A Comparative Evaluation of In Vitro Phytochemical Analysis, Antioxidant, Antibacterial and Anticancer Activity of Methanolic (MeOH) Crude Extract of Bark (GGB) and Leaf (GGL) of *Galphimia glauca*

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Abstract : The current in vitro exploration discovered the phytochemical components in MeOH extracts of bark (GGB) and leaf (GGL) parts of *Galphimia glauca* which were found to be effective in scavenging free radicals, obstructing the growth of MRSA, subduing the cancer cell proliferation (A549 and SW480) and displaying less toxicity towards the normal cell line (HEK293). The total flavonoids content (TFC) of GGB and GGL were 21.6 ± 0.34 and 20 ± 0.26 µg QE/ml, while total phenolic content (TPC) were 26.6 ± 0.5 and 21.8 ± 0.33 µg GAE/ml, respectively. However, the bark extract (GGB) showed elevated level of TPC and TFC than the leaf extract (GGL) and the same pattern was discovered in the evaluation of reducing power (FRP and FRAP) and total antioxidant activity (TAA). In DPPH assay, IC_{50} value of the extracts GGB and GGL were 43.7 ± 0.2 µg/ml and 48.8 ± 0.1 µg/ml respectively, while, IC_{50} value of quercetin was 25.2 ± 0.15 µg/ml but both the extracts exhibited slightly lower IC_{50} value in contrast to quercetin. The spectrum of antibacterial action of both the extract (GGB and GGL) was determined against the clinical and standard (ATCC 33591) methicillin-resistant *Staphylococcus aureus* (MRSA) through Agar well-diffusion assay by quantifying the Zone of Inhibition (ZI). ZI value for the extracts - GGB and GGL against the clinical MRSA strain was 16 ± 2 mm and 15 ± 1.5 mm respectively, while against the standard (ATCC 33591) MRSA strain was 15.3 ± 0.57 mm and 14 ± 1 mm respectively, at 10 mg/ml. Further, to evaluate and calculate IC_{50} value for anti-proliferative and cytotoxic effects of *G. glauca* plant extracts (GGB and GGL), MTT assay was utilized. The IC_{50} value for the GGB against A549, SW480, and HEK293 at 24 hours was 157.8 ± 2.44 µg/ml, 136.6 ± 2.73 µg/ml, and 388.67 ± 6 µg/ml, respectively, while IC_{50} value for the GGL against A549, SW480, and HEK293 at 24 hours was 194 ± 4.64 µg/ml 178 ± 3.1 µg/ml, and 317.2 ± 9.4 µg/ml, respectively. Hence, the extract of bark (GGB) and leaf (GGL) of *G. glauca* can be exercised as a therapeutic remedy against the detrimental diseases and disorders triggered by oxidative stress, an allergic reaction, and pathogenic invasion.

Keywords: *Galphimia glauca,* Antioxidant activity, Antibacterial, Methicillin-Resistant Staphylococcus *aureus* (MRSA), Anticancer.

I. Introduction

The emanation of several antibiotic resistance microbes and side effects of synthetic drugs have turned the attention to look into the alternative source of bioactive substances which must be safe, cost-effective, alimentary, and readily degradable. [7] One of the alternative sources is medicinal plants. India is the hub of diverse medicinal plants and vast knowledge of practicing traditional herbal treatment (Ayurveda) since 600 BC. [40-42] It encompasses numerous bioactive compounds, which are primarily secondary metabolites (phytoconstituents- such as phenols, anthraquinones, sterols, flavonoids, anthocyanins, etc.) and some of these metabolites are being consumed to combat a number of ailments such as cancer, neurodegenerative diseases, microbial infection, inflammatory diseases, etc. [1-6, 40-43] The *Galphimia glauca* plant was originated from Mexico and since then it is widespread throughout the world. In India, it is found in all states but especially grows in subtropical regions. [40] Pondicherry University harbors a diverse variety of plant species and consists of 450 to 500 floristic richness. Out of this 400 to 500 flora, *Galphemia glauca* or *Thryalis glauca* is one of the plant reported inside the campus. [45] *G. glauca* fall into the category of shrubs and family of Malpighiaceae/Malphegia. It nurtures up to 2-3 meters in height with small yellow flowering at terminal ends of branches. The leaves are usually oval in shape, glaucous green, and delicate. [40] *G. glauca* are pondered to be potential medicinal plant and are significantly being utilized for the medication of asthma [25], allergy [25, 40], depression and anxiety [24, 36, 43], central nervous system (CNS) disorders [40], and inflammatory diseases. [9, 23, 39, 44]

Since more than 25 years, *G. glauca* is being extensively studied but very little research has been done on its ethnomedicinal use. Only 40 to 50 research articles were found related to *G. glauca* till date and only one patent has been recorded regarding the usage of Galphimine B isolated from it. [40] Moreover, there is very limited data on the

exploitation of *G. glauca* plant regarding phytochemical screening, antioxidant, anticancer, and antibacterial activity. Further, there were no comparative studies has been reported for such ethnomedicinal properties between the bark and leaf extracts of *G. glauca*. Therefore, with this background, preliminary investigation was conducted to evaluate and compare the MeOH extract of bark (GGB) and leaf (GGL) for phytochemical constituents, antioxidant activity, anticancer/antiproliferative activity against SW480 (Colorectal adenocarcinoma), and A549 (Lung Carcinoma) cell lines, cytotoxic activity against human embryonic kidney/renal (HEK293) cell line, and antibacterial activity against clinical and standard (ATCC 33951) Methicilin-Resistant *Staphylococcus aureus* (MRSA) strains.

II. Materials

2.1 Plant Materials

The Bark and Leaf of *Glaphimia glauca* plant were identified and collected from Pondicherry University campus, Kalapet, Pondicherry. [45]

2.2 Bacterial Strains

Clinical and Standard ATCC 33591 Methicilin-Resistant *Staphylococcus aureus* (MRSA) were collected from the Biotechnology Department, Pondicherry University.

2.3 Cell lines

The following cell lines were used for the experiment: HEK293-Human Embryonic Kidney/Renal (normal cell), SW480-Colorectal adenocarcinoma, and A549-Lung Carcinoma and all such cell lines were attained from NCCS (National Centre for Cell Science)- Pune, India.

