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ANTIOXIDANT, BRINE SHRIMP LETHALITY AND ANTIMICROBIAL ACTIVITIES OF DIFFERENT EXTRACTS OF *GALPHIMIA GRACILIS* BARTL. STEM USING *IN VITRO* ASSAY MODELS

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ABSTRACT

Galphimia gracilis Bartl. (Family- Malpighiaceae) is known as gold shower and available almost everywhere in Bangladesh. This plant has some medicinal properties and is used as a source of vitamins. The current study was designed to evaluate *in vitro* antioxidant, brine shrimp lethality and antimicrobial activities of methanol (GSM), ethyl-acetate (GSEA) and petroleum ether (GSPE) extracts of *G. gracilis* stem. *In vitro* antioxidant activity of the extracts were studied using DPPH radical scavenging assay, NO scavenging assay, total phenol, total flavonoid content, total antioxidant capacity, 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. Presence of alkaloids, carbohydrates, flavonoids, steroids, terpenoids, tannins, saponins and glycosides were identified in the extracts. Ethyl-acetate extract showed highest activities in DPPH (IC_{50} 22.82±0.172 µg/ml), NO (IC_{50} 72.886±0.394 µg/ml) and total antioxidant assay (210.86±3.436 mg/gm Ascorbic Acid Equivalent). Methanol extract showed highest content in total phenol (972.02±4.56 mg/g Gallic Acid Equivalent), total flavonoid (135.98±1.103 mg/g Quercetin Equivalent) and total tannin content assays (111.454±1.462 mg/g Tannic Acid Equivalent). Ethyl acetate extract showed promising reducing capacity than other extracts in cupric reducing (correlation coefficient r= 0.99 and P<0.01) and reducing power capacity assays (r= 0.99 and P<0.001). Besides, it showed dose dependent activity in both assays. In brine shrimp lethality bioassay, methanol extract was found to be more potent than other extracts (LC_{50}=58.583 µg/ml, $\chi 2$=170.967, P<0.001). In disk diffusion technique methanol extract showed highest zone of inhibition against maximum number of microorganisms *Staphylococcus aureus, Salmonella typhi, Salmonella abony and Pseudomonas aeruginosa* (6.75±0.353 mm). The present findings suggest that the stem extracts of *G. gracilis* can be used as a source of potential candidates for lead compounds.

Keywords: *Galphimia gracilis,* Antioxidant, Brine shrimp lethality, Antimicrobial

INTRODUCTION

Free radical induced 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]. Reactive oxygen species cause damage to various bio-molecules including proteins, lipids, lipoproteins and DNA [2]. Many synthetic drugs protect against oxidative damage, but these drugs have adverse effects [3]. An alternative solution is to take natural antioxidants from food supplements and traditional medicines [4]. Infectious diseases comprise clinically evident illness resulting from the infection, presence and growth of pathogenic biological agents in an individual host organism. Infectious diseases are the world's leading cause of premature death, killing almost 50,000 people every day [5]. It has been reported that human infections are increasing at an alarming rate, especially in tropical and subtropical

developing countries during the last 20 years [6]. This is partly due to the indiscriminate use of antimicrobial drugs and the development of microbial resistance to some of the synthetic drugs [7]. The clinical efficiency of many existing antimicrobial drugs is being threatened by rapid development of multidrug resistant pathogens [8]. Many infectious diseases have been identified to be treated with herbal products throughout the history of mankind [9]. Natural products offer massive prospects for the development of new drugs, especially antimicrobial drugs, which can have therapeutic potential to treat infectious diseases. Antimicrobial compounds of plant origin have an enormous therapeutic potential to treat many infectious diseases [10].

Galphimia gracilis Bartl. is a native to eastern Mexico 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 ornamental shrub in flowering season November to February. *G. gracilis* has some medicinal properties and is used as a source of vitamins [11]. To the best of our knowledge no sporadic attempts have been taken to investigate antioxidant, brine shrimp lethality and antimicrobial properties of the stem part of this plant. That's why we have designed our current research project to explore possible aforementioned properties by using different types of *in vitro* models.

MATERIALS AND METHODS

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.

