangostin content in the Isolated Fraction:

Isolated fraction containing Alpha-mangostin is quantitatively analyzed with working standard by HPLC. Detailed method is mentioned in the Table No.2.

Table.2:

<table>
<tr><td colspan="3">HPLC INSTRUMENT</td></tr>
<tr><td>Instrument name</td><td>:</td><td>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, PDA/ UV-Vis detector and class VP Ver. 6.0 workstation is used for analysis.</td></tr>
<tr><td>Mobile phase</td><td>:</td><td>Solvent A: 0.1% TFA in water, Solvent B: 0.1% TFA in Methanol</td></tr>
<tr><td>Column</td><td>:</td><td>ODS, C-18. Phenomenex: Type: Luna 5µ C18 (2), Size: 250 x 4.60mm 5µ micron.</td></tr>
<tr><td colspan="3">CHROMATOGRAPHIC CONDITION</td></tr>
<tr><td>Detector</td><td>:</td><td>SPD- 10 AVP Photo diode array detector or Uv-Vis. detector.</td></tr>
<tr><td>Flow rate</td><td>:</td><td>1.0 ml /min</td></tr>
<tr><td>Wavelength</td><td>:</td><td>242nm for monitoring.</td></tr>
<tr><td>Injection volume</td><td>:</td><td>20 ul</td></tr>
<tr><td>Gradient condition</td><td>:</td><td>
<table>
<tr><th>Time</th><th>Solvent A (%)</th><th>Solvent B (%)</th></tr>
<tr><td>0.0</td><td>95.0</td><td>5.0</td></tr>
<tr><td>0.01</td><td>95.0</td><td>5.0</td></tr>
<tr><td>10.0</td><td>10.0</td><td>90.0</td></tr>
<tr><td>15.0</td><td>5.0</td><td>95.0</td></tr>
<tr><td>25.0</td><td>5.0</td><td>95.0</td></tr>
<tr><td>30.0</td><td>10.0</td><td>90.0</td></tr>
<tr><td>35.0</td><td>95.0</td><td>5.0</td></tr>
<tr><td>40.0</td><td>95.0</td><td>5.0</td></tr>
<tr><td>45.0</td><td>Stop</td><td></td></tr>
</table>
</td></tr>
<tr><td colspan="3">METHOD</td></tr>
<tr><td>Standard Preparation</td><td>:</td><td>100mg of working standard is dissolved in 10ml of Methanol. Filter the preparation through 0.45 Micron syringe filter before injection.</td></tr>
<tr><td>Sample preparation</td><td>:</td><td>100 mg of Alpha-mangostin rich isolated fraction is dissolved in 10 ml of Methanol. Filter the preparation through 0.45 Micron syringe filter before injection.</td></tr>
<tr><td>Procedure</td><td>:</td><td>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.

Area of sample X Wt. of std. (mg)__ X Dilution X 100 x Purity = %w/w
Area of Std. Wt. of sample (mg) Std. Dilution 100</td></tr>
</table>

3.0. Estimation of Total Polyphenolic Content in Partially Purified Extract:

Total Polyphenolics content was determined by Folin-Ciocalteu method [12]**.** In brief, 200 µL of test sample was added to a test tube that contained 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 gallic acid as standard. The total phenolics content of extract was expressed in terms of milligrams of gallic acid (mg GAE) per gram of dry extract.

4.0. In-vitro Pharmacological studies of partially purified Alpha-mangostin constituent:

Partially purified mangosteen constituents have demonstrated a variety of biological activities. The effects of partially purified Alpha- mangostin as antioxidants, antidiabetic and anti-inflammatory will be discussed in the subsequent paragraphs.

4.1. Antioxidant activity by DPPH (2, 2-diphenyl-1-picrylhydrazyl) method:

DPPH free radical activity of extract was measured by slightly modified method of Sanches-Moreno et al 1998. A simple method that has been developed to determine the antioxidant activity of extracts utilizes the stable 2,2-diphenyl- 1-picrylhydrazyl (DPPH) radical. The odd electron in the DPPH free radical gives a strong absorption maximum at 517 nm and is purple in color. The color turns from purple to yellow as the molar absorptivity of the DPPH radical at 517 nm reduces from 9660 to 1640 when the odd electron of DPPH radical becomes paired with hydrogen from a free radical scavenging antioxidant to form the reduced DPPH-H. The resulting decolonization is stoichiometric with respect to number of electrons captured [11].