2.4 Chemicals

Nutrient Agar (NA), Nutrient Broth (NB), Agar, Dulbecco's Modified Eagle Medium, Fetal Bovine Serum, Penicillin-Streptomycin Solution (1X), Trypsin–EDTA (1X), MTT (3-(4,5 dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) Reagent, Trypan Blue, Phosphate Buffered Saline (Ph-7.2), Sodium Chloride, Potassium Chloride, And Dimethyl Sulfoxide (DMSO), Dipotassium Hydrogen Phosphate, Disodium Hydrogen Phosphate, Potassium Hydrogen Phosphate, 2,2-Diphenyl-1-Picrylhydrazyl (DPPH), Folin Ciocalteu Phenol Reagent (2N), Gallic Acid Monohydrate, Quercetin, Aluminum Chloride, Sodium Nitrite, TPTZ (2,4,6 tripyridyl-*s*-triazine), Ammonium Molybdate, Ferric Chloride, Potassium Ferricyanide, Trichloroacetic Acid, and Sodium Carbonate were all attained from HiMedia Laboratories (Nasik, Maharashtra, India) and Methanol (HPLC Grade) were acquired from Merck (India) and all other chemicals used in the studies were of analytical grade and purity.

III. Methods

3.1 Preparation of MeOH extract

The bark and leaf were collected and washed thoroughly with distilled water and shade dried at 35-37 °C for 3 days. The dried leaves and barks were grinded using suitable food processing grinder into a fine powder and stored in a closed container at 4 C respectively. [28, 29] A 50 g of the fine grinded powder of the barks and leaves of the *Galphemia glauca* was added in a closed glass container and 500 ml of Methanol was added and subsequently agitated at 1800 rpm for 3 days at room temperature (RT), respectively. Each mixture was subsequently filtered through No. 1 Whatman filter paper and each filtrate was down concentrated and dried via rotary evaporator at 40 °C under reduced pressure. The obtained dried extract was weighed and the percentage of yield of extraction was quantified using the equation (1). After that, each dried extract was preserved in 4 °C in separate glass bottles designating as GGL for leaf and GGB for bark. [38, 46]

$$\textbf{Percentage of yield of extraction } (\%) = \frac{\textbf{The dry weight of crude extract (g)}}{\textbf{The initial weight of powdered plant material used (g)}} * 100\% \qquad \textbf{(Equation 1)}$$

3.2 Preliminary Screening of Phytochemicals

Qualitative analysis of phytochemicals of MeOH extract of bark (GGB) and leaf (GGL) of *G. glauca* was determined by adopting the standard protocol. [6, 41]

3.3 Quantitative Analysis of Flavonoids and Phenols.

3.3.1 Total Phenolic Content (TPC)

0.1 ml of the test sample (GGB and GGL {1 mg/ml}) was mixed with 0.9 ml of deionized H_2O and 1.8 ml of Folin-Ciocalteu's phenol reagent (10 times diluted) in respective test tubes and incubated for 5 min, then 1.2 ml of Na_2CO_3

solution (7.5% w/v) was added to the mixture. The reaction was incubated for 45 min in the dark at RT and O.D. of the blue color of each sample was quantified at 765 nm. TPC was enumerated as µg gallic acid equivalents (GAE) per milliliter on the basis of a standard curve of gallic acid (20–100 mg/L). The blank contained the same volume of deionized H_2O instead of the plant extract/gallic acid sample. [30, 31] All analysis was done in triplicate.

3.3.2 Total Flavonoids Content (TFC)

0.1 ml of the test sample (GGB and GGL {1 mg/ml}) was mixed with 0.9 ml of deionized H_2O and 0.1 ml of $NaNO_2$ (5% w/v) in a respective test tubes and then incubated for 5 min. after 5 min, 0.1 ml of $AlCl_3$ (10% w/v) was added and incubated for 5 min then 0.6 ml of NaOH (1 M) was added, followed by 1.2 ml of deionized water. The mixture was shaken vigorously and incubated for 30 min at RT. The O.D. of each sample was measured at 415 nm. TFC was enumerated as µg quercetin equivalents (QE) per ml on the basis of a standard curve of quercetin (20–100 mg/L). The blank contained an equivalent volume of deionized H_2O instead of the plant extract/quercetin sample. [32] All analysis was done in triplicate.

3.4 Determination of In vitro Antioxidant Activity

3.4.1 DPPH Free Radical Scavenging Assay (DFRS)

0.1 ml of extract (GGB and GGL)/Quercitin of different dilution (20-100 mg/L) were thoroughly fused with 0.9 ml of deionized H_2O in a respective test tube. 1ml of DPPH (5.9 mg in 100 ml of absolute CH_3OH) was added to the above suspension and kept for 15 min at RT in dark. O.D. was observed at 517 nm. DPPH radical scavenging ability was stated as a (%) of DPPH inhibition, which was enumerated via following equation (2). [18-20]

$$\% \text{ Inhibition of DPPH} = (A_{control} - A_{sample}/A_{control})*100 \qquad \text{(Equation 2)}$$

Where $A_{control}$ = absorbance of the suspension without the extract (DPPH only) and A_{sample} = absorbance of the suspension with the extract/Quercetin and DPPH. The blank contained an equivalent volume of deionized water instead of the plant extract/quercetin sample. All analysis was done in triplicate.

3.4.2 Total Antioxidant Activity (TAA)

1 ml of the test sample (GGB and GGL {0.25 mg/ml}) was mixed with 3 ml of phosphomolybdate reagent (0.6 M H_2SO_4, 28 mM $Na_2HPO_4{\cdot}2H_2O$ and 4 mM $(NH_4)_3PMo_{12}O_{40}$) in a respective test tube. The mixture was then incubated at 95 °C for 90 min and then allowed to cool to reach RT. The O.D. of each sample was recorded at 765 nm. TAA was stated as µg of Gallic Acid Equivalents (GAE) per ml on the basis of a standard curve of Gallic acid (50–250 mg/L). The blank contained an equal volume of deionized water instead of the plant extract/gallic acid sample. [17-19] All analysis was done in triplicate.

