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Research Article

Anti-proliferative Effect of the Root Bark of *Gardenia gummifera* L. f on HepG2 Cell Lines

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ABSTRACT

Gardenia gummifera L. f is a rarely explored medicinal plant species found in the dry forests of Karnataka, Tamil Nadu, Andhra Pradesh, and Kerala. The present work evaluates the anti-proliferative effect of the root bark of *G. gummifera* on HepG2 cell lines. With standard protocols, qualitative and quantitative phytochemical analysis of petroleum ether, chloroform, acetone, ethanol, and aqueous extracts were carried out. The acetone, ethanol, and aqueous extracts from the root bark of *G. gummifera* were subjected to antioxidant assays. The anti-proliferative assays such as methyl tetrazolium (MTT) assay, Neutral red assay, and lactate dehydrogenase (LDH) leakage were performed on HepG2 cell lines. The phytochemical examination indicated the presence of primary and secondary metabolites, especially polyphenolic compounds. Among the different extracts, acetone, ethanol, and aqueous extracts exhibited maximum availability of flavonoids and tannins. Also, ethanol extract exhibited the highest free radical scavenging potential with the lowest IC_{50}. Between the different extracts, the highest cytotoxic activity was observed in the ethanol extract of *G. gummifera.* Hence, this work highlights the significance of ethanol extract of *G. gummifera* L. f as a potent anti-cancer agent for clinical use in the battle against liver cancer.

Introduction

Hepatic cancer is considered one of the most common deadly malignant cancers in India, and it dangerously affects people's health. According to the most recent assessments, 746,000 people died among the 782,000 people who were diagnosed with liver cancer from global incidence in 2012.[1,2] Diagnosis of hepatic cancer is very difficult at the early stage because it does not show any signs and symptoms on this stage, but it shows a high rate of metastasis in the later stage. So, it could be diagnosed at the advanced stage of progression, and many patients with the advanced stage are not fit for the remedial therapies. This resulted in a high mortality rate in liver cancer. According to the complexity of the tumor stage, the extent of liver damage, and the patient's general health, multidisciplinary treatment methods like chemotherapy, radiation therapy, immune therapy, and targeted therapy are used. However, lack of a suitable cure, strident frequency of liver cancer, and severe side effects of synthetic drugs such as trouble breathing, neuropathy, weakened immune system, bleeding, vomiting, etc. have made it essential for the invention of new effective chemotherapeutic drugs.[2] Therefore, there is an urgent need to develop a novel agent that can protect or enhance the body's immune function while effectively treating HCC. Plant-derived natural products are useful to improve disease symptoms with less adverse effects due to their multiplex targets.

Plant origin drugs have formed the source of the conventional medical system that has been used for centuries in many countries including India. More than 50% of drugs identified as chemo preventive agents are either synthetic alternatives of the natural plant products or isolated from the plant sources. So natural compounds or pure extracts isolated from the plant parts have immense application for developing new drugs.[3]

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Due to lesser side effects, there has been a widespread demand for herbal drugs or plant-derived compounds worldwide. These compounds have tremendous uses like anti-cancer, antioxidant, and anti-inflammatory properties. Plant-derived compounds have been considered as natural substitutes for synthetic compounds. Various diseases can be treated with extracts of plants, which forms the root for all Indian practices of medicine.[4]

Some studies have reported the anti-cancer efficacy of the plant extracts and documented them as the source of drugs.[5]

G. gummifera Linn. f. belongs to the family Rubiaceae and is widely distributed in various parts of peninsular India. *G. gummifera* is considered one of the rare plant species of India in danger of extinction.[6] It is conventionally used in cardiac debility, obesity, lipolytic disorders, bronchitis, dyspepsia, flatulence for cleaning foul ulcers and wounds, neuropathy, splenomegaly, and is given to children in nervous disorders and diarrhea due to dentition.[7] It is also used in veterinary practice to keep off flies from wounds.[8,9] Reportedly, the major components obtained from Gardenia species are gum resin, volatile steam oil, and a coloring matter-gardenin. The resin obtained from the leaf bud is known to be pungent, astringent, thermogenic, carminative, antispasmodic, stimulant, diaphoretic, cardiotonic, antioxidant, antihyperlipidemic, antihelminthic, antiseptic, and expectorant. Indigestion, gas trouble, ulcer, cardiac troubles, and wound healing ability of the resin has also been widely stated.[9] Ayurveda mentions the ability of the resin to alleviate Kapha and Vata doshas. Also, the paste of the bark finds use as an antispasmodic and expectorant. It is traditionally given to children or infants for treating digestive disorders and dental problems during the eruption. Alcoholic extracts of the plant's root have been tested for its hepatoprotective, cardioprotective, and antioxidant properties.[10] Also, the anti-cancer effect of the methanol extracts of the leaves of *G. gummifera* Linn. f. on MDA-MB-231 cell lines was identified.[11] Antimicrobial, antiradical and insecticidal activity of *G. gummifera* L. f. was assessed by Prashith Kekuda.[12] Antiulcer and antioxidant activity of the methanol extracts of *G. gummifera* Linn. f. was reported by Pawan kumar chityala *et al*.[13] It was reported that the ethanolic extract of *G. gummifera* gum resin possesses cholesterol suppressive activity and antioxidant activity.[14]

The anti-cancer activity of *Gardenia gummifera L. f* is least studied, and its evaluation of activity can bring more light to the pharmacological activities of the same.[15] Encouraged from the reported activities, root bark extracts of various solvents of this plant are subjected to its phytochemical screening, antioxidant and cytotoxic properties for the identification and evaluation of the active extract. Considering the aforementioned traditional uses, it can be said that the medicinal effects rendered by the plant are numerous.[8] Since the pharmacologically relevant activities of the plant are a cumulative effect of its constituents, it can be postulated that identification, characterization, and evaluation of its phytoconstituents could pave the way for the discovery of many useful drugs against several diseases. This study attempts to unravel the antiproliferative effect of *G. gummifera* L.f root bark extracts on HepG2 cell lines.

