ORIGINAL ARTICLE

Evaluation of the Cytotoxic Effects of Thorn Extracts from Medicinal Plants on the sf21 Cell Line

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Abstract

Objective: The present study was aimed to screen the cytotoxic effect of thorn extracts of seven different plants. **Methodology:** The methanol extract of thorn of seven different plants were investigated for cytotoxic activity on Sf21 cell line using MTT (3-(4, 5-dimetylthiazol-2-yl)-2 and 5-diphenyltetrazolium bromide) assay. After exposure of the cell line at different concentrations to the plant extract (1.56–500 µg/mL), it was found that percentage of cell viability decreased in dose dependent manner. **Results:** The extract of *Acacia ferruginea* showed potential cytotoxic activity with IC50 value of 92.15 µg/mL against Sf21 cell line. Among the studied plants *Limonia aciddisima* (L), *Gymnosporia senegalensis* (L), *Acacia nilotica* and *Acacia catechu* (L) extracts were exhibited lesser cytotoxic activity, whereas *Acacia senegal*, *Aegel marmelos* and *Acacia ferruginea* were exhibited maximum cytotoxic activity on the Sf21 cell line and these 3 plant extracts has shown potent IC_{50} value of 188.5 µg/mL, 307.6 µg/mL, 92.15 µg/mL respectively on Sf21 cell lines when compared to control Benzonitrile drug. It is clear from the study that Acacia ferruginea is more cytotoxic to Sf21 cell lines compared to other plant extracts. The GC–MS study of *Acacia feruginea* showed that it has 37 bioactive components, of which some of the bioactive compounds are methyl mannose (57.14), phenol (8.07), cyclopropane carboxylic acid, benzene-di-carboxylic acid (0.31), hydroxy gamma butyrolactone (0.38), gamma sitosterol (3.52), Hexacosanol (3.83), and stigmasterol (4.65). **Conclusion:** The bioactive components found in *Acacia ferruginia* are cytotoxic and insecticidal to other pest. It shows that thorn not only defends themselves against the herbivores, but the specialized bioactive presence also plays a major defensive role. Hence, some of the bioactive can be isolated, characterized, and be used as a potential herbicides.

Key words: GC-MS, MTT assay, secondary metabolites, Sf21 cell line, thorn

INTRODUCTION

Herbivore is a major threat to plants growth and development, plants defend themselves by physical as well as chemical barrier. Chemical defense is by producing secondary metabolites such as phenolic compounds, flavonoids, alkaloids, and lignin's. They may act by being cytotoxic to plants by affecting the metabolic pathway or being unpalatable to insects, in which they are produced.[1]

Spinescence such as thorn and prickles is physical defense structures of plants.[2] Plant defends themselves against the herbivores not only by having mechanical structures on their surface such as hairs, trichomes, thorn, spines, and thicker leaves but also by production of toxic chemicals such as terpenoids, alkaloids, anthocyanins, phenols, and quinones that either kill or retard the development of the herbivores. Chemical defense was provided by secondary metabolites in them.[3] Morphological and biochemical defenses in plants are important to withstand insect attack. Although plants primarily defend them by having mechanical structure against insect pests, the biochemical-based defense is considered to be more effective as insect growth and development is directly affected.

Plants have evolved various mechanisms to defend themselves in response to insect's attack, such as structural traits acting as a barrier to attachment of insect pest, feeding, and oviposition in addition to production of specialized metabolites. Throughout

