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Preliminary phytochemical analysis and evaluation of cytotoxic profile of leaf and bark of *Garcinia morella* (Gaertn.) Desr. from Western Ghats

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Abstract
Phytochemicals are naturally occurring compounds in plants, play a very crucial role in protecting plants against diseases. Preliminary phytochemical analysis and cytotoxic studies in the leaf and bark of *Garcinia morella* collected from the Western Ghats were carried out. Hexane, chloroform, petroleum ether, water, and hydroalcohol were used for the extraction. The preliminary qualitative phytochemical analysis revealed the presence of alkaloids, flavonoids, tannins, terpenoids, phenols, carbohydrates, glycosides, saponins, and volatile oil. In the present study, the cytotoxic effect of hexane, chloroform, petroleum ether, water, and hydroalcoholic extract of leaf and bark was evaluated on HCT-116 using 3-(4, 5-dimethylthiazol-2-yl) 2, 5-diphenyl tetrazolium bromide (MTT) assay. The hydroalcoholic extract showed a remarkable decrease in the viable cell populations. Based on the observed results we could conclude that HAGM has a potent cytotoxic effect on cancer cells. Further detailed exploration is required to confirm its chemotherapeutic efficacy in different cancer cell lines. The chemical composition and medicinal properties of *G. morella* have already been studied and recorded. However environmental conditions influence the variations and accumulations of phytochemicals in plants. The type of solvents used in the extraction process will help other researchers to decide solvent composition for further work on the 7 on the leaf and bark of *Garcinia morella.*

Keywords: *Garcinia morella,* Western Ghats, phytochemicals, cytotoxic studies, hydroalcohol

Introduction

The pharmacological values of the plants lie in different chemical substances present in various parts of the plants such as root, stem, leaves, bark, fruits, and seeds [1]. The basis for the investigation of chemical compounds in plants from the fact that they are used in traditional, folk, and herbal systems of medicine. Isolation, purification, and clinical studies have shown that phytochemicals have behaved differently in in-vitro conditions, so preliminary studies on qualitative and quantitative phytochemical analysis are very essential. The phytochemical analysis is related to the traditional knowledge of the selection of a particular part of the plant for the administration of the crude drug [2]. It is difficult to explore the enormous number of organic compounds present in plants. Identification of chemicals present in plants has answered many taxonomical questions [3]. The second leading cause of death around the world is Cancer, accounting for an estimated 9.6 million deaths or one in six deaths, in 2018 [4]. According to WHO the most common types of cancer in men are lung, prostate, colorectal, stomach, and liver cancer, while breast, colorectal, lung, cervical, and thyroid cancer are the most common among women. Colorectal cancer is the third incidence after lung and breast cancers and accounts for almost 10% of total cancer cases and almost 8% of total cancer deaths. According to an estimation of WHO, more than 70% of all cancer deaths occurred in low and middle-income countries. Cancer-related deaths are projected worldwide and continue to rise to over 11 million in 2030 [5]. Hence there is an increased demand for cost-effective therapeutics and chemoprevention agents for various types of cancer. Several studies have shown natural products particularly medicinal plants have potential anticancer properties. Various anticancer agents have developed from plant products including cabazitaxel, campothecin, vinblastine, and vincristine [6]. However, treating cancer is complicated especially because the various pathways are being utilized to avoid cell death. *Garcinia morella* (Gaertn.) Desr. a member of the family Clusiaceae, distributed in India, the Philippines, and Srilanka. In India, it is widely distributed in Western Ghats, North-Eastern India, and Southern parts of India. It is a medium-sized evergreen tree, commonly known as Indian Gamboge because of its yellowish gummy exudation. The latex of the plant is dried and sold in the market as gamboge [7]. It is known as aduli, hardala and devanahuli in Kannada, tamal in Hindi, and Kankushta in Sanskrit. It is traditionally used in Ayurveda, Siddha, and folk systems of medicine in the treatment of jaundice, fever, and dysentery. The fruits are an excellent remedy for inflammation, gastritis, itching, and dizziness [8]. Previous pharmacological studies have stated that *G. morella* has potent antimicrobial, anti-inflammatory, anticancerous, antioxidant, free radical scavenging activities, hepatoprotective and larvicidal properties. Hence, to explore phytochemical constituents and cytotoxic properties of *G. morella* the present study has been carried out.