Materials and Methods

Chemicals and Reagents

Fetal bovine serum (FBS), Dulbecco's modified eagles medium (DMEM), Trypsin, 3-[4, 5-dimethylthiazol-2-yl]-2,5 diphenyltetrazolium bromide (MTT), and other cell culture reagents were procured from Sigma Aldrich. Chemicals used for phytochemical screening were obtained from High-Media Laboratories, India.

Sample Collection and Preparation of Plant Extracts

G. gummifera L. f root bark was collected from the Kanyakumari district, Tamilnadu, India. The collected plant specimen was identified and authenticated. A voucher specimen (SBSBRL 29) is maintained in the School of Biosciences, Mahatma Gandhi University, Kottayam.

G. gummifera L.f root bark was carefully washed using running tap water to remove adhering dust and soil followed by rinsing with distilled water. The root bark was then cut into small pieces, shade-dried, and powdered. The soxhlet extraction method was carried out through various solvents of increasing polarity, i.e., petroleum ether, chloroform, acetone, and ethanol. Water extract was made with hot water extraction. The extract was concentrated under reduced pressure using a rotary evaporator and the concentrated extract was kept under refrigeration and used for further studies. Then the percentage yield of each extract was duly calculated.

Preliminary Phytochemical Screening

Qualitative Phytochemical Analysis

Preliminary phytochemical screening of petroleum ether (PEGG), Chloroform (CHGG), acetone (ACGG), ethanol (ETGG), and aqueous (AQGG) extracts of *G. gummifera* Linn. f. root bark was conducted for the detection of bioactive compounds. The phytoconstituents tested were alkaloids, steroids, glycosides, flavonoids, anthocyanins, saponins, phenols, tannins, volatile oils, terpenoids, carbohydrates, and proteins using standard conventional protocols.[16-18]

Quantitative Estimation of Phenolics

Estimation of Flavonoids

The total flavonoids in the plant extracts can be quantified by measuring the optical density of the stable complex developed between aluminium chloride and ketone and hydroxyl groups of flavonoids at 510 nm.[19] The principle involved in the aluminium chloride ($AlCl_3$) colorimetric

method is based on the ability of AlCl3 to form acid-stable complexes with the C-4 keto groups and either the C-3 or C-5 hydroxyl group of flavones and flavonols. Besides, it also forms acid-labile complexes with the orthodihydroxyl groups in the A or B ring of flavonoids.

Varying volumes of quercetin (20, 40, 60, 80, 100 µL) were pipetted out from stock (1mg/mL) in test tubes labeled S1-S5 containing 4mL distilled water. 0.3 mL of 5% $NaNo_2$ was added to the above mixture. After 5 minutes, 0.3 mL of 10% $AlCl_3$ was added and incubated for 6 minutes. 2 mL of 1 M NaOH was added to the reaction mixture, and the total volume was made up to 10 mL with distilled water. The procedure was repeated with test samples, i.e., plant extracts. Then the solution was mixed well, and the absorbance was measured against a freshly prepared reagent blank at 510 nm.

Estimation of Tannins

Tannin like compounds reduces phosphotungstomolybdic acid in alkaline solution to produce a blue colour solution, the intensity of which is proportional to the amount of tannin. The intensity is measured in a spectrophotometer at 700 nm.[20]

The tannin content of the plant sample was estimated by following the standard procedure. The sample extract (1 mL) was mixed with Folin-Ciocalteau's reagent (0.5 mL), followed by the addition of saturated $Na2Co_3$ solution (1 mL) and distilled water (8 mL). The reaction mixture was allowed to stand for 30 minutes at room temperature. Absorbance was recorded at 725 nm using UV-visible Spectrophotometer.

Antioxidant Assays

DPPH Radical Scavenging Activity

The free radical scavenging activity of the different extracts was analyzed by using DPPH according to the modified method of Blois.[21] The molecule 1,1-diphenyl-2-picrylhydrazyl is characterized as a stable free radical and the delocalization of electrons in the DPPH gives its deep violet color. When DPPH solution was mixed with a substrate (AH), it turned to a reduced form with the loss of this violet color. To test the radical scavenging activity of the test solution, the change in optical density of DPPH was measured. Ascorbic acid was used as the antioxidant reference standard for comparison. The absorbance is measured at 517 nm.

ABTS Cation Free Radical Scavenging Activity

Free radical scavenging activity of different extracts of *G. gummifera* LF was evaluated in terms of radical scavenging activity by the standard protocol of Re *et al.* with some mild modifications.[22] When antioxidant was added to the blue-green chromophore of $ABTS^+$(2,2-azino-bis (3-ethylbenzthiazoline-6-sulfonic acid)), the solution became colorless and it was measured spectrophotometrically. The antioxidant reduces $ABTS^{\cdot+}$ to ABTS and decolorize it. ABTS solution was prepared by mixing 2.45 mM potassium persulphate with ABTS stock solution and allowed to keep at room temperature for 12 to 16 minutes. Different concentrations of *G. gummifera* L.f extracts (6.25, 12.5,25, 50, 100 µg/mL) were allowed to react with 180 µL of ABTS and kept for 12 minutes under room temperature. The absorbance was measured at 734 nm. The calibration curve was constructed using the ascorbic acid standard.

