The Antioxidant, Antimicrobial, and Cytotoxic Properties of *Garcinia mangostana* L. Peel Extracts and Their Alpha-Mangostin Content

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ABSTRACT

Garcinia mangostana L. (mangosteen) peel is widely used as a traditional medicine in Southeast Asia, known for its beneficial biological properties. This study assessed the optimal extraction solvent for α-mangostin from the peel of *G. mangostana* using 70 % ethanol, 70 % acetone, 80 % methanol, and water at room and boiling temperatures. Furthermore, the most optimal extract's antioxidant and antimicrobial properties were studied. The 70% ethanol and 70% acetone solvents produced the highest α-mangostin recovery, but the 70% ethanol showed higher flavonoid and anthocyanin contents than 70% acetone. The ethanolic extract showed moderate antimicrobial activity against *Escherichia coli* and *Staphylococcus aureus*. The brine shrimp larvae toxicity assay showed that the organic solvents were toxic at 100 µg/mL. The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay showed that 400 µg/ml of 70 % ethanolic extract was toxic to the HepG2 cells. When challenged with tert-butyl hydroperoxide (t-BOOH)-induced oxidative stress, the ethanolic *G. mangostana* extract from concentrations 12.5 to 50 µg/mL showed protective effects against this oxidative stimulation. This study showed that the 70% ethanolic extract of *G. mangostana* peel showed optimal recovery of α-mangostin and other phytochemicals while showing protective effects against oxidative stimulations in HepG2 cells.

Keywords: *Garcinia mangostana* L., α-mangostin, solvent extraction, antioxidants, cytotoxicity.

1. INTRODUCTION

Garcinia mangostana L., commonly known as purple mangosteen, is an evergreen tree native to Southeast Asia, including Malaysia, Thailand, Indonesia, the Philippines, and India (Figure 1.0 A) [1, 2]. The fruit is known for its purple peel and its sweet, tangy white flesh (Figure 1.0 B) [2]. Traditionally, the fruit is used as an anti-inflammatory agent for treating wounds and skin infections, urinary system disorders, dysentery, and several other diseases [3]. In addition, the peel was reported to have significant antioxidant activities, both *in vivo* and *in vitro*, and is increasingly being studied for its effects on biological processes and health conditions such as obesity, cancer, hepatic disorders, neurodegenerative diseases, and cardiovascular disorders [4-9]. Furthermore, the peel and seed of mangosteen, usually discarded as waste, can be used in cosmetic, medical, and healthcare applications [9]. For example, the fruit peel has been incorporated into biscuits to enhance its antioxidant properties [10], used as a cotton dye with antibacterial properties [11] and in skin care [12].

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There is an increasing interest in finding new sources of phytochemicals from underutilized parts of fruits, including the seed and peel. Several reports have shown that *G. mangostana* peel contains a significant amount of prenylated xanthones such as α-, β-, and γ-mangostin [13]. Although other plants also harbor xanthonic compounds [14]. Among all the reported xanthones, α-mangostin (Figure 1.0 C) is the first reported tetra-oxygenated xanthone and the most studied [3]. Alpha mangostin is yellowish and is insoluble in water, hence requiring solubilizers to enhance its bioactivity [15].

There are many extraction methods performed to extract α-mangostin from *G. mangostana* rind, but most of them require complex methods and materials [16, 17]. Usually, the traditional method of preparing medicinal plant-based formulations is maceration (with or without heating) in water. However, limited information is available to prove if this method is effective in extracting α-mangostin from the peel [18]. A study by Zhang et al. describes the necessity of developing efficient and selective methods for extracting bioactive natural products, given their typically low abundance [19]. However, recommending an optimal extraction solvent is often challenging due to variations in plant material, their solubility properties across different solvents, extraction temperature, and the specific compound targeted for [20-22]

The diversity of solvent types is crucial for maximizing the yield of target compounds and enhancing the biological activity of the extracts, as highlighted by Ajanal et al.[21] and Mahdi-Pour et al.[22]. These studies demonstrated that the extraction solvent directly impacts both yield and biological activity. The selection of polar and moderately polar solvents is based on α-mangostin's moderately polar characteristics, which are due to the presence of polar hydroxyl (OH) groups, a moderately polar methoxy group (R-O-CH_3), and a non-polar phenyl (3-methyl-2-buten-1-yl) group attached to its xanthone core.

Therefore, one of the aims of this research is to identify the most effective solvent for extracting α-mangostin from 70% ethanol, 80% methanol, 70% acetone, room temperature water, and hot water. Furthermore, using the most optimal extraction product, we aimed to assess the antimicrobial, *in vitro* antioxidant, and cytotoxicity using brine shrimp larvae and to assess the *in vitro* antioxidant properties in liver (HepG2) cells. These assays were chosen to assess whether the bioactivity of the selected extracts was comparable to the other extracts in the same fruit product reported in the literature and eventually suggest the most optimum extraction methods for industrial application.

2. MATERIALS AND METHODS

2.1 Materials and Chemicals

The chemicals used were Ethanol (Systerm, purity ≥ 99.9%), two (2) different grades of methanol, analytical reagent (AR) (Systerm, purity ≥ 99.8%) and gradient grade for liquid chromatography (Supelco, purity ≥ 99.8%), acetone (Fisher Scientific, purity ≥ 99.9%), ortho-phosphoric acid (Supelco, Purity = 85%), acetonitrile (Fisher Brand, Purity ≥ 99.9%), Folin-Ciocalteu's phenol reagent (Sigma Aldrich), sodium carbonate (Sigma Aldrich, purity ≥ 99.5%), aluminium chloride (Bendosen, purity ≥ 97%), potassium acetate (Sigma Aldrich, purity ≥ 99%), potassium chloride (Bendosen, purity ≥ 99.5%), sodium acetate (Bendosen, purity ≥ 99.2%), 2,4,6-tripyridyl-s-triazine (TPTZ) (Sigma Aldrich, purity ≥ 98%), ferric chloride (Acros Organics, purity ≥ 97%), 2,2-diphenyl-1-picrykhydrazyl (DPPH) (Sigma Aldrich), 2,2-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) (Sigma Aldrich, purity ≥ 99%), potassium persulfate (HmBG, purity ≥ 98%), and Luria Bertani (LB) agar (Difco). The standard used for this study is α-mangostin standard (Shaanxin Rainwood Biotech Co. Ltd., Purity 90%, Xian, China). For the antimicrobial test, *Escherichia coli* (ATCC no: 25922) and *Staphylococcus aureus* (ATCC no: 25923) were used. HepG2, a human liver cancer cell line, was obtained from the American Type Culture Collection (ATCC) (Cat no: HB-8065). Hybri-

max dimethylsulfoxide (DMSO) (Cat no: D2650), cell grade 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) powder (Cat no: M2128), dichloro-dihydro-fluorescein diacetate (DCFH-DA) (Cat no: D6883) were obtained from Sigma-Aldrich (USA). Alpha-Minimum Essential Medium (α-MEM) (Cat no: 21445-95) was procured from Nacalai Tesque (Japan), while fetal bovine serum (FBS) (Cat no: 10270-098) and the antibiotic, penicillin-streptomycin (Cat no: 15140-122) were products of Gibco (USA).