Plant Material and Preparation of the Extract:

The stems of *Galphimia gracilis* Bartl. were collected from Savar, Dhaka, Bangladesh during the dry season and authenticated by Md. Abdur Rahim, Technical Officer, Department of Botany, Jahangirnagar University. A voucher specimen (DACB Accession No. 38730) was deposited in the herbarium of Bangladesh National Herbarium for future reference. The collected plant parts of stem were cleaned and washed well with water. The cleansed stems were then partially dried by fan aeration and then fully dried in the oven at below 40 ºC for 4 days. The fully dried stems were then grinded to a powdered form and stored in suitable condition for few days. The powdered plant materials of stem (500 g) were used for extraction by soxhlet apparatus at elevated temperature (65 ºC) using petroleum ether, ethyl acetate and methanol consecutively (500 ml of each solvent). After each extraction the powder 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 stem were filtered individually through fresh cotton bed. The filtrates obtained were dried at temperature of 40±2 ºC 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.

Phytochemical screening:

The extracts of *Galphimia gracilis* stem underwent phytochemical screening to detect presence of potential phytochemical constituents like alkaloids, carbohydrates, glycosides, flavonoids, saponins, steroids, tannins and terpenoids [12].

Antioxidant Activity Evaluation

DPPH free radical scavenging assay [13]

Different concentrations (500, 200, 100, 50, 25 and 5 µg/ml) of stem extracts and standard were taken in test tube contains 1ml 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.

Nitric oxide scavenging capacity assay [14]

4.0 ml of each stem 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 ^{0}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. The percentage (%) inhibition activity was calculated using linear regression analysis.

Reducing power capacity assessment [15]

2.0 ml of each stem extracts or standard of different concentration solutions were taken and 2.5 ml of Potassium Ferricyanide [$K_3Fe(CN)_6$], 1% solution was added into each of test tubes. The test tubes were incubated for 10 minutes at 50 ^{0}C and 2.5 ml of Tri-chloro Acetic acid, 10% solution was added. The resultant mixtures were centrifuged at 3000 rpm for 10 min and 2.5 ml supernatant solution was withdrawn from each of the mixtures and mixed with 2.5 ml of distilled water. Then 0.5 ml of Ferric chloride ($FeCl_3$), 0.1% solution was added. The absorbance of the solutions was measured at 700 nm using a spectrophotometer against a typical blank solution.

Cupric reducing antioxidant capacity [16]

500 µl of each stem 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.

Determination of total phenol content [17]

1.0 ml of each stem extract (200 µg/ml), standard (gallic acid) of different concentrations and 5 ml of Folin–ciocalteu reagent (Diluted 10 fold) reagent solution were taken in marked test tubes and 4 ml of 7.5% Sodium carbonate solution was added. The test tubes were incubated at 20 °C (30 minutes for standard solutions and 1 hour for extract solution). Absorbance was determined using a UV-VIS spectrophotometer (Shimadzu UV PC-1600) at 765 nm against blank. Total phenol contents of the fractions were expressed as Gallic acid equivalents (GAE).

Determination of total flavonoid content [18]

Total Flavonoid was determined using the Aluminum chloride colorimetric method described by Wang and Jiao [13]. 1.0 ml stem extracts (200 µg/ml) and standard (Quercetin) were added to 3 ml of methanol and 200 µl of 10% aluminium chloride solution. 200 µl of 1 M potassium acetate solution and 5.6 ml of distilled water were added and then incubated for 30 minutes at room temperature to complete the reaction. Absorbance of the solution was measured at 415 nm using a spectrophotometer against blank. Total Flavonoid contents of the fractions were expressed as Quercetin equivalents (QE).

Determination of Total Antioxidant Capacity [19]

300 µl of each stem 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 ^{0}C 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).

Total tannin content [20]

0.1 ml of the stem 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).

Inhibition of erythrocyte lipid per-oxidation [21]

Venous blood was collected from 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 (100µM) to induce lipid per-oxidation. The test samples 200 µL was thereafter added. The contents were incubated for 1h at 37˚C. 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.

Brine Shrimp Lethality Bioassay [22, 23]

Brine shrimp eggs (*Artemia salina* leach) are hatched in simulated seawater to get nauplii. Sample solutions

are prepared by dissolving the test materials in pre-calculated amount of DMSO (Di-methyl sulphoxide). Ten nauplii are taken in vials containing simulated seawater. The samples of different concentrations are added and the volume was adjusted up to 5 ml. Survivors were counted after 24 hours. Vincristine sulphate is usually used as the reference cytotoxic drug. The mortality was corrected using Abott's formula [24]. After correcting the % mortality, probit analysis was performed and found out LC_{50} value calculated using Fenny probit analysis [25].

