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Original Article

Phytochemical Screening and Biological Evaluations of *Garcinia atroviridis*

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Abstract

Garcinia atroviridis (Clusiaceae) has been used as a postpartum medication agent in folkloric medicine. However, its biological potential has not been fully evaluated. The present study was aimed to evaluate the antioxidant efficacies, cholinesterase enzyme inhibitory and antimicrobial activity of the stem bark extracts of *G. atroviridis*. Successive extraction was done using solvents of varied polarity. The antimicrobial activity was performed using the disc diffusion method by determining the inhibition zone and further evaluated for their minimum inhibition concentration (MIC) and minimum lethal concentration (MLC). Anti-cholinesterase activity was determined by spectrophotometric method while antioxidant activity was evaluated by DPPH radical scavenging. The extracts showed significant inhibitory activity against food borne bacteria. However, only dichloromethane extract exhibited the anti-yeast potential against *Candida utilis* and none of the extracts tested showed anti-fungi activity. For cholinesterase enzyme inhibitory activity, ethyl acetate extract exhibited the strongest acetylcholinesterase (AChE) inhibitory activity with an IC_{50} value of 5.46 ± 2.91 µg/mL while water extract showed the lowest IC_{50} value (29.31 µg/mL) against butyrlcholinesterase (BChE). For antioxidant activity, methanol extract showed moderate activity against DPPH radicals with EC_{50} value of 71.96 µg/mL. The dichloromethane extract exhibited significant antimicrobial activity while ethyl acetate showed the strongest acetylcholinesterase (AChE) inhibitory activity. The present study showed the significance of this commercial plant as the potential source of bioactive compounds.

Keywords: antimicrobial, antioxidant, cholinesterase enzyme inhibitory, Clusiaceae, *Garcinia atroviridis*, phytochemical.

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1. Introduction

Since early human history, natural products have been utilized to alleviate disease and served as the most significant source of new leads for pharmaceuticals, agrochemicals, cosmetics, fine chemicals and nutraceuticals development [1]. Over the past few decades, plant-derived active constituents are gaining popularity due to their natural background [2]. Bioactive constituents are produced by plants as end products, by-products, or metabolites, making them as indispensable tools in biomedical research and unique prototypes for the development of drugs [2,3]. To date, the relative ease of access to plants has resulted in the discovery of a majority of plant-derived materials [4].

Garcinia atroviridis Griff. ex T. Anders., from the family of Clusiaceae, is a medium-sized fruit tree found wild in the forest of Peninsular Malaysia [5]. It is commonly known as ‘asam gelugor’ in Malaysia or ‘som-khaek’ in Thailand [6]. The tree can grow up to a height of 20 m, bearing highly acidic fruits which are orange-yellow [5]. Sun-dried slices of the fruits, known as ‘asam keping’, are commercially available and are commonly used as seasoning in culinary [5, 6].

G. atroviridis has been used as a medication agent to treat ear-ache, throat irritation, cough and dandruff in folkloric medicine [5]. In Peninsular Malaysia, *G. atroviridis* was used in a lotion made with vinegar, which is rubbed upon the abdomen of a woman after confinement [5]. The stem barks of the tree were traditionally used for the treatment for severe abdominal pain and diarrhea by the local community. In Thailand, *G. atroviridis* is used for improving blood circulation, as an expectorant, treatment of coughs and as laxative [6]. The plant can be used as complex mixtures containing a broad range of constituents (infusions, essential oils, tinctures, extracts) or as pure, chemically defined active principles [7]. In this study, we report the biological activities of the stem bark extracts from *G. atroviridis*, including the cholinesterase enzyme inhibitory, antimicrobial, and antioxidant as there is insufficient literature concerning it. This study could be beneficial to ascertain the pharmaceutical potential of this medicinal plant.