Into a set of eppendorf tubes arranged in the stand, labeled as Blank, Controls and Samples (different conc.).Added the reagents potassium Test Sample: 0-100 ul, Methanol: 375 to 625 ul, and DPPH: 625 ul (0.5mM) in the same order. Mixed thoroughly (Vortex) and Incubate at 37 ^{0}C for 30 minutes. The Control doesn't have any sample or test solution. A set of color control is run for test samples without DPPH but maintaining the volume with methanol. Blank reading is methanol alone. Read absorbance of all samples at 517 nm using spectrophotometer.

Ascorbic acid was used as standard antioxidant and blank of methanol was run with each assay. All determinations were carried out in triplicates. Same procedure was repeated using control sample i.e. DPPH without the sample. The inhibition of DPPH was calculated as a percentage according to the following equation [12,13]**.**

$$\% \text{ Of Scavenging activity} = \frac{(\text{Absorbance of control} - \text{Absorbance of sample})}{\text{Absorbance of control}} X\ 100$$

The percentage of scavenging activity was plotted against the sample concentration to obtain IC_{50}, defined as the concentration of sample necessary to cause 50% inhibition.

4.2. *Alpha-Glucosidase* inhibition activity:

Introduction:

α- glucosidase which is a membrane bound enzyme located in the epithelium of the small intestine, catalyzes the cleavage of glucose from disaccharides. Thus, the retardation of the action of *α-glucosidase* may be one of the most effective approaches to control diabetes [14]. Since carbohydrate intake influences obesity, *α-glucosidase* inhibition may be useful in obesity [15,16].

Principle:

α-glucosidase activity can be measured *in-vitro* by determination of the reducing sugar (Glucose) arising from hydrolysis of sucrose by *α-glucosidase* enzyme, isolated from small intestine of rat.

Materials required:-

- Enzyme: α-glucosidase isolated from rat intestine (store at -20°C).
- Substrate: Sucrose (RM 3063, Himedia, India, store at RT).
- Positive control: Acarbose, Glucobay (Bayer Pharma, India, store at RT).
- Total protein estimation kit (Biuret method) B-0211, Span diagnostics, store at 2-8°C.
- Other Reagents:

➢ Sodium dihydrogen orthophosphate dihydrate ($NaH_2PO_4.2H_2O$, RM1255, Himedia, India store at RT).

➢ Di-sodium hydrogen phosphate dihydrate ($Na_2HPO_4.2H_2O$, RM257, Himedia, India, store at RT).

➢ Glucose reagent (11208102, AGAPPE diagnostics, India, store at 2-8°C).

➢ Microwell plate - 96well flat, clear plate, Cat. no. 980040, Tarsons

Preparation of working solutions:

- Phosphate buffer (80mM, pH 7.0 at 25ºC):

Solution A: 1.248g of sodium dihydrogen orthophosphate dihydrate is dissolved and made up to 100ml with de-ionized water.

Solution B: 1.424g of di-sodium hydrogen phosphate dihydrate is dissolved and made up to 100ml with de-ionized water. Mix 39ml of solution A & 61ml of solution B and make up to 200ml with de- ionized water.

- Enzyme: Rats are sacrificed; intestine is removed and chilled with ice cold 80mM phosphate buffers (pH 7.0). The intestine is then cut open, the mucosa is scraped off with a piece of glass rod and homogenized in homogenizer with four parts (v/v) of cold 80mM buffer (pH 7.0). The tube is chilled with crushed ice during homogenization. Nuclei and large cell debris are removed by centrifugation at 2000–4000 rpm for 10minutes and supernatant is aliquot into 1.5ml vials and stored at –20ºC. [Protein content = approximately 0.5g/dl., by Biuret method].
- Substrate (37mM): 316 mg of sucrose is made up to 25ml with 80mM phosphate buffer pH 7.0.
- Positive control:

♦ Stock 1 (1mg/ml): 50mg of Acarbose dissolved in 50ml of 80mM Phosphate buffer, pH 7.0

♦ Working Stock: Dilute to a concentration of 5□ g/ml with 80mM Phosphate buffer pH 7.0.