2.2 Sample Collection and Extraction

Fully ripe mangosteen fruits were collected from a fruit orchard in Keningau, Sabah, Malaysia. The samples were collected and identified by Mr. Johnny Gisil, a botanist from the Institute for Tropical Biology and Conservation, Universiti Malaysia Sabah. The fruits were washed, then separated into peel and flesh. The peel was frozen at -20°C and then dried using a freeze dryer (Labconco). The dried peel was blended into powder (Figure 1.0 D) and kept at 4°C until further use. Then, 400 mg of dried peel powder was extracted by maceraction technique using 20 ml of the five different solvents each (70% ethanol, 80% methanol, 70% acetone, at room temperature water, hot water), respectively, by agitation at 250 rpm for 2 hours using a mechanical orbital shaker (Protech, Model No: 722), to make a final crude extract concentration of 20 mg/mL [23, 24]. Extraction with hot water was carried out by dissolving the peel powder into 100°C of distilled water for 10 minutes followed by agitation for 2 hours. After agitation, the solution was centrifuged for 10 minutes at 3000 rpm, then the supernatant was filtered using Whatman paper No. 1 and kept in 50- and 15-mL tubes at -20°C until further use (Figures 2.0 A-E). For the cytotoxicity and *in vitro* antioxidant assays using HepG2 cell lines, the 70% ethanolic extract solution was dried in an oven at 55°C and stored at -20°C until further use. The percentage yield for the dried ethanolic extract was 49.75%.

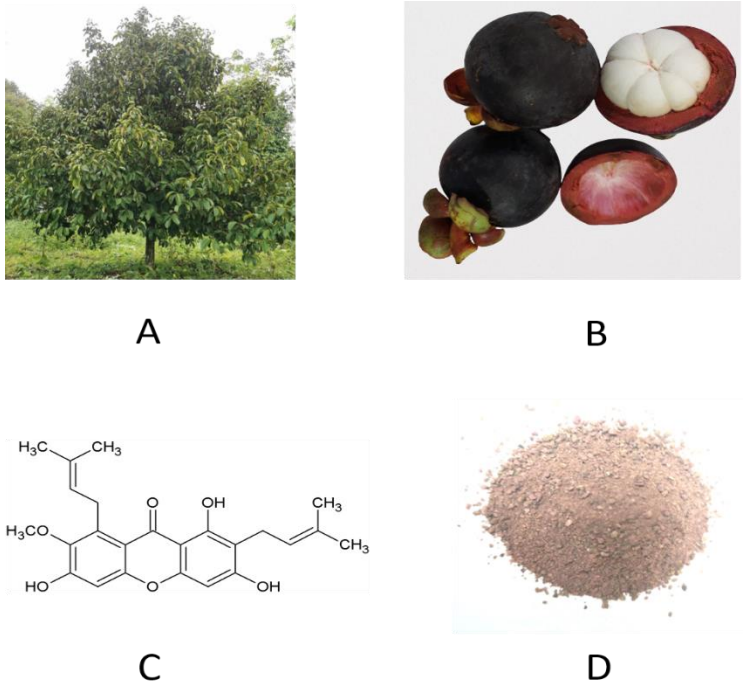

Figure 1: The *G. mangostana* tree (A), fruits and peel (B), chemical structure of alpha mangostin (C), peel powder of *G. mangostana* (D).

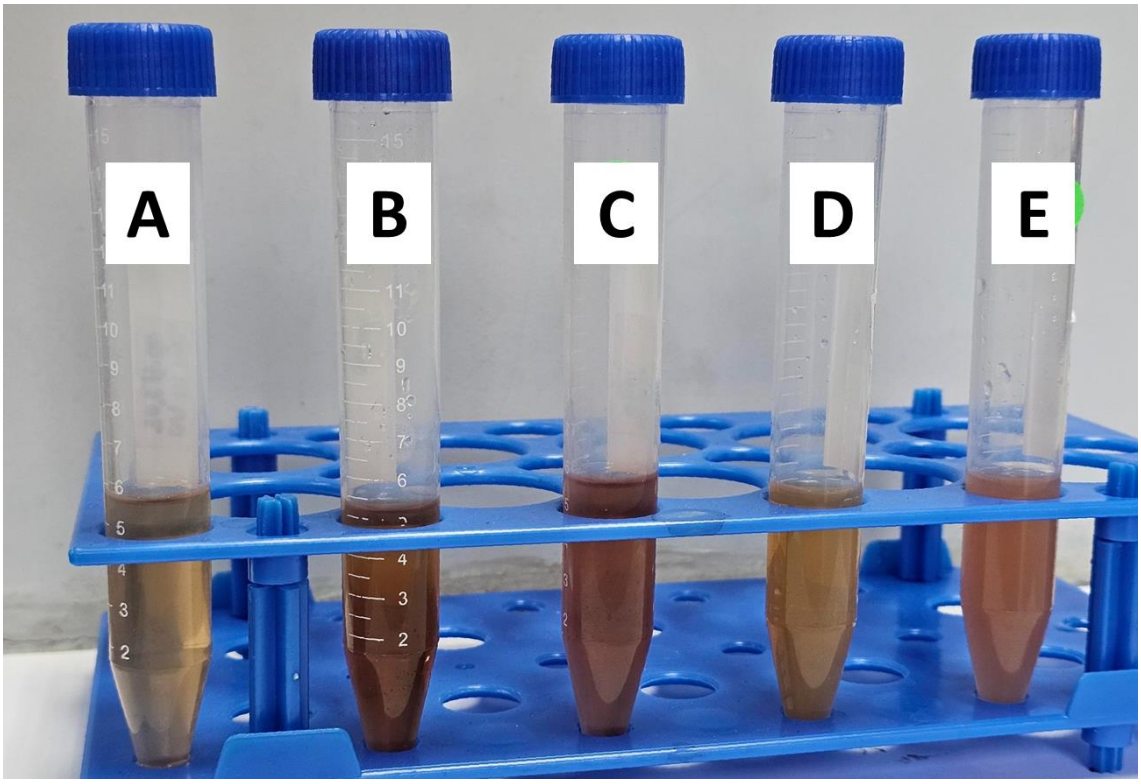

Figure 2: *G. mangostana* peel powder in different extraction solvents (A: 70% Acetone, B: 70% ethanol, C: 80% methanol, D: Room temperature water, E: Hot water)

2.3 Total Phenolic Content

Total phenolic content (TPC) was determined using the Folin-Ciocalteu method [25]. Briefly, 100 µL of sample extract was added with 750 µL of Folin-Ciocalteu reagent (diluted 10x with distilled water). The mixture was vortexed and left for 5 minutes in a dark environment to minimize photoreaction. Then, 750 µL of sodium carbonate (60 g/L) was added to the mixture and allowed to stand for 90 minutes. By using a microplate reader, the absorbance was measured at 725nm. Gallic acid was used as a standard (0 to 100 µg/ml) and results were expressed as mg gallic acid equivalent to 1.0 gram of dried sample (mg GAE/g).

2.4 Total Flavonoid Content

Total flavonoid content (TFC) was measured according to the method of Chang et al., with some modifications [26]. Briefly, 120 µL of extract or standard was mixed with 360 µL of methanol, then 24 µL of 10% (w/v) aluminium chloride was added to the mixture and left to stand for 6 minutes. Next, 24 µL of 1.0 M potassium acetate was added and left to stand for another 5 minutes. Finally, 680 µL of distilled water was added to the mixture. Exactly 300 µL of the mixture was pipetted into a microplate well, and the absorbance was read at 415 nm. A control blank was made by adding 120 µL of methanol to 180 µL of water. The control sample was prepared by mixing 30 µL of plant extract with 270 µL of distilled water in each well. Standard quercetin was used (0 – 100 µg/mL).

