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Evaluation of Antioxidant, Brine Shrimp Lethality and Antimicrobial Activities of *Galphimia gracilis* Bartl. Leaf Extracts Using *in vitro* Assay Models

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Authors' contributions

This work was carried out in collaboration between all authors. Authors MRH and NU designed the study, performed the statistical analysis, wrote the protocol and wrote the first draft of the manuscript. Authors MMH, MM and AP managed the analyses of the study. Author MMH managed the literature searches. Authors MMH and MSR supervised the work. All authors read and approved the final manuscript.

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ABSTRACT

Aims: *Galphimia gracilis* Bartl. (Family- Malpighiaceae) is known as gold shower or shower of gold. The current study was designed to evaluate *in vitro* antioxidant, brine shrimp lethality and antimicrobial activities of methanol, ethyl-acetate and petroleum ether extracts of *G. gracilis* leaf.
Study Design: Phytochemical screening, *in vitro* antioxidant, brine shrimp lethality and antimicrobial activities.
Place and Duration of Study: Department of Pharmacy, Jahangirnagar University, Savar, Dhaka-1342. The studies were carried out from November 2013 to February 2014.

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Methodology: *In vitro* antioxidant activity of the extracts were studied using DPPH radical scavenging assay, Nitric oxide (NO) scavenging assay, total phenol content, total flavonoid content, total antioxidant content, total tannin content and lipid peroxidation in human erythrocyte cell assays. Lethality bioassay was performed on *Artemia salina* Leach nauplii. Antimicrobial activity was investigated by disc diffusion technique.
Results: Presence of alkaloids, carbohydrates, flavonoids, steroids, terpenoids, tannins, saponins and glycosides were identified in the extracts. Ethyl-acetate extract (GLEA) showed highest activities in DPPH (IC_{50} of 21.70±0.51 µg/ml), NO (IC_{50} of 35.50±0.30 µg/ml), lipid peroxidation in human erythrocyte cells (IC_{50} of 10.38±0.34 µg/ml), total phenol (934.04±3.21 mg/g Gallic Acid Equivalent), total flavonoid (236.88±2.66 mg/g Quercetin Equivalent) and total antioxidant capacity assays (978.58±1.66 mg/g Ascorbic Acid Equivalent) among three different extracts. Methanol extract (GLM) showed promising reducing capacity than other extracts in cupric reducing (correlation coefficient, r= 0.95 and $P<0.05$) and reducing power capacity assays (r= 0.993 and $P<0.001$). Besides, it showed dose dependent activity in both assays. Methanol extract showed maximum content in total tannin assay (89.34±1.37 mg/g Tannic Acid Equivalent). In brine shrimp lethality bioassay, methanol extract was found to be more potent than other extracts (LC_{50}=64.46 µg/ml, $\chi 2$=39.87, $P<0.0001$). In disk diffusion technique, all the extracts showed poor activity.
Conclusion: The present findings suggest that leaf extracts of *G. gracilis* have promising antioxidant and cytotoxic activities. Moreover, the extracts can be used as potential candidates for lead compounds.

Keywords: Galphiamia gracilis; antioxidant activity; DPPH; total phenol; BHT; lethal activity.

1. INTRODUCTION

Free radicals can be very much fatal for our body when they react with important cellular components such as DNA, or the cell membrane. Cells may function poorly or die if this occurs. Free radical reactions are vital factors in the progression of chronic diseases such as cancers, hypertension, cardiac infarction, and atherosclerosis, as well as in rheumatism and cataracts [1]. Many synthetic drugs protect against oxidative damage, but these drugs have adverse side effects [2]. An alternative solution is to take natural antioxidants from food supplements and traditional medicines [3].