Procedure:

The assay is performed as per of *Vogel & Vogel* [3] with modifications. In brief, a pre-incubation mixture of 300µl contains 250µl of 80mM phosphate buffer pH 7.0/ vehicle buffer / positive control/test sample of various concentrations, add 50µl of enzyme. Mix and pre-

incubated at 37^0C for 30 minutes. Following pre-incubation, add 500µl substrate (37mM Sucrose) and incubate at 37^0C for 50minutes. Arrest the reaction by keeping in boiling water bath for 2 minutes, then cool. Add 250µl of glucose reagent to 50µl of reaction mixture and mix. Incubate at 25^0C for 10minutes, measure the absorbance at 510 nm spectrophotometrically (Molecular devices Versamax microplate reader). A control reaction is carried out without the test sample.

Calculation of results:

$$\% \text{ inhibition} = \frac{\text{Absorbance (Control)-Absorbance (test)}}{\text{Absorbance (control)}} \times 100$$

Note:

- IC_{50} is calculated using log-probit analysis.
- Appropriate solvent and color corrections should be done (in case of colored samples & non- aqueous solutions).
- Methanol can be used up to 2.5% of the pre-incubation volume.
- DMSO can be used up to 2.5% of the pre-incubation volume.

Critical Control Points:

- PH, incubation time, temperature and storage conditions.
- Boiling water bath to be used to arrest the reaction.

Final assay concentration:

In 800µl reaction volume, the final concentrations are 80mM of phosphate buffer pH 7.0, 23.12mM of Sucrose and 50 µl of *α-glucosidase.*

Assay performance measures:

- Z'-factor: 0.94
- S/B ratio: 182

4.3. *α-Amylase* inhibition assay

Introduction:

In humans, the digestion of starch involves several stages. Initially, partial digestion by the salivary *amylase* results in the degradation of polymeric substrate into shorter oligomers. Later on in the gut, these are further hydrolyzed by pancreatic *α-amylase* into maltose, maltotriose and small maltooligosaccharides. The digestive enzyme *(α-amylase)* is responsible for hydrolyzing dietary starch to maltose, which breaks down to glucose, prior to absorption. Inhibition of the *α-amylase* should reduce the unfavorable high postprandial blood glucose peak in diabetes. *α-amylase* inhibition is also a useful target in obesity [17,18].

Principle:

Pancreatic α-amylase hydrolyses the 2-chloro-4- nitrophenol α-D - maltotriose (CNP-G3) to release 2-chloro-4-nitrophenol and form 2-chloro-4- nitrophenol α-D - maltoside (CNPG2), maltotriose and glucose. The rate of formation of the 2-chloro-4-nitrophenol can be measured at 405nm.

Materials required:

- Enzyme: α-amylase (EC 3.2.1.1) (Type VI-B: From porcine pancreas, 500,000 units [30 units/mg solid at pH 6.9 (lot specific)], A3176, Sigma, USA, store at 2-8°C).
- Substrate: CNP-G3 reagent [2-chloro-4-nitrophenol α-D-maltotrioside] Futura System S.r.l, Apparecchi Scientifici diagnostics, store at 2-8°C.
- Positive control: Acarbose, Glucobay (Bayer Pharma, India, store at RT).
- Other reagents:

➢ Sodium dihydrogen orthophosphate dihydrate ($NaH_2PO_4.2H_2O$, RM1255, Himedia, India, store at RT).

➢ Di-sodium hydrogen phosphate dihydrate ($Na_2HPO_4.2H_2O$, RM257, Himedia, India, store at RT).

➢ Microwell plate: Costar 384 well polystyrene, non-treated. (Cat. no. 3702, Corning)

Preparation of working solutions:

- **Phosphate buffer (40mM, pH 6.9 at 25ºC):**

➢ **Solution A:** 6.24g of sodium dihydrogen orthophosphate dihydrate is dissolved and made up to 1000ml with de-ionized water.

➢ **Solution B:** 7.12g of di-sodium hydrogen phosphate dihydrate is dissolved and made up to 1000ml with de-ionized water.

➢ Mix 45ml of solution A & 55ml of solution B & make up to 200ml with de- ionized water.

