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Evaluation of Phytochemical, Antiproliferative and Larvicidal Activity of Gliricidia Sepium Leaves

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ABSTRACT

Medicinal plants based drugs and chemicals used for curing enormous ailments and it help for the invention of modern pharmaceuticals. The target of this present study is to find out the bioactive compounds and to evaluate antiproliferative and larvicidal effect on *Gliricidia sepium* leaves. Phytochemical determination was oriented to search secondary metabolites and this preliminary study shows *Gliricidia sepium* it has alkaloid, flavanoids, phytosteroids, steroids, tannins, terpenoids, mucilage, and coumarins. In this Research a significant cytotoxic effect was observed in *Gliricidia sepium* methanolic extract against Adenocarcinoma gastric cells. The larvicidal activity was observed by using aqueous and methanolic plant extracts on Anopheles mosquitoes and shows a high mortality rate on 48 hours after the plant extracts exposure. The result of this study shows this plant has significant phytochemicals and potential antiproliferative and Larvicidal effects.

Keywords: Pharmaceuticals, Adenocarcinoma Gastric cells, *Gliricidia sepium,* Mortality rate, Larvicidal and Antiproliferative

I. INTRODUCTION

Phytochemical screening is a method which exposes or reveals certain components or properties readily available in plants for bio-activity or ethno-medical applications. The medicinal plants play an important role in the development of newer drugs because of their effectiveness, less side effects and relatively low cost when compared with synthetic drugs (**Raj *et al.*,2011**)The present study aims in exploring the phytochemical constituentsof the crude leaf extracts by using their crude extracts and fraction of *Gliricidia sepium.*

Binomial name:

Gliricidia sepium

Taxonomic classification:

Kingdom	Plantae
Species	G.Sepium
Genus	Gliricidia
Family	Fabaceae
Subfamily	Faboideae
Tribe	Robinieae

Vernacular names

Tamil	Seemai agathi
English	Mother of cocoa
Bengali	Saranga
Kannada	Gobbardamara
Malayalam	Siima konna
Marathi	Giripushpa
Telugu	Madri

(*G. sepium*) was introduced to the Philippines and is native to the American continent. It is used as shade for cocoa and coffee plantations in Mexico that is why it is called madre de cacao. It is also used as a poison for rodents **(Delizo RL *et al.*,1974)** The traditional use of branches and leaves of *G. sepium* is against pruritic ailments, fever and it is one of the most frequently used plants for skin infections **(Gomez-estrada H *et al.*,2011)**. This study aimed to evaluate the cytotoxic activities of *G. sepium* leaves against selected human cancer cell.

Gastric cancer is a malignant tumor with the fifth incidence and third mortality worldwide. There were 951,000 new cases and about 723,000 patients died of it in 2012. Undoubtedly, Adenocarcinoma gastric cancer has been affecting people's living standards, and a major public health problem **(Torre La *et al.*,2015)**. The five-year survival rate of people is still very low in patients with serious gastric cancer. The main reason may be lack of high specificity and high sensitivity for early detection while the pathogenesis of the disease is still not fully understood **(Dang y *et al.*,2017)** Therefore, there is a dire need to discover some early detection methods or biomarkers to increase the detection rate of gastric cancer and reduce the incidence and mortality. Without doubt, gastric cancer puts lots of pressure on human life. Its epidemiology trend was affected by region, age and gender **(Bray F *et al.*,2018).**

In the past several decades, a number of synthetic chemical insecticides have been developed and effectively used to control mosquitoes. Unfortunately, the application of such chemical insecticides has resulted in long term harmful effects on the environment and non-target organism including human beings. In addition, the management of these disease vectors using synthetic insecticides has failed in part due to their efficiency in attaining physiological resistance. Herbal plants or botanical medicines have been used traditionally by herbalist worldwide for the prevention. Plants are rich source of bioactive chemical compounds with insecticidal properties. The activity of crude plant extracts is often attributed to the complex mixture of active compounds. Crude extracts of leaves or bark of these plants have been tested earlier by several investigators. The plant *Gliricidia sepium* (fabaceae) traditionally claimed to possess larvicidal activity **(Jayakumar *et al.*,2016) (Shad.A *et al.*,2013) (Kumar kp *et al.*,2014)**. Therefore, the potential for exploiting these essential oils for vector control, can be taken into account.