Materials and Methods

Collection and processing of plant material

Fresh leaves and bark of *G. morella* were collected from the Thattapura forest, Thirthahalli taluk, Shimoga district, Karnataka, India. The collected plant material was identified and authenticated by the taxonomist Prof. Shiva Moorthy, Mysore. The collected plant material is washed under running tap water to remove the dust and other impurities, then shade dried under room temperature, finally the dried plant material was grounded into coarse powder using mixer grinder. Exactly 5g of the coarse powder of leaf and bark were taken in 25ml of different solvents like hexane, chloroform, petroleum ether, water and hydroalcohol and then placed on water bath for 4 hours at 50^oC. The extract was filtered through Whatmann No.1 filter paper and kept for evaporation. For further investigation the condensed extracts were stored in refrigerator in air tight containers at 4^0C.

Chemicals: Analytical grade chemicals were used in the present investigation.

Qualitative phytochemical screening

The preliminary qualitative phytochemical analysis was performed to detect the presence of various chemical groups in five different solvent extracts, based on the standard procedures [9].

Test for Alkaloids

Dragendoff's test: To 0.2ml of sample, 0.2ml of HCl was added. Followed by the addition of 2-3 drops of Dragendoff's reagent, the appearance of orange or red precipitate and turbid solution indicates the presence of alkaloids.

Test for Carbohydrates

Molisch's test: Few drops of Molisch's reagent (α- napthol dissolved in alcohol) was added to 0.2 ml of sample and mixed with.0.2 ml of sulphuric acid added along the sides of the test tube and observed for the appearance of a purple colour ring for positive test.

Test for Tannins

Braymer's test: 2 ml water was mixed with 0.2 ml of plant extract and heated on water bath for 10 minutes. The mixture was filtered and $FeCl_2$ was added to the filtrate and observed for dark green solution which indicates the presence of tannin.

Test for Terpenoids

Salkowski's test: 0.2 ml of plant extract was taken in a test tube with 0.2 ml of chloroform, followed by the addition of concentrated sulphuric acid to form a layer. Presence of reddish-brown colour at the interface would show would show the presence of terpenoids.

Test for Glycosides

0.2 ml of sample was mixed with 0.2 ml of chloroform.0.2ml of acetic acid was added to this solution and the mixture was cooled on ice. Sulphuric acid was added carefully and the colour change from violet to blue to green indicates the presence of steroidal nucleus (A glycone portion of glycoside).

Test for Steroids

Lieberman Burchardt test: 0.2 ml of sample was mixed with 0.2 ml of chloroform. To this 0.2ml of concentrated sulphuric acid was added. Red colour appears in the lower layer of chloroform indicates the presence of steroids.

Test for Saponins

Test for Saponins (Foam test) 0.2mls of extract was added 0.6ml of water in a test tube. The vigorous shaking of the mixture results in the formation of persistent foam that confirms the presence of saponins.

Test for Flavonoids

Alkaline reagent test: 0.2 ml of plant extract was mixed with dilute sodium hydroxide solution in a clean and dry test tube. To this diluted hydrochloric acid was added. Observation of yellow solution that turn colourless later would indicate the presence of flavonoids.

9. Mucilage test (Glycoprotein): 0.2ml of extract was taken in a test tube and 0.2ml of absolute alcohol was added and allowed to try. Formation of precipitation indicates the occurrence of mucilage.

10. Volatile oil: 0.2ml of extract was treated with few drops of dilute hydrochloric acid. The formation of white precipitate indicates the presence of volatile oils.

11. Test for phenols: To 0.2 ml of extract 0.4 ml of distilled water followed by a few drops of 10% aqueous ferric chloride solution were added. Appearance of blue or green colour indicates the presence of phenols.

Cytotoxic Studies

Materials

1. MTT Powder (the solution is filtered through a 0.2 µm filter and stored at 2–8 °C for frequent use or frozen for extended periods).
2. DMSO
3. SpectraMax i3X

Cell lines and culture medium

HCT-116 cell line was purchased from ATCC, stock cells were cultured in DMEM supplemented with 10% Foetal Bovine Serum (FBS), penicillin (100 IU/ml), and streptomycin (100µg/ml) in a humidified atmosphere of 5% CO_2 at 37^oC until confluent. The cell was dissociated with cell dissociating solution (0.2 % trypsin, 0.02 % EDTA, 0.05 % glucose in PBS). The cells viability was checked and centrifuged. Further, 50,000 cells /well were seeded in a 96 well plate and incubated for 24 hrs at 37^oC, 5 % CO_2 incubator. Media components were purchased from Invitrogen bioservices.

