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In-Vitro Cytotoxic and Antioxidant Activity of *Gnidia glauca* (Fresen) Gilg Root Extract

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ABSTRACT

Cytotoxic and antioxidant activity of petroleum ether, chloroform and ethanol root extract of Gnidia glauca have been carried out. The MTT assay was used for in-vitro cytotoxic activity on two human cancer cell lines HT-29 and A-549. Results of MTT assay showed that chloroform extract exhibited excellent cytotoxic activity on A-549 and ethanol extract showed excellent activity on HT-29 with IC_{50} value less than 10µg ml^{-1}. All the extracts exhibited a dose dependent growth inhibitory effect on both the cell lines. The antioxidant activity of the extracts has been evaluated using DPPH radical scavenging, reducing power and nitric oxide method. The results of the study indicate that, ethanol extract of the roots of Gnidia glauca possess promising activity in DPPH radical scavenging, reducing power and nitric oxide scavenging methods. The petroleum ether and chloroform extracts also showed moderate antioxidant activity in all the three models. The cytotoxic and antioxidant activities may be attributed to the presence of phenolic and flavonoids present in the extracts.

Keywords: *Gnidia glauca*, MTT assay, DPPH assay, reducing power, nitric oxide activity.

INTRODUCTION

Medicinal plants have been used as remedies for human diseases for centuries. A large number of medicinal plants and their purified constituents have shown beneficial therapeutic potentials. The reason for using them as medicine lies in the fact that they contain chemical components of therapeutic value[1]. The medicinal value of plants lies in some chemical substances usually secondary metabolites that produce a definite physiological action on human body. Herbal remedies used in folk medicine provide an interesting and still largely unexplored source for the creation and development of potentially new drugs for the chemotherapy, which help to overcome the growing problem of resistance and toxicity of currently available drugs. The most important bioactive phytochemicals are alkaloids, flavonoids, tannins and phenolics, which possess various bioactivities including anticancer, antioxidant and anti-inflammatory activities[2,3].

Numerous studies have been focused on natural anticarcinogenic agents. Within the sphere of cancer plants have played an important role as a source of effective anticancer agents and it is significant that over 60% of the currently used anticancer agents are derived in one way or other from natural sources including plants, marine organisms and microorganisms[4,5]. They include vinblastine, vincristine, taxanes, etoposide and teniposide and the semisynthetic derivatives of epipodophyllotoxin, camptothecin, irinotecan, topotecan and several others[6]. Chemotherapy still is a major challenge to the cancer patients, because such highly potent drug can be toxic and less than 1% of injected drug molecules can reach their target cells and tissues[7]. Sometimes cancer cells also develop resistance to the treatment through mutations. It is worthy to mention the vivid current interest in the discovery of natural drugs for cancer treatment and chemoprevention. Huge numbers of plant species have been screened worldwide and bioassayed for this purpose.

Oxidation is a basic part of aerobic life and metabolism. During oxidation, many free radicals are produced such as superoxide anion ($O_2^{\cdot-}$), hydrogen peroxide (H_2O_2), peroxyl ($ROO^{\cdot}$) radicals and reactive hydroxyl ($OH^{\cdot}$) radicals. The nitrogen derived free radicals are nitric oxide (NO_2) and peroxynitrate anion ($ONOO^{-}$). All these radicals are known as reactive oxygen species (ROS), which are capable of reacting with lipids, nucleic acids, proteins, enzymes and other micro molecules resulting in cellular damage[8]. Free radicals are involved in the development of degenerative diseases. They have also been implicated in the pathogenesis of diabetes, liver damage, nephrotoxicity, inflammation, cancer, cardiovascular disorders, neurological disorders and in the process of ageing[9]. To protect these free radical induced damage, antioxidants are most popular agents that interact and synergistically neutralize the reactive species. Hence there has been an increased interest in food industry as well as in the preventive medicine in the development of "natural antioxidants" from plant materials. However the use of synthetic antioxidants such as butylated hydroxyl anisole (BHA) and butylated hydroxyl toluene (BHT) has been widely restricted because of their reported toxic and carcinogenic effects[10, 11]. Furthermore, natural products of plant origin have been proposed as a potential source of natural antioxidants with strong activity, which is mainly due to the phenolic compounds like flavonoids, phenols, flavonols and pronanthocyaninidins and their consumption has contributed in the prevention of distractive process caused by oxidative stress[12].
Gnidia glauca (Fresen) Gilg (Syn: *Lasiosiphon eriocephalus*) belongs to Thymeliaceae family. It is a shrub growing about 3 meters height and widely distributed in India, Srilanka and Africa. It is used in traditional African medicine for cancer, sore throat, abdominal pain, wounds, burns and snake bites[13]. Leaves have been applied to treat contusions, swelling, back ache and joint aches[14]. It is considered as a power full vesicant. The roots of this plant are used as antiviral agent against rabies in Ethiopia[15]. It also has agrochemical application as a molluscicide, insecticide, pesticide and even larvicidal agents[16-18]. It has been shown that several Gnidian species possess remarkable antineoplastic activity[19].