Galphimia gracilis Bartl. is a native to eastern Mexico, deciduous lowland forest, acahuales, roadsides, often in wet situation such as stream banks and ditches but widely cultivated throughout the tropical and sub-tropical regions and has become naturalized in many areas. It is known as gold shower or shower of gold and available in all parts of Bangladesh. It is a cultivated attractive ornamental shrub (4 m long) in flowering season November to February. *G. gracilis* has some medicinal properties and is used as a source of vitamins [4]. *G. gracilis* has been widely misapplied to many other species of *Galphimia,* particularly to those with deciduous petals. It is often confused with the partly sympatric *G. glauca*, which is easily separated by its persistent petals and smaller fruits (2.5 mm long vs. 4.5-5 mm long in *G. gracilis*) [5]. The biological activities of this plant are very much unknown. To the best of our knowledge no sporadic attempts have been taken to investigate the antioxidant, brine shrimp lethality and antimicrobial properties of the leaf part of this plant. That is why we have designed our current research project to explore the antioxidant, brine shrimp lethality and antimicrobial properties by using different types of *in vitro* models. These activities will help the researchers to establish the therapeutic functions of this plant.

2. MATERIALS AND METHODS

2.1 Chemicals and Reagents

Folin-Ciocalteu reagent, Methanol, Sodium Phosphate (Na_3PO_4) and Ammonium molybdate were purchased from Merck, Germany. Sodium carbonate, Potassium Acetate and Concentrated H_2SO_4 (98%) were purchased from Merck (India) Limited. Gallic acid, Quercetin and 1, 1-diphenyl-2-picrylhydrazyl (DPPH) were purchased from Sigma Chemicals, USA. Aluminium Chloride and Ascorbic acid were purchased from SD Fine Chem. Ltd., Biosar, India. Vincristine Sulphate was obtained from Techno Drugs Ltd., Bangladesh. All chemicals and reagents used were of analytical grade.

2.2 Plant Material and Preparation of the Extract

The leaves of *Galphimia gracilis* Bartl. were collected from Jahangirnagar University campus, Savar (located at 23.8583°N 90.2667°E), Dhaka, Bangladesh during the dry season. The plant is widely distributed in this area and the land of this area is composed of alluvium soil of the river. The plant was authenticated by Md. Abdur Rahim, Technical Officer, Department of Botany, Jahangirnagar University. A voucher specimen (DACB Accession No. 38730) was deposited in Bangladesh National Herbarium for future reference. The collected plant parts of leaf were cleaned and washed well with water. The cleansed leaves were then partially dried by fan aeration and then fully dried in the oven at below 40°C for 4 days. The fully dried leaves were then grinded to a powdered form and stored in suitable condition for few days. The powdered plant materials of leaf (500 g) were used for extraction by soxhlet apparatus at elevated temperature (65°C) using petroleum ether, ethyl acetate and methanol consecutively (500 ml of each solvent). After each extraction the powder (leaf) was dried and used again for the next extraction. Extraction was considered to be complete when the plant materials become exhausted of their constituents that were confirmed from cycles of colorless liquid siphoning in the soxhlet apparatus. All three extracts of leaf were filtered individually through fresh cotton bed. The filtrates obtained were dried at temperature of 40±2°C to have gummy concentrate of the crude extracts. Each extract was kept in suitable container with proper labeling and stored in cold and dry place.

2.3 Phytochemical Screening

The extracts of *Galphimia gracilis* leaf underwent phytochemical screening to detect presence of potential phytochemical constituents like alkaloids, carbohydrates, glycosides, flavonoids, saponins, steroids, tannins and terpenoids [6].

2.4 Antioxidant Activity Evaluation

2.4.1 DPPH free radical scavenging assay [7]

Different concentrations (500, 200, 100, 50, 25 and 5 µg/ml) of leaf extracts and standard were taken in test tube contains 1 ml of each concentration and is properly marked. Then 2 ml of 0.004% DPPH solution in the solvent was added to each test tube to make the final volume 3 ml. The mixture incubated in room temperature for 30 minutes in a dark place. Then the absorbance was measured at 517 nm. IC_{50} value was calculated using linear regression analysis.

2.4.2 Nitric oxide scavenging capacity assay [8]

4.0 ml of each leaf extracts and standard of different concentration (200, 100, 50, 25 and 5 µg/ml) solutions were taken in different test tubes and 1.0 ml of Sodium nitroprusside, (5 mM) solution was added into the test tubes. The test tubes were incubated for 2 hours at 30°C to complete the reaction. 2.0 ml solution was withdrawn from the mixture and mix with 1.2 ml of griess reagent and the absorbance of the solutions were measured at 550 nm using a spectrophotometer against blank. A typical blank solution contained the distilled water. IC_{50} value was calculated using linear regression analysis.