- Enzyme (0.4998units/ml): 1.66mg of α-amylase is made up to 100 ml with 40 mM phosphate buffer pH 6.9. (Enzyme units are lot specific).
- Substrate (1.51mM): CNP-G3 reagent ready to use.
- Positive control:

➢ Stock 1: (1mg/ml): 50mg of Acarbose dissolved in 50ml of 40mM Phosphate buffer, pH 6.9

➢ Stock 2: Dilute to a concentration of 2.5µg/ml with 40mM Phosphate buffer, pH 6.9.

➢ Working stock: Dilute to a concentration of 0.25µg/ml with 40mM Phosphate buffer, pH 6.9.

Procedure:

The assay is performed as per Gella *et al* [19] with modifications. In brief, a pre-incubation mixture of 22.5µl contains 14.5µl 40mM phosphate buffer pH 6.9 / vehicle buffer/ positive control / test sample of various concentrations and 8µl of enzyme (0.4998units/ml). Mix and pre-incubate at 37^0C for 10 minutes. Following pre-incubation, add 31µl substrate (2.3mM CNP-G3) and incubate at 37°C for 8 to 14 minutes. Measure the absorbance at 405 nm in kinetics mode (Pherastar). A control reaction is carried out without the test sample.

Calculation of results:

$$\% \text{ inhibition} = \frac{\text{Absorbance (Control)-Absorbance (test)}}{\text{Absorbance (control)}} \times 100$$

Note:

- IC_{50} is calculated using log-probit analysis.
- Appropriate solvent and color corrections should be done (in case of colored samples & non- aqueous solutions).
- Methanol can be used up to 1% of the pre-incubation volume.
- DMSO can be used up to 5 % of the pre-incubation volume.

Critical Control Points:

- PH, incubation time, temperature and storage conditions.
- Ensure that the substrate is colorless before addition.
- Keep the enzyme cold before dispensing (use ice bucket).
- Pipetting of reagents and samples.

Final assay concentration:

In a 53.5µl reaction volume, the final concentrations are 16.82mM of phosphate buffer pH 6.9, 0.87mM of CNP-G3 and 4.998 milliunits of *α-amylase* enzyme.

4.4. Anti-Inflammatory Activity by Carrageenan induced paw edema:

Anti-inflammatory activity was measured by using the assay-"Carrageenan induced rat paw edema" (Winter et al., 1962; Adeyemi et al., 2002). An acute model of inflammation experiment i.e., Carrageenan-induced paw edema in mice was used to evaluate the anti-inflammatory effect of partially purified Alpha-mangostin. Into the three groups of mice (n=4), inflammation was induced by 50ul of 1% carrageenan in normal saline by subplanator administration into the right paw of each mouse. Different groups were administered orally with 20 mg/ml each concentration of Alpha-mangostin, Sulindac and the control vehicle (10% Ethanol) 1 hr before the injection of carrageenan. The linear paw circumference was measured at hourly interval for 3 h (Bamgbose and Noamesi, 1981) after the injection of carrageenan. The increment in paw thickness due to carrageenan administration was expressed as edema and anti-inflammatory activity was measured as the percentage reduction in edema level when drug was present, relative to control (Duffy et al., 2001) as shown in **Table 3**. Results are expressed as the mean ± standard deviation (S.D.). The one-way analysis of variance (ANOVA) was used for comparing the paw thickness among the induced, and

test groups. P-values < 0.05 were considered significant. Each experiment was performed in triplicate.

Statistical analysis

The values are Mean ± standard deviation (SD) of 3 experiments. The IC50 was calculated by log-probit Analysis.

RESULTS AND DISCUSSION

5.0. Identification of Alpha-mangostin rich fraction 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 105^0C (Figure No.2). Identification of Alpha-mangostin in partially purified extract was confirmed against standard Alpha-mangostin at Rf value 0.91 ± 0.05.