The objective of this study is to investigate the cytotoxic activity of crude extracts from roots of *Gnidia glauca* (Fresen) Gilg towards cancer cells HT-29, Human colorectal adrenocarcinoma and A-549, Human lung carcinoma using MTT assay. In addition, the antioxidant activity of crude extracts was also examined through DPPH scavenging, nitric oxide scavenging and reducing power method.

MATERIALS AND METHODS

Collection and Identification of Plant Material: *Gnidia glauca (*Fresen*)* Gilg was collected in Tunga river basin of Central Western Ghats of Karnataka. The plant was authenticated in Dept. of studies and research in applied Botany, Jnana Sahyadri, Shankaraghatta and voucher specimen (KU/AB/KSV/237) was deposited in the department for future reference.

Extraction of Plant Material: The roots of *Gnidia glauca* (Fresen) Gilg were washed thoroughly 2-3 times with running tap water and once with sterile water. The material was shade dried, coarsely powdered

and used for extraction. Weighed amount (500g) of the material was successively extracted using solvents of varying polarity namely, petroleum ether (60-80^{0}C), chloroform and ethanol using soxhelt extractor. Each extraction was carried out nearly 48 cycles. The extracts were filtered and concentrated using rotary flash evaporator under reduced pressure and at controlled temperature. The extracts obtained were dried, packed and stored at 4^{0}C in refrigerator.

Phytochemical analysis: All the extracts were subjected to preliminary phytochemical analysis using standard procedure to identify the various phytoconstituents[20].

Cytotoxic Activity: The cytotoxic effect of crude extract of the plant was evaluated by 3-(4,5-dimethyl-2-thiazoyl)-2, 5-diphenyl-2H-tetrazonium bromide (MTT) assay[21] using Human colorectal adrenocarcinoma (HT-29) and Human lung carcinoma (A-549) cell lines. The cell lines were obtained from National Center for Cell Sciences (NCCS), Pune, India. Cells were cultured in minimum essential medium (MEM) supplemented with glutamine (0.6g L^{-1}), gentamicin (25 mg ml^{-1}) and 10% fetal calf serum at 37^{0}C and in humidified 5% CO_2. For the assay 5 µl of the dilute cell suspension (1X10^5 cells per well) was plated in to 96 well plates and incubated for 24 hours. After 24 hours, when the monolayer formed, the supernatant was flicked off and test compounds of different concentrations (10, 20, 30 µg ml^{-1} in DMSO) was added to each wells and kept for incubation at 37^{0}C in 5% CO_2 atmosphere for 72 hours and cells were periodically checked for granularity, shrinkage and swelling. After 72 hours, the sample solution in the well was flicked off and 50 µl (5mg ml^{-1}) of MTT dye was added to each wells. The plates were gently shaken and incubated for 4 hours at 37^{0}C in 5% CO_2. The supernatant was removed, 50 µl of propanol was added and plates were gently shaken to solubilize the formed formazan. The absorbance was measured by using spectrophotometer at 490 nm (Shimadzu 1700). All the assays were performed in triplicates.

***In-vitro* Antioxidant Activity:** The free radical scavenging activity of the petroleum ether, chloroform and ethanol extract of the roots of *Gnidia glauca* was determined using various *in-vitro* assays such as DPPH radical scavenging, nitric oxide scavenging and reducing power method.

DPPH Radical Scavenging Activity: The antioxidant activity of the plant extracts namely petroleum ether, chloroform and ethanol and the standard was assessed on the basis of radical scavenging effect of stable 1,1-diphenyl-2-pecryl-hydrazyl (DPPH) free radical[22].

About 0.3mM solution of DPPH was prepared in methanol and 1ml of this solution was added to 3ml of crude extracts dissolved in methanol at different concentrations (400, 800, 1200, 1600 µg ml^{-1}). The mixture was kept at room temperature for 30 minutes and the absorbance was measured at 517nm using a spectrophotometer (Shimadzu 1700). The percentage scavenging activity at different concentrations was determined and the IC_{50} value of the fractions was compared with that of gallic acid, which was used as the standard. All the tests were performed in triplicates and percentage inhibition was calculated by comparing absorbance values of control and test samples.

$$\text{Percentage inhibition} = \{(A_{Control} - A_{Test}) / A_{Control}\} \times 100$$

Where $A_{Control}$ is the absorbance of control (without extract) and A_{Test} is the absorbance with extracts.