3.4.3 Ferric ion Reducing Antioxidant Potential (FRAP) assay

The reagent was ready by mixing in 50 ml of $C_2H_3NaO_2$ buffer (pH 3.6, 30mM), 5 ml of iron (III) chloride solution (20 mM), 5 ml of TPTZ solution (10 mM) prepared in 40 mM HCl. The reagent mixture was incubated for 15 min at 37 °C in dark prior to the experiment. Then 0.015 ml of the test sample (GGB and GGL {0.25 mg/ml}) was mixed with 2.85 ml of FRAP reagent in a respective test tube. The mixture was then kept for 30 min at RT in the dark. The O.D. of each sample was recorded at 593 nm. The ferric reducing antioxidant ability was enumerated as µg gallic acid equivalents (GAE) per ml on the basis of a standard curve of gallic acid (50–250 mg/L). The blank contained an identical amount of deionized water instead of the plant extract sample/gallic acid. [12, 26] All analysis was done in triplicate.

3.4.4 Ferric Reducing Power (FRP)

1 ml of the test sample (GGB and GGL {0.25 mg/ml}) was mixed with 2.5 ml of $Na_2HPO_4{\cdot}2H_2O$ buffer (0.2 M, pH 6.6) and 2.5 mL of $K_3Fe(CN)_6$ (1% w/v) in a respective tube. The reaction mixture was vortexed and incubated at 50 °C for 20 min. after 20 min, 2.5 ml of Cl_3CCOOH (10% w/v) was added to halt the reaction and the mixture was centrifuged at 3000 rpm for 10 min. Next, 2.5 ml of aliquots from the mixture was separated from each test tube and added to 2.5 ml of deionized H_2O and 0.5 ml of iron (III) chloride solution (0.1% w/v). The mixture was then incubated for 30 min at RT in the dark. The O.D. of each sample was recorded at 700 nm. The FRP of the extracts (GGB and GGL) was quantified as µg of gallic acid equivalents (GAE) per mL on the basis of a standard curve of gallic acid (50–250 mg/L). The blank contained a similar volume of deionized water rather than the plant extract sample/gallic acid. [27, 33] All analyses were evaluated in triplicate.

3.5 Antibacterial Activity using Agar-well Diffusion Assay.

The spectrum of antibacterial ability was enumerated via agar-well-diffusion assay. [2] 15 ml of NA (nutrient agar) medium containing 1% (w/v) agar was inoculated with respective 10^6 CFU/ml (6 hours old nutrient broth (NB) culture) of clinical and standard ATCC 33591 methicillin-resistant *Staphylococcus aureus* strain. The inoculated medium was poured into a separate sterile Petri dish and left for 20 min to get dried. Then wells were bored in each plates using 6 mm sterile borer and the well-bottom was sealed with 10 µl of soft agar (1% w/v). 100 mg of each plant extracts GGB (MeOH extracts of bark) and GGL (MeOH extracts of the leaf) was dissolved in 1 ml of 70% DMSO to make a stock solution. Now, 50 µl of each extracts GGB, GGL, 70% DMSO, and distilled H_2O was instilled into the wells and incubated for 2 hours at 4 °C to get dried, respectively. After drying, the plates were incubated for 12-15 hours at 37 °C and the Zone of Inhibition (ZI) was measured. 80% DMSO and distilled H_2O were used as a negative control. All analysis was done in triplicate.

3.6 Antiproliferative and Cytotoxic activity

Antiproliferative and cytotoxic activity of the plant extracts was measured using MTT assay as described by Ghagane SC *et al*. [21] with slight modification. Briefly, all the cells were subcultured in a complete DMEM medium containing 10% FBS, 1% penicillin-streptomycin, 1% glutamine solution and maintained in an incubator with 95% humidity and 5% CO_2 atmosphere at 37 °C. After reaching 80% confluence, cells were trypsinized and the cell count was done using trypan blue staining, loaded onto the hemocytometer. A density of 5 x 10^3 cells were seeded into a cell culture plate (96 well plate) and incubated in the same condition for 16-24 hours to obtain a monolayer adherent culture. The old media was aspirated carefully from each well and each cell lines were treated with different concentration (50-800 mg/L) of MeOH extracts of bark (GGB) and leaf (GGL) parts of G. *glauca* and incubated for 24 hours, respectively. The subsequent treatment sets are established to study the anticancer effect of the plant extracts: against A549 and SW480: (i) Negative control: cells only, and (ii) Test samples: cells + methanol extract of bark (GGB); cells + methanol extract of the leaf (GGL). The same treatment sets were monitored to check the cytotoxic effect against HEK293 normal cell lines. After 24 hours, the drug media was aspirated carefully from each well. Now, each well was filled with 50 µl of MTT (1 mg/ml) and incubated at 37 °C for 3 hours in a dark and then 100 µl of DMSO was instilled to each well and again incubated in the same condition for 30 minutes to solubilize the formazan product. The O.D. was recorded at 540 nm using ELISA microplate reader. The results were plotted as % viability vs concentration of extracts (GGB and GGL).
Cell Viability (%) was calculated using the following equation:

$$\textbf{\% of cell viability} = (A_{sample}/A_{control})*100 \qquad \textbf{(Equation 3)}$$

Where A_{sample} = absorbance of the mixture with extracts and $A_{control}$ = absorbance of the mixture without the extract.

IV. Statistical Analysis

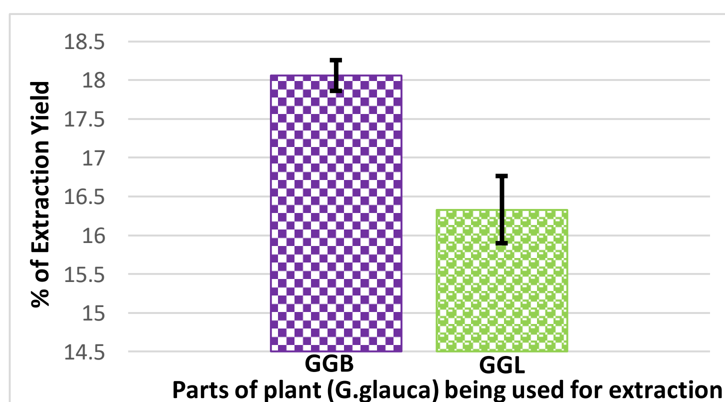
All analysis was conducted in triplicate (n = 3**)**. The data was quantified as the statistical mean value ± standard deviation using MS (Microsoft) Excel 2013.