Ferric-reducing/Antioxidant Power (FRAP) Assay

FRAP assay was carried out according to the method explained by Bensie and Strain.[23] It is based on reducing the complexity of ferric iron and 2,3,5-triphenyl-1,3,4-triaza-2-azoniacyclopenta-1,4diene chloride (TPTZ) to the ferrous form at low pH, which gives purple-blue color, measured at 593 nm. Different concentrations of *G. gummifera* L.f extracts (6.25, 12.5, 25, 50, 100 µg/mL) were allowed to react with 180 µL of FRAP reagent and kept for 12 minutes under room temperature. The mixture was allowed to stand for 6 minutes, and the absorbance of the sample was measured at 593 nm. Ascorbic acid was used as the antioxidant reference standard.

Cell Culture and Treatments

HepG2 (Human Hepatic Cells) cells were initially procured from National Centre for Cell Sciences (NCCS), Pune, India and maintained by Dulbecco's modified Eagle's medium, DMEM (Sigma Aldrich, USA). The cell line was maintained in a 25 cm^2 tissue culture flask with DMEM complemented with 10% FBS, sodium bicarbonate (Merck, Germany), L-glutamine. An antibiotic solution of Penicillin (100 U/mL), Streptomycin (100 µg/mL), and Amphotericin B (2.5 µg/mL) was also added to the solution. Cultured cell lines were kept at 37°C in a moistened 5% CO_2 incubator (NBS Eppendorf, Germany) and sub-cultured three times a week to attain the confluency.

Anti-proliferative Activity of *G. gummifera* L. f Root Bark Extracts

Preparation of Plant Extracts:

1 mg of plant extract was mixed with 1 mL DMEM using a cyclomixer. 0.22 µm millipore syringe filter was used to filter the sample solution to ensure a sterile condition. The extracts were serially diluted appropriately to obtain solutions of various concentrations i.e. 100, 50, 25, 12.5, 6.25 µg in 500 µL of 5% DMEM.

Determination of Anti-proliferative Activity

Two days old confluent monolayer of cells were trypsinized, and the cells were suspended in 10% growth medium, 100 µL cell suspension ($5x10^3$ cells/well) were seeded in 96 wells tissue culture plate and incubated at 37ºC in a humidified 5% Co_2 incubator. After 24 hours. incubation of the cells in the 96 well plate, 100 µL extract in various

concentrations were added in triplicates to the respective wells and incubated at 37°C in a humidified 5% Co_2 incubator. Untreated control cells were also kept. The cells were observed for morphological changes at fixed intervals using an inverted phase-contrast microscope. Any detectable changes in the morphology of the cells, such as rounding or shrinking of cells, granulation, and vacuolization in cytoplasm, were considered as cytotoxicity indicators.

Cytotoxicity Assay by MTT Method

15 mg of MTT (Sigma, M-5655) was mixed with 3 mL PBS until fully dissolved and filtered to ensure sterilization and incubated for 24 hours. Then the sample content in wells was removed, and all test and cell control wells were mixed with 30 µL of reconstituted MTT solution, the plate was lightly shaken well, then incubated at 37ºC in a humidified 5% CO_2 incubator for 4 hours. The supernatant was removed after the incubation period and 100 µL of MTT Solubilization Solution (Dimethyl sulphoxide, DMSO, Sigma Aldrich, USA) was added and the wells were mixed gently by pipetting up and down to solubilize the formazan crystals. The absorbance values were measured by using a microplate reader at a wavelength of 540 nm.[24]

The percentage of growth inhibition was calculated using the formula:

$$\%\,viability = \frac{OD\ sample}{OD\ control} \times 100$$

The cytotoxicity was taken in IC50 values intended by regression analysis using Graph Pad Prism 5.

The values were represented as the mean value ± standard deviation (SD) for three observations.

Neutral Red assay

The ability of the live cell to uptake and bind neutral red is used to distinguish between dead cells and live cells within a population. Therefore, the amount of accumulated neutral red is a determinant of cell viability.

Confluent cells were trypsinized for 2 minutes and maintained in T flasks in complete aseptic conditions and treated with varying concentrations of the sample (100, 50, 25, 12.5, 6.25 µg in 500 µL of 5% DMEM) and incubated for 24 hours.

Treated and untreated cells were mixed with 10 µL of the neutral red solution and incubated for 3 hours in a Co_2 incubator at 37˚C. Cells were then washed with PBS and fixed by using 200 µL of fixing solution (50% ethanol and 1% acetic acid). The fixation solution was discarded after 1 minute after which 200 µL of extraction buffer was added and mixed well. The plates were incubated for 20 minutes at room temperature. The absorbance was measured using a microplate reader at 540 nm and the percentage viability was calculated.[25]

The percentage of growth inhibition was calculated using the formula:

$$\%\,viability = \frac{OD\ sample}{OD\ control} \times 100$$

Lactate Dehydrogenase Assay

Lactate dehydrogenase activity assesses cellular glycolytic capacity and is used as a quantitative marker enzyme for the intact cell. Quantification of LDH leakage is an important and commonly applied test for detecting severe irreversible cell damage.