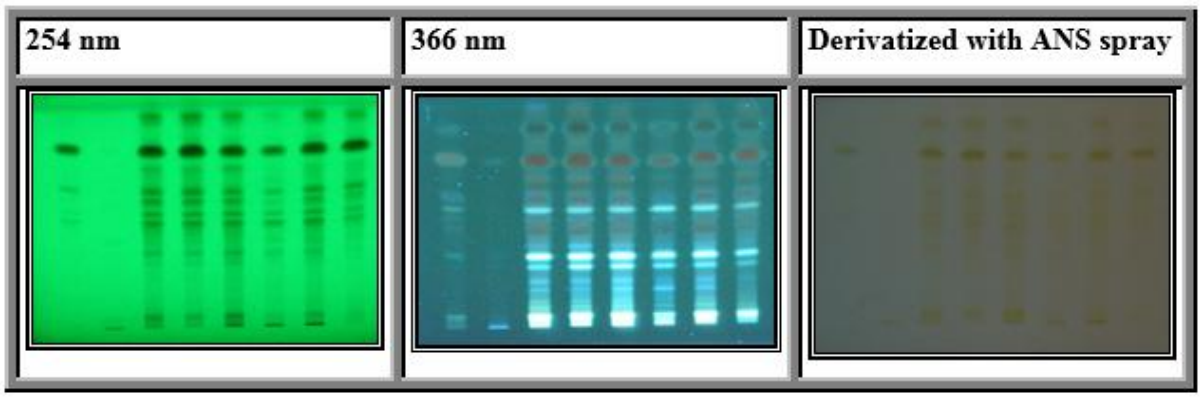


Figure No 1: HPTLC fingerprint profile of Fraction E3 (From Track no; 3-8) and Standard Alpha-mangostin (Track no;1). Track no-2 is the blank.

6.0. Quantification of the Alpha-Mangostin in the rich fraction by HPLC:

Quantification of Alpha-mangostin in the purified extract was done by HPLC. Major peak corresponds to Alpha-mangostin at Retention time 15.2 min was identified and calculated as 66.0 ± 0.62 % 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 (36.18 ± 0.71% w/w) [20].

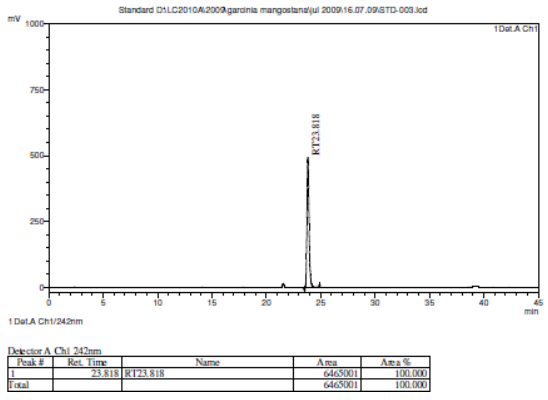
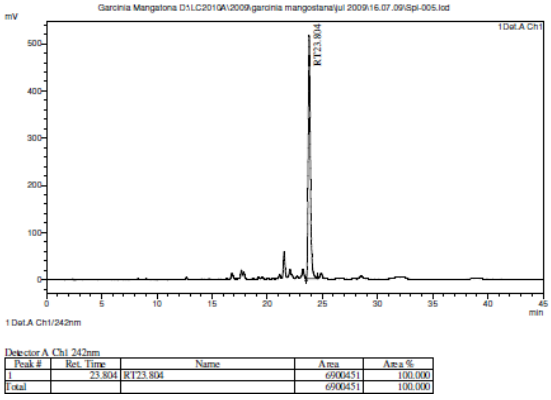
A. Alpha-Mangostin working Standard Chromatogram	B. Alpha-Mangostin from isolated Fraction
	

Figure No 2: HPLC chromatograms of a) Standard Alpha-mangostin and b) Purified Alpha-mangostin rich fraction.

7.0. 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 [20]. Folin-Ciocalteu phenol reagent was used to obtain crude estimation of a number of phenolic compounds present in the extract. The total polyphenolic content in the partially purified extract was found to be 52.2 ± 2.5 mg/g of dry extract (in Gallic acid equivalents).

8.0. *In-vitro* Pharmacological activity studies.

8.1. Antioxidant Activity of partially purified Alpha-mangostin by DPPH Method;

DPPH radical is a commonly used substrate for fast evaluation of antioxidant activity because of its Stability in the radical form and simplicity of the assay [21, 22] The principle behind this assay in the color change of DPPH solution from purple to yellow as the radical is quenched by the antioxidant [23] color changes can be measured quantitatively by spectrophotometer absorbance at 517 nm. Partially purified Alpha-mangostin was screened for the antioxidant activity according to the method described and results were showed **Table 4** comparable with known antioxidant standard Ascorbic acid. Partially purified extract is showing reducing power activity almost equal to the Ascorbic acid. The partially purified Alpha-mangostin extract exhibit a significant dose-dependent inhibition of DPPH activity

with 50% of inhibition (IC50) at concentration of **7.4 ug/ml** (**Figure 3a**) and IC50 value of Ascorbic acid was **4.5ug/ml** (**Figure 3b**).