2.4.3 Reducing power capacity assessment [9]

2.0 ml of each leaf extracts and standard of different concentration solutions (200, 100, 50, 25 and 5 µg/ml) were taken in different test tubes and 2.5 ml of Potassium ferri-cyanide [$K_3Fe(CN)_6$], 1% solution was added into each of test tube. The test tubes were incubated for 10 minutes at 50°C to complete the reaction and 2.5 ml of Tri-chloro Acetic acid, 10% solution was added into each of the test tubes. The total mixtures were centrifuged at 3000 rpm for 10 min. 2.5 ml supernatant solution was withdrawn from each of the mixtures and mixed with 2.5 ml of distilled water. 0.5 ml of Ferric chloride ($FeCl_3$), 0.1% solution was added to each of the test tubes. The absorbance of the solution was measured at 700 nm using a spectrophotometer against distilled water. A typical blank solution contained the same solution mixture without leaf extracts or standard and it was incubated under the same conditions as the rest of the samples solution. Increased absorbance of the reaction mixture indicated increase reducing power.

2.4.4 Cupric reducing antioxidant capacity [10]

500 µl of each leaf extracts or standard of different concentration solutions (200, 100, 50, 25 and 5 µg/ml) were taken in different test tubes. 1.0 ml of 0.01 M $CuCl_2.2H_2O$ solution, 1.0 ml of ammonium acetate buffer, pH 7.0, 1.0 ml of

0.0075 ml of neocaproin solution and 600 µl of distilled water were added and the final volume of the mixture was adjusted to 4.1 ml. The total mixtures were incubated for 1 hour at room temperature. The absorbance of the solutions was measured at 450 nm using a spectrophotometer against blank. A typical blank solution contained the reagent mixture without extract or standard and treated as same.

2.4.5 Determination of total phenol content [11]

1 ml of leaf extracts or standard of different concentrations were taken in a test tube. 0.5 ml of folin-ciocalteu (Diluted 10 fold) reagent solution was added to the test tubes. 7.5% Sodium carbonate solution (4 ml) was added to the same test tubes and mixed well. Test tubes containing standard solutions were incubated for 30 minutes at 20℃ to complete the reaction but the test tubes containing extract solution were incubated for 1 hour at 20℃ to complete the reaction. Then the absorbance of the solution was measured at 765 nm using a spectrophotometer against blank. A typical blank solution contained the solvent used to dissolve the plant extract. The total content of phenolic compounds in leaf extracts was expressed in mg/g gallic acid equivalents (GAE).

2.4.6 Determination of total flavonoid content [12]

1ml of leaf extracts or standard of different concentrations was taken in a test tube and 3 ml of methanol was added. Then 200 µl of 10% aluminium chloride solution was added into the same test tube followed by the addition of 200 µl of 1 M potassium acetate. Finally, 5.6 ml of distilled water was mixed with the reaction mixture. The reaction mixture then Incubated for 30 minutes at room temperature to complete the reaction. Then the absorbance of the solution was measured at 415 nm using a spectrophotometer (UV 1601-PC, Shimadzu) against blank. Methanol served as a blank. The Total content of flavonoid compounds in leaf extracts was expressed in mg/g quercetin equivalent (QE).

2.4.7 Determination of total antioxidant capacity [13]

300 µl of each leaf extracts or standard of different concentration solutions were taken into different test tubes and 3 ml of reagent solution was added into each of the test tubes. The test tubes were incubated at 95℃ for 90 minutes to complete the reaction. The absorbance of the solutions was measured at 695 nm using a spectrophotometer against blank after cooling to room temperature. A typical blank solution contained 3 ml of reagent solution and the appropriate volume (300 µl) of the same solvent used for the sample and incubated under the same conditions as the rest of the samples solution. The antioxidant activity was expressed as the number of equivalents mg/g of ascorbic acid (AAE).