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history, the specialized bioactives have been exploited by man for their anti-insecticidal properties. Furthermore, commercial insecticides are often based on plant-secondary metabolites and adapted further to give them the physicochemical properties that render them systemic. Some of the bioactives that are toxic to arthropods which are produced constitutively or induced by herbivore damage include alkaloids, benzoxazinoids, glucosinolates, and terpenoids.[4-6] Specialized metabolites for which insecticidal or anti-insect activity has most often been proven are isoprene derived terpenoids followed by alkaloids and phenolic compounds.[7] Moreover, synergistic effect among different defensive components enhances the defensive system of plants against the herbivores invaders.[8,9] Among the endogenous secondary metabolites, phenol is considered as one of the most common and widespread group of defensive compounds, which play a major role in HPR against herbivores, including insects.[10,11] Oxidation of phenols catalyzed by polyphenol oxidase (PPO) and peroxidase (POD) is a potential defense mechanism in plants against herbivorous insects. Quinones formed by oxidation of phenols bind covalently to leaf proteins and inhibit the protein digestion in herbivores.[12] In addition, Quinone's also exhibits directtoxicity to insects.[13] When phenol is ingested by the herbivores insect it exert toxicity and certain phenolic compound was produced by the larvae of the oriental leaf-worm *Spodoptera litura*, when feeding on *capsicum annum*.[14] Flavonoids are said to be cytotoxic and interact with different enzymes through complexation, protect the plant against insect pests by influencing the behavior, growth, and development of insects.[15] Moreover, synergistic effect among different defensive components enhances the defensive system of plants against the herbivores invaders. Terpenoids are said to exhibit acute toxicity to insects. Terpenoids along with other metabolites exhibited synergistic effect with ten times increase in mortality on *Spodoptera litura* to plants are able to identify the compounds in insect oral secretions, which elicit more intense volatile responses than mechanical damage alone.[16-18] Plant also defend by the formation of fatty acid-amino acid conjugates (FACs), the precursor of both plant and herbivores which were the first compound to be identified in *Spodoptera exigua* (beet armyworm) oral secretions. As *Spodoptera littorals* (Lepidoptera:Noctuid) is one of the most serious pests which are highly polyphagia, voracious and its worldwide spread. It can attack 87 species of economically important plants. Hence, Spodoptera insect cell line was used for the cytotoxic studies.

MATERIALS AND METHODS

Collection and processing of thorn

The plants having thorn belonging to different families were collected from Dhanvantri forest, Bangalore, authenticated from the Department of Botany, St. Joseph Autonomous College, Bangalore, India. The thorn to be investigated was dismantled from the collected plants, washed with tap water, wiped with tissue paper and allowed to shade dry. Around 20 g of thorny plant material was weighed, powdered, and dissolved with 125 ml of methanol, kept for 4h extraction on water bath at 50°C, filtered using Whatmann filter paper No.1. Methanol filtrate was reduced by evaporation and stored in refrigerator for further use.

Cell lines and culture medium

Sf-21 cell line procured from American type culture collection, stock cells were cultured and supplemented with 10% inactivated Fetal Bovine Serum (FBS), penicillin (100 IU/ml), and streptomycin (100 µg/mL) in a humidified atmosphere of 5% CO_2 at 37°C until confluent. The cell was dissociated with cell dissociating solution (0.2% trypsin, 0.02% EDTA, and 0.05% glucose in PBS). The viability of the cells is checked and centrifuged. Further, 50,000 cells/well was seeded in a 96 well plate and incubated for 24 h at 37°C, 5% CO_2 incubator. For cytotoxicity studies, 32 mg/ml stocks were prepared using DMSO. Serial two fold dilutions were prepared from 320 µg/mL to 10 µg/mL using Mitsuhashi and Maramorosch plain insect media for treatment.

MTT cell viability assay

Cell viability of Sf21 cells was determined by MTT (3-(4, 5-dimetylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide) assay.[19] Raw cells of Sf21 was dissociated with cell dissociating solution (0.2% trypsin, 0.02% EDTA, and 0.05 % glucose in PBS). The viability of the cells was checked and centrifuged. The cells were seeded at the density of 5×10^4 cells/well in 96-well culture plates and incubated for 24 h (at 37°C and 5% CO_2), 100 µl of different concentrations of test drugs or plant extract were added on to microtiter plates. The plates were then incubated at 37°C for 24 h in 5% CO_2 atmosphere. After incubation, the test solutions in the wells were discarded and 100 µl of MTT (5 mg/10 ml of MTT in PBS) was added to each well. The plates were incubated for 4 h at 37°C in 5% CO_2 atmosphere. The supernatant was removed and 100 µl of DMSO was added and the plates were gently shaken to solubilize the formed formazan. The absorbance was measured using a microplate reader at a wavelength of 590 nm. The percentage growth inhibition was calculated using the following formula and the concentration of the extract needed to inhibit cell growth by 50% (IC_{50}) values is generated from the dose-response curves for the cell line. The % inhibition is calculated using this formula.