COLLECTION OF PLANT MATERIALS

The selected medicinal plant *Gliricidia sepium* was collected from Thiruvarur (Dt), Tamil Nadu, India. The plant leaf was first washed well and cleaned leaves were dried at room temperature. These dried materials were macerated to powder form with a mixer grinder and stored in air tight container for further use.

PREPARATION OF THE EXTRACT

The coarsely powder was packed into soxhlet column and extracted with 70% methanol for 48 hours (64.5-65.5^{o}c). The extract was concentrated under reduced pressure at 40^{o}c using rotary evaporator and stored in a refrigerator at 2-8^{o}c for use in subsequent

experiment. Aqueous extract was prepared by using distilled water it also used in experiment purpose.

QUALITATIVE ANALYSIS OF PHYTOCHEMICALS

Chemical tests for the screening and identification of phytochemicals in methanolic and aqueous plant extract were carried out using standard procedures as described.

TEST FOR ALKALOIDS

The extract was evaporated in a test tube. To the residue dilute HCL was added, shaken well and filtered. With the filtrate following tests were performed:

- **Hager's test**

To the 2-3 ml of filtrate, Hager's reagent was added. Yellow precipitate was formed showing the presence of alkaloids.

- **Mayer's test**

To the 2-3 ml of filtrate, Mayer's reagent was added. Formation of yellow precipitate showed the presence of alkaloids.

TEST FOR FLAVONOIDS

- **Shinoda test**

To the extract, added 5 ml of 95% ethanol and few drops of conc.HCL. To this solution, 0.5g of magnesium turnings was added. Observance of pink coloration indicated the presence of flavonoids.

- **With lead acetate**

To the small quality of extract, lead acetate solution was added. Formation of yellow colour. This yellow colour gets decolorized after the addition of acid.

- **With sodium hydroxide**

On addition of an increasing amount of sodium hydroxide, the extract showed yellow colour. This yellow colour gets decolorized after the addition of acid.

TEST FOR CARBOHYDRATES

- **Fehling's test**

1ml of Fehling's A, and 1ml of Fehling's B solution were mixed and boiled for one minute. Now the equal amount volume of test solution was added to the above mixture. The solution was heated in boiling water bath for 5-10 minutes. First yellow, then brick red precipitate was observed, indicates the presence of carbohydrates.

- **Benedict's test**

Equal volume of Benedict's reagent and test solution were mixed in a test tube. The mixture was heated in boiling water bath for 5 minutes. Solution appeared green showing the presence of reducing sugar.

TEST FOR PHENOLS

- **$FECl_3$ solution test**

On addition of 5% $Fecl_3$ solution to the extract, a deep blue black colour appeared.

TEST FOR GLYCOSIDES

- **Borntrager's test**

To the 3ml of extract H_2SO_4 was added. The solution was then boiled and filtered. The filtrate was cooled and to it equal volume of benzene was added. The solution was shaken well and the organic layer was separated. Equal volume of dilute ammonia solution was added to the organic layer. The ammonical layer turned pink showing the presence of glycosides.

- **Legal's test**

To the concentrated extract few drops of 10% NAOH were added, to make it alkaline. Then freshly prepared sodium nitroprusside was added to the

solution. The presence of blue coloration indicated the presence of glycosides in the extract.

- **Keller-killiani test**

To 2ml of the extract glacial acetic acid, one drop 5% $Fecl_3$ and concentrated H_2SO_4 was added. Reddish brown colour appeared at junction of two liquid layers and upper layer turned bluish green indicating the presence of glycosides.

TEST FOR SAPONINS

To 2ml of plant extract, 2ml of distilled water was added and shaken in a graduated cylinder for 15 minutes lengthwise. Formation of 1cm layer of foam indicates the presence of saponins.

TEST FOR PHYTOSTEROIDS

To 1ml of plant extract, equal volume of chloroform was added and subjected with few drops of concentrated sulphuric acid. The appearance of bluish brown ring indicates the presence of phytosteroids

TEST FOR STEROIDS

The test solution was treated with minimum amount of chloroform to which three drops of acetic anhydride and two drops of concentrated sulphuric acid were added. Appearance of purple colour changing to blue or green denotes the presence of steroids.