Nitric Oxide Scavenging Activity: Nitric oxide[22] generated from sodium nitroprusside in aqueous solution at physiological pH interacts with oxygen to produce nitrate ions which were measured by Griess reaction.

3ml of 100 mM sodium nitroprusside in phosphate buffer (0.5ml) was added to 1ml of different concentrations (100, 200, 300, 400, 500 and 600 µg ml^{-1}) of extract and standard. The resulting solution was then incubated at 25^{0}C for 60 minutes. 5ml of Griess reagent (1% sulphanilamide, 0.1% naphthyl ethylene diamine dihydrochloride, 2% H_3PO_4) was then added to incubated sample. The absorbance of the chromophore formed during the diazotization of nitrate with sulphanilamide and with subsequent coupling with naphthyl ethylene diamine dihydrochloride was measured at 546nm using spectrophotometer. The

same reaction mixture without plant extracts but with 0.5M phosphate buffer served as control. Vitamin C was used as standard. All the tests were performed in triplicates. The percentage inhibition of nitric oxide generated was measured by comparing the absorbance values of control and test.

$$\text{Percentage inhibition} = \{(A_{Control} - A_{Test})/ A_{Control}\} \times 100$$

Where $A_{Control}$ is the absorbance of control (without extract) and A_{Test} is the absorbance in presence of extracts.

Reducing Power Activity: The extracts (1ml) of various concentrations (250, 500, 750, 1000 and 1250 µg ml^{-1}) was mixed with phosphate buffer (2.5ml, 0.2M, pH 6.6) and 1% potassium ferricyanide (2.5ml). The mixture was incubated at 50^0C for 20 minutes. Aliquot of trichloroacetic acid (2.5ml, 10%) was added to the mixture, which was then centrifugated at 3000 rpm for 10 minutes. The upper layer of the solution (2.5ml) was mixed with distilled water (2.5ml) and freshly prepared ferric chloride solution (0.5ml, 0.1%). The absorbance was measured at 700nm. Gallic acid was used as standard and all the tests were performed in triplicates. The increased absorbance of the reaction mixture indicated increased reducing power[22].

Statistical Analysis: All the data were presented as mean±SEM. The Statistical Analysis was carried out by one way analysis of variance (ANOVA). P values <0.05 were considered as significant. The IC_{50} values were obtained by linear regression method of plot using Microsoft Excel 2007 software.

RESULTS AND DISCUSSION

Phytochemical Screening: Preliminary phytochemical screening of the crude extracts of *Gnidia glauca* revealed the presence of various phytochemical constituents. Analysis showed the presence of alkaloids, flavonoids, steroids, triterpenes, tannins and phenolics, carbohydrates and glycosides. During fractionation, these compounds distributed in solvents of different polarities on the basis of solubility as shown in table 1.

Table 1: Phytochemical constituents in the extracts of *Gnidia glauca*.

Phytochemical Test	Pet-ether Extract	Chloroform Extract	Ethanol Extract
Alkaloids	+ve	-ve	-ve
Flavonoids	-ve	+ve	+ve
Steroids	+ve	+ve	+ve
Triterpenes	+ve	+ve	-ve
Tannins and phenolics	-ve	+ve	+ve
Saponins	-ve	-ve	-ve
Carbohydrates	-ve	-ve	+ve
Glycosides	-ve	-ve	+ve
Proteins and Amino acids	-ve	-ve	-ve

+ve: Present -ve: Absent

Cytotoxic Activity: The Cytotoxic Activity of pet-ether, chloroform and ethanol extracts of *Gnidia glauca* was investigated using MTT assay on two human cancer cell lines HT-29 and A-549. A mitochondrial enzyme in living cells, succinate-dehydrogenase, cleaves the tetrazonium ring, converting the MTT to an insoluble purple formazan. Therefore the amount of formazan formed is inversely proportional to the number of lysed cells.