2.4.8 Total tannin content [14]

0.1 ml of the sample extract is added with 7.5 ml of distilled water in a test tube. 0.5 ml of folin Phenol reagent was added. Then 1 ml of 35% sodium carbonate solution was added. The volume was adjusted up to 10 ml with distilled water. The mixture was shaken well, kept at room temperature for 30 min and absorbance was measured at 725 nm. Blank was prepared with water instead of the sample. A set of standard solutions of tannic acid is treated in the same manner as described earlier and read against a blank. The results of tannins were expressed in terms of mg/g tannic acid equivalent (TAE).

2.4.9 Inhibition of erythrocyte lipid peroxidation [15]

Venous blood was collected from a healthy volunteer after obtaining informed consent and delivered into heparinized tubes. Whole blood was centrifuged at 4000 rpm, for 10 min, washed three times with desired phosphate buffered saline (pH 7.4) and suspended in the same buffer to obtain desired hematocrit level. A portion of 200 µl erythrocyte was delivered in a test tube followed by 100 µl hydrogen peroxide (100 µM) to induce lipid peroxidation. The test samples 200 µl was thereafter added. The contents were incubated for 1 hour at 37℃. The reaction was stopped by thio-barbituric acid stock reagent (0.375% TBA, 15% TCA, 0.2 M HCl). After cooling the solution was centrifuged at 3000 rpm for 5 min. The absorbance of the supernatant was measured at 532 nm. IC_{50} value was calculated using linear regression method.

2.5 Brine Shrimp Lethality Bioassay [16,17]

The brine shrimp lethality bioassay is very useful tool for the isolation of bioactive compounds from

plant extracts. The eggs of Brine shrimp (*Artemia salina* Leach) were collected and hatched in a tank at a temperature "around 25℃" with constant oxygen supply. Two days were allowed to hatch and mature the nauplii. Stock solutions of the samples were prepared by dissolving required amount of extracts in specific volume of pure dimethyl sulfoxide (DMSO) and sea water. Then specific volumes of sample were transferred from the stock solution to the test tubes to get final sample concentrations of 1, 5, 10, 20, 50, 100, 200 and 500 µg/ml. In the control tubes same volumes of DMSO (as in the sample tubes) were taken. With the help of a Pasteur pipette 10 living nauplii were put to each of the test tubes. Vincristine sulfate was used as positive control and evaluated at very low concentration (10, 5, 1, 0.5, 0.25, 0.125 and 0.06 µg/ml). After 24 hours the test tubes were observed and the number of nauplii survived in each test tube was counted. From this, the percentage of mortality of brine shrimp nauplii was calculated for each concentration of the extract and then corrected using Abott formula [18]. After correcting the % mortality, probit analysis was performed and found out LC_{50} value calculated using Fenny probit analysis [19].

2.6 Antimicrobial Activity

2.6.1 Microorganisms

Two Gram positive bacteria *Bacillus subtilis*, *Staphylococcus aureus* and four Gram negative bacteria *Escherichia coli*, *Salmonella typhi*, *Salmonella abony* and *Pseudomonas aeruginosa* were used for the study.

2.6.2 Experimental procedure

Antimicrobial activity of the plant extracts were investigated by disc diffusion technique described by Bauer et al. [20]. Subcultures prepared from pure cultures of six microorganisms were used for the sensitivity test. In an aseptic condition under laminar air hood cabinet, the test organisms were transferred from the subculture to 5 ml of nutrient broth contained in screw-capped test tubes using a transfer loop and then incubated for 24 hours at 37℃ for their optimum growth $5x10^5$ cfu/ml. Leaf extracts (400 µg/disc) were used for this investigation. Standard disc of Azithromycin (30 µg/disc) and blank discs (impregnated with solvents followed by evaporation) were used as positive and negative control respectively. Bacterial cell suspension was spread throughout the plates by using sterile 'L' shape spreader. The sample discs, the standard antibiotic discs and the control discs were placed gently on the previously marked zones in the nutrient agar plates. The plates were kept in an incubator at 37℃ for 48 hours to facilitate bacterial growth. After incubation, the antimicrobial activities of the test materials were determined by measuring the diameter of the zones of inhibition in millimeter with a transparent scale.