TEST FOR TANNINS

To 1ml of plant extract, 2ml of 5% ferric chloride was added. Formation of dark blue or greenish black indicates the presence of tannins.

TEST FOR TERPENOIDS

To 0.5ml of extract, 2ml of chloroform was added and concentrated sulphuric acid was added carefully. Formation of red brown colour at the interface indicates presence of terpenoids.

TEST FOR FIXED OILS AND FATS

Small quantities of extracts were pressed between two filter papers. An oily stain on filter paper indicates the presence of fixed oil.

TEST FOR GUMS AND MUCILAGE

About 10 ml of extract were slowly added to 25ml of absolute alcohol under constant stirring, precipitation indicates the presence of gums and mucilage.

TEST FOR COUMARINS

To 1ml of extract, 1ml of 10% NaOH was added. Formation of yellow colour indicates presence of coumarins.

TEST FOR CHLOROGENIC ACID

The aqueous and alcoholic powder solution is treated with aqueous ammonia and are exposed to air with gradually develops a green colour indicates the presence of chlorogenic acid.

MTT ASSAY

The cytotoxicity of *Gliricidia sepium* treated on AGS cells was determined by the method of **Mosmann, (1983).**

PRINCIPLE

The yellow 3-4, 5dimethylthiozol-2-Yl)-2,5diphenyltetrazoliumbromide (MTT) is reduced by mitochondrial dehydrogenase of viable cells yielding a measurable purple formation product. Viable cells contain NAD (P) H-dependent reductase, which reduce the MTT reagent to formazon, with a deep purple colour. Formazon crystals are then dissolved using solubilizing solution and absorbance is measured at 500-600 nm by plate reader.

REAGENTS

MTT STOCK SOLUTION

MTT (50 mg) dye was dissolved in 10 mL of PBS. After vortexing for 1 min, it was filtered through 0.45 micro filters. The bottle was wrapped with aluminium foil to prevent light, as MTT was light sensitive. The preparation was stored at 4°C.

PROCEDURE

Cell viability assay, AGS viable cells were harvested and counted using hemocytometer diluted in DMEM medium to a density of 1×10^4 cells/ml was seeded in 96 well plates for each well and incubated for 24 h to allow attachment. After AGS cells treated with control and the containing different concentrations of *Gliricidia sepium* methanolic extract 50 to 300 µg/ml were applied to each well. AGS cells were incubated at 37°C in a humidified 95% air and 5% CO_2 incubator for 24 h. After incubation, the drug-containing cells wash with fresh culture medium and the MTT (5 mg/ml in PBS) dye was added to each well, followed by incubated for another 4 h at 37°C. The purple precipitated formazan formed was dissolved in 100 µl of concentrated DMSO and the cell viability was absorbance and measured 540 nm using a multi-well plate reader. The results were expressed at the percentage of stable cells with respect to the control. The half maximal inhibitory concentration (IC_{50}) values were calculated and the optimum doses were analyzed at different time period.

$$\text{Inhibitory of cell proliferation (\%)} = \frac{\text{Mean absorbence of the control} - \text{Mean absorbence of the sample}}{\text{Mean absorbence of the control}} \text{ X 10}$$

LARVICIDAL ACTIVITY

SELECTION, COLLECTION AND CULTURE OF MOSQUITOES

Vector species *Mosquito* was selected for the present study. *Mosquito* is an epidemiologically important vector involved in the transmission of many viral pathogens including yellow fever, dengue and chikungunya. As a vector species, the growth and multiplication of *Mosquito* has proven to be very difficult to suppress or control due to their remarkable ability to adapt to various environments, their closeness with humans and their effective reproductive biology. Mosquito larvae was cultured and maintained in the bottle at room temperature with potato extract kept at varying distances around households, were used in the present study. Collected larvae were taken into the laboratory and subjected to species level identification using standard manual .The screened larvae were maintained at 28 ± 2 °C and subjected for larvicidal activity.