V. Results and Discussions.

5.1 Preparation of Methanolic (MeOH) Extract.

The bark (GGB) and leaves (GGL) of G. glauca were extracted with Methanol respectively. The percentage of yield of extraction was calculated, which is shown in Figure 1 and Table 1. As from Figure 1 and Table 1 MeOH extract of barks (GGB) had a higher percentage of yield of extraction of 18 ± 0.2 % as compared to the percentage yield of MeOH extract of leaves (GGL) i.e., 16.3 ± 0.43 %.

Figure 1: The percentage yield of MeOH extracts of G. *glauca* bark (GGB) and leaves (GGL). The data are represented as



the mean value ± standard deviation of triplicates (n=3).

Table 1: The percentage (%) yield of MeOH extracts of G. *glauca* bark (GGB) and leaves (GGL)

The initial weight of powdered plant material used (g)		The dry weight of crude extract (g)		% yield of extraction (Equation 1)	
GGB	GGL	GGB	GGL	GGB	GGL
50	50	9 ± 0.2	8.1 ± 0.43	18 ± 0.2	16.3 ± 0.43

The data are represented as the mean value ± standard deviation of triplicates (n=3).

5.2 Preliminary Screening of Phytochemicals

The existing screening of phytochemicals of MeOH extracts of bark (GGB) and leaf (GGL) parts of G. *glauca* had shown the presence of various bioactive compounds as summarized in Table 2.2. Both the extracts, GGB, and GGL have similar bioactive constituents except gums and mucilage which was found to be present in the extract of the bark (GGB) while it was absent in the extract of the leaf (GGL).

Table 2: Phytochemical screening of the MeOH extracts of G. glauca bark and leaf

Phytochemicals	Tests performed	MeOH Extract	
		GGB	GGL
Alkaloids	Mayer's Test	+	+
	Wagner's Test	+	+
Carbohydrates	Molisch's Test	+	+
	Benedict's Test	+	+
	Fehling's Test	+	+
Flavonoids	Alkaline Reagent Test	+	+
	Ammonium Test	+	+
	Aluminium Chloride Test	+	+
Phenolic compounds	Ferric Chloride Test	+	+
Steroids	Salkowski test	+	+
	Libermann Burchard's test	+	+
Protein & aminoacids	Biuret Test	+	+
	Ninhydrin Test	+	+
Anthraquinone		+	+
Gums And Mucilage		+	-
Glycosides	Legal's Test	+	+
	Borntrager's test	+	+
	Keller-Kiliani test	+	+
Tannins	Ferric Chloride Test	+	+
Saponins	Foam test	+	+

(+) **Presence of particular phytochemical** (-) **Absence of particular phytochemical**

5.3 Quantitative Analysis of Flavonoids and Phenols

As phenolics and flavonoids are very powerful antioxidants due to presence of polar hydroxyl groups in their structures, therefore the presence of these two bioactive compounds in the MeOH extract is reasonable and also directly related to the antioxidant activity, antibacterial, anticancer activity, etc. [17, 35]

5.3.1 Total Phenolic Content (TPC)

The TPC was analyzed via the FC (Folin-Ciocalteu) reagent test. In this assay, FC reagent get reduced by a phenolate ion which is produced from phenol by losing an H^+ ion under the alkaline condition and hence produces blue colored compounds which are monitored using spectrophotometrically. The TPC was determined as µg gallic acid equivalents (GAE) per mL on the basis of a standard curve of gallic acid (20–100 mg/L, y = 0.0171 x + 0.0493, R^2 = 0.9972, where y= $Absorbance_{765\ nm}$ and x= µg GAE/ml) which were represented in Figure 3 and Table 3. Based on table 3, the TPC of MeOH bark extract (GGB) i.e. 26.6 ± 0.5 µg/ml was higher than the TPC of MeOH leaf extract (GGL) i.e. 21.8 ± 0.33 µg/ml.

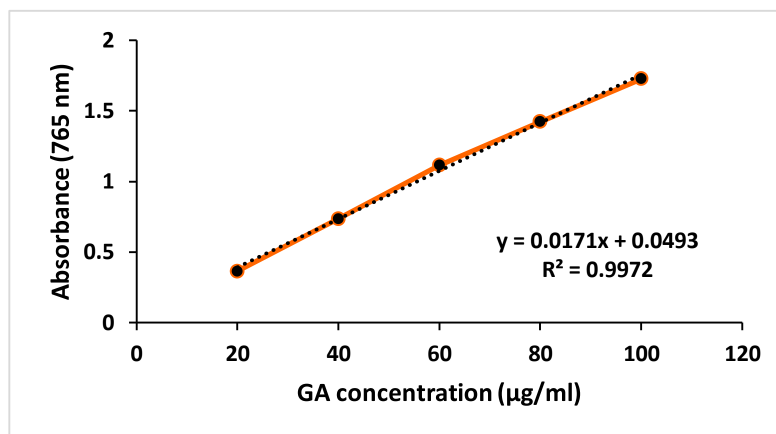


Figure 3: Standard Curve for calculating TPC in the extracts of GGB and GGL of G. *glauca,* keeping gallic acid as standard.

Table 3: TPC inclusion in the MeOH extracts of G. *glauca.*

Extracts Name	Total Phenolic Contents (TPC)*
GGB	26.6 ± 0.5
GGL	21.8 ± 0.33

*** TPC was quantified via equation y = 0.0171x + 0.0493 (Figure 3), where y= $Absorbance_{765\ nm}$ and x= µg GAE/ml. The data are represented as the mean value ± standard deviation of triplicates (n=3).**

5.3.2 Total Flavonoid Contents (TFC)

The TFC was analyzed by using aluminum chloride colorimetry test. In this reaction, aluminum ions interact with the C-4 C=O functional group and either the C-3 or C-5 OH functional group of flavones and flavonols or sometimes it also interacts with the ortho-di-OH functional group which appear at the A or B ring of flavonoids ensuing in the creation of stable/weak complexes. The TFC was quantified as µg quercitin equivalents (QE) per mL on the basis of a standard curve of quercetin (20–100 mg/L, y = 0.0101 x + 0.0448, R^2 = 0.9925, where y= $Absorbance_{415\ nm}$ and x= µg QE/ml) which were represented in Table 4 and Figure 4. Based on table 4, the TFC of MeOH bark extract (GGB) i.e. 21.6 ± 0.34 µg/ml was similar to the TFC of MeOH leaf extract (GGL) i.e. 20 ± 0.26 µg/ml.

