



Anti-cancer effect of *Gymnema sylvestre* Leaf Extract against MG63, Human Osteosarcoma cell line - An *in vitro* analysis

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ABSTRACT

Background: *Gymnema sylvestre* also known as 'GURMAR' is one of the important herbs in the Ayurvedic system of medicine and Indian proprietary medicines.

Objective: In the present investigation, phytochemical screening, free radical scavenging, anti-inflammatory and anti-cancer activities were evaluated *in vitro* in the aqueous extract from the leaves of *Gymnema sylvestre* (AGS).

Methods: Leaf samples were subjected with four different solvents (ethanol, acetone, ethyl acetate and distilled water) and the four extracts were screened for phytochemicals using different biochemical tests. AGS was subjected to fourier-transform infrared (FT-IR) spectroscopy to analyze its functional groups. Antioxidant activity of AGS was determined by measuring nitric oxide (NO) radical scavenging activity. The anti-inflammatory activity was studied using the inhibition of protein denaturation assay and anti-cancer activity was evaluated using MTT assay against MG63 cell lines.

Results: Phytochemical screening confirmed the presence of phytoconstituents like alkaloids, carbohydrates, triterpenoids, proteins, phenols and flavonoids. The FT-IR spectrum with different peak values revealed the presence of various functional compounds in AGS and it showed significant NO radical scavenging activity with the EC50 value of 401.66 µg/mL. AGS displayed strong anti-inflammatory activity (IC50 = 17.5 µg/mL). Moreover, AGS also exhibited significant dose-dependent anti-cancer activity against MG63 cell line and the IC50 value was 19.5 µg/mL.

Conclusion: The results indicate that *Gymnema sylvestre* can act as a promising free radical scavenger, anti-inflammatory and anticancer agent against MG63 human osteosarcoma cell lines. Hence, *G. sylvestre* can be used as natural antioxidants for preventing the oxidative stress related degenerative diseases..

Key Words: Anti-cancer, Anti-inflammatory, Free radicals, *Gymnema sylvestre*, MG63, Osteosarcoma

INTRODUCTION

Many important valuable medicinal compounds are present in the herbal plants which were proven in the ancient time by some traditional methods¹. There are about 45,000 plant species are found in India, in that majority of plant species possess pharmacological significance². Many plant species consist of bioactive constituents which are used directly as drugs or pharmacological agents. Phytochemicals act as antioxidants by neutralizing free radicals which damage DNA, proteins and lipids. 80% of human population relies on traditional system of medicine for primary health needs and these

systems are mainly based on medicinal plants^{3,4}. Due to the rise in population, there is demand in supply of drugs and also cost for treatments are increased. Sometimes, several synthetic drugs cause side effects and development of resistance to presently using drugs by microbes also increased, recently⁵. To overcome these problems, now a day, plant materials are used as a source of medicine for a wide range of human ailments. Today plant compounds provide the models for 50% of western drugs⁶.

Gymnema sylvestre (*G. sylvestre*) is a large tropical plant which is native to central and western India and also found

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in Africa and in Australia. *G. sylvestre* is one of the ayurvedic and medicinal herb, have been used for more than 2,000 years in India to treat diabetes. Now a day, there is a demand for *G. sylvestre* leaves are in pharmaceutical trade because it is an important anti-diabetic medicinal plant. This plant contains pentriacontane, hentriacontane, phytin, chlorophyll a, chlorophyll b, tartaric acid, formic acid, butyric acid, anthraquinone derivatives, inositol, d-quercitol, gymnemic acids⁷.

Osteosarcoma, the primary bone malignancy, is one of the most common cancers worldwide. Generally, chemotherapy with agents such as cisplatin, methotrexate, and cyclophosphamide is widely used for treating osteosarcoma⁸. Chemotherapy may result in drug resistance, as well as several side effects including drug-cytotoxicity which causes damage to normal tissues. Therefore, alternative treatments for osteosarcoma need to be considered. At present, cancer-fighting foods are being discussed as potential therapeutic products against osteosarcoma. Various nutritional and functional phytochemicals have been extracted from plants. These plant-derived substances also act as natural anticancer agents⁹.

Pharmacological studies reveal that *G. sylvestre* has some medicinal properties such as anti-diabetes, anti-microbial effect, anti-inflammatory, anti-cancer, anti-atherosclerotic effect, larvicidal effect and anti-hepatoprotective activity^{10,11}. However, the anticancer effects of *G. sylvestre* on human osteosarcoma have not been studied so far. Therefore, in the present study, we investigated No radical scavenging activity and anti-inflammatory activity of aqueous extract of *G. sylvestre* (AGS). This study was also focused on investigating the anticancer effects of AGS on human osteosarcoma MG63 cells.

MATERIALS AND METHODS

Plant material

The healthy and fresh leaves of *G. sylvestre* were collected from the farms of Coimbatore in the month of December 2018, Tamil Nadu.