2. Materials and Methods

2.1. Plant Material and Extraction

Stem bark of *G. atroviridis* was collected from Yan Kedah, Malaysia. The plant specimen was identified by the botanist and a voucher specimen (USM 11201) was deposited in herbarium of the university. Air-dried stem bark of *G. atroviridis* was ground and sequentially extracted in a Soxhlet apparatus with *n*-hexane, dichloromethane and

methanol. The concentrated methanol extract was further partitioned with chloroform and then ethyl acetate while the water extract was obtained using maceration. All the extracts (*n*-hexane, dichloromethane, chloroform, ethyl acetate, methanol and water) obtained were concentrated *in vacuo* at 40 °C prior to drying with nitrogen gas.

2.2. Chemicals and Reagents

Dragendorff's reagent, hydrochloric acid, sulfuric acid, ferric chloride hexahydrate ($FeCl_3.6H_2O$), Folin-Ciocalteu reagent, gallic acid, catechin, 2, 2'-diphenyl-1-picrylhydrazyl (DPPH), ascorbic acid, acetylcholinesterase from electric eel, 5, 5'-dithiobis(2-nitrobenzoic acid), acetylthiocholine iodide, butyrylcholine esterase from equine serum, *S*-butyrylthiocholine chloride, physostigmine, *p*-iodonitrotetrazolium violet and RPMI 1640 agar plates were purchased from Sigma-Aldrich (St. Louis, USA). McFarland standard and Mueller Hinton agar medium were obtained from HiMedia (Mumbai, India). All the other solvents and reagents used were of analytical grade.

2.3. Phytochemical Analysis

The crude extracts of the stem bark were assessed for the presence of alkaloids, tannins, saponins and terpenoids using the standard procedures as described by Iqbal et al., 2015 [8].

2.3.1. Test for Alkaloids

15 mg of each crude extract was separately stirred with 1% HCl on a water bath for 5 min and filtered. Dragendorff's reagent was added into the filtrate, an orange red precipitate shows the presence of alkaloids.

2.3.2. Test for Tannins

Each crude extract of 0.5 g was separately stirred with distilled water and then filtered. A few drops of 5% ferric chloride were then added. Black or blue-green coloration or precipitate indicates the presence of tannins.

2.3.3. Test for Saponins

0.5 g of each crude extracts was separately shaken with distilled water. The formation of frothing in a warming water bath for 5 min shows the presence of saponins.

2.3.4. Test for Terpenoids

Each crude extract of 0.1 g was separately shaken with chloroform followed by the addition of concentrated H_2SO_4. A reddish brown coloration of the interface indicates the presence of terpenoid.

2.4. Determination of Total Phenolic Content (TPC)

The total phenolic content of various extracts of *G. atroviridis* were measured using Folin-Ciocalteu assay with some modifications [9]. Stock solutions of 1 mg/ml of various stem bark extracts and standard, gallic acid (Sigma-Aldrich, USA) were prepared in methanol. A series of working standard (0.3125 to 3.125 μg/ml) was prepared for gallic acid by diluting the stock solution with distilled water. In individual test tube, 0.5 ml of Folin-Ciocalteu reagent was added into 1 ml

of test substance. The test tubes were shaken thoroughly and left at room temperature for 5 minutes. Then, 1 mL of 20 % sodium carbonate and 6 ml of distilled water were added, shaken well and were allowed to stand for another 2 hours in dark at room temperature. After 2 hours, 0.2 ml of the reaction mixture was transferred from each test tube into a 96-well plates and absorbance was measured at 760 nm using microplate reader. A standard curve was constructed for gallic acid. The experiment was done in triplicate. Total phenolic content in the samples was expressed in milligrams of gallic acid equivalent (GAE) per gram of extract (mg GAE/g extract).

2.5. Determination of Total Flavonoid Content (TFC)

Total flavonoid content was determined following the procedures of Dewanto et al [10] and Sultana et al [11] with some modifications. Stock solutions of all stem bark extracts at 2 mg/ml and standard, catechin (Sigma-Aldrich, USA) at 1 mg/ml were prepared in methanol. A series of working standard of catechin (1 to 10 μg/ml) was prepared by diluting the stock solution with distilled water. In individual test tube, 1 ml of each concentration of test substance was transferred and 4 ml of distilled water was then added, followed by 0.3 ml of sodium nitrite. The mixtures were allowed to stand for 5 minutes at room temperature. A volume of 0.3 ml of 10% aluminium chloride was added and the mixture was transferred into 10 ml volumetric flask. It was then followed by addition of 2 ml of 1 M sodium hydroxide and the volume made up to 10 ml with distilled water. The resulting solution was mixed well and transferred back into test tube. Once solutions were prepared, 0.2 ml of each solution was transferred into a 96-well plates and its absorbance was measured at 510 nm using microplate reader. A standard curve was generated for standard, catechin. Total flavonoid content of the samples was expressed as milligrams of catechin equivalent (CE) per gram of extract (mg CE/g extract).