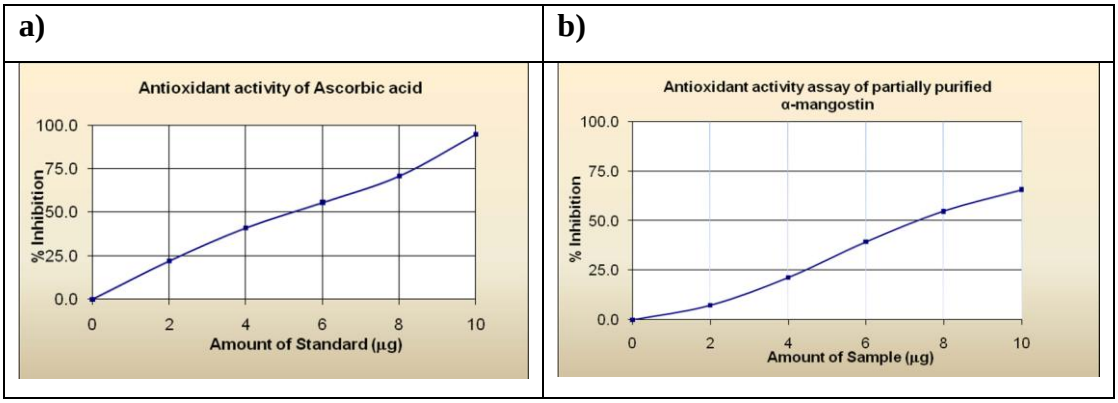


Fig.3: Standard curve for determination of IC50 value of (a) Ascorbic acid *and* (b) partially purified Alpha-mangostin

8.2. *Alpha-glucosidase* and *Alpha-amylase* inhibition activity by Alpha-mangostin:

One of the most critical control points is the control of plasma postprandial glucose levels in the early treatment of diabetes mellitus (Oritz et al., 2007). Controlling the absorption of glucose produced from the breakdown of starch by hydrolysis by inhibiting the pancreatic *Alpha-amylase* and by limiting the absorption of glucose by inhibition of intestinal *Alpha-glucosidase* enzymes (Krentz and Bailey, 2005) are the two available therapeutic approaches for the Type II diabetes management.

The partially purified Alpha-mangostin extract exhibit a significant dose dependent inhibition of *Alpha-Glucosidase* and *Alpha-amylase* activity with 50% of inhibition (IC50) at concentration of 45.2 ug/ml and 30.58 ug/ml respectively (Standard Acarbose was 11.6 ug/ml for the Inhibition of Alpha-glucosidase and 5.4 ug/ml for the *Alpha-amylase* inhibition).

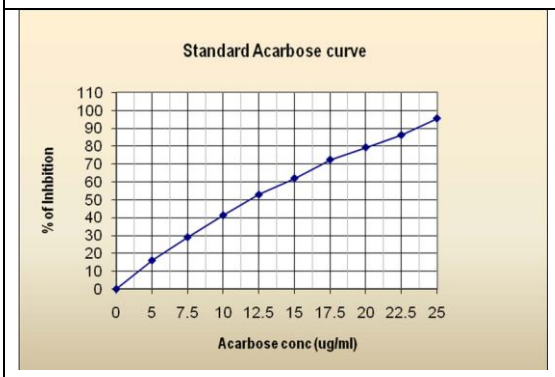
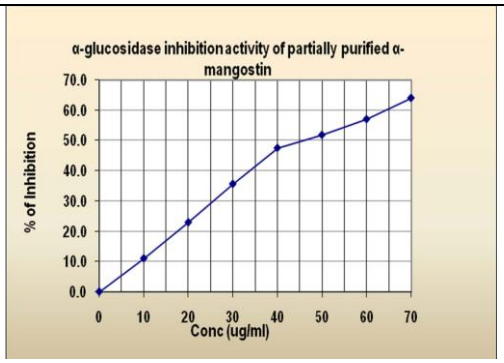
a)	b)
	