Preparation of extracts

The collected leaves of *G. sylvestre* were shade dried and powdered, and extracted in a Soxhlet extractor using four different solvents such as distilled water (AGS), ethanol (EGS), acetone (AcGS) and ethyl acetate (EaGS). The solvent was evaporated under reduced pressure using a rotary evaporator at 45°C and the dried extracts obtained were stored at -20°C until further use.

Cell lines and Chemicals

MG63 cell lines (Human Osteosarcoma cell) were procured from National centre for cell sciences (NCCS), Pune, In-

dia. Cell lines were cultured in minimal essential medium supplemented with fetal bovine serum (FBS). Antibiotics, EDTA and glucose were purchased from Hi-Media laboratories Ltd., Mumbai. 3-(4,5-dimethyl thiazol-2-yl)-5-diphenyl tetrazolium bromide (MTT), MEM medium, trypsin, Fetal bovine serum, Phosphate buffered saline and other chemicals were purchased from Sigma-Aldrich (Bangalore, India)..

Qualitative analysis of phytochemicals

For preliminary phytochemical analysis, standard procedures were performed in different extracts of *G. sylvestre*. Phytoconstituents such as alkaloids, flavonoids, triterpenoids, phenols, carbohydrates, proteins, glycosides, saponins and steroids were examined by performing different phytochemical tests¹² [Edeoga *et al.*, 2005].

Fourier Transform Infrared spectral analysis

Fourier Transform Infrared Spectrophotometer (FT-IR) is the most powerful tool to identify the types of chemical bonds or functional groups present in the phytochemicals. The wavelength of light absorbed is the salient feature of the chemical bonds seen in the annotated spectrum. Dried powder of AGS was used for FT-IR analysis (IR-affinity 1, Sigma, Japan) using KBr pellet method¹³ with a Scan range from 4000 to 400cm⁻¹.

Estimation of total phenolic content

The total phenol in AGS was measured according to the method of Singleton and Rossi¹⁴. 1.0 mL of the extract was mixed with 1.0 mL of Folin-Ciocalteu's phenol reagent and 1.0 mL of saturated sodium carbonate (35%) was added to the mixture after 3 min. The mixture was made up to 10 mL by adding deionised water and kept for 90 min at room temperature in the dark. The absorbance was measured immediately against the prepared blank at 725 nm. Gallic acid was used as the reference standard. The total phenol content is expressed as milligrams of gallic acid equivalents (GAE) per gram of extract.

Estimation of total flavonoid content

Total flavonoid content in AGS was evaluated as described by Jia *et al*¹⁵. 0.25 mL of extract was diluted with 1.25 mL of distilled water and 75 µL of a 5% sodium nitrite were added. After 6 min, 150 µL of 10% aluminium chloride was added and mixed. After 5 min, 0.5 mL of 1 M sodium hydroxide was added. The absorbance was measured at 510 nm against the blank. Rutin was used as the reference standard. The total flavonoid content is expressed as milligrams of rutin equivalents (RE) per gram of extract.

Total antioxidant activity assay (Phosphomolybdenum assay)

The antioxidant activity of the sample was determined by phosphomolybdenum method¹⁶. An aliquot of 0.1 mL of sample solution was mixed with 1 mL of the reagent solution (0.6 M sulphuric acid, 4 mM ammonium molybdate and 28 mM sodium phosphate). The tubes were capped with silver foil and kept at 95°C for 90 min and cooled to room temperature. The absorbance was measured at 695 nm against a blank. Ascorbic acid was used as a standard and total antioxidant capacity was expressed as ascorbic acid equivalents (GAE) per gram of extract.

Nitric oxide scavenging activity assay

Nitric oxide (NO) released from sodium nitroprusside was evaluated using Griess reaction¹⁷. Different concentrations of AGS (dissolved in dimethyl sulfoxide) was added with sodium nitroprusside (5 mM) in phosphate-buffered saline and incubated at room temperature for 2.5h. After incubation, 500 µL of sample was diluted with 500 µL of griess reagent. The absorbance was measured at 695 nm against a blank. Gallic acid was used as the positive control. The percentage nitrite radical scavenging activity of AGS and gallic acid were calculated using the following formula: $[(A_{\text{control}} - A_{\text{test}}) / A_{\text{control}}] \times 100$, where A_{control} was the absorbance of the control, and A_{test} was the absorbance of the test.