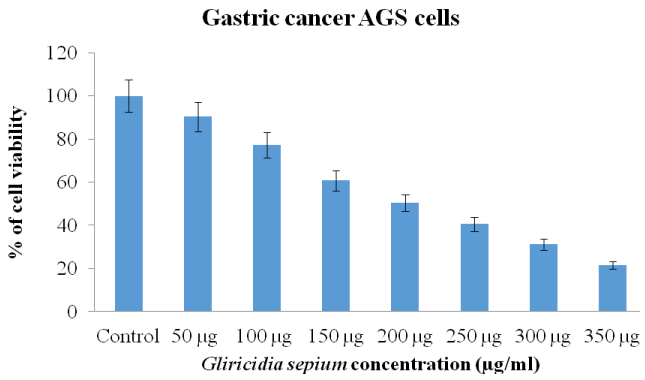
LARVICIDAL BIOASSAY:

Bioassay for the larvicidal activity was carried out using WHO procedure with minor modifications. Twenty larvae, each were introduced into treatment trays containing 50ml of water. To the treatment set, respective concentrations of the plant extract (20µl, 40 µl, 60 µl, 80 µl and 100µl) were added from the stock solution; a control was maintained, containing only larvae and 1 ml of methanol. Mortality counts of larvae were monitored at regular intervals i.e. 6, 12, 24, 48 Hours after treatment. Larvae were considered dead if they settle and remain motionless in the bottom of the test beaker with no response to light or mechanical stimulus or not recovering life functions even after being transferred to their growth medium.

Table 1. 5.0 Qualitative phytochemical screening of leaves of ***GLIRICIDIA SEPIUM***

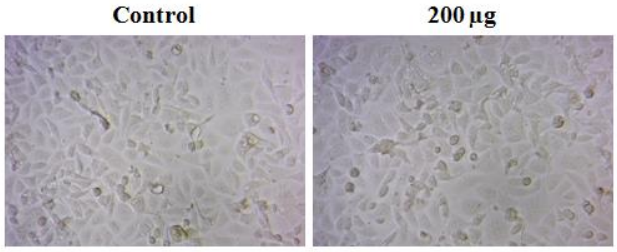
Table 1 represented the qualitative analysis of phytochemical constituents of *Gliricidia sepium* And the result revealed that the presence of Alkaloids, Flavonoids, Coumarins, Terpenoids, Tannins, Steroids, Phytosteroids, and absence of Carbohydrates, saponin, Phenols, Glycosides Chlorogenic acid, in both methanolic and aqueous solution.

Anti-proliferative effects of *Gliricidia sepium* on the activity of cytotoxicity in AGS cells.



The AGS cells were treated with increasing concentration of *Gliricidia sepium* (50-350 µg/ml) for 24 h and the results are expressed as a percentage of the control value in presenting as a cell cytotoxicity ratio for AGS cells using MTT assay. Data were presented as mean ± SD asterisks indicate statically different experiments compared to control.

Morphological changes in control and *Gliricidia sepium* treated AGS cells for 24 h



S.NO	CONCENTRATION (ppm)	MORTALITY RATE (24 h)(100%)	MORTALITY RATE(48h)(100%)
1.	2000ppm	10%	20%
2.	4000ppm	25%	30%
3.	6000ppm	37%	40%
4.	8000ppm	45%	50%
5.	10000ppm	57%	60%

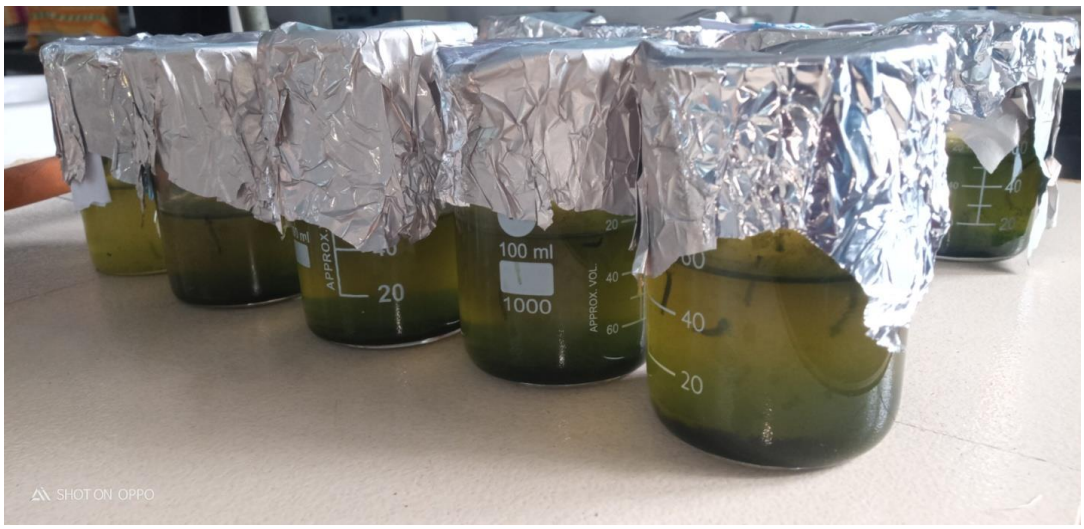
Photomicrograph represents morphological changes in AGS cells such as shrinkage, detachment, membrane blebbing and distorted shape induced by *Gliricidia sepium* methanolic extract of treatment (200 µg/ml for 24 h) as compared with control. Control showed normal intact cell morphology and their images were captured by light microscope.