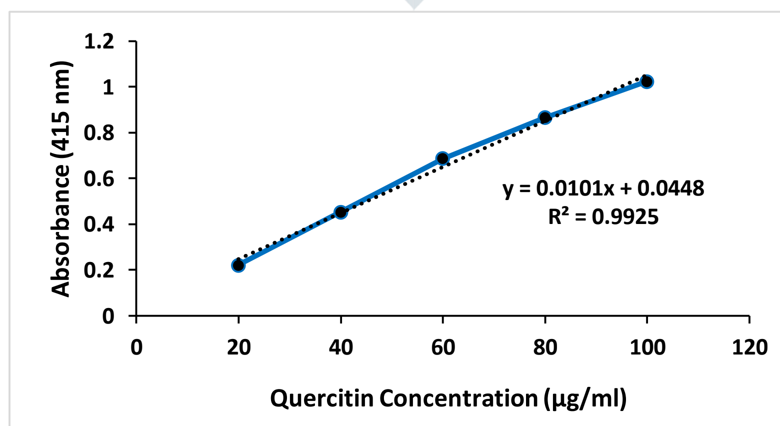


Figure 4: Standard Curve for quantifying TFC in the extracts of GGB and GGL of G. *glauca,* utilizing Quercetin as a Standard.

Table 4: TFC inclusion in the MeOH extracts of G. *glauca*.

Extracts Name	Total Flavonoid Contents (TFC)*
GGB	21.6 ± 0.34
GGL	20 ± 0.26

***TFC was quantified via equation y = 0.0101x + 0.0448, R^2 = 0.9925 (Figure 3), where y= Absorbance$_{415\ nm}$ and x= µg QE/ml. The data are represented as the mean value ± standard deviation of triplicates (n=3).**

Furthermore, in each extract, GGB, and GGL, the TPC was found to be higher than the TFC as most flavonoids are also phenolics (table 3 and 4). [30]

5.4 Determination of In vitro Antioxidant Activity

Plants are the enormous repository of natural antioxidant due to the inclusion of diverse bioactive phytochemicals such as phenolics, tannins, tocopherol, flavonoids, vitamin C, A, E, etc. [37] These antioxidants have a central role in scavenging free radicals which cause oxidative stress which ultimately leads to several chronic diseases (cancer, inflammatory, cardiovascular, and neurodegenerative). [34, 40] Therefore, the antioxidant capability of MeOH crude extract of bark and leaf of G. *glauca* is being explored to evaluate their potency to scavenge the highly reactive free radicals using the following assay:

5.4.1 DPPH (1, 1-Diphenyl-2-Picrylhydrazyl) Free Radical Scavenging Assay (DFRS)

DFRS is the simplest, sensitive, and extensively used assay for discerning the antioxidant activity of the plant extracts even at low concentration. DPPH is a stable free radical with unpaired electrons producing a deep violet color in alcoholic solution which has an absorbance at 517 nm. Due to the presence of unpaired electrons, it has the potential to accept H^+ ions from the proton donor which results in the reduction of DPPH solution from deep violet to the yellow color and this change is determined using spectroscopically. [13, 30] A number of studies have publicized that the plant extracts have the power to donate protons as it encompasses a number of bioactive compounds like phenols, flavonoids, etc. and therefore, acting as a free radical scavenger. [22, 27] Antioxidant potential of the MeOH extracts of bark (GGB) and leaf (GGL) parts of G. *glauca* are determined using DFRS. The results are expressed as a percentage of DPPH inhibition, which was enumerated using the following equation (2) and was displayed in Figure 5. Based on Figure 5, the MeOH extract of bark (GGB) showed slightly higher activity than the MeOH extract of the leaf (GGL) but both the extracts showed slightly lower scavenging activity than the standard (Quercetin). Further, IC_{50} (half maximal inhibitory concentration) value of the Quercetin, GGB and GGL are quantified by utilizing the equation y = 6.687x + 32.6, y = 0.737x + 17.769, and y = 0.8004x + 10.935 from the dose-response curve (Figure 5) and were found to be 25.2 ± 0.15 µg/ml, 43.7 ± 0.2 µg/ml and 48.8 ± 0.1 µg/ml respectively.