Preparation of test sample

For cytotoxicity studies, 32mg/ml stocks were prepared using DMSO. Serial two-fold dilutions were prepared from 320µg/ml to 10µg/ml using DMEM plain media for treatment.

MTT assay

MTT is a basic enzymatic assay used to measure the ability of living cells based on mitochondrial activity [10]. HCT-116 cell lines were seeded separately in 96-well plates at 5.0 x

10^5 cells/ml (50,000cells/well). Cells were incubated for 24 hours before exposing to varying concentrations of plant samples. A partial monolayer was formed and the supernatant was flicked off and washed after 24 hours of incubation. The monolayer was once washed with medium and microtiter plates were added with 100 µl of different test concentrations of test drugs on the partial monolayer. Doxorubicin an anticancerous drug and DMSO were added and the cells were incubated for 24 hours at 37ºC 24hrs in 5% CO_2 atmosphere. After incubation the spent media was discarded and 100 µL of MTT (5 mg/10 ml of MTT in PBS) was added to each well and again incubated for 4 hours. The supernatant was removed and the resulting formazan crystals produced by the reduction of MTT by the live cells were then dissolved by the addition of 100 µl of DMSO. By using a microplate reader the absorbance was measured at 590 nm. The percentage growth inhibition was calculated using the following formula

$$\text{Inhibition \%} = \left(\frac{\text{Optical density of control-Optical density of sample}}{\text{Optical density of control}}\right) \times 100$$

Using the inhibition per concentration, the half maximal concentration (IC_{50}) values of the samples were computed with Graph Pad Prism 6, which employs non-linear regression curve fit.

IC50 Value

The measure of effectiveness of drug or a compound in inhibiting a biological or a biochemical process is known as half-maximal inhibitory concentration (IC_{50}). This indicates the quantity of a particular drug or a compound needed to inhibit a biological process or a component of a process such as an enzyme, cell, cell receptor or microorganism by half. By constructing a dose-response curve the IC_{50} value of a drug can be determined by examining the effect of different concentrations of antagonist on reversing agonist activity. IC50 values of a given drug can be calculated by determining the concentration needed to inhibit half of the maximum biological response of the agonist.

Results

Qualitative Phytochemical analysis

Phytochemical analysis for different solvent extracts of leaf and bark of *G. morella,* showed differences in the presence or absence of phytoconstituents in various solvents because of their different polarity (Table-1&2).

Cytotoxicity studies

HAGM at graded dose has shown decrease in the percentage of viability of HCT-116 cells. HAGM at 10, 20, 40, 80, 160 and 320 µg/ml concentrations has shown the % of inhibition of 7.74, 18.52, 34.98, 49.09, 67.56, and 84.48 with IC50 value of 82.24, where Petroleum ether and hexane extract of leaf showed IC50 value of 88.94 and 100.2 µg/ml respectively, where as the % of inhibition of 7.70, 14.77, 38.53, 51.16, 65.72 and 88.27 with the IC50 value of **79.09** for bark extract, where chloroform, hexane and petroleum ether extracts of bark showed IC50 value of 96.48, 121.5 and 150.6 µg/ml respectively, when compared with the standard doxorubicin 25.36 µg/ml (Fig.1, 2&3).

Discussion

The present study was conducted to screen the phytochemicals and to evaluate cytotoxic potentials of various extracts of leaf and bark of *Garcinia morella* at various concentrations.

Screening of phytoconstituents from the leaves and bark of *G. morella* majorly showed the presence of alkaloids, tannins, terpenoids, glycosides, carbohydrates, volatile oil and phenols. Alkaloids have antimicrobial activities and many health benefits [3]. Pet.ether, water and hydroalcoholic extracts showed the presence of alkaloids. Hexane and chloroform extracts did not showed presence of alkaloids for leaf, where as all solvent extracts showed presence of alkaloids in bark extracts. Tannins are potential anti-fungal, antibacterial and antiviral agents, they reduces the risk of coronary artery diseases in humans [11]. All solvent extracts of leaf showed the presence of tannins. Tannins were observed only in water and hydroalcoholic extracts of bark.