2.6. Free Radical Scavenging Activity

The scavenging effects of the extracts for DPPH radicals (Sigma-Aldrich, USA) were tested according to the method by Duan et al [12], with slight modifications. All the extracts including ascorbic acid were dissolved in DMSO to obtain stock solution samples to the desired concentration (3.91 to 4000 μg/ml). Ascorbic acid was used as a positive control. The ability to scavenge the DPPH radical was calculated using the equation as stated below:

$$\text{Scavenging Effect (\%)} = [1 - (A_{sample} - A_{sample\ blank}) / A_{control}] \times 100$$

The crude extracts with the highest scavenging activity were further diluted into eight different concentrations. Serial dilutions for the samples and ascorbic acid were done using DMSO in order to obtain 6 different concentrations. The method used was the same as the assay of DPPH free radical scavenging activity described above. Inhibitory concentration (IC_{50}) of the results obtained was determined.

2.7. Cholinesterase Enzyme Inhibitory Activity

Acetylcholinesterase (AChE) and butyrylcholinesterase (BChE) inhibitory potential of the samples was determined using a spectrophotometric method as described by Khaw et al [13]. For the AChE assay, 140 µl 0.1M Na_2PO_4 buffer (pH 8) was added to a 96-well microplate, followed by the addition of 20 µl test sample and 20 µl AChE (0.09 U/ml). Ten microliters of 10mM 5, 50-dithiobis(2-nitrobenzoic acid) was then added to each well, followed by addition of 10 µl acetylthiocholine iodide as the substrate (14mM). The absorbance of the colored end-product was measured at 412 nm at designated intervals for 30 minutes after initiation of the enzymatic reaction. For the BChE assay, the same procedure as described for AChE was followed; however, the enzymes and substrates were substituted with BChE from equine serum and *S*-butyrylthiocholine chloride, respectively. Experiments were performed in triplicates. The extracts were initially tested at a fixed concentration of 100 µg/ml. Subsequently, a set of five concentrations of each extract in the range of 6.25 to 100 µg/ml was used to estimate the 50% inhibitory concentration (IC_{50}). Physostigmine, a cholinesterase inhibitor, was used as the reference standard. Absorbance of the test samples was corrected by subtracting the absorbance of their respective blank. Percentage inhibition was calculated using the following formula:

$$\text{Inhibition}\ (\%) = (A_{control} - A_{sample})/(A_{control}) \times 100\ \%$$

2.8. Antimicrobial Activity

2.8.1. Test Microorganisms

The test microorganisms used in the study include 5 Gram-positive bacteria [*Proteus mirabilis*, *Streptococcus epidermidis, Bacillus cereus*, *Bacillus subtilis* and Methicilin-resistant *Staphylococcus aureus* (MRSA)], 5 Gram-negative bacteria (*Escherichia coli*, *Citrobacter freundii, Pseudomonas aeruginosa, Klebsiella pneumonia* and *Shigella boydii*), 2 yeasts (*Candida utilis* and *Crytococcus neoformans*) and 6 fungi (*Fusarium solani, Rhizopus stolonifer, Trichoderma viridae, Aspergillus fumigatus, Microsporum gypseum* and *Trichophyton rubrum*). The cultures were previously isolated from contaminated food samples. All microbial cultures were maintained at 4 °C until further use. The inoculum was prepared by adding 5 ml of sterile physiological saline to the agar slant and was shook vigorously to get the cell or spore suspension. The turbidity of the bacteria and yeast suspension was adjusted to match 0.5 McFarland Standard. The spore count of fungal suspension was determined with hemocytometer (Neubauer) under light microscope. The microbial inoculums were adjusted with sterile saline to the inoculum size recommended by Clinical and Laboratory Standards Institute [14].