2.5 Total Anthocyanin content

Total anthocyanin content (TAC) was determined according to the method by Giusti and Wrolstad, with

slight modifications [27]. Briefly, 25 µL of the sample was pipetted into a microwell plate and mixed with 175 µL of potassium chloride buffer (0.025 M; pH 1.0). The mixture was mixed well and left for 15 minutes. The absorbance values were measured at 515 and 700 nm against distilled water as a blank solution. In another plate, 25 µL of the sample was pipetted into a microwell plate and mixed with 175 µL of sodium acetate buffer (0.025 M; pH 4.5) and rested for 15 minutes. The absorbance values were measured at 515 nm and 700 nm. Then the TAC was calculated by using the following formula.

Total anthocyanin content (mg/kg of dried sample)
$= A \times Mw \times DF \times 10 / (\varepsilon \times C)$

Where *A*, absorbance = $(A_{515}\text{-}A_{700})$ pH 1.0 – $(A_{515} – A_{700})$ pH 4.5, *Mw* is the molecular weight for cyanidin-3-glucoside = 449.2, *DF* is the dilution factor of the samples, ε = is the molar absorptivity of cyanidin-3-glucoside = 26900, and *C* is the concentration of the buffer in mg/mL. The result of TAC was expressed as mg cyanidin-3-glucoside equivalent to kg dried weight sample.

2.6 FRAP (Ferric Reducing/Antioxidant Power) Assay

Ferric reducing/antioxidant power (FRAP) assay was done according to Russo et al. [28]. Briefly, 20 µL of the extract sample or standard was mixed with 180 µL of FRAP reagent in each 96-well microplate. FRAP reagent was prepared by mixing 100 mL of acetate buffer (300 mM, pH 3.6) with 10 mL of 10 mM TPTZ solution and 10 mL of 20 mM Ferric chloride solution. The mixture was incubated in the dark for 40 minutes at 37°C to minimize photoreaction, and the absorbance was read at 593 nm.

2.7 2,2-Diphenyl-1-picrylhydrazyl (DPPH) Assay

The scavenging activity of the extract was measured using DPPH as a free radical model using an assay reported by Clarke et al. and Baliyan et al., with slight modification [29, 30]. About 0.3 mM DPPH solution was prepared by dissolving 0.0118 g DPPH powder into 100 mL of absolute methanol. Then, 100 µL of the extract with different concentrations (4 µg/mL – 5000 µg/mL) was added into 100 µL of DPPH solution and incubated in the dark for 30 minutes, to minimize photo-reaction. Absorbance was measured at 540 nm using a Multiskan Microplate reader. The percentage of antioxidant activity in the samples was calculated as follows:

$AA (\%) = [(A_c - A_s)/A_c] \times 100\%$

AA = Antioxidant activity percentage

A_c = Absorbance of methanol only with DPPH reagent

A_s = Absorbance of a sample with DPPH reagent

The AA (%) of all samples is plotted and the result of DPPH is expressed as IC_{50} value (half maximal inhibitory concentration).

2.8 2,2'-Azino-bis (3-ethylbenothiazoline-6-sulphonic acid) (ABTS) Assay

The ABTS decolorization assay was done according to Panyatip et al and Teggar et al. with slight modifications [31, 32]. About 7.0 mM of ABTS solution and 2.45 mM of potassium persulfate solution were mixed to give a blue-green color of ABTS. The mixture was rested for 16 hours and diluted with 80% methanol to produce an absorbance of 0.7 at 734 nm, a working solution. Then, 100 µL of the sample was added and mixed with 100 µL working ABTS solution in a 96-well plate and incubated for 30 minutes. The absorbance of the mixture was read by a microplate reader at 734 nm. The IC_{50} value was calculated from the graph according to the percentage inhibition of ABTS.

The ABTS radical scavenging activity was calculated using the following equation:

ABTS scavenging activity $= [(A_B - A_S)/A_B] \times 100\%$

A_B = Absorbance of the blank with ABTS reagent

A_S = Absorbance of the sample with ABTS reagent

2.9 High-Performance Liquid Chromatography (HPLC) Analysis

HPLC analysis was carried out according to the method by Mohammad et al. with some modifications [17]. Briefly, each extract solution was diluted to 400

μg/mL using the respective solvent, filtered using a 0.45 μm filter, and aliquoted into HPLC vials. The analysis was done using an isocratic system using an Agilent 1200 HPLC system, operated using a C18 column (100 mm × 4.6 mm × 5μm particle size). The mobile phase was 5% of 0.1% ortho-phosphoric acid in water and 95% of acetonitrile. The flow rate was adjusted to 0.8 mL/min at 25°C and was run for 18 minutes. The sample volume was 20 μL, and the eluate was monitored by a UV-Vis detector at 240 nm. The α-mangostin content in the samples was calculated based on the standard calibration curve of α-mangostin plotted by peak area versus α-mangostin concentration. The results were reported as milligrams of α-mangostin per gram dry peel powder (mg α-mangostin/g dry peel powder).

2.10 Brine Shrimp Toxicity Assay

The brine shrimp toxicity assay was done according to the method by Saito and Tamrin, and Elshazly et al. with slight modifications [33, 34]. Briefly, brine shrimp eggs (*Artemia salina*) purchased from a local aquarium store were hatched in filtered and sterilized seawater, equipped with an aerator for 24 hours. The hatched shrimp larvae were then separated from the unhatched eggs and shells into a separate container under the same conditions and were further reared for another 24 hours. After that, 10 healthy larvae were put in petri dishes containing various concentrations of extracts of 70% ethanol, 70% acetone, 80% methanol, room temperature water, and hot water extracts with water. Sea water and solvents only were used as controls. Then, 24 hours post-incubation, the number of living larvae in each dish was counted. Each assay was repeated in triplicate.

2.11 Antimicrobial Test

Based on the results of the phytochemical antioxidant assays, the 70% ethanol extract was further selected for the antimicrobial test due to its better phytochemical antioxidant activities and less toxicity compared to other solvents. The antimicrobial test was done using the disk diffusion method [35]. Briefly, a stock solution of 10 mg/mL and 5 mg/mL of 70% ethanolic extract of *G. mangostana* was used. For the negative control, only 70% ethanol solvent was used. The positive control was 2.0 mg/mL of Kanamycin dissolved in 70% ethanol [36]. A 20 μL of each solution was pipetted into a sterile paper disc and allowed to dry. The final concentration of extracts used was 200 and 100 μg/mL, and the final concentration of drug used was 40 μg/mL.

The bacteria used were Gram-negative: *E. coli* and Gram-positive: *S. aureus*, all wild-type. Each bacterium was cultured on a petri dish and further selected from single colonies. The subsequent culture solution was adjusted to a 0.5 MacFarland concentration by dissolving the bacteria in sterile normal saline. Each bacterium was streaked evenly on Luria-Bertani agar in a round petri dish and the paper discs with different treatments were each firmly placed on top of the dish. The antibacterial activity was determined by measuring the zone of inhibition (ZI) around the disc, expressed in mm, after 24hours of incubation. The experiment was repeated three times.