MTT assay								
	Control	50 µg	100 µg	150 µg	200 µg	250 µg	300 µg	350 µg
Mean	109	92.75	76.46	61.37	49.14	41.31	31.73	22.94
	91	77.43	63.84	51.23	41.02	34.49	26.49	19.16
	100.1	85.18	70.22	56.36	45.13	37.94	29.14	21.07
Average	100.0333	85.12	70.17333	56.32	45.09667	37.91333	29.12	21.05667
SD	9.000185	7.660176	6.310129	5.070118	4.060103	3.410078	2.620057	1.890035

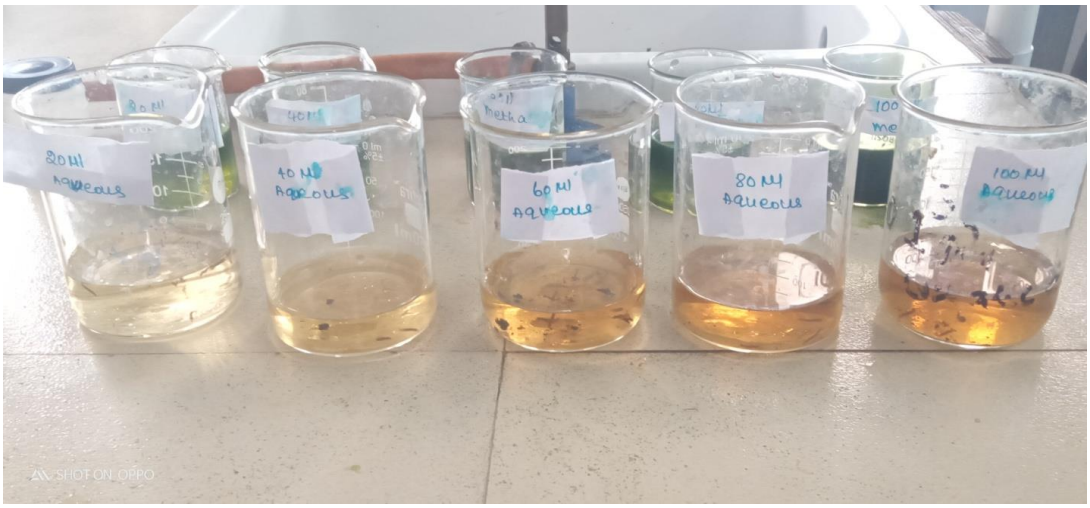
LARVICIDAL BIOASSAY

LARVICIDAL EFFECT OF AQUEOUS EXTRACT OF GLIRICIDIA SEPIUM

S.NO	CONCENTRATION(ppm)	MORTALITY RATE(24h)(100%)	MORTALITY RATE(48h)(100%)
1.	2000ppm	10%	25%
2.	4000ppm	20%	35%
3.	6000ppm	30%	45%
4.	8000ppm	40%	55%
5.	10000ppm	50%	65%