2.7 Statistical Analysis

Values are presented as mean ± SEM (Standard error of the mean) and mean ± SD (Standard deviation). One was analysis of variance followed by Bonferroni and Tukey multiple comparisons, probit analysis and pearson correlation analysis were performed to analyze different data set in these experiments. $P<0.05$, $P<0.01$ and $P<0.001$ were considered statistically significant. Statistical programs used were GraphPad Prism version 6 and SPSS version 16.

3. RESULTS AND DISCUSSION

3.1 Phytochemical Screening

The active components found in the methanol extract of *G. gracilis* (GLM) includes alkaloids, carbohydrates, glycosides, flavonoids, tannins, saponins, steroids and terpenoids; ethyl acetate extract (GLEA) includes alkaloids, carbohydrates, glycosides, flavonoids and terpenoids; petroleum ether extract (GLPE) includes alkaloids, saponins and terpenoids. Results are further summarized in (Table 1).

3.2 *In vitro* Antioxidant Assays

In DPPH assay among three extracts, GLM and GLEA were found to show good IC_{50} values of 22.87±0.35 µg/ml and 21.70±0.51 µg/ml respectively (Table 2). It was noted that when DPPH accepts an electron donated by an antioxidant compound, the DPPH is decolorized, which is quantitatively measured from the changes in absorbance. High polyphenolic contents of the extracts contribute to decolorize DPPH. In NO radical scavenging method, among the three extracts highest NO radical scavenging was demonstrated by GLEA with IC_{50} value of 35.50±0.30 µg/ml (Table 2). In both experiments GLEA presented the highest activity among three different extracts. Nitric oxide (NO) is a physiologically important chemical mediator

generated by endothelial cells, macrophages, neurons and involved in the regulation of various biochemical processes. Excess generation and accumulation of nitric oxide are implicated in the cytotoxic effects observed in various disorders like AIDS, cancer, Alzheimer's, and arthritis [21]. Over production of NO can mediate toxic effects such as DNA fragmentation, cell damage and neuronal cell death [22]. Several reports have pointed out the role of phenolic compounds in NO scavenging [23,24]. The leaf extracts may have capacity to undermine the effects produced by NO formation as well as can prevent the chain of reactions that is caused by excess NO generation.

Values are presented as mean ± SEM (for DPPH, NO and LPO) and mean ± SD (for total phenol, total flavonoid, total antioxidant and total tannin) (n=3). One way ANOVA followed by Tukey and Bonferroni multiple comparisons was performed to analyze the data sets. Values in same row with different superscripts are significantly different from one another ($P<0.05$).

In reducing power capacity assessment, the GLM exhibited the slightly higher reducing power (correlation coefficient, r= 0.99 and $P<0.001$) than other extracts and standard ascorbic acid (Fig. 1). In case of CUPRAC assay at low concentrations same extract showed low absorbance than ascorbic acid and GLEA but in higher concentrations it showed maximum reducing capacity (r= 0.95 and $P<0.05$) when compared with ascorbic acid and the rest (Fig. 1). In both cases GLM presented dose dependent activity. The extract possess

Table 1. Phytochemical constituents identified in three extracts of *G. gracilis* leaf

Phytochemicals	Name of the test	Observed changes	Results		
			GLPE	GLEA	GLM
Alkaloids	Mayer's test	Creamy white precipitate	—	+	—
	Hager's test	Yellow crystalline precipitate	+	+	+
	Wagner's test	Brown or deep brown precipitate	+	+	+
	Dragendorff's test	Orange or orange-red precipitate	—	+	+
Carbohydrates	Molisch's test	A red or reddish violet ring is formed at the junction of two layer and on shaking a dark purple solution is formed	+/—	+	+
Glycosides	General test	Yellow color	+	+	+
	Bromine water test	yellow precipitate	—	++	+/—
	Test for Glucoside	Production of brick-red precipitation	—	—	+
Flavonoids	General test	Red color	—	—	+
	Shinoda test (Magnesium Hydrochloride reduction test)	Green to blue color	+/—	+	+
	Zinc Hydrochloride reduction test	Red color after few minutes	—	—	+
Saponins	Frothing test	Formation of stable foam	+	—	++
Steroids	Libermann-Burchard's test	Greenish color	—	—	++
Tannins	Lead acetate test	A yellow or red precipitate	—	—	+
	Ferric chloride test	Blue green color	—	—	++
	Alkaline reagent test	Yellow to red precipitate	+/—	—	+
Terpenoids	Salkowski test	Yellow color appears at the lower layer	+	+	+