2.12 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) Assay

HepG2 cells were seeded at a density of 1×10^4 cells/well in a 96-well plate and incubated at 37°C, 5% CO_2 until 70% confluency was reached. The cells were then washed with 1× PBS prior to treatment with different concentrations of 70% ethanolic *G. mangostana* dried extract diluted in DMSO. After an overnight incubation, the culture medium was removed, and DMEM supplemented with 10% FBS was added to each well before the addition of 10 μL of MTT solution. Incubation was carried out in the CO_2 incubator at 37°C for 2.5 hours. Then, SDS with HCl (0.01 M) was pipetted into every well before incubating the plate again for 24 hours. Subsequently, the formation of the formazan crystals was measured using the microplate reader at an absorbance of 540 nm. The control used was diluted DMSO without extract. Results were expressed as percentage (%) cellular viability [37]. This experiment was repeated three times.

2.13 *In vitro* Antioxidant Assay

2.13.1 Reduction of innate ROS level after extract treatment

The innate ROS level was determined based on the method published by Wang and Joseph (1999) [38]. Briefly, the cells were first seeded in a 24-well plate at a density of 2×10^5 cells / well. Medium was replaced with serum-free medium before the commencement of the assay when 70% confluency was achieved. After an overnight incubation, DCFH-DA (5 μM) was pipetted into every well and left for 45 min. Next, the extracts at different concentrations (12.5, 25.0, and 50.0 μg/mL), negative control (medium only), and positive control tert-butyl hydroperoxide (t-BOOH) (420 μM)) were added into the wells. The fluorescence emitted from the cells was determined using a fluorescence microplate reader [λ_{ex} = 485 nm, λ_{em} = 530 nm]. Cellular ROS level was measured at 30 minutes interval for 120 minutes.

2.13.2 ROS reduction after induced oxidative stress on cells pre-treated with the extract

Cells were seeded similarly for the measurement of innate ROS. After removal of media, cells were treated with the ethanolic *G. mangostana* peel extracts for 20 h at 12.5, 25.0, and 50.0 μg/mL concentrations. Then, DCFH-DA (5 μM) was pipetted into each well prior to induction with oxidative stress using t-BOOH (420 μM). The 24-well plate was measured with a fluorescence microplate reader (λ_{ex} = 485 nm, λ_{em} = 530 nm) immediately. ROS production was measured every 30 min for 2 hours [38].

2.14 Statistical Analysis

Data are presented as mean ± standard deviation (SD) of at least three independent experiments. One-way analysis of variance (ANOVA) was used for analysis and followed by Tukey's multiple comparison post hoc test. For the MTT assay, an unpaired sample T-test was performed to assess the differences between different doses. Data were analyzed with GraphPad Prism (version 8.0, San Diego, CA, USA) and SPSS for *in vitro* antioxidant assays (version 2.0, IBM). All data were examined at the level of significance where $p < 0.05$.

3. RESULTS

3.1 Phytochemical contents and antioxidant activities of *G. mangostana* peel extracts in Different Solvents

Our analysis showed that 70% acetone and 70% ethanol extracted the most phenolic compounds, followed by 80% methanolic extract and hot water. Meanwhile, 80% methanol was able to extract the most flavonoid (TFC), followed by 70% ethanol and 70% acetone. Water, i.e. the most polar solvent, was not able to effectively extract flavonoids because the latter are relatively less polar (Table 1). The 80% methanolic extract contained the highest anthocyanins, followed by 70% ethanolic extract, hot water and 70% acetone extract. This finding is interesting as there was a significant difference between anthocyanin content in hot water and room temperature water, suggesting that anthocyanin extraction is more efficient in hot water (Table 1).

The FRAP, DPPH, and ABTS assays were done to assess the *in vitro* antioxidant status of the different extracts of *G. mangostana* peel. For the FRAP assay, 70% acetone extract showed the highest potential (111.5 μg/mL Trolox eq) compared to other solvents. The DPPH assay produced similar results in 70% acetone, 80% methanol and 70% ethanol extracts (IC_{50} = 22.46 – 25.56). The same pattern was also observed in the ABTS assay, where 70% acetone, 80% methanol, and 70% ethanol extracts produced the same results (IC_{50} = 22.82 – 28.25). We noted that room-temperature water did not exhibit high antioxidant activities compared to hot water extraction (Table 1).

Table 1: Phytochemicals content and antioxidant activities of different extracts of *G. mangostana*

Solvents	70% Ethanol	80% Methanol	70% Acetone	RT Water	Hot Water
TAM	95.32 ± 2.33^{a}	86.94 ± 0.41^{b}	94.26 ± 3.68^{a}	0.00 ± 0.00^{c}	0.96 ± 0.04^{c}
TPC	255.40 ± 6.10^{ab}	243.6 ± 0.92^{b}	269.60 ± 12.64^{a}	5.87 ± 3.39^{d}	141.58 ± 11.48^{c}
TFC	90.99 ± 1.97^{b}	105.93 ± 4.61^{a}	73.38 ± 3.77^{c}	9.43 ± 1.13^{d}	8.52 ± 0.47^{d}
TAC	455.32 ± 4.41^{ab}	506.09 ± 11.97^{a}	409.5 ± 31.64^{b}	33.18 ± 17.34^{c}	443.08 ± 29.93^{ab}
FRAP assay	98.60 ± 1.47^{b}	101.75 ± 4.29^{b}	111.58 ± 2.58^{a}	5.15 ± 0.27^{d}	68.66 ± 2.54^{c}
DPPH assay	25.56 ± 0.79^{c}	24.72 ± 0.88^{c}	22.46 ± 0.62^{c}	333.50 ± 9.57^{a}	106.39 ± 9.26^{b}
ABTS assay	24.25 ± 0.49^{c}	$22.82 \pm 1.25c^{c}$	28.25 ± 0.58^{bc}	736.78 ± 20.95^{a}	49.48 ± 6.24^{b}

This table compares the influence of different solvents on various bioactive contents and antioxidant activities. Different letters within rows denote significant differences ($p < 0.05$) among solvents for each parameter. All values were expressed as means ± standard deviation (N=3). One-way ANOVA was used to compare the variance between different samples.

Total Alpha Mangostin (TAM) content expressed as µg/mg of dried sample

Total Phenolic Content (TPC) expressed as Gallic acid equivalent µg/ml

Total Flavonoid Content (TFC) expressed as Quercetin equivalent µg/ml

Total Anthocyanin Content (TAC) expressed as mg/kg of dried sample

Ferric Acid Reducing Potential (FRAP) assay expressed as Trolox equivalent (µg/ml).

DPPH and ABTS assays expressed as IC_{50}.

3.2 HPLC Analysis of *G. mangostana* Peel Extracts in Different Solvents

HPLC chromatograms of the sample extracts in different solvents and in water at room and at boiling temperatures are shown in Figure 3.0 (A – E) and α-mangostin standard Figure 3.0 (F). The peak of α-mangostin compound is found at the range of 5.8 to 6.0 mins in the organic solvent extracts, and 6.3 min in aqueous extract (hot temperature). The slight variation in the retention time of α-mangostin peak is most likely due to the polarity difference between the sample extract solution and the HPLC mobile phase (95 % acetonitrile). Based on the standard calibration curve, the amount of α-mangostin extracted using each solvent were; 95.3 µg/mg for 70% ethanol extract, 94.26 µg/mg for 70% acetone extract, 86.94 µg/mg for 80% methanol extract 0.96 µg/mg for hot water extract and not detected for room temperature water extract (Table 1). Considering the results of α-mangostin content, phytochemical content and antioxidant activities, it was noted that 70% ethanol was the most suitable green solvent due to its lesser toxicity than methanol and acetone. Hence, the ethanolic extract was chosen for further antimicrobial and *in vitro* antioxidant assays using HepG2 cell line.