% Inhibition = (OD of Control – OD of sample/OD of Control) × 100

GC–MS analysis

GC–MS analysis of *Accacia ferruginea* extract was performed using a thermo GC–MS Clarus 500 (Perkin Elmer). For MS detection, the MS DSQ II electron ionization mode with ionization energy of 70 eV was used, with a mass range at m/z 50–650. Restek RtxR-5M Scapillary

column (30 m × 0.25 mm, film thickness = 0.25) 5% diphenylamine/95% dimethyl polysiloxane) was used for the analysis. The initial column temperature was programmed at 60°C/5 min, respectively. The GC injector and MS transfer line temperatures were set at 280°C and 290°C, respectively. GC was performed in the splitless mode. Helium (at flow rate = 1.0 ml/min) was used as the carrier gas. A 1.0 µL injection volume was used. Major and essential compounds were identified by retention times and mass fragmentation patterns using data of standards from the National Institute of Standards and Technology (NIST).

Statistical evaluation

IC_{50} values for cytotoxicity tests were derived from a non-linear regression analysis (curve fit) based on sigmoid dose response curve (variable) and computed using Graph Pad Prism 6 (Graphpad, San Diego, CA, USA). All data values are expressed as mean (±SD).

Non-linear regression

In statistics, non-linear regression is a form of regression analysis, in which observational data are modeled by a function which is a nonlinear combination of the model parameters and depends on one or more independent variables. The data are fitted by a method of successive approximations

RESULTS

Cytotoxicity assay of plant extracts

To evaluate the cytotoxic effect of seven different methanolic extracts of the thorn of plants, MTT assay was employed. After 24 h of incubation, cell viability was determined by the MTT assay. Among all the tested samples, *Acacia ferruginea* had shown the highest % inhibition of 81.27 on Sf21 cell line in comparison with Benzonitrile as control which has shown 87.07% inhibition at 320 µM concentration. The treatment was carried out in triplicates. All data values are expressed as mean (±SD). The extracts induced cell cytotoxicity in a dose dependent manner. The results of cytotoxicity assay are presented in [Table 1 and Figure 1]. *Acacia ferruginia* showed highest toxicity than other plants extract probably due to presence of the good number of toxic bioactives which supports its activity. The other extracts showed average activity, whereas *Gymnosporia senegalensis* has shown least activity with 36.22 % inhibition.

GC–MS analysis of *Acacia ferruginea*

The GC–MS chromatogram of the extract is shown in Figure 2. GC–MS analysis resulted in identification of 36 different metabolites. Compound identification was done in comparison with the reference standards present in NIST and Wiley 9.0. It is found that some of the bio-actives components are notably responsible for the cytotoxicity activity shown. Some of the bioactive were analyzed with their respective percentage area present. Important compounds identified include methyl mannose (57.14), phenol (8.07), benzene-di-carboxylic acid (0.31), hydroxy gamma butyrolactone (0.38), gamma sitosterol (3.52), 1-hexacosanol (3.83), imidazole, 2-amino-5-[(2-carboxy) vinyl] (0.60), stigmasterol (4.65), Ergost-5-en-3-ol, (3.beta.) (2.53), and Lupeol (3.60).

Table 1: Cytotoxicity study of standard Benzonitrile

Compound	Concentration in µM	OD at 590 nm	% inhibition
Control	0	0.758	0
Benzonitrile	10	0.648	14.51
	20	0.594	21.63
	40	0.464	38.78
	80	0.337	55.54
	160	0.247	67.41
	320	0.098	87.07

DISCUSSION

Seven different methanolic extracts were screened for cytotoxic assay. *Acacia ferruginea* had shown the highest % inhibition of 81.27 on Sf21 cell line at 320 µM concentration and *Gymnosporia senegalensis* has shown least activity with 36.22% inhibition. Few researchers also worked on the cytotoxic studies using *in vitro*, *in vivo,* and *in silico* methods supporting the use of selected plants for its activity.[20-23]

MTT assay is most commonly and effectively employed assay due to its simplicity and also effective. It is best method to be applied for anti-inflammatory and anti-cancer studies for almost any type of the test samples at initial stages.

The water-soluble MTT can be readily taken up by the cells which are viable. MTT is dependent on the ability of mitochondrial enzyme dehydrogenase to be taken up living cells. During the reaction, MTT is reduced to blue color formazan which is water insoluble, and hence, it has to be dissolved further for measurement in calorimeter. The dissolvants include propanol, ethanol, acid- isopropanol, acid- isopropanol- 10% Triton X, DMSO, or mineral oil. DMSO is the most preferred choice of most researchers.