++: Strong presence, +: Presence, —: Absence, +/—: Presence or absence not ascertained

Table 2. Antioxidant potential of three different extracts of *G. gracilis* leaf

In vitro antioxidant models	GLM	GLEA	GLPE	Ascorbic acid
DPPH (IC_{50})	22.87±0.35[b]	21.70±0.51[b]	842.33±0.58[a]	17.66±0.34[c]
NO (IC_{50})	40.32±0.27[c]	35.50±0.30[d]	45.07±0.49[b]	89.14±0.44[a]
Total phenol (mg/g GAE)	917.72±3.40[b]	934.04±3.21[a]	72.02±0.35[c]	-
Total flavonoid (mg/g QE)	145.54±0.95[c]	236.88±2.66[a]	218.59±2.83[b]	-
Total antioxidant (mg/g AAE)	939.63±1.37[b]	978.58±1.66[a]	258.52±2.95[c]	-
Total Tannin (mg/g TAE)	89.34±1.37[a]	81.58±0.66[b]	67.13±2.95[c]	-
LPO in Human erythrocyte (IC_{50})	17.87±0.23[b]	10.38±0.34[c]	21.39±0.50[a]	1.94±0.12[d]

polyphenolic compounds that usually show reducing power. The reducing ability of a compound generally depends on the presence of polyphenolic reductants [25], which have been reported to exhibit antioxidant potential by breaking the free radical chain, donating a hydrogen atom [26]. They also prevent the formation of peroxide by reacting with some of its precursors. These effects of reductants in the sample indicate their contribution in the antioxidant effect. Cuprac reducing power is also associated with antioxidant activity [27]. Now, it can be speculated that the presence of reductants (i.e. antioxidants) in the leaf extract account for this reducing capacity (Table 1).

In total phenol, total flavonoid and total antioxidant capacity assays GLEA was found to exhibit good content than other extracts (978.58±1.66 mg/g Gallic Acid Equivalent, 236.88±2.66 mg/g Quercetin Equivalent and 978.58±1.66 mg/g Ascorbic Acid Equivalent respectively) (Table 2).The values differ significantly (P<0.05) in different *in vitro* assay models (Table 2). Antioxidant properties of polyphenols arise from their high reactivity as hydrogen or electron donors which can stabilize and delocalize the unpaired electron (chain-breaking function) and from their potential to chelate metal ions (termination of the Fenton reaction) [28]. In this study, all extracts have been found to possess considerable amount of gallic acid equivalent substances (polyphenolic compounds). Flavonoids play an important role in antioxidant system in plants. Phenolic compounds or polyphenols, represented in majority by flavonoids, are a major area of research, because they are considered as potent antioxidants, anti-inflammatory, antiviral, anti-bacterial and anti-cancer agents [29,30]. The antioxidant properties of flavonoids are due to several different mechanisms, such as scavenging of free radicals, chelation of metal ions, such as iron and copper, and inhibition of enzymes responsible for free radical generation [31]. The total antioxidant assay has been successfully used to quantify vitamin E in seeds and, being simple and independent of other antioxidant measurements commonly employed; it was decided to extend its application to plant extracts [13]. In this study all extracts possess considerable antioxidant constituents. In total tannin content assay, GLM showed maximum capacity (89.34±1.37 mg/g Tannic Acid Equivalent). Tannins like polyphenol compounds are used as a sword to destroy the free radicals and saves cellular macromolecules. Free radical scavenging activity of glycosides was also proved in a previous study [31]. Lipid peroxidation was known to cause destabilization and disintegration of the cell membrane, leading to liver injury, arteriosclerosis and kidney damage [32]. Per-oxy radicals are important agents that mediate lipid peroxidation thereby damaging cell membrane. Hydrogen peroxide when comes in contact with hemoglobin causes the degradation of hem and release Fe ions which could further initiate the production of free radicals via the Fenton reaction and consequently lipid peroxidation which is a mechanism for cell deterioration and subsequently death [33,34]. In this model among three different extracts maximum activity (IC_{50}= 10.38±0.34 mg/ml) was exerted by GLEA whereas ascorbic acid was found to show IC_{50} of 1.94±0.12 mg/ml. We can correlate this activity with antioxidant effect of the extract. The extract can scavenge the free radical and stop peroxidation process. Therefore, we can assume that presence of different phytochemicals (alkaloids, carbohydrates, glycosides, saponins, steroids, tannins, terpenoids and flavonoids identified from preliminary phytochemical group evaluation tests are presented in (Table 1) in the leaf extracts may account for the aforementioned antioxidant activities assessed by *in vitro* protocols.