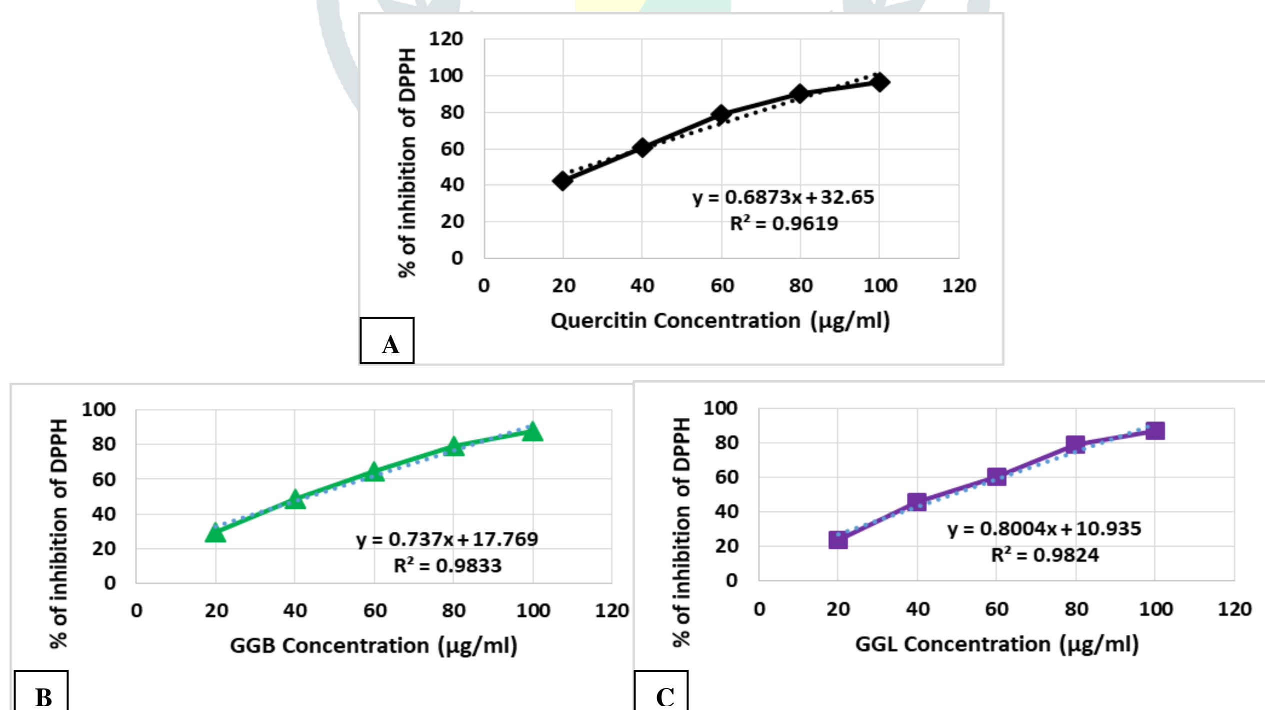


Figure 5: A, B, and C represent the graph of percentage (%) of inhibition of DPPH in a dose-dependent manner by Quercetin, GGB, and GGL, respectively.

5.4.2 Total Antioxidant Activity (TAA)

TAA was analyzed by using the phosphomolybdate colorimetry method. In this assay, the phosphomolybdenum blue color complex in which the molybdenum (Mo) oxidation state is (VI) is reduced to Mo (V) resulting in the formation of green color phosphocomplex. This reduction is caused due to the presence of reducing bioactive compounds such as phenolics, flavonoids, sterols, vitamin C and carotenoids in the plant extracts and can be detected by spectroscopically at 765 nm. [33, 30] TAA was determined as µg gallic acid equivalents (GAE) per ml on the basis of a standard curve of gallic acid (50–250 mg/L, y = 0.0058x - 0.0879, where y= $Absorbance_{765\ nm}$ and x= µg GAE/ml)) which were represented in Figure 6 and Table 5. Based on Table 5, the MeOH extract of bark (GGB) showed slightly higher total Antioxidant activity i.e. 223 ± 0.1 µg/ml than the MeOH extract of the leaf (GGL), 194.1 ± 0.1 µg/ml.

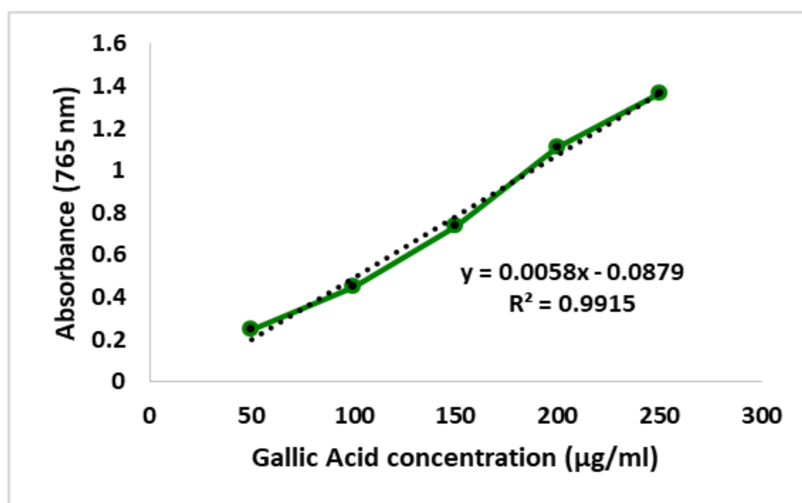


Figure 6: Standard Curve for calculating Total Antioxidants Activity (TAA) in the extracts of GGB and GGL of G. *glauca,* using gallic acid as standard.

Table 5: Total Antioxidants Activity (TAA) present in the MeOH extracts of G. *glauca.*

Extracts Name	Total Antioxidants Activity (TAA)*
GGB	223 ± 0.1
GGL	194.1 ± 0.1

*** TAA was valuated using the equation, y = 0.0058x - 0.0879 (Figure 6), where y= $Absorbance_{765\ nm}$ and x= µg GAE/ml. The data are represented as the mean value ± standard deviation of triplicates (n=3).**

5.4.3 Ferric ion Reducing Antioxidant Potential (FRAP) Assay

FRAP assay is routinely utilized to analyzed the antioxidant capability of the plant extracts by reducing Fe^{3+}-TPTZ complex to blue color Fe^{2+}-TPTZ complex at acidic pH which is recorded spectroscopically at 593 nm. This reduction is caused due to the presence of reducing agents (phenolic, flavonoids, etc.) in the plant extracts which has the potential to donate protons in a redox-based colorimetric reaction. [19] FRAP is determined as µg gallic acid equivalents (GAE) per ml on the basis of a standard curve of gallic acid (50–250 mg/L, y = 0.0049x + 0.1069, where y= $Absorbance_{593\ nm}$ and x= µg GAE/ml) which were represented in Figure 7 and Table 6. Based on Table 6, the result demonstrates the MeOH extract of bark (GGB) showed slightly higher antioxidant activity than the MeOH extract of the leaf (GGL). The FRAP value of GGB was 155.3 ± 0.1 µg/ml while FRAP value of GGL was 132.3 ± 0.02 µg/ml.

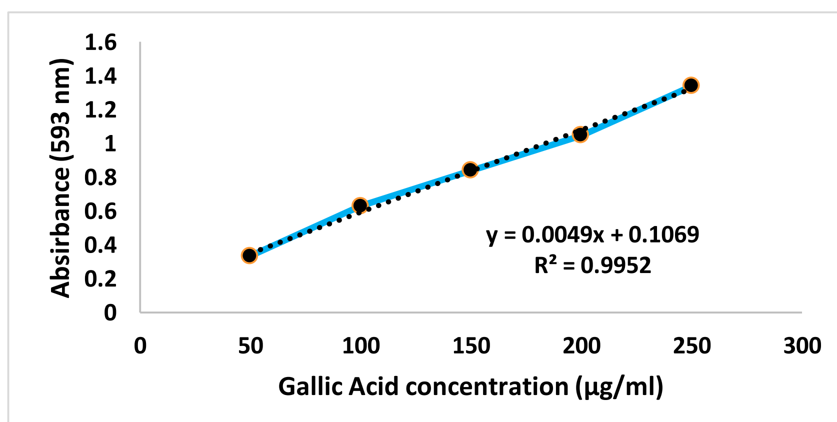


Figure 7: Standard Curve for calculating FRAP in the extracts of GGB and GGL of G. *glauca*, using gallic acid as standard.