Antimicrobial activity

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.

Experimental procedure

Antimicrobial activity of the stem extracts were investigated by disc diffusion technique described by Bayer et al, (1966) [26]. Subcultures prepared from pure cultures of six microorganisms were used for the sensitivity test. In an aseptic condition under laminar air flow, 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 ^{0}C for their optimum growth $5x10^6$ cfu/ml. Stem 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 ^{0}C 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.

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 Graph Pad Prism version 6 and SPSS version 16.

RESULTS AND DISCUSSION

Phytochemical screening

The active components found in the methanol extract of *G. gracilis* stem (GSM) include alkaloids, carbohydrates, glycosides, flavonoids, tannins, saponins and steroids. Ethyl acetate stem extract (GSEA) includes carbohydrates, flavonoids, saponins, steroids, tannins and terpenoids. Petroleum ether stem extract (GSPE) includes alkaloids, saponins and terpenoids. Results are further summarized in table 1.

In vitro antioxidant assays

In DPPH assay among three extracts, GSM and GSEA were found to show good IC_{50} values 25.9±0.230 µg/ml and 22.82±0.172 µg/ml respectively whereas ascorbic was found to exhibit very good IC_{50} value of 17.663±0.335 µg/ml (Table 2). In NO radical scavenging method, among the three extracts highest NO radical scavenging was demonstrated by GSEA with IC_{50} value of 72.886±0.394 µg/ml. Other two extracts showed poor IC_{50} values whereas IC_{50} value of standard ascorbic acid was 89.135±0.438 µg/ml (Table 2). In both experiments GSEA presented good activity among three different extracts. It was noted that when DPPH accepts an electron donated by an antioxidant compound, the DPPH is decolorized. This is quantitatively measured from the changes in absorbance. 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 activities observed in various disorders like AIDS, cancer, Alzheimer's, and arthritis [27]. Several reports have pointed out the role of phenolic compounds in NO scavenging [28, 29]. The stem extracts may have capacity to undermine the effects produced by NO formation as well as can prevent the chain reactions that is caused by excess NO generation.

In cuprac reducing power capacity assessment, the GSEA exhibited the highest reducing power activity than other extracts (correlation coefficient r= 0.99 and $P<0.01$) and standard ascorbic acid (r= 0.96 and $P<0.01$) (Figure 1). In case of reducing power capacity assay same extract showed maximum reducing capacity (r= 0.99 and $P<0.001$) when compared with ascorbic acid (r= 0.99 and $P<0.0001$)

and the rest (Figure 1). In both cases GSEA presented dose dependent activity. The extract may possess poly-phenolic compounds that usually show reducing power. The reducing ability of a compound generally depends on the presence of poly-phenolic reductants [30], which exhibit antioxidant potential by breaking the free radical chain via donating a hydrogen atom [31]. They also prevent the formation of peroxide by reacting with some of its precursors. So, it can be speculated that the presence of reductants (i.e. antioxidants) in the stem extract may account for this reducing capacity.

In case of total phenol, total flavonoid and total tannin capacity assay, GSM was found to exhibit good content than other extracts (972.02±4.56 mg/g Gallic Acid Equivalent, 135.98±1.103 mg/g Quercetin Equivalent and 111.454±1.462 mg/gm Tannic Acid Equivalent respectively). In total total antioxidant content assay, GSEA showed maximum capacity (210.86±3.436 mg/g Ascorbic Acid Equivalent). In erythrocyte lipid peroxidation method, among three different extracts maximum activity (IC_{50}= 16656.6±5.45 mg/ml) was exerted by GSPE but all the extracts showed poor activity. Ascorbic acid was found to show IC_{50} 1.9377±0.117 mg/ml (Table 2). For the aforementioned experiments the values differ significantly (P<0.05). 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 terminate Fenton reaction [32]. In this study, all extracts have been found to possess considerable amount of gallic acid equivalent substances (polyphenol compounds). 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 [33].

Tannins like polyphenol compounds are also used to neutralize the free radicals and saves cellular macromolecules. Free radical scavenging activity of glycosides was proved in a previous study [34]. 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 [19]. In this study all extracts possess considerable antioxidant constituents. 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) in the stem extracts may account for the aforementioned antioxidant activities assessed by *in vitro* protocols.