2.8.2. Antimicrobial Susceptibility Testing

The assay was conducted as per the procedure defined by Jorgensen & Turnidge [14]. All the extracts were prepared at

concentration of 20 mg/ml in 5% dimethyl sulfoxide (DMSO). Mueller Hinton agar medium (HiMedia) was used for test bacteria and yeasts, RPMI 1640 (Sigma-Aldrich) agar plates were used for filamentous fungi. By using a sterile cotton swab, the microbial inoculum was streaked on the agar medium. Then, 6 mm antibiotic assay disc (Whatman) coated with the extract was placed on the inoculated medium. 5% DMSO was included as solvent control. 30 µg/ml chloramphenicol was used as the reference antibiotic control for bacteria, and 30 µg/ml Amphotericin B for fungi and yeasts. The plates were incubated at 30 °C for 48 to 96 hours for fungi, and at 37 °C for 24 hours for bacteria and yeasts. The diameter of the clear zones surrounding the disc was measured after the incubation.

2.8.3. Determination of Minimal Inhibitory Concentration (MIC) and Minimal Lethality Concentration (MLC)

Broth microdilution assay was conducted per protocol defined by Tong et al [15]. Only microorganisms that susceptible to the extract on disc diffusion assay were tested. Sterile Muller Hinton broth was used for test bacteria whereas RPMI 1640 medium containing 0.2% dextrose buffered with 0.165 M MOPS to a pH of 7.0 at 25 °C were used for test fungi. Sterile 96-well plate with flat bottom (Fisher, USA) was used in this assay. The extract was dissolved in 5% DMSO to the concentration of 2 mg/ml, which was then diluted to the highest test concentration (1 mg/ml) after the addition of 100 µl inoculums. Then, serial two fold dilution of the extract was carried out to yield a concentration ranging from 500 to 15.63 µg/ml. The well containing only 5% DMSO and inoculum was served as growth control. The plates were incubated at 30 °C for 96 hours for test fungi, and at 37°C for 24 hours for bacteria. The *p*-iodonitrotetrazolium violet salt (Sigma-Aldrich, USA) dissolved in 99.5% ethanol was added as microbial growth indicator after the incubation. The formation of red formazan indicates the microbial growth. The MIC was defined as the lowest concentration of extract that prevents microbial growth. Then, to determine its MLC, the mixture in each well was suitably streaked on agar plates to judge the viability. The MLC was defined as the lowest concentration of extract that kill the microbial cells.

2.9. Statistical Analysis

All data were analysed and expressed as mean ± standard deviation of three replicates (n=3). The differences between the assayed values of the various extracts were analysed using One-way analysis of variance (ANOVA) which is a parametric test, followed by Tukey's honestly significant difference (HSD) Test at 95 and 99% confidence interval. Results with $P < 0.05$ were considered significant, while those with $P < 0.01$ were regarded as very significant. This analysis was carried out using Statistical Package for Social Sciences (SPSS) software, version 18.0 (SPSS Inc., Chicago, USA).

3. Results and Discussion

3.1. Phytochemical Analysis

Biologically active compounds usually occur in low concentration in various plants. Thus, extraction solvent and compositions of sample are known as the most important parameters [16, 17]. In this work, *G. atroviridis* stem bark extracts were obtained sequentially in a Soxhlet using *n*-hexane, dichloromethane and methanol. The concentrated methanol extract was further partitioned with chloroform and then ethyl acetate while the water extract was obtained using maceration. The selection of solvent is the crucial factor in the extraction process. It determines the amount and type of bioactive compounds extracted. The phytochemical screening of crude extracts of the stem bark of *G. atroviridis* revealed the presence of bioactive secondary metabolites such as alkaloids, tannins, saponins and steroids as shown in table 1.