Saponins have been suggested to have anticancerous properties [3, 12]. Saponins were detected only in chloroform extract of leaf, saponins were not detected in any extracts of bark. Phenols are the most abundant and ubiquitous plant metabolites have antioxidant and free radical scavenging activities, phenolic acids, flavonoids and tocopherols are the naturally occurring phenols in plants [3, 13]. Hexane and Pet.ether extracts of the leaf showed the presence of phenols and partially they were detected in chloroform and hydroalcoholic extracts. All solvent extracts of bark showed the presence of phenols. Flavonoids are the greater important compounds, were detected only in hexane extract of leaf, where as flavonoids were observed in all solvent extracts of bark. Molisch's test showed the presence of carbohydrates in all the solvent extracts of leaf and bark.

Proteins are present in water and hydroalcohol extracts of both leaf and bark. Steroids are anti-inflammatory and analgesic agents [14]. Steroids were not observed in any of the solvent extracts of leaf. Steroids were detected in aqueous and hydroalcoholic extracts of bark. Terpenoids are the important biomolecules present in plants responsible for plant survival and also having biological properties [3]. Terpenoids were positive in water and hydroalcoholic extracts of leaf. All solvent extracts of bark showed the presence of terpenoids. Cardiac glycosides were found positive in all solvent extracts of leaf and they were not detected in any solvent extracts of bark. Volatile oils were found positive in water and hydroalcoholic extracts of leaf. Volatile oils were detected in all extracts of bark except chloroform and petroleum ether. The aim of this study to determine the effect of *G. morella* leaf and bark extracts on HCT-116 cell lines. Cell culture was exposed to leaf extracts in different doses of 10, 20, 40, 80, 160 and 320µg/ml and incubated for 24 hours based on previous studies. Results of cytotoxic studies showed that hydroalcoholic extract was comparatively effective against HCT-116 with the IC_{50} value of 82.24 and 79.09µg/ml for leaf and bark extracts respectively. According to American National Cancer Institute (NCI), cytotoxic extracts must have IC_{50} values less than 30µg mL^{-1} to be considered active [15]. The lower the IC_{50} value, the more effective the anticancer potential of the pure compound or crude extract. Since IC_{50} for different extracts of leaf and bark obtained were more than 30µg mL^{-1}, it can be concluded that the extracts pose weak cytotoxic activity. However in terms of cytotoxicity against HCT-116 cells IC_{50} value for hydroalcoholic extract of bark was the lowest (79.09μg/mL) as compared to leaf other solvent extracts of bark. Similarly, previous investigations on *Garcinia* extracts of different species did exhibit selective cytotoxic activity against HCT-116 [5]. Hydroalcoholic extract of leaf and bark showed comparatively significant activity against HCT-116. This is in contrast with the findings of [16] where in phytochemicals from *G. rubra* leaves working significantly against HCT-116 and A549 but not against MCF-7. Another aspect needed to be considered that this present study is carried out based on crude extracts, there is a possibility of variety of compounds exists in the plant extract and the mixture of compounds and impurities might hide the potential anticancer activity. The investigation of drug interaction in multicompound remedies is difficult because of interaction among compound where some may show less specific activity and some may reduce the toxicity of more therapeutically effective plant [17]. Thus, the effect of potential anti-cancer compound can be fractionated and isolated from the crude extracts. Many species of Garcinia are reported to have anticancer activity against different types of cancer cell lines. But to our knowledge the activity of *G. morella* has not investigated against HCT-116 cell lines, but fruits of *G. morella* have remarkable efficacy against neuroblastoma and Dalton's lymphoma [18]. Other species of Garcinia have already been established to induce apoptosis. Methanolic extracts of *G. dulcis* fruits were induced apoptosis of liver cancer cell lines HepG2 [19]. The leaves of *G. cowa* induced apoptosis and activated autophagy in cervical cancer cell line HeLa, pancreatic cancer cell line PANC-1 and A549 [20]. An attempt has been made to show the presence of various phytoconstiuents and cytotoxic efficacy of crude extracts of leaf and bark. Presence of impurities in the crude extract may negatively modifies its effects, hence further purifications and fractionations will be carried out as a future aspect to prove the efficacy of *G. morella* on HCT-116.

Tables and Figures

Table 1: Phytochemicals detected in various solvent extracts of leaf of *Garcinia morella*.