Fig.4: Standard curve for determination of IC50 value of (a) standard Acarbose *and* (b) purified Alpha-mangostin fraction

8.3. Alpha-mangostin rich fraction on Anti-Inflammation:

Mechanism of edema development after the injection of carrageenan, in the paw of the rat, is due to release of inflammatory metabolic substances like histamine, serotonin and prostaglandin. Carrageenan-induced rat paw edema is widely used as a working model of inflammation in the screening for a new anti-inflammatory drug. In this study, the anti-inflammatory activity of the partially purified Alpha-mangostin extract was evaluated by this method as described by the Winter et al., 1962 and Adeyemi et al., 2002. Concentration of extract used for the evaluation at the dose of 20 mg/Kg body weight, showed almost same potency of inhibition as that of standard drug. These results revealed the truth that partially purified Alpha-mangostin extract with a dose of 20 mg/kg body weight has very good anti-inflammatory activity compared to the reference drug Sulindac

Anti-inflammatory activity of our partially purified Alpha-mangostin extract may be due to the Inhibition of inflammation mediators like histamine, serotonin and prostaglandin [25, 25] (Vinegar et al., 1969).

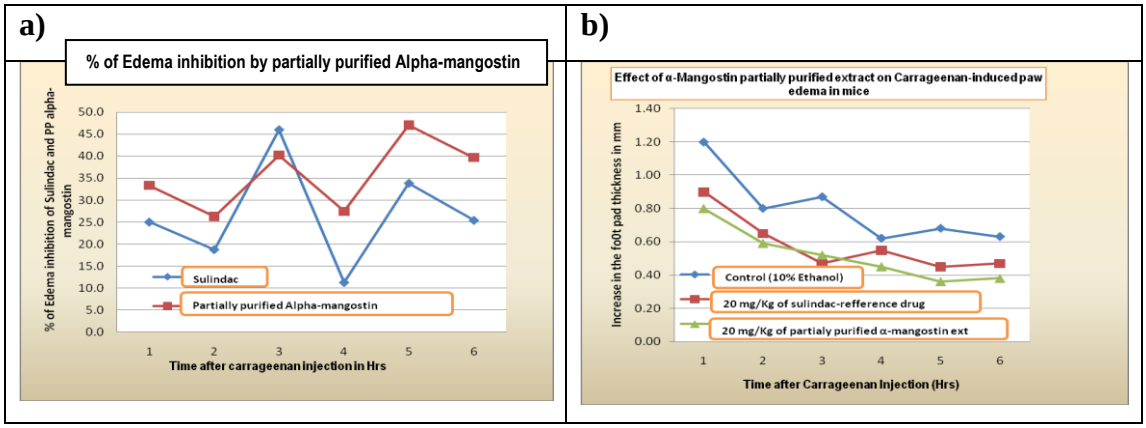


Fig.5. Anti-inflammatory effects of purified α-mangostin and sulindac on carrageenan-Induced paw edema in mice. Expressed values are the mean of four animals. Reference drug used was Sulindac. Sulindac and α-mangostin treated animals showed significant difference when compared with control group (Control: solvent control (10% EtOH, α-mangostin vs. control, p = 0.002; sulindac vs. control, p = 0.006).

CONCLUSION

As an outcome of the present *in-vitro* study, purified fraction containing rich content of Alpha-mangostin was showing potent antioxidant and Anti-inflammatory activity could be attributed to the presence of high concentrations of xanthones (Alpha-mangostin) and Phenolic compounds. These two compounds might be responsible for the very good antioxidant activity related to the health protecting benefits against nascent oxygen radical damage. Present study also gave us strong insight that, purified fraction containing rich content of Alpha-mangostin can be a potent phytochemical entity for the development drug products; particular for the disease related to free radical damage such as Cardiovascular, Arthritis, Atherosclerosis and inflammation etc. Primary results on the Anti-diabetic activity of the rich fraction were also showing good inhibition of Alpha-glucosidase and Alpha-amylase. Though the inhibition of these two enzyme activity is not much appreciable compared to the standard Acarbose but can be used as a safe and effective phytochemical candidate for the inhibition of this carbohydrate metabolizing enzymes at higher dosage without any side effect.

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