The IC_{50} values of extracts used in this study are given in table 2. Among the tested extracts, the ethanol and chloroform extracts exhibited highest cytotoxicity on both the cell lines HT-29 and A-549 with IC_{50} value <10 µg ml^{-1}. The pet-ether and chloroform extracts of the plant also showed considerable cytotoxicity on HT-29 with IC_{50} value of 10 µg ml^{-1}. But Pet-ether and ethanol extracts are moderately active towards A-549 with IC_{50} values of 20 µg ml^{-1} and 30 µg ml^{-1} respectively. In the present study all

the three extracts showed dose dependent growth inhibitory effect on both the cell lines. The cytotoxic activity of chloroform and ethanol extract of *Gnidia glauca* may be due to the presence of flavonoids and phenolic compounds. Polyphenolic compounds might inhibit the cancer cells by xenobiotic metabolizing enzyme that alter metabolic activation of potential carcinogens, while some flavonoids could also alter hormone production and also inhibit aromatase to prevent the development of cancer cells[23]. According to American National Cancer Institute (NCI) plant screening programme[24], a crude extract is generally considered to have *in-vitro* cytotoxic activity if the IC_{50} value is less than 30 µg ml^{-1}. Since IC_{50} values of root extracts were not more than 30 µg ml^{-1}, they were potent anticancer therapeutic agents.

Table 2: IC_{50} values of cytotoxic activity on HT-29 and A-549 by MTT assay

Extracts	IC_{50} value in µg ml^{-1}	
	HT-29	A-549
Pet-ether	10	20
Chloroform	10	<10
Ethanol	<10	30

Most of the anticancer drugs have been discovered through random screening of plant materials. Nowadays, isolation and elucidation of novel compounds have become an important part of cancer research for the development of potent anticancer agents[25]. Hence due to the potential anticancer activity of chloroform and ethanol extracts, isolation and elucidation of active components are recommended.

***In-Vitro* Antioxidant Activity**

a) DPPH Radical Scavenging Activity: Free radicals are known to be a major factor in biological damages and DPPH has been used to evaluate the free radical scavenging activity of natural antioxidants[26, 27]. DPPH, which is a free radical with a purple colour, changes into a stable compound with yellow colour by reacting with an antioxidant and the extent of reaction depends upon the hydrogen donating ability of the antioxidant. The free radical scavenging activity of pet-ether, chloroform and ethanol extracts as well as standard (gallic acid) are as shown in figure 1. The ethanol extract showed scavenging activity close to gallic acid, where as pet-ether and chloroform extracts showed a weak radical scavenging activity. The IC_{50} values for DPPH radical scavenging activity for gallic acid, ethanol, pet-ether and chloroform extracts are 244.3±2.33, 255.3±4.33, 1240.6±9.24, 1500±15.0 µg ml^{-1} respectively.

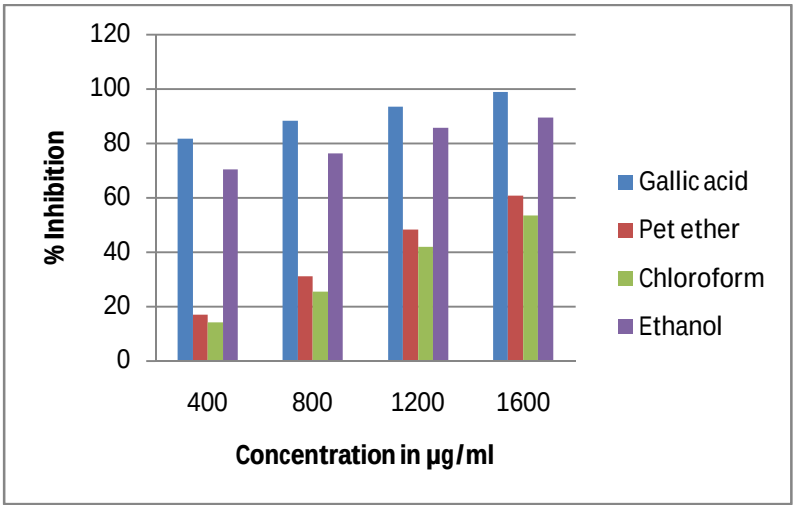

Figure 1: DPPH radical scavenging activity of different extracts of *Gnidia glauca* and gallic acid

b) Nitric Oxide Radical Scavenging Activity: The nitric oxide assay is based on the principle that sodium nitroprusside in aqueous solution at physiological P^H spontaneously generates nitric oxide, which

reacts with oxygen to produce nitrate ions that can be estimated by Griess reagent. Scavengers of nitric oxide compete with oxygen, leading to reduce production of nitrate ions. The percentage inhibition was increased with increase in concentration of extracts. Figure 2 shows the nitric oxide scavenging activity of standard (Vitamin C) and samples tested. Among the tested samples, ethanol extract showed promising nitric oxide scavenging activity. The IC_{50} value was found to be 490.33±6.06µg ml^{-1} for ethanol extract and 225.3±3.17µg ml^{-1} for Vitamin C. The pet-ether and chloroform extracts showed weak scavenging activity with IC_{50} values >600 µg ml^{-1}.