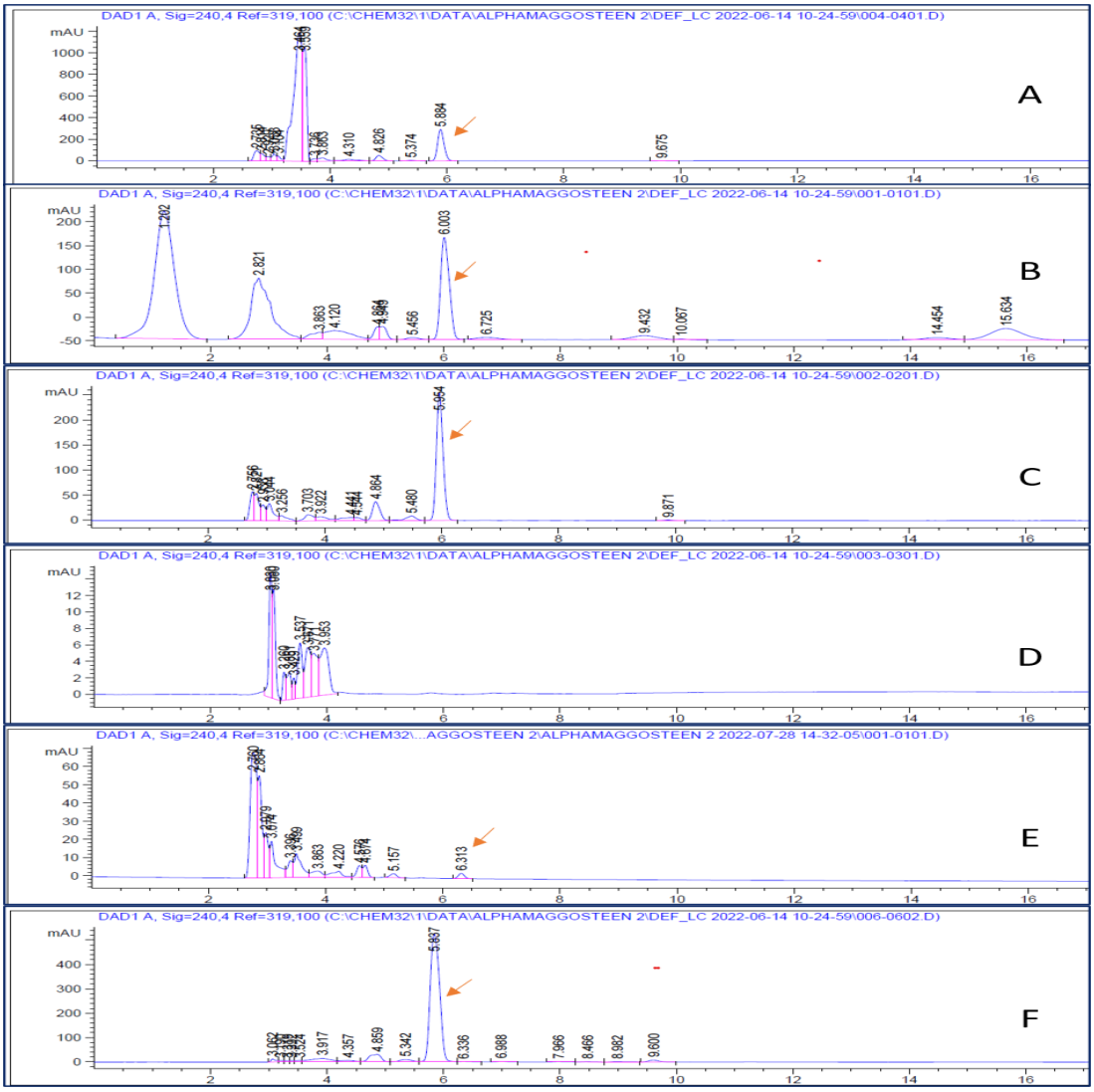

Figure 3: HPLC chromatogram of different extracting solvents of *G. mangostana* peel. (A: 70% Acetone, B: 70% ethanol, C: 80% methanol, D: Room temperature water, E: Hot water, F: Alpha mangostin standard).

3.3 Antimicrobial Activities of Ethanolic Extract of *G. mangostana* peel

The GM extract (10mg/mL) produced a ZI of 9.75 mm in an *E. coli* cultured dish, which is significantly higher than the negative control ethanol, 7.33 mm, but less than the positive control Kanamycin, 17.6 mm. The treatment of GM extract produced ZI of 9.33 mm in *S. aureus* cultured dish, higher than the negative control ethanol, 6.66 mm, but lesser than the positive control Kanamycin, 14.3 mm. This result indicates that GM ethanolic extract produced no activity and moderate antimicrobial activities against *E. coli* and *S. aureus*, respectively, based on the ZI (Table 2).

Table 2: Antimicrobial activity of 70% ethanolic extract of *G. mangostana* peel against *E. coli* and *S. aureus* based on disk diffusion assay, measured by diameter of inhibition zone of extract (cm)

Substance	*E. coli*	*S. aureus*
G. mangostana (10mg/ml)	9.75 ± 0.50^{b}	9.33 ± 0.67^{b}
Kanamycin (10mg/ml)	17.6 ± 0.57^{a}	14.3 ± 0.33^{a}
Ethanol	7.33 ± 0.57^{b}	6.66 ± 0.50^{c}

All values were expressed as means ± standard deviation (N=3). One-way ANOVA was used to compare the variance between different samples. Different letters within columns, indicate significant difference at $p < 0.05$

3.4 Cytotoxic Effects of *G. mangostana* Extracts on Brine Shrimp Larvae and HepG2 Cell Line

In the brine shrimp larvae toxicity assay, the solvents (70% ethanol, 80% methanol and 70% acetone) all showed LD_{50} values of less than 100 µg/mL; the negative control seawater showed LD_{50} of more than 2000 µg/mL. Similarly, all the extracts of 70% ethanol, 80% methanol and 70% acetone) all showed LD_{50} values of less than 100 µg/mL. The hot water mangosteen and room temperature mangosteen peel extract showed LD_{50} of 1750 µg/mL and 1633 µg/mL, respectively (Table 3). This result corresponds to the phytochemical contents of each extract, and also the effects of solvents on the brine shrimps' viability.

The dried ethanolic *G. mangostana* peel extracts were not toxic to HepG2 liver cells when tested at a concentration range of 12.5 µg/mL to 200 µg/mL. However, cellular viability was decreased by approximately 10-fold when treated with the extract at concentrations of 400 µg/mL to 1000 µg/mL, indicating a cytotoxic effect. Therefore, non-toxic concentrations 12.5, 25 and 50µg/mL were selected for subsequent antioxidant assay to determine ROS level in HepG2 liver cells (Figure 4.0).

Table 3. Brine Shrimp Toxicity Assay

Substance		LD_{50} (µg/mL)
Solvent	Sea Water	$> 2000^{a}$
	70% Ethanol, 80% Methanol, 70% Acetone	$< 100^{d}$
Extracts	Hot water	1750 ± 57^{b}
	Room temperature water	1733 ± 57^{c}
	70% Ethanol, 80% Methanol, 70% Acetone	$< 100^{d}$

Notes:

All values were expressed as means ± standard deviation (N=3). One-way ANOVA was used to compare the variance between different samples. Different letters within columns, indicate significant differences at $p < 0.05$. LD_{50} (µg/mL): Lethal dose for 50% of the brine shrimp population, expressed in micrograms per milliliter. Values indicate the toxicity level of each substance, with lower LD_{50} values representing higher toxicity.