3.2. Determination of Total Phenolic and Total Flavonoid Contents

The effect of different extraction solvents on TPC and TFC of the stem bark of *G. atroviridis* were also determined. Generally, chloroform, ethyl acetate, methanol and water extracts showed the high level of phenolic and flavonoid contents with no significant difference among them. Phenolic compounds are important for their excellent scavenging activity due to the presence of hydroxyl group [18]. The phenolic content of stem bark extracts of *G. atroviridis* was expressed as mg GAE/g of extract as shown in table 2. Standard curve of gallic acid was linear (y = 92.836x + 0.003) with regression value (R^2) of 0.9977. The results revealed that methanol was the best solvent for extracting phenolic compounds followed by water, ethyl acetate, chloroform, dichloromethane and *n*-hexane. A variation in the total phenolic content ranged from 10.78 ± 0.93 to 53.26 ± 2.91 (mg GAE/g) from the *n*-hexane to methanol extracts, respectively. The results were in agreement with Yu et al. [19] where they reported methanol was the most effective in extracting phenolic compounds.

Solvent used for extraction significantly affect the TFC of the sample. The water extract from the stem bark of *G. atroviridis* exhibited the highest TFC with the value of 136.89 ± 5.32 mg CE/g. The corresponding flavonoid content of the various stem bark

Table 1. Phytochemical screening of stem bark extracts of *G. atroviridis.*

Extracts	Alkaloids Test	Tannins Test	Saponins Test	Terpenoids Test
n-Hexane	−	−	+	+
Dichloromethane	+	+	+	+
Chloroform	+	+	+	+
Ethyl Acetate	+	+	+	+
Methanol	−	+	+	−
Water	+	+	+	−

+ = presence; − = absent.

Table 2. Phenolic and flavonoid content of various crude extracts of *G. atroviridis*.

Extracts	Total phenolic content (mg GAE/g)	Total flavonoid content (mg CE/g)
n-Hexane	10.78 ± 0.93[b]	52.54 ± 3.33[b]
Dichloromethane	13.31 ± 0.32[b]	61.29 ± 8.37[b]
Chloroform	31.17 ± 2.59[a]	125.04 ± 20.96[a]
Ethyl Acetate	44.11 ± 6.18[a]	116.03 ± 12.61[a]
Methanol	53.26 ± 2.91[a]	128.88 ± 4.49[a]
Water	48.79 ± 2.34[a]	136.89 ± 5.32[a]

Results are mean values of 3 replicates ± SD. Means not sharing the same letter are significantly different at $P < 0.05$ probability in each column.

Table 3. DPPH radical scavenging activity (IC_{50}) of the crude extracts.

Extracts	IC_{50} (µg/ml)
n-Hexane	328.3
Dichloromethane	91.17
Chloroform	97.44
Ethyl Acetate	77.01
Methanol	71.96
Water	330.3
Ascorbic Acid	7.40

extracts decreased in the order of water > methanol > chloroform > ethyl acetate > dichloromethane > *n*-hexane (Table 2). Standard curve of catechin was linear ($y = 0.284x + 0.0059$) with regression value of 0.999. The TFC present in the methanol extract of the stem bark was significantly higher than the leaf extract as reported by Abdullah et al. [20], suggesting the stem bark of *G. atroviridis* could be a potential source of flavonoid compounds.

3.3. Free Radical Scavenging Activity

DPPH is a stable free radical which contributes to the maximum absorbance within 515 to 528 nm. It turned into yellow by losing its chromophore upon receiving proton from any hydrogen donors [18]. Hence, the reduction of DPPH by antioxidants results in the loss of absorbance. The results of DPPH free radical scavenging assay is shown in table 3. The free radical scavenging efficacies of the extracts were reported as IC_{50}, which is the concentration of the tested samples required to scavenge 50% of DPPH radical [21]. Of all the extracts of *G. atroviridis*, the methanol, ethyl acetate, dichloromethane and chloroform extracts showed moderate activity against DPPH radicals. The other tested extracts (*n*-hexane and water), however, displayed a rather low activity with IC_{50} ranging from 328.3 to 330.3 µg/ml, and thus required much higher concentrations to reduce 50% of free radical concentrations. Thus, phenolic compounds present in the methanol extract contributed to the overall antioxidant activity of the extract. According to a study by Mackeen et al., the strongest antioxidant activity was reported on the methanolic fruit extract of this plant using ferric thiocyanate and thiobarbituric acid methods [22]. With the exception of water extract, the extracts from all other solvents