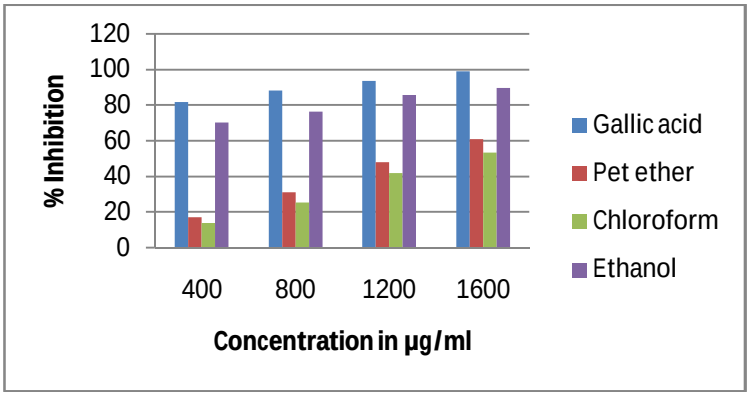

Figure 2: Nitric oxide radical scavenging activity of different extracts of *Gnidia glauca* and vitamin C

c) Reducing Power Activity: In the reducing power assay, the presence of antioxidants in the sample would results in the reduction of Fe^{3+} to Fe^{2+} by donating an electron. This reducing capacity of compounds could serve as an indicator of potential antioxidant properties and increase in absorbance could indicate an increase in reducing power[28, 29].The reducing power of various extracts and standard (gallic acid) are shown in figure 3. Ethanol extract showed a moderate reducing ability when compared with standard. The IC_{50} values of gallic acid and ethanol are 87.5±2.59µg ml^{-1} and 687.8±5.77µg ml^{-1} respectively. However pet-ether and chloroform extracts showed a weak reducing ability with IC_{50} values 1249.3±19.05µg ml^{-1} and 1612.6±8.51µg ml^{-1} respectively.

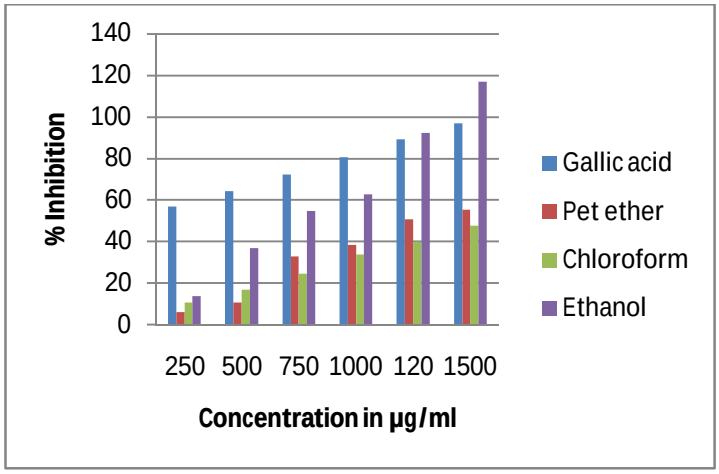

Figure 3: Reducing power of different extracts of *Gnidia glauca* and gallic acid

APPLICATIONS

The present study is focused on the cytotoxic and antioxidant activity of crude extracts of *Gnidia glauca*. However the study is helpful in the identification of active anticancer and antioxidant compounds present in the potent crude fractions.

CONCLUSIONS

The results of MTT assay showed that the chloroform extract of roots of *Gnidia glauca* exhibited excellent cytotoxicity on cancer cell line A-549 and ethanol extract showed excellent activity on HT-29. The ethanol extract of the plant also exhibited promising reducing power, DPPH radical scavenging and nitric oxide scavenging activity. The overall antioxidant activity of these extracts might be attributed due to the presence of flavonoid, phenolic and other phytochemical constituents. Many antioxidants have been identified as anticarcinogens. The flavonoids have reported for their cytotoxicity due to the presence of phenolic groups. The cytotoxicity of chloroform and ethanol extracts may also be due to the presence of flavonoids having mono to polyphenolic groups in the structure.

The potent cytotoxicity and antioxidant activity of ethanol extract indicating that most of the active components are extracted with polar solvent ethanol in higher concentration. However, there is a need for further investigation of this plant in order to identify and isolate its active antioxidant and anticancer principles. The results of the study will also need to be confirmed using *in-vivo* models.

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