Different concentrations of samples such as 6.25, 12.5, 25, 50, 100 µg/mL were added to cell-free supernatant collected from tissue culture plates for performing LDH release assay. 2.7 mL potassium phosphate buffer, 0.1 mL sodium pyruvate solution, and 0.1 mL, 6 mM NADH solution were added to samples taken in a cuvette and mix well. The decline of OD was noted at 340 nm in a spectrophotometer, thermostatic at 25°C. Instead of sample enzyme dilution buffer was prepared as a blank solution.[26]

Following formula used to calculate the activity of lactate dehydrogenase,

$$Volume\ of\ activity\ (U/mL) = [(Abs - Ab0) \times 3\ (mL) \times df] \div [6.2 \times 0.1\ (mL)]$$

Results

Preliminary Phytochemical Evaluation

The results found in the present evaluation are summarized in Table.1. The quantitative phytochemical analysis showed alkaloids, glycosides, flavonoids, anthocyanins, saponins, phenols, carbohydrates, proteins, tannins, volatile oils, terpenoids, and steroids in the extract of root bark of *G. gummifera* L.f. The crude ethanol extract of root bark of *G. gummifera* L.f (ETGG) with the most phytochemicals also showed the highest yield of 13.5% (w/w) compared to other extracts, whereas acetone (ACGG), aqueous (AQGG), chloroform (CHGG), petroleum ether (PEGG) extracts which yielded 7.5, 3.8, 1.2 and 0.95% (w/w), respectively. The phytocompounds like flavonoids, anthocyanins, saponins, phenols, carbohydrates, proteins, tannins, and steroids were found in the acetone, ethanol, and aqueous extract. Petroleum ether and chloroform extracts showed less availability of prominent phytoconstituents.

Quantitative Estimation of Phenolics

Phenolic compounds are very important plant metabolites with potent antioxidant activity. The total phenolic content of acetone (ACGG), ethanol (ETGG), and aqueous (AQGG) extracts was measured in terms of milligrams of quercetin equivalent per gram of extracts. Ethanol extract showed the highest phenolic content (Table 2).

Antioxidant Assays

DPPH Radical Scavenging Activity

Plant extract's free radical scavenging activity was determined by their ability to scavenge DPPH radical, as

Table.1: Phytochemical screening of root bark of G. gummifera Linn. F extracts

Constituents	*Petroleum ether*	*Chloroform*	*Acetone*	*Ethanol*	*Water*
Alkaloids	+	+	-	+	+
Flavanoids	+	+	+	+	+
Steroids	+	-	+	+	-
Anthocyanins	-	-	+	+	+
Glycosides	-	-	+	+	+
Saponins	-	-	+	+	+
Phenols	-	-	+	+	+
Tannins	+	+	+	+	+
Volatile oils	+	+	-	-	-
Terpenoids	+	-	+	+	+
Carbohydrates	-	-	+	+	+
Proteins	-	-	+	+	-

"+" designates the presence of constituents. "-" designates the absence of constituents.

Table 2: Quantitative estimation of flavonoids and tannins.

Sl. No.	*Quantitative estimation*	*Ethanol extract (ETGG)*	*Acetone extract (ACGG)*	*Aqueous extract (AQGG)*
1.	Flavonoid content (mg of quercetin equivalent per gram of extract)	21.0 ± 1.1	19.6 ± 0.93	13.3 ± 0.54
2.	Tannin content (mg of quercetin equivalent per gram of extract)	18.4 ± 0.86	15.4 ± 0.63	4.3 ± 0.3

The values are expressed as Mean± standard deviation of the 6 independent determinations

depicted in Fig 1. A dose-dependent elevation in quenching of free radicals with an increase in the concentration of extracts was noted. The ethanol extract was most effective with the lowest IC_{50} value, followed by acetone and aqueous extracts, as shown in Table 3. Ethanol and acetone extracts exhibited a good antioxidant activity with IC_{50} values of 7.05 ± 0.08 and 11.15 ± 1.9 µg/mL when compared to the standard ascorbic acid value of 5.16 ± 0.02 µg/mL. But the aqueous extract showed reduced antioxidant status with an IC_{50} value of 55.15 ± 2.5 µg/mL.

The Fig. 1 represents DPPH scavenging activity exhibited by 3 different extracts of *G. gummifera* L.f root bark. The values were expressed as the mean ± standard deviation of 6 independent measurements (n=6)

ABTS Cation Free Radical Scavenging Activity

Fig. 2 depicts the ABTS radical scavenging activity of different extracts of *G. gummifera* L. f. root bark. ABTS radical scavenging activity of ethanol, acetone, and aqueous extracts was found to be 8.3 ± 0.09, 47.63 ± 2.3, 115.9 ± 5.6 µg/mL, respectively in Table 3. The ethanol extract was found to be the most effective radical scavenger among the three extracts.

The Fig. displays ABTS cation free radical scavenging activity exhibited by 3 different extracts of *G. gummifera* L.f root bark. The values were expressed as the mean ± standard deviation of 6 independent measurements (n=6).

Ferric-reducing/Antioxidant Power (FRAP) Assay

FRAP activity was measured based on the reduction of a ferric ion into ferrous ion. The antioxidant activity of ethanol, acetone, and aqueous extracts was 18.76 ± 2.06, 48.10 ± 2.4, 204.7 ± 10.5, respectively. Which indicates the antioxidant ability of the ethanol extract followed by acetone and aqueous extracts.

The Fig. 3 represents ferric reducing antioxidant power (FRAP) exhibited by 3 different extracts of *G. gummifera* L. f root bark . The values were expressed as the mean ± standard deviation of 6 independent measurements (n=6)

Since the plant's acetone, ethanol, aqueous extracts showed higher phytochemical contents and antioxidant capacity when compared to other extracts, they were selected for cytotoxic and anti-proliferative activity study against HepG2 cell lines using the MTT, Neutral red, and lactate dehydrogenase assay.