Types of tests	Hexane	Chloroform	Pet.ether	Water	Hydroalcoholic
Alkaloid	-	-	+	+	+
Carbohydrate	+	+	+	+	+
Tannin	+	+	+	+	+
Terpenoid	-	-	-	+	+
Glycoside	+	+	+	+	+
Steroid	-	-	-	-	-
Saponin	-	+	-	-	-
Flavanoid	+	-	-	-	-
Proteins (Myllon's test)	-	-	-	+	+
Glycoprotein test	+	-	-	-	-
Volatile oil	-	-	-	+	+
Phenol test	+	P.P	+	-	P.P

*PP- Partially present

Table 2: Phytochemicals detected in various solvent extracts of bark of *Garcinia morella*.

Types of tests	Hexane	Chloroform	Pet.ether	Water	Hydroalcoholic
Alkaloid	+	+	+	+	+
Carbohydrate	+	+	+	+	+
Tannin	-	-	-	+	+
Terpenoid	+	+	+	+	+
Glycoside	-	-	-	-	-
Steroid	-	-	-	+	+
Saponin	-	-	-	-	-
Flavanoid	+	+	+	+	+
Proteins (Myllon's test)	-	-	-	+	+
Glycoprotein test	-	+	+	-	-
Volatile oil	+	-	-	+	+
Phenol test	P.P	+	+	+	+

*PP- Partially present

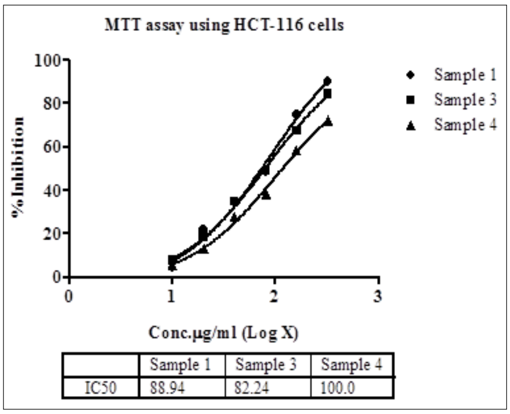

Fig 1: MTT assay leaf extract using HCT-116 (Sample-1-Pet.ether, Sample-3-Hydroalcohol and 4-hexane).

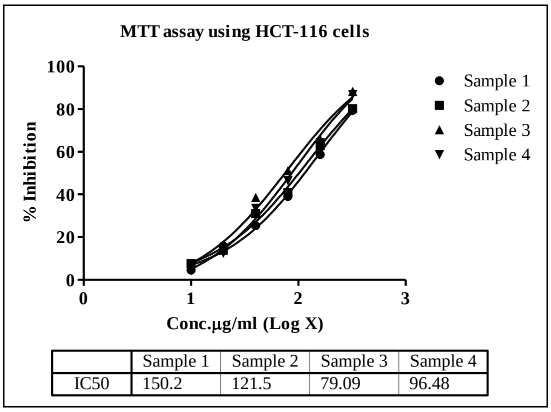

Fig 2: MTT assay leaf extract using HCT-116 (Sample-1-Pet.ether, Sample-2-chloroform, Sample-3-Hydroalcohol and 4-hexane).

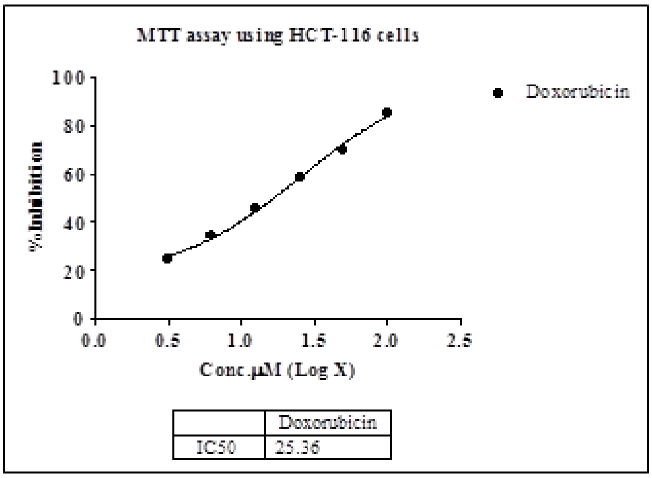

Fig 3: MTT assay using Standard

Conclusion

Based on the results and discussions on this study it can be concluded that leaf and bark extracts of *G. morella* showed the presence of various secondary metabolites and dose dependent cytotoxic activity towards HCT-116 cell line. Future efforts may be directed towards purifying the active principle and determining the specific cell signalling pathways involved in cancer cell toxicity.

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