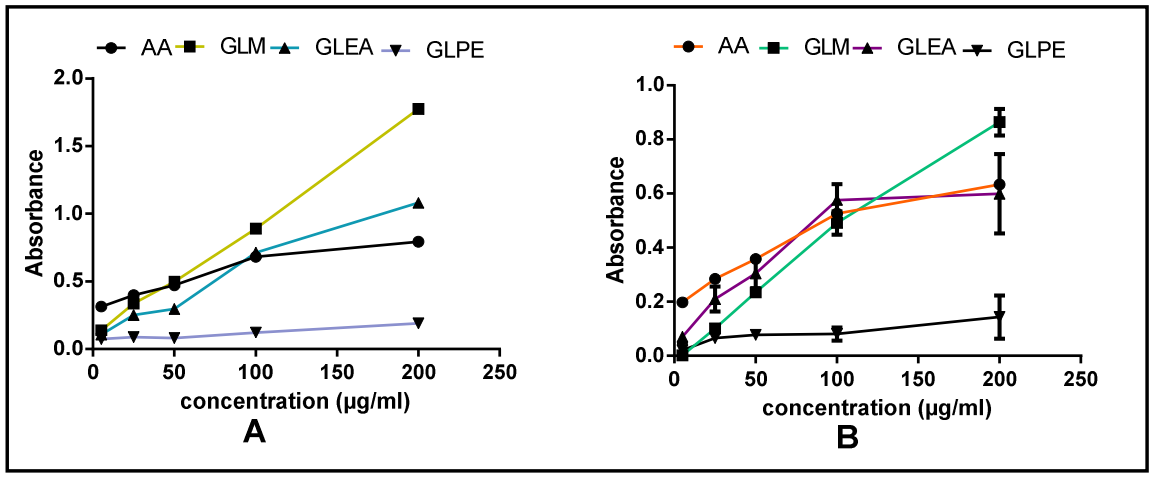

Fig. 1. (A) Cuprac reducing capacity assessment of three different leaf extracts and standard. (B) Reducing power capacity assessment of three different leaf extracts and standard

(A) Values are presented as mean ± SEM (n=3). Pearson Correlation analysis was performed between different concentrations and absorbance of each leaf extract and standard. For GLM, correlation coefficient, r= 0.999 and P<0.0001; for GLEA, correlation coefficient, r= 0.987 and P<0.01; for GLPE, correlation coefficient, r= 0.981 and P<0.01 for ascorbic acid, r= 0.961 and P<0.01. (B) Values are presented as mean ± SEM (n=3). Pearson Correlation analysis was performed between different concentrations and absorbance of each leaf extract and standard. For GLM, correlation coefficient, r= 0.996 and P<0.001; for GLEA, correlation coefficient, r= 0.897 and P<0.05; for GLPE, correlation coefficient, r= 0.943 and P<0.05; for ascorbic acid, r= 0.963 and P<0.01