Determination of the Anti-proliferative Effect

Cytotoxicity Effects of Extracts

Cytotoxic effects of the various extracts of root bark of *G. gummifera* Linn. f. on HepG2 cell lines were evaluated by MTT assay. Conversion of yellow tetrazolium salt MTT to purple Formazan crystals by metabolically active cells is the basic principle of MTT assay. The number of viable cells found is proportional to the amount of formazan crystal. Cell viability of 6.25 µg/µL of the ethanol extract of

G. gummifera L f displayed 85.99%, and 100 µg/µL of the same extract showed 40.77% cell viability. The LD50 of the ETGG was found to be 43.872 µg/mL. Cell viability of 6.25 µg/µL of the acetone with the lowestxtract displayed 87.39%, and 100 µg/µL of the same fraction showed 43.6% cell viability. The LD_{50} of the ACGG was found to be 46.236 µg/mL. Cell viability of 6.25 µg/µL of the aqueous extract displayed 90.7%, and 100 µg/µL of the same fraction showed 65.52% cell viability. The LD_{50} of the AQGG was found to be 152.707 µg/mL (Fig. 4).

G. gummifera Linn. f. extracts treatment was found to bring about morphological changes to HepG2 cells such as rounding or shrinking of cells, granulation, and vacuolization in the cytoplasm, which are indicative of cytological damage relating to cell death/apoptosis (Fig. 5 to 7).

Effect of Extracts on Neutral Red Assay

The neutral red assay showed reduced cell viability in HepG2 cell lines upon treatment with ACGG, ETGG, and AQGG. 6.25 µg/µL of the ACGG, ETGG, and AQGG showed 87.39%, 87.99%, 90.70% of viability and 100 µg/µL showed 43.60%, 40.77%, and 65.52% of cell viability, respectively (Fig. 8).

Effect of EEFH on Lactate Dehydrogenase Assay

LDH has proven to be highly functional *in vitro* marker for cellular toxicity. In our study, increased LDH value was observed for ETGG compared to the other two samples, i.e. ACGG and AQGG (Fig. 9). Increased LDH leakage indicates loss of membrane integrity of cells which confirms the cytotoxic ability of the plant extract.

Discussion

Nowadays, lifestyle diseases, including cancer, especially hepatocellular carcinoma, have become a major challenge for humankind. One of the most widespread malignant tumors across the globe is hepatocellular carcinoma. It comes around 5.6% of all new cancer cases detected

Table 3: Results of antioxidant and free radical scavenging activity IC50 (µg/mL)

S.No.	*IC50 (µg/mL)*	*Ethanol extract (ETGG)*	*Acetone extract (ACGG)*	*Aqueous extract (AQGG)*
1	DPPH	7.05 ± 0.08	11.15 ±1.9	55.15 ±2.5
2	ABTS	8.3 ± 0.09	47.63 ±2.3	115.9 ±5.6
3	FRAP	18.76 ± 2.06	48.10 ±2.4	204.7 ±10.5

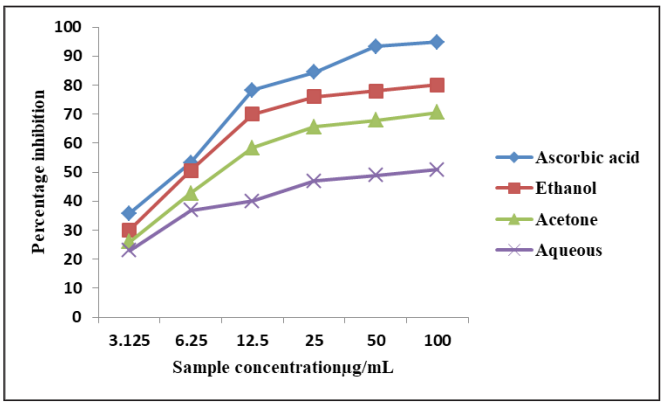

Fig.1: DPPH radical scavenging activity of crude extracts of G. gummifera L. f root bark

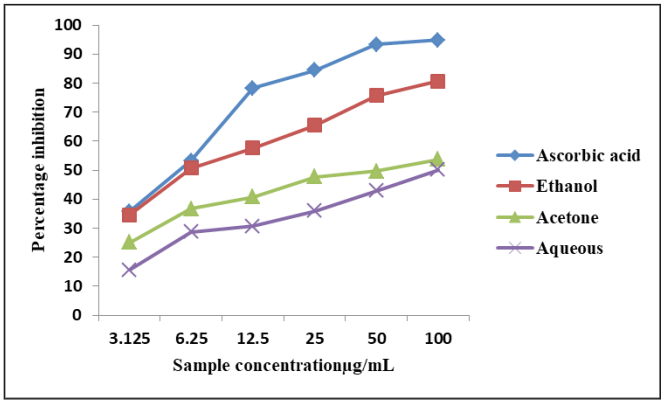

Fig. 2: ABTS free radical scavenging activity of crude extracts of G. gummifera L. f root bark

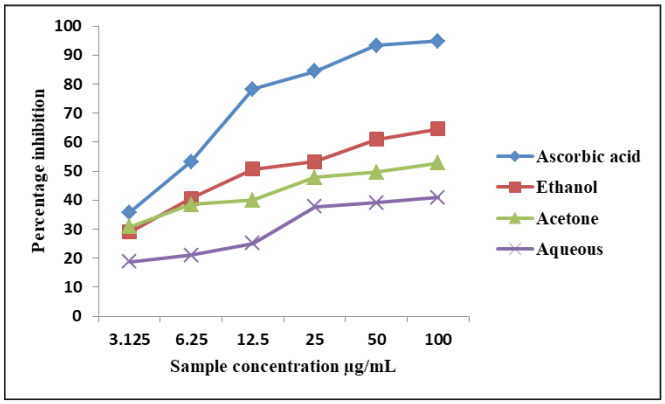

Fig. 3: Ferric reducing antioxidant power of crude extracts of G. gummifera L. f root bark