In vitro anti-inflammatory activity – Protein denaturation method

Aqueous extract of *G. sylvestre* (0.05 mL) at various concentrations (20, 40, 60, 80, 100 µg/mL) and standard drug, diclofenac sodium (0.05 mL) at different concentrations (20, 40, 60, 80, 100 µg/mL) were taken separately and 0.5% BSA (0.45 mL) was added to both test and standard drug solution. Test control consisted of 0.05 mL of distilled water and 0.45mL of BSA. The samples were incubated at 37°C for 20 min and the temperature was increased progressively up to 57°C for 3 min. After cooling, add 2.5 mL of phosphate buffer to the above solutions after 20 min. The absorbance was measured at 416 nm using UV-Visible spectrophotometer¹⁸. The results were compared with the standard drug, diclofenac sodium. The percentage inhibition of protein denaturation can be calculated as.

Percentage Inhibition = $100 - [(A_{\text{test}} - A_{\text{standard}}) / A_{\text{test}}] \times 100$.

In vitro anti-cancer activity – MTT assay

MTT assay is a cytotoxic assay which is frequently used to identify the toxicity level of the test sample. The antiproliferative activity of AGS was measured using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. MG63 cell lines were used to evaluate the anti-cancer

activity. The different concentrations of AGS (10, 20, 30, 40, and 50 µg/mL) were supplemented to cell lines in 96-well plates. After the removal of exhausted media, the MTT reagent (5 µg/mL) added in all wells, and plates were incubated at 37 °C for 3 h. Subsequently, the MTT solution was replaced with dimethyl sulfoxide. The absorbance was measured at 540 nm using a microplate reader¹⁹. The graph for percentage of cell viability was plotted to calculate IC₅₀ value.

Statistical analysis

All analyses were carried out in triplicates and the results are presented as mean±SD. Data were analysed using Analysis of variance (ANOVA) and Duncan's multiple range test (DMRT) with least significance difference (LSD), $p < 0.05$ as a level of significance.

RESULTS AND DISCUSSION

Phytochemical screening

Phytochemicals such as alkaloids, terpenoid, steroids, saponins were processed the standard methods, phytochemical analysis of plant was need to discover and extended to novel therapeutically agents with improved efficiency.

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The secondary metabolites such as phenols, flavonoids, tannins and alkaloids are reported to have many biological and therapeutic properties through free radical scavenging activity²⁰. The analysis and characterization of these bioactive compounds from plants is important to determine their medicinal value. The phytochemicals extraction depends on the diffusion of each compound in the plant material matrix into the solvent²¹. The factor that needs to be considered when selecting the solvent for phytochemicals extraction is the solubility of the target compound^{22, 23}.

Preliminary phytochemical screening was done in AGS, EGS, AcGS and EaGS extracts to find out the existence of certain phytoconstituents such as phenols, carbohydrates, proteins, glycosides, alkaloids, flavonoids, triterpenoids, saponins and steroids and the results were shown in Table-1.

AGS showed the presence of alkaloids, carbohydrates, triterpenoids, proteins, phenols and flavonoids. EGS showed the presence of carbohydrates, triterpenoids, phenols and flavonoids. AcGS showed the presence of carbohydrates, glycosides, proteins, phenols and flavonoids. Alkaloids, phenols and proteins were present in EaGS. AGS was found to possess more phytochemicals compared to other extracts. Thus further studies were carried out on AGS.

Fourier-transform infrared spectroscopy (FT-IR)

FT-IR spectroscopy was done in order to find out the molecular properties and functional groups of AGS. The FT-IR spectrum of AGS is shown in Fig. 1. The presence of variety of functional groups made the AGS spectra very complex. The absorption peaks at 3819-3000 cm^{-1} was for O-H stretching, 2924 cm^{-1} was for C-H stretching vibrations in fatty acids, 1620 cm^{-1} was for amide I and amide II absorptions of proteins, 1460-1150 cm^{-1} was for esters and aliphatic chains of fatty acids and 1200-800 cm^{-1} was for C=O and C-C stretching of acids²⁴. A very intense peak at 3387 cm^{-1} was due to the stretching of N-H. The strong absorption peaks at 2376 & 2330 cm^{-1} was due to the stretching vibration of O=C=O. The signal at 2059 cm^{-1} was an indicative of multiple bonded CO group²⁵. The very intense peak at 1033 cm^{-1} indicated C-N stretching. The absorption peak near 500 cm^{-1} to 600 cm^{-1} was for C-I stretching. The signal at 833 cm^{-1} was for aromatic C-H out of plane bend²⁶.

Quantification of phytochemical components

Phenols and flavonoids are natural antioxidants and have also been proven to significantly increase antioxidant activities²⁷. Phenolics are widely distributed in the plant kingdom and it exhibited several biological activities including anti-cancer, antibacterial, anti-inflammatory and antiviral activities in several studies²⁸. Flavonoids are known as nature's drug which is excellent free radical scavengers and has also been proven to have numerous pharmacological and biological activities²⁹. Total phenols and flavonoid content of AGS was listed in table 2. The total phenolic and flavonoid contents were found to be 3.57 mg GAE/g extract and 7.41 mg RE/g extract respectively.