Table 6: Ferric ion Reducing Antioxidant Potential present in the MeOH extracts of G. *glauca*.

Extracts Name	Ferric ion Reducing Antioxidant Potential (FRAP)*
GGB	155.3 ± 0.1
GGL	132.3 ± 0.1

***FRAP was determined using the equation, y = 0.0049x + 0.1069 (Figure 7), where y= $Absorbance_{593\ nm}$ and x= µg GAE/ml. The data are represented as the mean value ± standard deviation of triplicates (n=3).**

5.4.4 Ferric Reducing Power (FRP)

FRP assay of a plant extract is a vital determinant of its antioxidant capacity because of the inclusion of reducers (phenolics, flavonoids, carotenoids, etc) which causes reduction of yellow color Fe^{3+}-cyanide complex to Pearl's Prussian blue color Fe^{2+}-cyanide complex which is recorded spectroscopically at 700 nm. [5, 30] FRP was determined as µg gallic acid equivalents (GAE) per ml on the basis of a standard curve of gallic acid (50–250 mg/L, y = 0.0049x + 0.1069, where y= $Absorbance_{700\ nm}$ and x= µg GAE/ml) which were represented in Figure 8 and Table 7. Based on Table 7, the result exhibits that the MeOH extract of bark (GGB) showed slightly higher reducing activity than the MeOH extract of the leaf (GGL). The FRP value of GGB was 168.8 ± 0.1 µg/ml while FRP value of GGL was 158 ± 0.1 µg/ml.

Figure 8: Standard Curve for calculating FRP in the extracts of GGB and GGL of G. *glauca*, using gallic acid as standard.

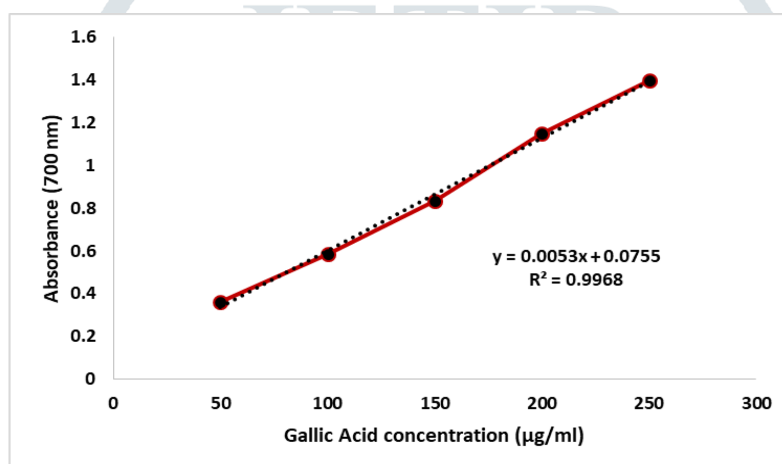


Table 7: Ferric Reducing Power/antioxidant capacity present in the MeOH extracts of G. *glauca*.

Extracts Name	Ferric ion Reducing Power (FRP)*
GGB	168.8 ± 0.1
GGL	158 ± 0.1

***FRP was determined using the equation, y = 0.0053x + 0.0755 (Figure 8), where y= $Absorbance_{700\ nm}$ and x= µg GAE/ml. The data are represented as the mean value ± standard deviation of triplicates (n=3).**

5.5 Antibacterial Activity using Agar well Diffusion Assay.

The emergence of several antibiotic resistance bacteria and side effects of synthetic drugs have turned the attention to look into the alternative sources of antimicrobial substances which must be safe and easily degradable. [7] To overcome this problem, researchers are looking into natural remedies to treat such infectious diseases by using medicinal plants. [8] Plants are the hub of several bioactive/phytochemical constituents which possess several ethnomedicinal uses. [15, 16] Therefore, in present study MeOH extract of bark and leaf of G. *glauca* is being used to determine the antibacterial action against the clinical and standard (ATCC 33591) methicillin-resistant Staphylococcus *aureus* (MRSA), as there is lack of information regarding the antibacterial effect of the G. *glauca* plant. The spectrum of antibacterial action was quantified via Agar-well-diffusion assay by quantifying the Zone of Inhibition (ZI). The results are represented in Table 8. Based on table 8, the result exhibits that both the MeOH extract of bark (GGB) and MeOH extract of the leaf (GGL) showed significant antibacterial activity against the clinical and the standard (ATCC 33591) MRSA strains. ZI value for the extracts - GGB and GGL against the clinical MRSA strain was 16 ± 2 mm and 15 ± 1.5 mm respectively while against the standard (ATCC 33591) MRSA strain was 15.3 ± 0.57 mm and 14 ± 1 mm respectively. ZI value for 70% DMSO against the clinical and the standard (ATCC 33591) MRSA strains was 2.7 ± 1.5 mm and 3 ± 1 mm while distilled water showed no zone of inhibition (NZ).

Table 8: Antibacterial Activity of bark (GGB) and leaf (GGL) extract of G. *glauca* based on the zone of inhibition (ZI) calculated in millimeter (mm).

Pathogenic Name	Extract (10mg/ml)		Negative control	
	GGB	GGL	70% DMSO	Distilled water
MRSA 01 (Clinical isolate)	16 ± 2	15 ± 1.5	2.7 ± 1.5	NZ
ATCC 33591 MRSA (Standard)	15.3 ± 0.57	14 ± 1	3 ± 1	NZ

ZI was calculated by subtracting 6 mm (size of the borer used) from the actual zone of inhibition. The data are represented as the mean value ± standard deviation of triplicates (n=3). Here, NZ= No zone of inhibition.