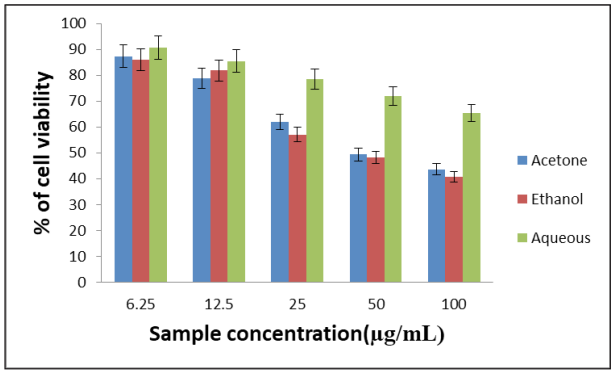

Fig. 4: MTT cell viability assay of AQGG, ETGG, and ACGG root bark extracts on HepG2 cell line. Data were represented as mean ± SEM of three separate experiments.

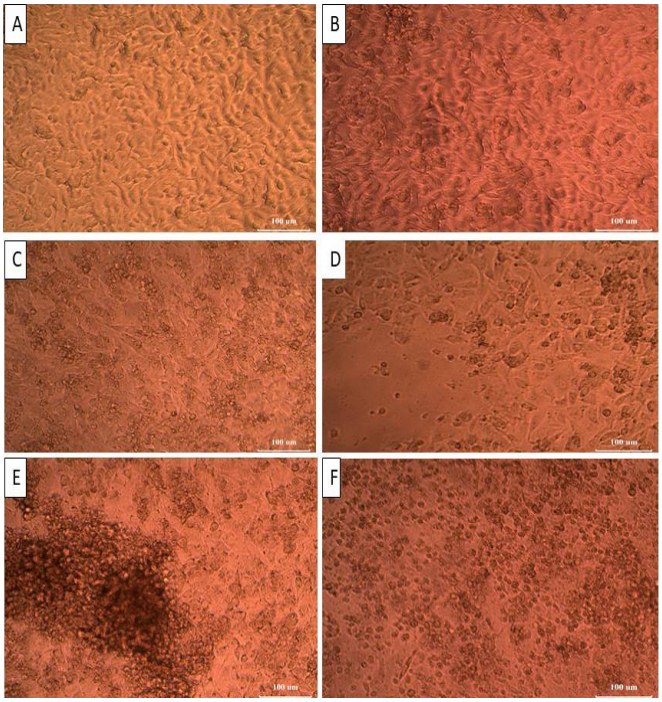

Fig. 5: Effect of ethanol extract (ETGG) on HepG2 cell line. A-Control, B-6.25 µg/mL, C-12.5 µg/mL, D-25 µg/mL , E- 50 µg/mL, F- 100 µg/mL. Data represented as mean ± S.E.M. of three separate experiments.

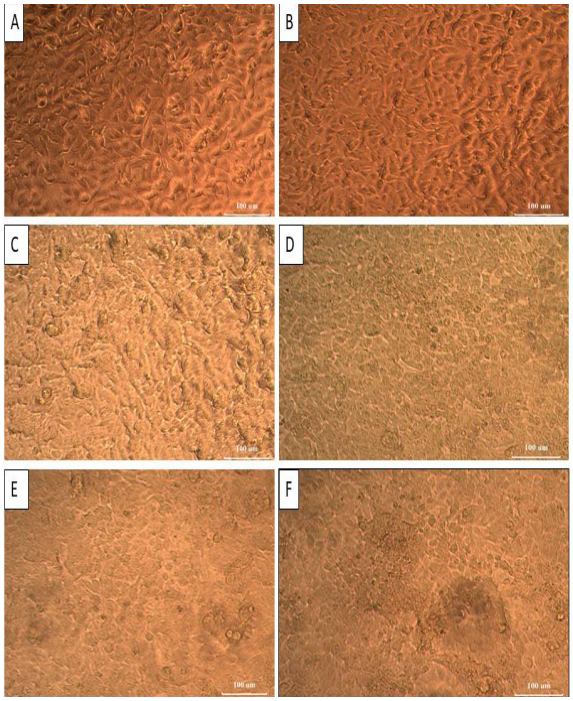

Fig. 6: Effect of aqueous extract (AQGG) on HepG2 cell line. A-Control, B-6.25 µg/mL, C-12.5 µg/mL, D-25 µg/mL , E- 50 µg/mL, F- 100 µg/mL. Data represented as mean ± S.E.M. of three separate experiments.