3.3 Brine Shrimp Lethality Bioassay

In Brine shrimp lethality bioassay, GLM was found to be the most toxic to brine shrimp nauplii, with LC_{50} of 64.46 µg/ml ($\chi 2$=91.34, P<0.001) whereas anticancer drug vincristine sulphate showed LC_{50} value of 1.89 µg/ml ($\chi 2$=14.198, P<0.01). GLEA also showed potent toxicity. Data set are illustrated in (Table 3). The brine shrimp bioassay has been established as a safe, practical and economic method for determination of bioactivities of synthetic compound as well as plant products [16,35]. The correlation between the Brine shrimp assay and *in vitro* growth inhibition of human solid tumor cell lines demonstrated by the national Cancer Institute (NCI, USA) is significant because it shows the value of this bioassay as a pre-screening tool for antitumor drug research [36]. According to Meyer et al. (1982) extracts derived from natural resources which have $LC_{50} \leq 1000$ µg/ml using brine shrimp bioassay were claimed to contain bioactive principles [16]. Criteria of brine shrimp toxicity for compound or plant extract was established as LC_{50} values above 1000 µg/ml are non-toxic, between 500 & 1000 µg/ml are weak toxic, and below 500 µg/ml are toxic [37]. In this study methanol and ethyl-acetate extract showed very good LC_{50} values of 14.58 and 19.13 µg/ml respectively (Table 3). According to the National Cancer Institute (NCI), the conditions and criteria of cytotoxic activity for the crude extracts is an LC_{50} value ≤ 20 µg/ml, is considered to be very cytotoxic [38]. Therefore GLM is considered toxic. Among the three extracts the highest lethality potential was found for GLM. It was reported that natural polyphenolic compounds are known as normal cell differentiation promoter, tumor production and proliferation cell inhibitor, and apoptosis inducer [39,40]. Toxicity presented by GLM may attribute to the phytochemicals such as alkaloids, carbohydrates, glycosides, flavonoids, tannins, saponins, steroids and terpenoids.

The experiments were done in triplicate (n=3). Fenny probit analysis was performed to find out LC_{50} values, confidence interval limit, chi square and P value.

3.4 Antimicrobial Activity

In antimicrobial study GLM proved efficacy against maximum number of microbes (*E. coli, S. typhi* and *P. aeruginosa*). Among three extracts, GLEA showed highest zone of inhibition against *S. typhi* and *P. aeruginosa* (8.26±0.35 mm). The standard, Azithromycin, exhibited good zone of inhibition against all tested pathogenic organisms. All results are presented in (Table 4). In comparison among the three extracts GLEA showed highest antimicrobial activity. But the activity of all the extracts was poor.

Table 3. Brine shrimp lethality of different extracts of *G. gracilis* leaf

Extracts/standard	LC_{50} (µg/ml)	CI	$\chi 2$	*P* value
GLM	64.46	53.89-76.54	91.34	<0.001
GLEA	131.88	113.81-155.98	94.68	<0.001
GLPE	225.42	157.41-341.71	282.72	<0.001
Vincristine sulphate	1.89	0.63-8.61	14.198	<0.01

CI= Confidence Interval, $\chi 2$= Chi square

Table 4. Antimicrobial activity of different extracts of *G. gracilis* leaf in disc diffusion method

Test Organisms	Zone of inhibition (mm)			
	Azithromycin	GLM	GLEA	GLPE
Bacillus subtilis	12.05±0.07^{b}	0.00±0.00^{a}	0.00±0.00^{a}	0.00±0.00^{a}
Staphylococcus aureus	27.50±0.71^{c}	0.00±0.00^{a}	0.00±0.00^{a}	7.50±0.71^{b}
Escherichia coli	25.50±0.71^{c}	7.25±0.35^{b}	0.00±0.00^{a}	0.00±0.00^{a}
Salmonella typhi	27.50±0.71^{b}	7.50±0.71^{a}	8.25±0.35^{a}	7.75±0.35^{a}
Salmonella abony	27.75±0.35^{b}	0.00±0.00^{a}	0.00±0.00^{a}	0.00±0.00^{a}
Pseudomonas aeruginosa	17.00±1.41^{c}	7.50±0.71^{b}	8.25±0.35^{b}	0.00±0.00^{a}

Values are presented as mean ± SD (n=3). Values with different superscript in each row are significantly different from one another (P<0.05). One way ANOVA followed by Tukey multiple comparisons was performed to analyze this data set

4. CONCLUSION

There is a possibility to suggest that the presence of different phytochemicals is responsible for the activities. However further co-ordinated and well-structured studies would be required to characterize and isolate the bioactive compounds responsible for these activities and determine their underlying molecular mechanism action to find out novel lead candidates.

CONSENT

Not applicable.

ETHICAL APPROVAL

Not applicable.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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