Larvicidal activity by methanol extract of Gliricidia sepium leaves



Larvicidal activity by aqueous extract of Gliricidia sepium leaves

II. RESULTS AND DISCUSSION

The phytochemical constituents present in the leaf extracts of *Gliricidia sepium*. The phytochemical studies of all the three extracts conclude that acetone and water extracts of leaf samples had more positive results for glycosides, oils, sapponins and flavonoids .Preliminary phytochemical analysis revealed the presence of six compounds viz. flavanoids, glycosides, oils, sapponins, phenolics, gum and mucilage. Acetone and chloroform extracts gave positive results for flavanoids, glycosides, phytosterols, oils and sapponins. Traditionally sapponins have been extensively used as detergents, pesticides as well as mollucides, in addition to their industrial application such as foaming, surface active agents etc and also found to have beneficial health effects **(Arunasalam JK 2004**). The plant is reported to contain glycosides, alkaloids, sapponins, flavonoids, tannins, carbohydrates, phenolic compounds and phytosterols by premier workers.

Human gastric cancer AGS cell lines were procured from the cell repository of National Centre for Cell Sciences (NCCS), Pune, India. Dulbecco`s Modified Eagle Media (DMEM) was used for maintaining the cell line, which was supplemented with 10% Fetal Bovine Serum (FBS). Penicillin (100 U/ml), and streptomycin (100 µg/ml) were added to the medium to prevent bacterial contamination. The medium with cell lines was maintained in a humidified environment with 5% CO_2 at 37°C.

The IC_{50} values were determined from the *Gliricidia sepium* dose responsive curve where inhibition of 50% cytotoxicity compared to control cells. All experiments were performed at least three times in triplicate.

The AGS cells were treated with increasing concentration of *Gliricidia sepium* methanolic extract (50-350 µg/ml) for 24 h and the results are expressed as a percentage of the control value in presenting as a cell cytotoxicity ratio for AGS cells using MTT assay. Data were presented as mean ± SD asterisks indicate statically different experiments compared to control (**Mosmann T 1983)**

Statistical analysis

The values are expressed as mean ± SD. The statistical comparisons were performed by one way analysis of variance (ANOVA) followed by Duncan's Multiple Range Test (DMRT), using SPSS version 12.0 for windows (SPSS Inc. Chicago; http://www.spss.com). The values are considered statistically significant if the p value was less than 0.05.

In the present study, an attempt has been made to untap one of the fabaceae family that is *Gliricidia sepium* for its larvicidal potential. In this study the efficacy of the volatile oil from the leaves was evaluated against fourth Instar larvae of Aedes aegypti at various concentrations of 10, 25, 50, 100, 250 mg/ml. The results were shown in table 1. The essential oil was evaluated at 0, 1^{st}, 2^{nd}. 3rd, 4 th, 6th, 12th and 24th hour. The evaluation clearly shows volatile oil exhibits larvicidal property dose-dependently. A dose-dependent effect on mortality was recorded with increasing concentrations of essential oil and compounds increasing the mortality of the larvae. Larvicidal bioassays revealed that 24 h mortality rate of the whole essential oil were 250 mg/ml. Plant-derived toxicants are valuable source of potential insecticides. They play a major role in mosquito control programs in near future. So, there is always a tremendous need in plant insecticides throughout the globe. These plant-derived insecticides are effective against specific target insects, less expensive, easily biodegradable and to non-toxic products. In the present study the methanolic extract of leaves of *Gliricidia sepium* showed the higher larvicidal activity than aqueous extract.

III.SUMMARY AND CONCLUSION

Methanol and aqueous extract of *Gliricidia sepium* was prepared based on standard protocols and they were analysed for their following tests.

Preliminary phytochemical analysis of aqueous extract and methanol extract of *Gliricidia sepium* showed positive results for Alkaloids, Flavonoids, Coumarins, Terpenoids, Tannins, Steroids, Phytosteroids, Saponins.
MTT assay was performed in the methanolic extract of *Gliricidia sepium* leaves.

For larvicidal bioassay, WHO standard procedure was followed with slight modifications and observed after 24 hours and 48 hours. At the end of 24 hours according to extract concentration the mortality rate, at 2000ppm and 3000ppm concentration only 10% to 25% of mortality was observed. In 10000ppm concentration 60% of mortality was observed, whereas after 48hours all the larvae were died irrespective of concentrations. Based on the present study, methanol extract of *Gliricidia sepium* could be used as a best larvicidal drug candidate.

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