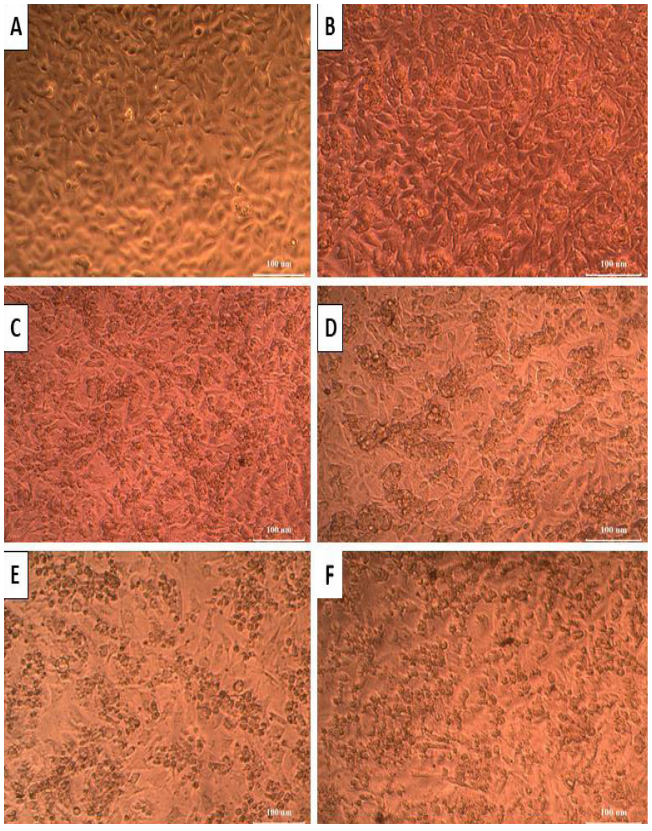

Fig. 7: Effect of acetone extract (ACGG) on HepG2 cell line. A-Control, B-6.25 µg/mL, C-12.5 µg/mL, D-25 µg/mL, E- 50 µg/mL, F- 100 µg/mL. Data represented as mean ± S.E.M. of three separate experiments.

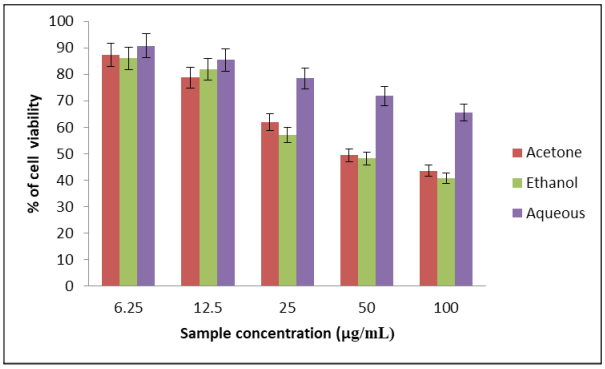

Fig. 8: Neutral red assay of ACGG, ETGG, and AQGG root bark extracts on the HepG2 cell line. Data were represented as mean ± SEM of three separate experiments.

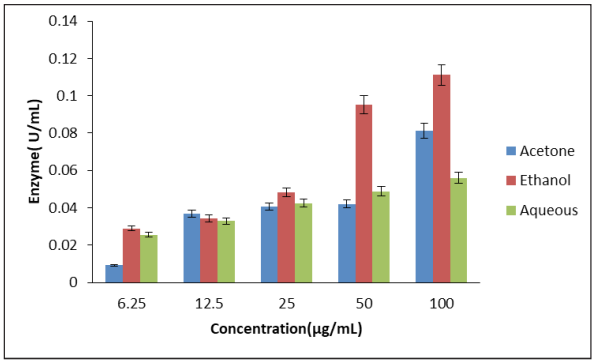

Fig. 9: Lactate Dehydrogenase Assay of ACGG, ETGG, and AQGG root bark extracts on the HepG2 cell line. Data represented as mean ± SEM of three separate experiments.

every year and nearly 9.1% of all cancer-related deaths worldwide.[27] Genetic predisposition, exposure to environmental carcinogens, and unhealthy lifestyles are the various factors that lead to cancer development. Normally, depending on the extent of damage, the cells are repaired or trigger senescence or eliminated themselves, but instead, the above said factors donate to tumorigenesis by destroying genetic material. Cellular damage occurred by oxidative stress through the oxidation of complex molecules is one of the major causes of carcinogenesis and other chronic diseases. Antioxidant and anti-cancer activities of phytoconstituents can counteract this cellular damage and thereby act as anti-proliferative agents.

Medicinal plants impart beneficial pharmacological effects on animal bodies due to their ability to synthesize and store secondary metabolites such as phenolics, alkaloids, glycosides, tannins, and volatile oils.[28] Most of these compounds can act as antioxidants and anti-cancer agents. The infinite and resourceful medicinal properties of medicinal plants fundamentally rely on their phytochemical constituents. Hence, modern drug discovery techniques rely heavily upon isolation, characterization, structural elucidation followed by bioactivity guided screening of phytochemicals for pharmacological properties. *G. gummifera* Linn. f. is a well-known plant found in the tropical natural forest with extensive traditional uses. Since most people today are unaware of the importance and specific use of this species, identification, characterization, and evaluation of its phytoconstituents could pave the way for discovering many useful drugs against several diseases.