Brine shrimp lethality bioassay

In Brine shrimp lethality bioassay, GSM was found to be the most toxic to brine shrimp nauplii, with LC_{50} of 58.583 µg/ml ($\chi 2$=170.967, P<0.001) whereas anticancer drug vincristine sulphate showed LC_{50} value 1.891 µg/ml ($\chi 2$=14.198, P<0.01). Other two extracts also showed potent toxicity. Data sets 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 [22, 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 [22]. 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 extract showed good toxicity against the nauplii. According to the National Cancer Institute (NCI), the conditions and criteria of cytotoxic activity for the crude extracts is an LC_{50} values ≤ 20 µg/ml, is considered to be very cytotoxic [38]. Therefore GSM is considered toxic. It was reported that toxicity of plant extracts is attributed to different types of secondary metabolites such as saponins, terpenoids, steroids, tannins, alkaloids etc. [39]. Toxicity presented by GSM may attribute to the phytochemicals such as alkaloids, carbohydrates, glycosides, flavonoids, tannins, saponins and steroids.

Antimicrobial activity

In antimicrobial study GSM proved efficacy against maximum number of microbes S.aureus, *S. typhi, S. abony and P. aeruginosa* (6.75±0.353 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 GSM has moderate antimicrobial activity. Presence of different phytochemicals such as alkaloids, carbohydrates, glycosides, flavonoids and terpenoids may be responsible for this antimicrobial activity. There are many reports on the antimicrobial activity of plant phytochemicals. Plant alkaloids may be responsible for potential antibacterial activity [40-42]. Cryptolepine, an indoloquinoline alkaloid, was

studied in detail to find out possible antibacterial potential. Antimicrobial effect of the alkaloid is thought to be through a different mechanism as the compound inhibits topoisomerase to intercalate DNA and to inhibit DNA synthesis [43, 44]. The tannin contents of the plant extracts may also contribute to the antimicrobial activity. The antimicrobial mechanisms of tannins may be due to their astringent property that can induce complexation with microbial enzymes or substrates, iron deprivation, hydrogen bonding or non-specific interaction with microbial enzymes, toxic action on microbial membranes, complexation of metal ions [45, 46]. The antimicrobial activity may also be due to the presence of flavonoids and glycosides [39, 47]. Therefore we can posit that, phytochemicals present in the stem extracts may account for this activity.

CONCLUSIONS

We are still not sure about how the stem extracts exerted the aforementioned therapeutic activities. There is possibility to suggest that activity may be due to the presence of different phytochemicals. 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.

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

Phytochemicals	Name of the test	Observed changes	Result		
			GSPE	GSEA	GSM
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* stem

In vitro Antioxidant Models	GSM	GSEA	GSPE	Ascorbic Acid
DPPH(IC_{50})	25.9±0.230[c]	22.82±0.172[b]	770.95±0.225[d]	17.80±0.356[a]
NO (IC_{50})	1156.94±0.378[d]	72.886±0.394[b]	125.86±0.202[c]	89.76±0.542[a]
Total phenol (mg/g GAE)	972.02±4.56[c]	876.121±0.987[b]	50.575±0.813[a]	-
Total flavonoid (mg/g QE)	135.98±1.103[c]	121.757±1.788[b]	78.345±1.28[a]	-
Total antioxidant (mg/g AAE)	137.555±1.562[a]	210.86±3.436[c]	144.7±0.707[b]	-
Total Tannin (mg/g TAE)	111.454±1.462[c]	85.7±0.705[b]	54.836±1.436[a]	-
LPO in Human erythrocyte(IC_{50})	34220.6±3.98[d]	22326.63±9.86[b]	16656.6±5.45[c]	1.9377±0.117[a]

Values are presented as mean± SEM (for DPPH, NO and LPO assays) and mean ±SD (for total phenol, total flavonoid, total antioxidant and total tannin content assays) (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).

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

Extracts/Standard	LC_{50} (µg/ml)	CI	$\chi 2$	*P* value
GSM	58.583	43.036-76.596	170.967	<0.001
GSEA	110.881	91.182-137.519	116.699	<0.001
GSPE	108.865	90.746-132.957	31.840	>0.05
Vincristine sulphate	1.891	0.626-8.611	14.198	<0.01

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

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.