The GC–MS study of *Acacia feruginea* showed that it has 37 bioactive components, of which some of the bioactive compounds are methyl mannose (57.14), phenol (8.07), cyclopropane carboxylic acid, benzene-di-carboxylic acid (0.31), hydroxy gamma butyrolactone (0.38), gamma sitosterol

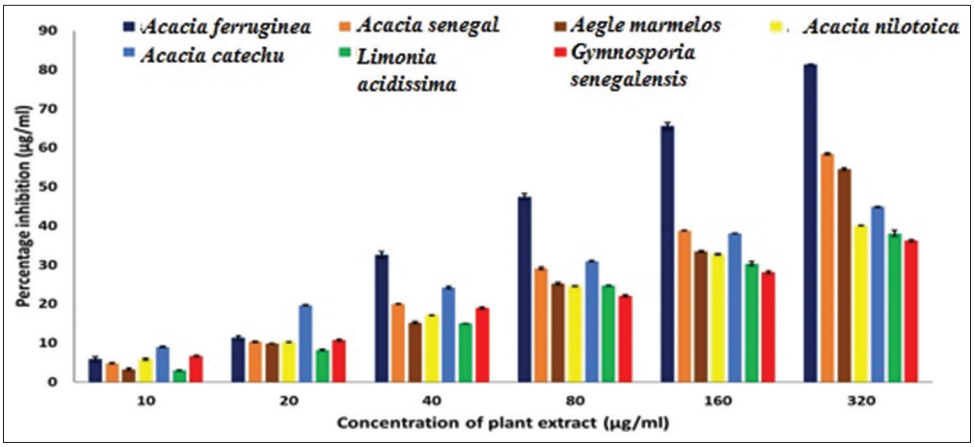

Figure 1: Comparison of % inhibition of all plant extracts against Sf21 cell line

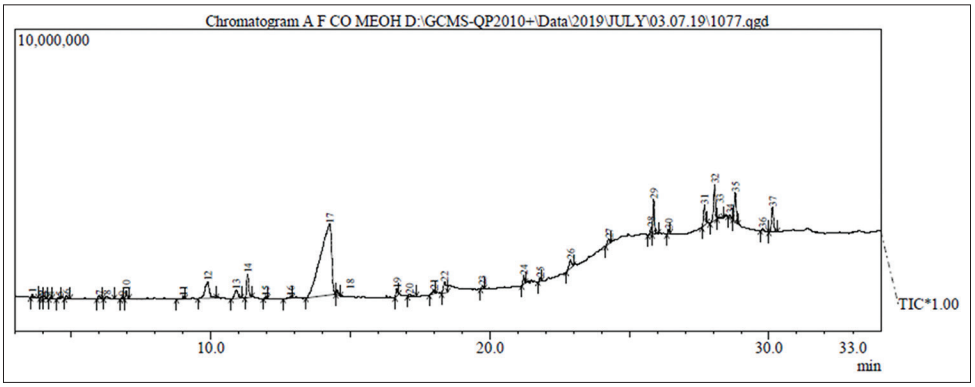

Figure 2: GC–MS analysis of methanolic extract of *Acacia ferruginea*

(3.52), hexacosanol (3.83), and stigmasterol (4.65). There are no reports found associated with all these compounds with the selected plant extract. Patil *et al*.[24] reported similarly compounds in petroleum ether extract in *Citrus medica* seeds. The use of such constituents or the application of these novel dietary supplements to prevent human physiological issues due to presence of potential antioxidants.

The essential target of cancer through chemotherapy primarily is to target them specifically against only cancer cells but not affecting the normal body cells. However, it is still important step to further analyze the cytotoxic effects of the extracts on other cell lines of cancer in have a holistic understanding of their action. Furthermore, the chemical principle which is accountable for its cytotoxic effects is yet to be substantiated.

CONCLUSION

The present study involved the use of methanol extracts to evaluate its cytotoxic activity against Sf21 cell line. It was observed that the methanol extracts of Acacia ferruginea displayed noticeable cytotoxicity against the insect cell lines. This study reveals Acacia ferruginea as synthesizer of secondary metabolites with significant bioactivities, which are cytotoxic to insect cell lines. Hence, some of the bioactives can be isolated, characterized further to be used as potential cytotoxic agents in cancer treatment.

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