Primary phytochemicals analysis of the *G. gummifera* Linn. f. extracts showed desired phytochemicals such as alkaloids, glycosides, flavonoids, anthocyanins, saponins, phenols, carbohydrates, proteins, tannins, volatile oils, terpenoids, and steroids. The above-said phytochemicals are effective antioxidants, and they are strong anti-cancer agents.[29] Ethanolic extract of the *G. gummifera* Linn. f. contained higher levels of total phenolics, flavonoids, steroids, saponins, and terpenoids, making its synergistic antioxidant properties to absorb and neutralize free radicals as well as quenching reactive oxygen species.[30] Quantitative estimation of flavonoids and tannins were expressed as quercetin equivalents in mg per gram dry extract and revealed its highest proportion in ethanol followed by acetone and aqueous extracts. The highest total flavonoid content of 21.0 ± 1.1 mg QE/g and tannin content of 18.4 ± 0.86 mg QE/g was observed for ethanol extract than the other two extracts. Hence ethanol extracts showed a notable amount of flavonoid and tannin content of significant medicinal potential. Several research works reported the antioxidant effect and free radical scavenging property of phenolics and flavonoids.[31] Hence it is cleared that the identified components of the extract might be capable of suppressing oxidative stress, multiplication of cancer cells, control the inflammatory and immune response, and arrest lipid peroxidation.[32] Antioxidant and free radical scavenging activity of the ethanol, acetone, and aqueous extracts of *G. gummifera* was evident from DPPH Radical Scavenging Activity, ABTS Cation free radical scavenging activity, and Ferric-reducing/antioxidant power (FRAP) assays. These assays are based on the electron transfer between the sample extracts and reagent, and the resultant color change is evaluated, spectrometically. DPPH is a standard method to assess the radical scavenging ability of natural products, and it is a stable molecule, readily accepts hydrogen radical, and becomes a diamagnetic molecule, which can be measured at 517 nm. When it gets to come upon antioxidants, reduced to yellow colored compound diphenylpicryl hydrazine. Among the three extracts, ETGG exhibited the highest DPPH radical scavenging activity in comparison with ascorbic acid, and hence it acts as a potent free radical scavenger.[33] The FRAP method is centered on reducing colorless complex, Fe^{3+} TPTZ, to a blue-colored compound-complex, Fe^{2+}-tripyridyltriazine made by the action of antioxidants at low pH and can be measured spectrometrically. The maximum ferric reduction was observed in the ethanol extract, which indicates ferric reducing antioxidant power of the sample. The comparative antioxidant activity to scavenge ABTS was compared with standard ascorbic acid. The ethanol and acetone extracts act as good radical scavengers of ABTS, but ethanol shows an improved activity. Therefore, antioxidant assays confirmed the potent antioxidant activity of ethanol extract of *G. gummifera* L. f, and its effective radical scavenging potential could reveal significant application in therapeutic applications.

The antineoplastic property of ethanolic extract of the *G. gummifera* Linn. f. (ETGG) in HepG2 cell lines confirmed through viz MTT assay, Neutral red uptake, and LDH leakage assay. These colorimetric cytotoxicity or cell viability assays are meant to evaluate the metabolic activity of the cells. These assays were chosen to assess the reliability of the obtained result. MTT assay measures the activity of the mitochondrial enzyme, and in this assay, MTT is reduced to purple formazan by NADH, then the product is measured. At the same time, LDH is an indicator of irreversible cell death due to cellular damage. NRU is a highly sensitive indicator of cell viability and is a good marker for lysosomal damage. The cytotoxic effect of ETGG by MTT assay in HepG2 cell lines was established by applying colony count. Among the different extracts, the highest cytotoxic activity was observed in ETGG treated cells, followed by ACGG and AQGG. In this assay, ETGG at the 6.25 µg/mL concentration showed 85.99%, and 100 µg/mL showed 40.77% cell viability and an LD50 value of 43.872 µg/mL. Morphological changes in the cells indicate the signs of apoptosis. So our outcomes endorse that the plant extracts induce apoptosis in cancer cells. A dose-dependent growth inhibition observed in the treated cells revealed its cytotoxic potential against HepG2 cell

lines. ETGG displayed a very good cytotoxic effect against HepG2 cell lines based on the outcome of the neutral red and lactate dehydrogenase assay studies. The present investigation shows that ETGG exerts inhibitory effects on HepG2 cell lines in a concentration-dependent manner.

On the other hand, ETGG extracts prevent the proliferation of selected cancer cells considerably, therefore suggesting their cytotoxic properties. So these cytotoxic assays confirm the apoptosis-inducing potential of the ethanol extract. *G. gummifera* is rich in flavonoids and tannins so that the cytotoxic effect might be due to its rich availability of phenolics i.e. flavones.[8,34] Taken together with the result of all assays of ethanol extract, it is confirmed that the root bark of this plant is a potential source of flavonoids and tannins compounds with antioxidant and cytotoxic effects. Hence the findings of the present study specified and proved the antioxidant and cytotoxic effect of ethanolic extract of the *G. gummifera* on HepG2 cell lines.

Oxidative stress is regarded as the imbalance between synthesis and degeneration of ROS and RNS species. When antioxidant resistances fail to neutralize ROS and RNS properly, these free radicals remain in the body for more extended periods. It oxidizes susceptible biomolecules like DNA, proteins, lipids and leads to the production of many dreadful diseases, including autoimmune diseases, cancer, neurodegenerative diseases, or infection by the human immunodeficiency virus (HIV).[35] Plant-derived antioxidants can effectively boost the characteristic antioxidant defense system of the body and thereby eradicate the free radical-mediated pathophysiology of several diseases. Researchers have proposed that antioxidants can also be used to eradicate cancer cells. It is reported that the use of plant-based antioxidants is an effective strategy to suppress oxidative stress-associated disorders like cancers.[36] The present work proved the antioxidant efficacy of the ETGG, and revealed the extract's impact on cytotoxicity and anti-proliferative effects on liver cancer cells.

Based on this preliminary screening, ethanolic extract of the *G. gummifera* Linn. f is considered an agent with potential anti-cancer activity, and therefore can be a good candidate for further screening stages *in vivo* and or *in vitro* clinical trials. This study confirms the potent anti-cancer activity of ETGG, which can find application in therapeutic regimens. From the current investigations, it can be concluded that the ethanolic extract of the *G. gummifera* Linn. f is an accessible source of bioactive compounds, which exhibits considerable cytotoxic activity against liver cancer cell line HepG2. The present study may be beneficial for the identification of potent natural molecules against liver cancer.

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