exhibited significant correlations between DPPH scavenging activity and its TPC and TFC. The similar trends were also reported by Xu & Chang in the extracts of legumes [23]. In addition, we noticed that the antioxidant activity was affected by the solvent used for extraction, by influencing the TPC and TFC of the extracts. Thus, extraction solvent must be carefully selected for the antioxidant studies of *G. atroviridis* stem bark. In a study conducted by Tan et al., they reported the isolation of antioxidant compounds from the dichloromethane extract of the stem bark of *G. atroviridis* [24]. However, future isolation works on methanol extract are warranted since it exhibited the highest scavenging activity against DPPH than the dichloromethane extract.

3.4. Cholinesterase Enzyme Inhibitory Activity

Alzheimer's disease (AD), a chronic neurodegenerative brain disorder that results in dementia, cognitive impairment, and memory loss, is accompanied by severe deficiency in choline acetyltransferase activity in the hippocampus and cerebral cortex [25]. To date, the only therapeutic agent that consistently proven to be efficacious in treating the cognitive and functional symptoms of AD is cholinesterase inhibitors [26]. It was proposed to enhance the function of central cholinergic neurons by permitting acetylcholine to remain in the synaptic cleft longer through inhibiting the enzyme with inhibitors [27]. In searching for cholinesterase inhibitors, stem bark extracts of *G. atroviridis* have been screened. In table 4, the ethyl acetate extract showed the highest AChE inhibitory activity with an IC_{50} value of 5.46 µg/ml, while the chloroform extract exhibited a moderate AChE inhibitory activity with an IC_{50} value of 56.09 µg/ml, followed by the methanol extract (IC_{50} = 69.65 µg/ml), water extract (IC_{50} = 72.32 µg/ml), dichloromethane extract (IC_{50} = 150.01 µg/ml) and *n*-hexane extract (IC_{50} = 282.89 µg/ml). It was believed that the presence of tannins in the ethyl acetate extract which contributed to the enzyme inhibition activity as reported by Goncalves et al. [28]. On the other hand, water extract and chloroform extract showed moderate inhibitory activity against BChE with the IC_{50} values of 29.31 µg/ml and 45.90 µg/ml, respectively.

The selectivity indices of the extracts

Table 4. Acetylcholinesterase (AChE) and butyrylcholinesterase (BChE) enzymes inhibitory activity of the various crude extracts.

Extracts	Percentage inhibition at 100 µg/ml (AChE)	IC_{50} µg/ml (AChE)	Percentage inhibition at 100 µg/ml (BChE)	IC_{50} µg/ml (BChE)	Selectivity Index	
					AChE	BChE
n-Hexane	37.36 ± 4.399	282.89 ± 15.87	53.76 ± 1.94	70.42 ± 8.68	0.24	4.01
Dichloromethane	41.31 ± 4.85	150.01 ± 4.00	52.45 ± 1.40	88.63 ± 13.14	0.59	1.69
Chloroform	57.16 ± 1.70	56.09 ± 2.45	69.51 ± 0.48	45.90 ± 1.62	0.81	1.22
Ethyl Acetate	52.10 ± 0.49	5.46 ± 2.91	57.70 ± 5.32	84.76 ± 14.19	15.52	0.06
Methanol	57.70 ± 2.66	69.65 ± 2.162	45.75 ± 1.15	175.29 ± 33.50	2.51	0.39
Water	57.36 ± 2.33	72.32 ± 8.37	71.95 ± 4.40	29.31 ± 4.05	0.40	2.46
Physostigmine (Control)	-	0.045±0.0067	-	0.14±0.015	3.11	0.32