Table 4: Antimicrobial activity of Methanol (GSM), Ethyl acetate (GSEA) and Pet-Ether (GSPE) extracts of *G. gracilis* stem in disc diffusion method

Test Organisms	Zone of inhibition (mm)			
	Azithromycin	GSM	GSEA	GSPE
Bacillus subtilis	12.05±0.070[b]	0.00±0.00[a]	0.00±0.00[a]	0.00±0.00[a]
Staphylococcus aureus	27.5±0.707[c]	6.75±0.353[b]	0.00±0.00[a]	6.75±0.353[b]
Escherichia coli	25.5±0.707[c]	0.00±0.00[a]	6.75±0.353[b]	0.00±0.00[a]
Salmonella typhi	27.5±0.707[c]	6.75±0.353[b]	0.00±0.00[a]	0.00±0.00[a]
Salmonella abony	27.75±0.353[c]	6.75±0.353[b]	6.75±0.353[b]	0.00±0.00[a]
Pseudomonas aeruginosa	17.00±1.414[c]	6.75±0.353[b]	0.00±0.00[a]	0.00±0.00[a]

Values are presented as mean± SD (n=2). 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.

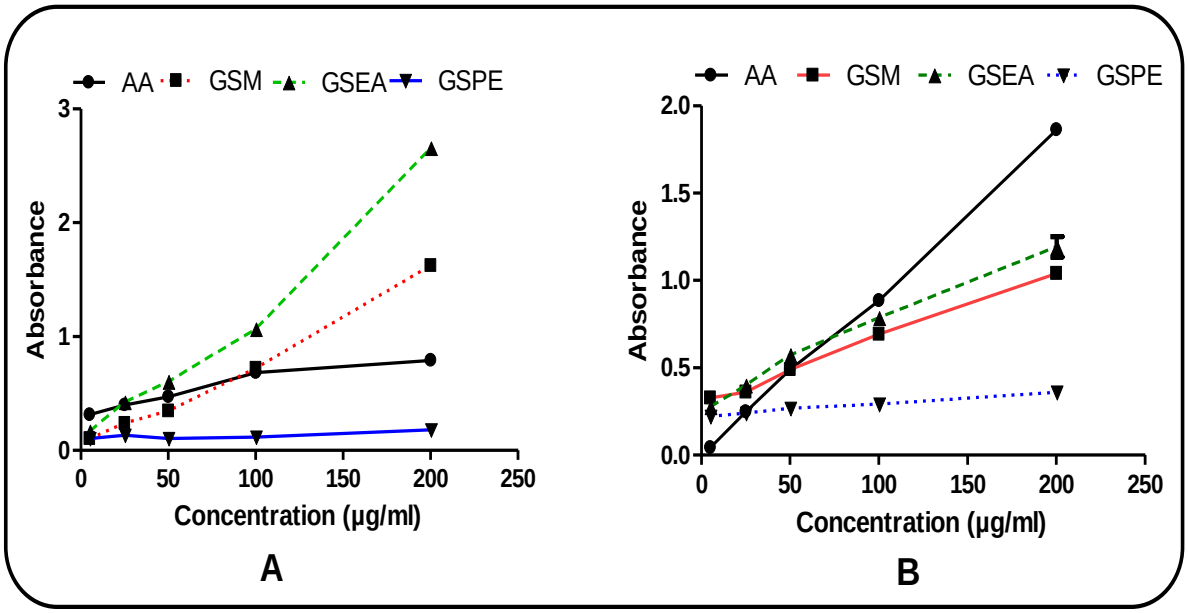

Figure 1: AA= Ascorbic Acid A. Cuprac reducing capacity assessment of three different stem extracts and standard. Values are presented as mean±S.E.M (n=3). Pearson Correlation analysis was performed between different concentrations and absorbance of each stem extract and standard. For GSM, correlation coefficient, r= 0.995 and *P*<0.001; for GSEA, correlation coefficient, r= 0.99 and *P*<0.01; for GSPE, correlation coefficient, r= 0.822 and *P*>0.05; for ascorbic acid, r= 0.96 and *P*<0.01. B. Reducing power capacity assessment of three different stem extracts and standard. Values are presented as mean±S.E.M (n=3). Pearson Correlation analysis was performed between different concentrations and absorbance of each stem extract and standard. For GSM, correlation coefficient, r= 0.997 and P<0.001; for GSEA, correlation coefficient, r= 0.994 and P<0.001; for GSPE, correlation coefficient, r= 0.994 and P<0.001; for ascorbic acid, r= 0.999 and P<0.0001.

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