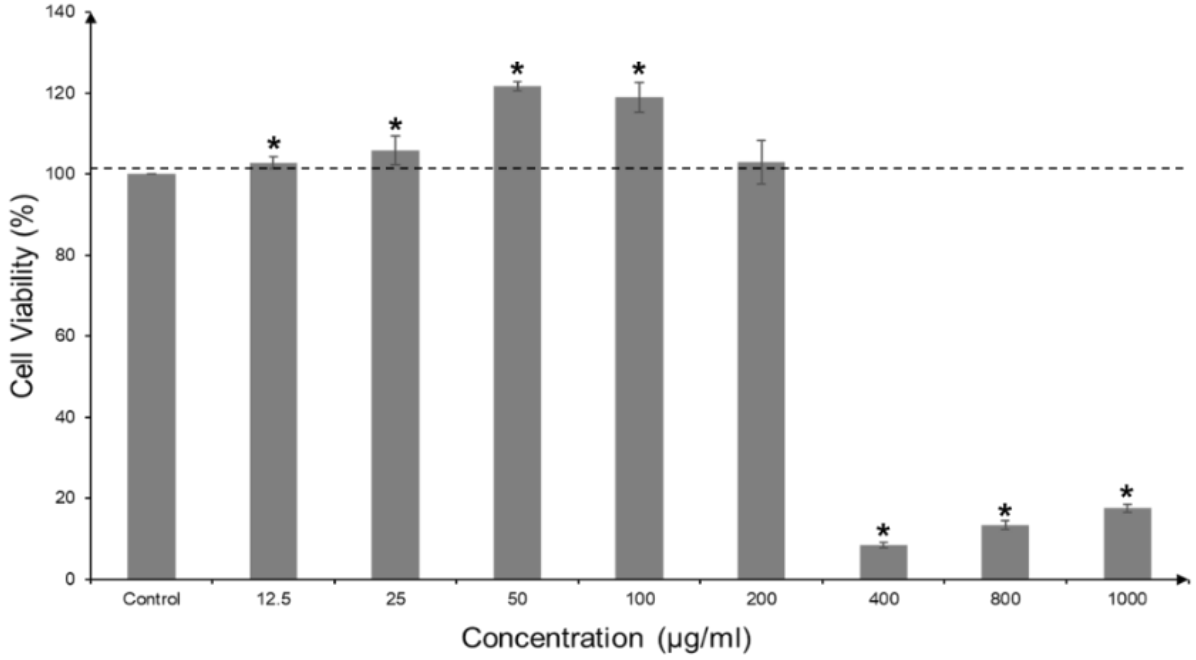

Figure 4. Cellular viability of HepG2 cells after pre-treatment with *G. mangostana* peel extract at various concentrations (12.5 to 1000µg/mL). All measurements were performed in triplicates and expressed as means ± standard deviation. Data were analyzed by comparing samples of different concentrations with untreated control using unpaired sample t-test. Values with symbol *, are significantly different at $p < 0.05$.

3.5 Direct Effects of Ethanolic *G. mangostana* peel Extract on Intracellular Reactive Oxygen Species (ROS) Generation of HepG2 cell line.

The effects of *G. mangostana* peel extracts on innate reactive oxygen species (ROS) levels were determined using the selected non-toxic concentrations (12.5, 25.0 and 50.0 µg/mL). It was found that cells treated with different concentrations of ethanolic *G. mangostana* peel extract maintained basal ROS levels as compared to the control. On the other hand, the positive control, tert-butyl hydroperoxide (t-BOOH), increased intracellular ROS exponentially as expected, which showed that the cell model system was working (Figure 5.0).

3.6 Protective Effects of Ethanolic *G. mangostana* Peel Extract towards tert-butyl hydroperoxide (t-BOOH)-induced Oxidative Stress

The protective effects of *G. mangostana* peel extracts were evaluated during t-BOOH-induced oxidative stress in HepG2 cells. As shown by Figure 6.0, cells pre-treated with different concentrations of extracts, 12.5 to 50 µg/mL, were able to reduce ROS level by 63.6%, 53.2% and 49.0%, respectively, when monitored for 120 min.

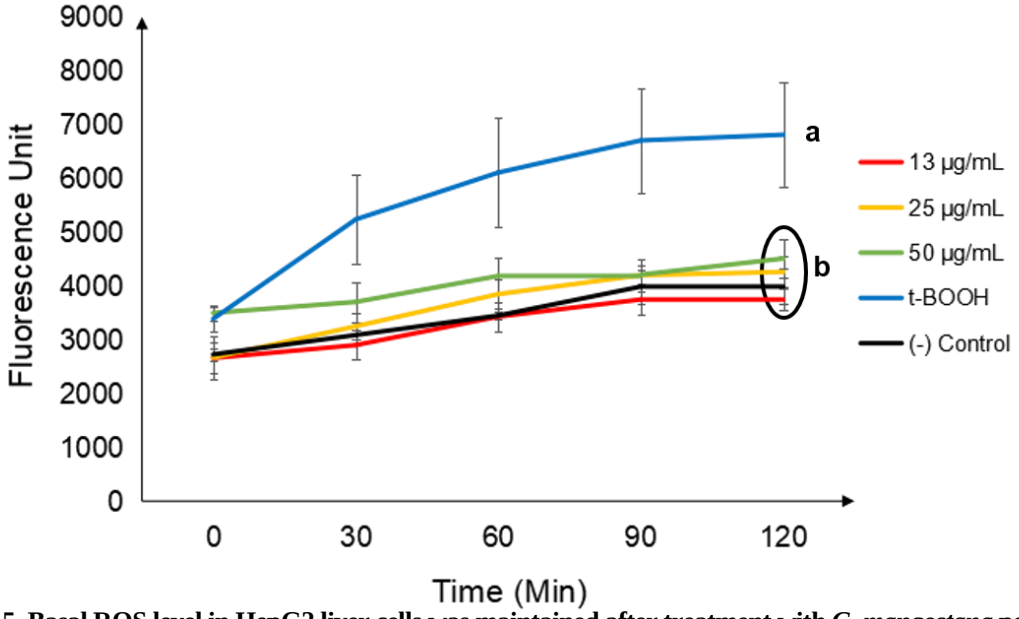

Figure 5. Basal ROS level in HepG2 liver cells was maintained after treatment with *G. mangostana* peel extract at various concentrations. ROS production was monitored for 120 min using DCFH-DA assay. All values were performed in triplicates and expressed as means ± standard deviation. One-way ANOVA was used to compare the variance between different concentration of samples at 120 min. Means with a common letter are significantly similar at $p < 0.05$.