towards AChE and BChE were also calculated, as shown in table 4. It is noteworthy that ethyl acetate extract showed the selectivity index of 15.52 for AChE over BChE, which was higher than that of physostigmine (3.11). Regarding the selectivity towards BChE over AChE, most of the extracts (*n*-hexane, dichloromethane, chloroform, methanol and water) exhibited higher selectivity for BChE than physostigmine (0.32). Physostigmine, which acts as a reference drug, was more active against AChE than BChE, which is not surprising since physostigmine is a selective AChE inhibitor [29]. Previous study by Tan et al. reported the isolation of two new cholinesterase inhibitors, garcineflavanone A and garcineflavonol A from the chloroform extract of *G. atroviridis* [30]. Surprisingly, the cholinesterase inhibitory activity of the ethyl acetate extract obtained in the present study was significantly higher than the isolated pure compounds, suggesting that there is synergistic effect of cholinesterase inhibitors present in the extract. Thus, further works in ethyl acetate extract are crucial in an attempt to search for novel cholinesterase enzymes inhibitors.

3.5. Antimicrobial Activity

Table 5 showed the antimicrobial activity of the extracts on disc diffusion assay. In the screening test, 5 of the 6 extracts exhibited significant inhibitory activity on at least one of the tested bacteria. Methanol extract exhibited the most significant antibacterial activity by inhibiting all the Gram-positive bacteria tested. Thus, indicating the potential of the extract as a source for antibacterial compounds. The *n*-hexane extract did not exhibit any antimicrobial activity on all the tested microorganisms, indicating the antimicrobial compounds produced by *G. atroviridis* were polar in nature. The methanol extract also exhibited significant antibacterial activity on MRSA, indicating the potential of the extract in combating multidrug-resistant microorganism. However, the activity was considered as moderate, relative to the drug control based on the size of the inhibition zones.

All the crude extracts exhibited antimicrobial activity on both Gram-positive and Gram-negative food borne bacteria which were positively associated with the TPC and TFC of the extracts. Hence, they had a broad spectrum of biological activity. However, most of the crude extracts showed better antimicrobial activity on Gram-positive bacteria as compared to Gram-negative bacteria. This is due to the differences in their cell wall composition. The cell walls of Gram-negative bacteria contain an outer membrane which constitutes the outer surface of the cell wall. This outer membrane acts as a coarse sieve and exerts little control over the movement of antibiotic substances into the bacterial cell [31]. Based on the results, only dichloromethane extract exhibited anti-yeast activity, and none of the extracts exhibited antifungal activity. This finding revealed that the extracts from *G. atroviridis* exhibited inhibitory activity only on unicellular prokaryotes, but not on eukaryotic microorganisms. Mackeen et al. [22] reported

Table 5. Antimicrobial activity of the crude extracts of *G. atroviridis* by disc diffusion method.

Test Organisms	Diameter of clear zone (mm)						
	n-Hexane	Dichloromethane	Chloroform	Ethyl Acetate	Methanol	Water	Positive Control
Gram Positive Bacteria							
P. mirabilis	-	18.4 ± 2.5	12.4 ± 0.6	-	15.0 ± 3.4	17.7 ± 1.9	22.7 ± 1.2
S. epidermidis	-	21.7 ± 3.1	15.0 ± 1.1	13.3 ± 2.6	12.6 ± 1.8	17.2 ± 1.3	19.7 ± 0.6
B. cereus	-	20.8 ± 2.7	15.3 ± 1.8	-	18.2 ± 0.9	11.8 ± 0.4	22.3 ± 1.0
B. subtilis	-	13.2 ± 0.8	17.3 ± 1.2	-	23.3 ± 1.2	-	19.7 ± 2.1
MRSA	-	-	-	-	17.3 ± 1.2	-	17.0 ± 1.0
Gram Negative Bacteria							
E. coli	-	13.2 ± 1.4	-	-	-	14.7 ± 1.1	17.3 ± 1.2
C. freundii	-	-	-	-	-	-	19.0 ± 1.7
P. aeruginosa	-	21.7 ± 2.3	12.7 ± 2.1	11.3 ± 1.5	15.3 ± 1.6	22.7 ± 2.6	22.3 ± 1.6
K. pneumonia	-	-	-	-	-	-	24.3 ± 1.5
S. boydii	-	24.7 ± 2.5	11.6 ± 1.1	20.4 ± 1.4	13.4 ± 1.3	22.1 ± 1.7	15.0 ± 2.0
Yeasts							
C. utilis	-	17.2 ± 1.3	-	-	-	-	27.0 ± 1.0
C. neoformans	-	-	-	-	-	-	22.3 ± 0.6
Filamentous Fungi							
F. solani	-	-	-	-	-	-	18.0 ± 1.0
R. stolonifer	-	-	-	-	-	-	23.0 ± 1.2
T. viridae	-	-	-	-	-	-	15.7 ± 1.2
A. fumigates	-	-	-	-	-	-	19.3 ± 0.6
M. gypseum	-	-	-	-	-	-	19.6 ± 1.8
T. rubrum	-	-	-	-	-	-	19.7 ± 1.6