5.6 Anti-proliferative and Cytotoxic Activity

Several studies have revealed that the plant extracts have the potential to inhibit cell proliferation of cancer and this due to the existence of multiple bioactive phyto-components including phenols, anthraquinones, sterols, flavonoids, anthocyanins, etc., and it is also considered to be safe in comparison to synthetic drugs which shows several side effects and also enables to determine the cytotoxic effects of the plant extracts on normal cells due to acute overdose. [10, 14] To measure the anti-proliferative and cytotoxic effects of G. *glauca* plant extracts, MTT assay was used. In this assay, MTT – yellow tetrazolium bromide is reduced to purple formazan derivative by mitochondrial succinate dehydrogenase. This assay is commonly utilized as an in vitro model for several drug screening and toxicity testing of plants extracts various cancer and normal cell lines. [12] The two extracts, GGB (MeOH extract of bark) and GGL (MeOH extract of leaf) of G. glauca has shown relative reduction in the viability of the two cancer cell lines (A549 AND SW480) in a dose-dependent manner, while there was an insignificant/less cytotoxic effect in the viability of the normal cell line (HEK293) in comparison to the cancerous cell lines as represented in figure 9. Further, IC_{50} (50% inhibition concentration) of both the extracts (GGB and GGL) against HEK293, A549, and SW480 cell lines at 24 hours was estimated and the results were represented in table 9. Moreover, from figure 9 and table 9 the result shows that the MeOH extract of bark (GGB) sample are more potent as IC_{50} value shows less cytotoxic effect against normal cell line (HEK293) i.e. 388.67 ± 6 µg/ml and more anti-proliferative effect against A549 and SW480 cancer cell lines i.e. 157.8 ± 2.44 µg/ml and 136.6 ± 2.73 µg/ml and respectively, in comparison to MeOH extract of leaf (GGL).

Figure 9: (A) - represent % viability of cells after the treatment of different concentration of MeOH extract of bark (GGB) parts of G. *glauca*; (B) - represent % viability of cells after the treatment of different concentration of MeOH extract of leaf (GGL) parts of G. *glauca.* The data are represented as the mean value ± standard deviation of triplicates (n=3).

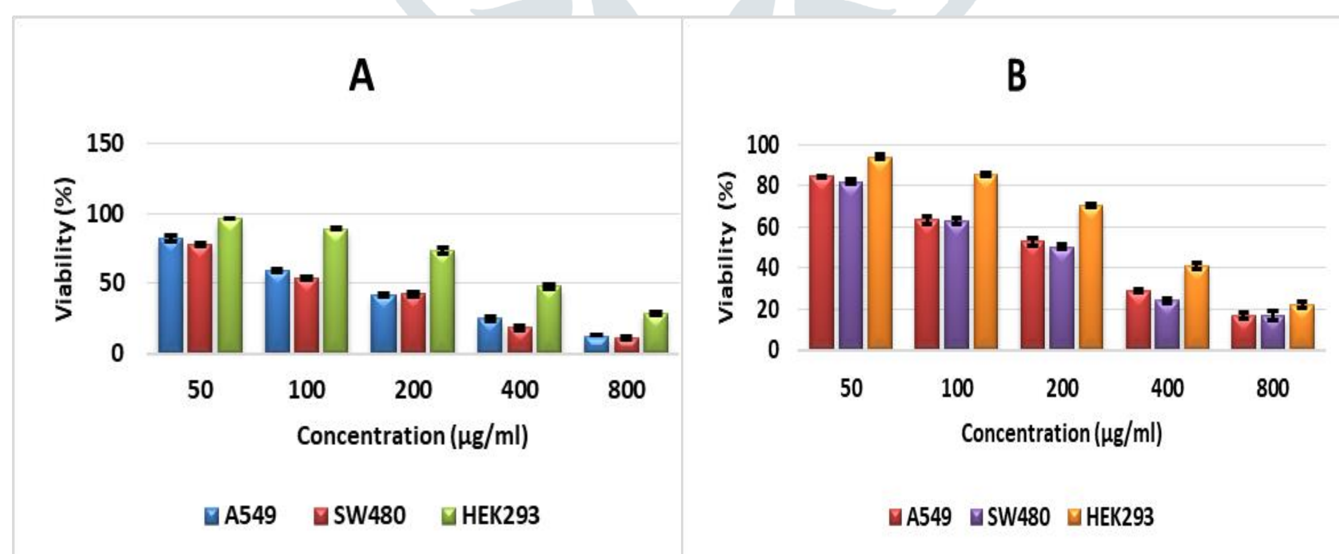


Table 9: IC_{50} values of % inhibition of cell proliferation by G. *glauca* (μg/ml).

Cells	MeOH extract of bark (GGB)	MeOH extract of the leaf (GGL)
Colorectal adenocarcinoma (SW480)	136.6 ± 2.73	178.1 ± 3.1
Lung Carcinoma (A549)	157.8 ± 2.44	194 ± 4.64
Human Embryonic Kidney/Renal Cells (HEK293)	388.68 ± 6	317.2 ± 9.4

The data are represented as the mean value ± standard deviation of triplicates (n=3).

VI. Conclusion

The current findings are preliminary but provide a shred of significant evidence that the MeOH extract of bark (GGB) and leaf (GGL) parts of G. *glauca* exhibited a strong in vitro antioxidant, anticancer, and antibacterial (against MRSA) activity. However, bark extract (GGB) possess the highest antioxidant, anticancer, and antibacterial activity than the leaf extract (GGL) and this could be due to the presence of high contents of phenolics and flavonoids in the extract. Hence, there was a substantial correlation between the total content of phytochemicals (phenolics and flavonoids), antioxidants, anti-proliferative and antibacterial effect but to confirm this hypothesis, an additional investigation is needed. Further, the study has also given a new insight for the development of anodyne, economical, and less toxic natural drugs and also seals the gap between the lack of information regarding the phytochemical screening, anti-proliferative action against cancer, and antioxidant potential of the extracts of G. *glauca*. Hence, more studies are needed to isolate, purify, and identify the novel bioactive compounds which are responsible for such biological properties and to form whole phytochemical profiling of *G. glauca* using current metabolomics tools.

VII. Acknowledgement

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VIII. Conflict of interest

All authors declare that there is no conflict of interest.

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