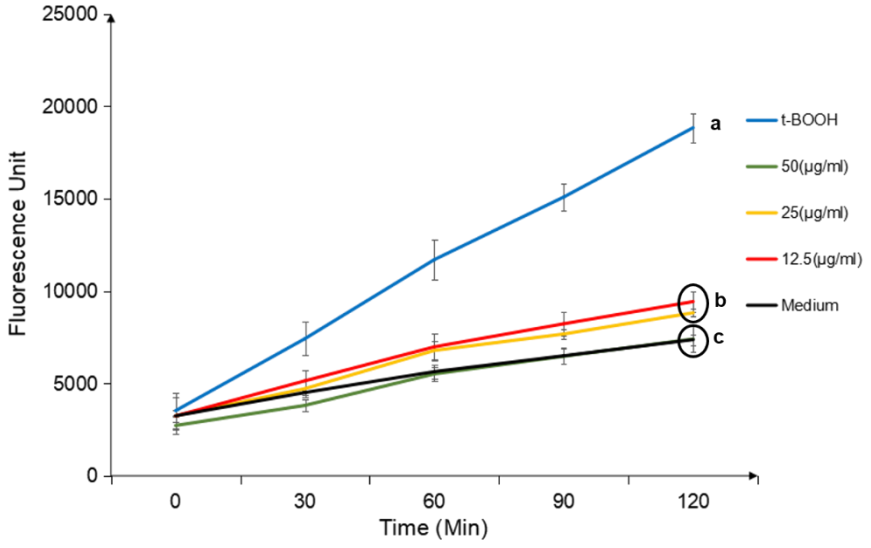

Figure 6. Reduction of ROS level in oxidative stress-induced HepG2 cells after pre-treatment with different concentrations (50, 25 and 12.5µg/mL) of *G. mangostana* peel extracts. All values were performed in triplicates and expressed as means ± standard deviation. One-way ANOVA was used to compare the variance between different concentration of samples at 120 min. Means with a common letter are significantly similar at $p < 0.05$.

4. DISCUSSIONS

In this study, 70% ethanol was selected as the most optimum solvent to extract α-mangostin from *G. mangostana* peel (Figure 3). This solvent was also able to extract high amount of phenolic, flavonoid and anthocyanins from the *G. mangostana* sample (Table 1). Ethanol is a polar solvent, making it a suitable choice for extracting relatively polar compounds like α-mangostin, whereas water is not suitable due to α-mangostin's low solubility in aqueous conditions. Previous reports also showed ethanol as the preferred green solvent for extracting xanthones from mangosteen peel [16, 39]. The highest amount of flavonoid and anthocyanin recovery was obtained by using 80% methanol solvent, and the highest amount of phenolic content was by 70% acetone (Table 1). However, the 70% ethanolic extract was selected for further antimicrobial and cytotoxicity assays because of its efficiency in extracting α-mangostin and its lower toxicity. Furthermore, the usage of ethanol as a green solvent to extract α-mangostin was also supported by a previous report [40]. As expected, water extracts showed lower content of flavonoids and phenolic compounds as reported previously [41]. Interestingly, our findings showed that extraction using hot water was efficient in recovering anthocyanins. As the traditional way of extracting phytochemicals from mangosteen was usually through boiling, this may partly explain its beneficial effect as more anthocyanins can be recovered through this method. A recent study also supported the use of hot water extraction to extract anthocyanins from blue pea flowers [42]. Additionally, the use of hot water-based extraction was also effective in extracting phytochemicals in *Pleurotus* samples [43].

The antioxidant assays of the 70% ethanol, 70% acetone and 80% methanol extracts were almost similar (Table 1). A previous report showed that mangosteen peel extracted by ethanol for 24 hours produced the best antioxidant yield. The study reported that the xanthone was best extracted using a long extraction period but the antioxidant yield was optimal at short extraction times [44]. However, the effect of extraction time was not assessed in the present study. Additionally, a study by Tran *et al*, showed that ethyl acetate solution was able to effectively extract α-mangostin and its derivatives from mangosteen peel and showed good antioxidant activity [45].

The results of HPLC analyses showed all the extracts, except the room temperature water, contained α-mangostin (Figure 3.0, Table 1). Effective absorption of α-mangostin at 240 nm was also reported by a previous study [46]. A previous study by Pothithirat *et al.*, the extraction with 50% ethanol was reported to be suitable for extracting phenolic compounds, free radical-scavenging components and tannins, while 95% ethanol was recommended for the extraction of α-mangostin [47]. In this study, we also found that water-based extraction with high temperature was more suitable for extracting α-mangostin compared to using room-temperature water. This finding was also supported by Mulia et al., although in their study, they used different parameters (65°C for 30 minutes) [18]. As we used 100°C extraction for 10 minutes, this might explain the lesser recovery of α-mangostin due to the potential degradation of this compound with higher temperature, or due to shorter extraction time. Another study showed that Soxhlet extraction is more effective than the maceration technique in extracting xanthones from *G. mangostana* peel using 95% ethanol. The study also showed that the conditions for the highest xanthone recovery using ultrasonic-assisted extraction were at 33°C, 50% ethanol and at 50% amplitude [48]. The Box-Behnken design showed that the highest xanthone recovery conditions were at 33°C, 80% ethanol and 75 amplitudes. Although ethanol was selected as the solvent of choice in this study, α-mangostin recovery was not specifically mentioned in the study [48]. In this study, we have compared the types and percentage of solvents and the α-mangostin contents in each extracts.

In the antimicrobial test (Table 2), similar findings

demonstrated that the ethanolic extract of *G. mangostana* produced a clear zone of inhibition in *S. aureus* and *E. coli* [49]. Additionally, *G. mangostana was* effective in reducing cariogenic microorganisms [50]. Consistent reports have shown that *G. mangostana* was effective in reducing *S. aureus*, which is an opportunistic pathogen in immunocompromised individuals [51]. Additionally, it was demonstrated that α-mangostin was effective in reducing both biofilms of methicillin-resistant *S. aureus* and methicillin-sensitive *S. aureus* [52]. These results further support the application of α-mangostin and/or its derivative for industrial applications e.g. incorporation of α-mangostin in bioplastic material for its antimicrobial properties [52].

The brine shrimp toxicity assays of the *G. mangostana* peel extracts diluted in 70% ethanol, 80% methanol and 70% acetone, were not distinguishable compared to solvents only unless lower concentrations (< 100 µg/mL) were used, showing the toxicity of these solvents on brine shrimps (Table 3). However, between the water extracts, it was noted that the LD_{50} values did not differ significantly between hot and room temperature water extracts, probably due to the lower α-mangostin or other phytochemical contents in the water extracts (Table 3). A previous study showed that the toxicity of *G. mangostana* extract is correlated with the α-mangostin content [18]. Though in their study, the heating time used was 30 minutes compared to 10 minutes in our study. This further implies the effect of extraction time in extracting α-mangostin using water-based extraction methods. Based on the literature, our study was the first to explore the toxic effects of different extracts of *G. mangostana* peel in brine shrimps, simultaneously, using the described solvents.

The cytotoxicity of *G. mangostana* peel extracts was also examined using HepG2 liver cell line (Figure 4.0). We found that *G. mangostana* peel extracts were not cytotoxic between 12.5 µg/mL to 200 µg/mL. Nonetheless, the cell viability was reduced significantly at the concentration range of 400 µg/mL to 1000 µg/mL (Figure 4.0). The *G. mangostana* peel extract at concentrations of 50 to 100 µg/mL was toxic to murine macrophage cells (RAW 264.7), whereas lower concentrations (5 to 25 µg/mL) were non-toxic to these cells, showing that the toxicity of this extract varies according to cell type [53]. The acute oral toxicity experiment of *G. mangostana* peel extracts on a rat model system was also performed and no morbidity or mortality was observed during the entire experimental period, even at a high dosage of 5000mg/kg of *G. mangostana*. This indicates that *G. mangostana* peel is safe for human consumption at the right dose [54].