Results are mean ± standard deviation of 3 experiments.

the similar findings where all the crude extracts (methanol) from *G. atroviridis* failed to inhibit the growth of filamentous fungi. However, the roots of *G. atroviridis* exhibited the most significant antimicrobial activity among all the plant parts tested. The data justified the rationale of the traditional usage of the stem barks for the treatment for food-related illness.

Table 6 showed the MIC and MLC values of the crude extracts on selected microorganisms. In general, the results obtained were in agreement with the data obtained from the disc diffusion assay, where the MIC and MLC values were lower for the tested microorganisms that showed larger inhibition zone on disc diffusion assay. The results showed that the MIC values of the tested microorganisms were ranged from 31.3 to 500 µg/ml while the MLC values ranged from 62.5 to 1000 µg/ml. This data suggesting the antimicrobial activity of the extract is concentration-dependent and different test microorganisms showed different

Table 6. Antimicrobial activity of the crude extracts of *G. atroviridis* by determining MIC and MLC.

Test Organisms	MIC/MLC (µg/ml)				
	Dichloromethane	Chloroform	Ethyl Acetate	Methanol	Water
Gram Positive Bacteria					
P. mirabilis	31.3/125	250/1000	-	62.5/250	250/500
S. epidermidis	31.3/62.5	125/500	500/1000	125/500	31.3/125
B. cereus	31.3/62.5	250/500	-	62.5/125	500/1000
B. subtilis	62.5/125	62.5/250	-	31.3/62.5	-
MRSA	-	-	-	125/500	-
Gram Negative Bacteria					
E. coli	250/1000	-	-	-	125/500
C. freundii	-	-	-	-	-
P. aeruginosa	31.3/125	125/500	62.5/500	125/500	31.3/62.5
K. pneumonia	-	-	-	-	-
S. boydii	31.3/250	125/500	31.3/125	250/500	31.3/125
Yeasts					
C. utilis	125/1000	-	-	-	-
C. neoformans	-	-	-	-	-

susceptibility to the extract. Higher concentration from the extract is needed to inhibit the microbial growth if the microbial cells are less susceptible to the extract. In this study, the MLC values obtained were significantly higher than the MIC values, indicating a higher concentration of the extract was needed to kill the microbial cells, instead of inhibiting the growth. According to Levison, the MLC value for bactericidal drug is usually the same as or not more than fourfold higher than the MIC value [32]. Based on the results, the activity of the extracts on the tested microorganisms was bactericidal, where the MLC values were not fourfold higher than the MIC values for all of the tested microorganisms.

4. Conclusion

In conclusion, various stem bark extracts of *G. atroviridis* were examined for their antioxidant, cholinesterase enzyme inhibitory and antimicrobial activities. Among all the extracts tested, methanol extract showed the lowest IC_{50} in DPPH free radical scavenging assay, suggesting a positive correlation between the antioxidant activity and its TPC and TFC.

On the other hand, ethyl acetate extract showed the strongest AChE inhibitory activity (IC_{50} = 5.46 ± 2.91 µg/ml) and the selectivity index of 15.52 for AChE over BChE, which was higher than that of physostigmine, a reference drug. The extracts (except *n*-hexane) exhibited antimicrobial activity against food borne bacteria. In short, the plant could be the potential source for various bioactive compounds and thus provide information for the desirable pharmacological outcomes.

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