To determine the effects of *G. mangostana* peel ethanolic extracts on intracellular reactive oxygen species (ROS) level, the DCFH-DA assay was initiated on HepG2 cell line. ROS are chemically reactive molecules that are produced within the biological system. Increased ROS accumulation promotes oxidative stress and causes damage to primary cellular components such as lipids, proteins and DNA [55]. Based on Figure 5.0, cells treated with different concentrations (50, 25 and 12.5 µg/mL) of *G. mangostana* peel extract did not affect basal ROS level and maintained its production as compared to untreated control. A similar observation was observed in mouse preadipocytes treated with *Gouania mauritiana*, *Antirhea borbonica* and *Doratoxylon apetalum* plant extracts, where the basal ROS level was not affected by the extracts alone [56]. Subsequently, the extract was further investigated for its protective effect during induced oxidative stress.

In this study, the protective effects of *G. mangostana* peel ethanolic extracts during tert-butyl hydroperoxide (t-BOOH) induced oxidative stress were also determined. Based on Figure 6.0, all concentrations of *G. mangostana* peel extracts were able to significantly reduce ROS production in t-BOOH-treated HepG2 liver cells. This model system was also used by Zhu et al. to investigate the protective potentials of the ethanolic extract of *Clerodendrum cyrtophyllum* Turcz and its derivative fractions [57]. Their results indicated that cells pre-treated

with these extracts had reduced t-BOOH-induced ROS generation, similar to our work. On the other hand, Ribeiro and colleagues also tested the protective effects of *Cheiloclinium cognatum*, *Guazuma ulmifolia* Lam., *Hancornia speciosa* Gomes and *Hymenaea stigonocarpa* Mart. aqueous extracts on HepG2 cells [58]. All extracts showed protective properties against acetaminophen (APAP) induced oxidative stress at concentrations ranging from 50 to 100 µg/mL [58]. This was supported by Moongkarndi et al. where the protective effect of mangosteen extract was examined against β-amyloid-induced oxidative stress in SK-N-SH neuronal cells [59]. It was demonstrated that SK-N-SH neuronal cells pre-treated with 400 µg/mL of mangosteen extract was able to reduce β-amyloid-induced ROS level by 46%. A previous study by Raghavendra et al. also showed mangosteen as a potential source of natural antioxidant and as anticholinesterase agent [60]. To our best knowledge, this study is the first to test mangosteen peel ethanolic extract for its antioxidative and liver protective potentials *in vitro.*

Despite the favorable results obtained from this study, there are several limitations identified that need to be addressed in further studies. In this study, we only measured the antioxidant activity based on fluorescence markers in HepG2 cells; however, the mechanistic pathways on how the extract affects the biological antioxidant pathway were not assessed. Hence, future studies should explore the role of this extract on the cellular antioxidant pathways, such as the NRF2-antioxidant element response signaling pathway in this particular cell line. Furthermore, the antioxidant, antimicrobial and cytotoxic activities were based on the combination of phytochemicals present in the peel. Therefore, the interactions between the different phytochemicals, for instance, anthocyanin and α-mangostin, were not assessed. This could be further considered in the future if detailed single-compound studies are planned. Based on our findings, further investigations could focus on isolating, purifying, and characterizing α-mangostin and other related compounds in the *G. mangostana* peel, to increase our understanding of its properties and potential applications. Further, the effects of extraction time and extraction temperature could be further explored to find the most optimized parameters for α-mangostin extraction.

5. CONCLUSIONS

G. mangostana peel is widely used in traditional medicine and is known for its beneficial biological properties. In this study, we found that extraction by 70% ethanol produced the most optimal recovery of α-mangostin and polyphenols, and exhibited robust phytochemical, antimicrobial and antioxidant activities. These observations point towards the potential of extracting phytochemical compounds using ethanol as a green solvent from the waste part of *G. mangostana* fruit for human consumption, and the ability of ethanolic *G. mangostana* peel extract to counteract *in vitro* oxidative stress processes. Further studies should explore the role of extraction time and temperature for further optimization of α-mangostin extraction. Additionally, mechanistic studies to explore the antioxidant properties of α-mangostin in HepG2 cell lines could answer some key questions to further support its application in health and industrial settings.

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Conflict of Interest

The authors declare that they have no potential conflict of interests.

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Author Contributions

All authors contributed to the conception, experimentation, writing and approved the final submission.

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الخصائص المضادة للأكسدة، والمضادة للميكروبات، والسامة للخلايا لمستخلص قشرة فاكهة الجارسينيا مانجوستانا L (Garcinia mangostana L) ومحتواها الألفا–مانجوستين

أوليفر دين جون[1, 2]، مونجيا بيليزا كوزماس موجولات[1]، يونيس لوا هانري[1]، كاي سونج نج[3]، شيري–آن تان[3]، نوومي سوروقاو[1]*

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ملخص

تُستخدم قشرة فاكهة الجارسينيا مانجوستانا (المانجوستين) L , (Garcinia mangostana L) على نطاق واسع كدواء تقليدي في جنوب شرق آسيا، وتُعرف بخصائصها البيولوجية المفيدة. هدفت هذه الدراسة إلى تقييم أفضل مذيب لاستخلاص الألفا–مانجوستين مانجوستين – (α) من قشرة الجارسينا مانجوستانا(G. mangostana(باستخدام الإيثانول بنسبة 70٪، والأسيتون بنسبة 70٪، والميثانول بنسبة 80٪، والماء في درجة حرارة الغرفة ودرجة الغليان. بالإضافة إلى ذلك، تمّت دراسة النشاط المضاد للأكسدة والمضاد للميكروبات في المستخلص الأكثر فاعلية. أظهر الإيثانول 70٪، والأسيتون ٧٠٪ أعلى نسبة استخلاص للألفا–مانجوستين، مانجوستين –(α) إلا أن الإيثانول 70٪ . أظهر محتوى أعلى من مركبات الفلافونويد والأنثوسيانين مقارنة بالأسيتون 70٪. أظهر مستخلص الإيثانول نشاطًا مضادًا للميكروبات بمستوى متوسط ضد الإشريكية القولونية *(Escherichia coli)* العنقودية الذهبية . (*Staphylococcus aureus*)أظهرت اختبارات السمية على يرقات الجمبري الملحي أن المذيبات العضوية كانت سامة عند تركيز 100 ميكروجرام/مل. ك ما أظهر اختبار [3–(4,5–ثنائي ميثيل ثيازول–2–يل)–2,5–ثنائي فينيل تترازوليوم بروميد] . MTT أن مستخلص الإيثانول بنسبة 70٪ عند تركيز 400 ميكروجرام/مل كان سامًا لخلايا. HepG2 وعند الخلايا للإجهاد التأكسدي الناتج عن تربوتيل هيدروبيروكسيد،(t-BOOH) *tert-butyl hydroperoxide* أظهر مستخلص الإيثانول لقشرة الجارسينيا مانجوستانا) G. mangostana(عند تركيز يتراوح من 12.5 إلى 50 ميكروجرام/مل. تأثيرًا وقائيًا ضد هذا الإجهاد التأكسدي. تُشير نتائج هذه الدراسة إلى أن مستخلص الإيثانول بنسبة 70٪ من قشرة الجارسينيا مانجوستانا) G. mangostana(يُعطي أفضل معدل استرجاع للألفا–مانجوستين والمركبات النباتية النشطة الأخرى، كما يُظهر تأثيرًا حماية ضد الإجهاد التأكسدي في خلايا.HepG2

الكلمات الدالة: جارسينيا مانجوستانا L ،(Garcinia mangostana L)، ألفا–مانجوستين، مانجوستين، –(α)، استخلاص بالمذيبات، مضادات الأكسدة، والسامة للخلايا.

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