Total antioxidant capacity assay

Reactive oxygen species (ROS) are very toxic and play an important role in the development of many human diseases. Many secondary metabolites like polyphenols, flavonoids and alkaloids serve as sources of antioxidants and perform radical scavenging activity. The total antioxidant capacity of AGS was measured spectrometrically by phosphomolybdenum method, which is based on the reduction of Mo (IV) to Mo (V) by the analyte and the subsequent formation of green phosphate/Mo (V) compounds with a maximum absorption at 695 nm³⁰. AGS exhibited different degrees of antioxidant

capacity (Table 2) and the value was 23.07 mg AAE/g. The excellent antioxidant capacity might be due to the presence of phytochemicals present in AGS.

NO radical scavenging assay

Nitric oxide plays an important role in various inflammatory processes. It is a chemical mediator produced by macrophages, neurons and endothelial cells which is involved in the regulation of different physiological processes³¹. When NO reacts with free radicals, it will become very toxic and reactive peroxy nitrate anion and may contribute to the development of various carcinomas³². AGS exhibited a strong scavenging activity of NO. At concentrations of 100-500 $\mu\text{g}/\text{mL}$, the scavenging abilities were between 12.61% - 60.72% for AGS and 29.14% - 94.25% for gallic acid. The standard drug, gallic acid ($\text{EC}_{50} = 259.31 \mu\text{g}/\text{mL}$) exhibited significantly ($P < 0.05$) high NO radical scavenging activity when compared with AGS ($\text{EC}_{50} = 401.66 \mu\text{g}/\text{mL}$). This may be due to the antioxidant phytochemicals present in AGS by preventing the formation of nitrite. *Hyriopsis cumingii* extracts showed more NO scavenging activity, which may be due to the presence of phenolics³³.

In vitro anti-inflammatory activity (Protein denaturation method)

Proteins denaturation is an important cause of inflammation. Protein denaturation is a process in which proteins lose their structure because of the external stress which leads to the loss of biological function³⁴. The effects of AGS on inhibition of protein denaturation are shown in Figure 3. AGS at different concentrations (20 – 100 $\mu\text{g}/\text{mL}$) provided significant protection against denaturation of BSA. In this study, AGS showed maximum inhibition, 72.61% at 100 $\mu\text{g}/\text{mL}$. There was a significant difference in percentage inhibition of protein (BSA) denaturation between the concentrations tested ($P < 0.05$). The IC_{50} value of AGS was found to be 63.67 $\mu\text{g}/\text{mL}$ and the IC_{50} value of diclofenac sodium was found to be 51.28 $\mu\text{g}/\text{mL}$. From these results, it can be stated that AGS is capable of controlling the auto-antigen production and inhibits protein denaturation in arthritic disease³⁵. Anti-inflammatory activity of AGS is attributed to alkaloids, carbohydrates, triterpenoids, proteins, phenols and flavonoids.

In vitro anti-cancer activity

The MTT assay is one of the most widely used assays to evaluate preliminary anticancer activity of synthetic products and natural extracts³⁶. It is a highly reliable and colorimetric assay to assess cytotoxicity and could be performed in any type of cell lines. To evaluate the cytotoxic effect of AGS, a MTT assay was performed on human osteosarcoma MG63 cell line. MG63 cell lines were treated with AGS at different concentrations (10 μg to 50 μg) and IC_{50} (50% growth inhibition) value was determined³⁷. The cancer cell lines were

inhibited significantly with the increasing of AGS concentration (Fig. 4). A chart was plotted using the concentration of the AGS in X-axis and the percentage of cytotoxicity in Y-axis. AGS showed maximum cytotoxicity, 72.56% at 50 µg/ml and the IC₅₀ value was found to be 19.5 µg/mL. Control did not show any cytotoxicity. The Limit of cytotoxic activity for the crude extract according to National Cancer Institute (NCI), are IC₅₀ < 20 µg/mL³⁸. The IC₅₀ of AGS fall within the NCI guidelines limit, thus it has potential anticancer activity against MG63 cells. Preliminary phytochemical investigation revealed the presence of secondary metabolites in AGS. Therefore, the cytotoxic effect of AGS might be due to the presence of these secondary metabolites.

CONCLUSIONS

In conclusion, the results obtained in the present study indicated the presence of alkaloids, carbohydrates, triterpenoids, proteins, phenols and flavonoids in the aqueous extract of *G. sylvestre*. The extract effectively scavenged the nitrite radical and also showed potent anti-inflammatory and anti-cancer activity against MG63. These activities may be due to the strong occurrence of polyphenolic compounds. This study gives an idea that this extract can be used to design a potent anti-inflammatory and anti-cancer drug.

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Table 1: Preliminary phytochemical analysis of *G. sylvestre* extracts

S. No.	Phytochemical compounds	Extracts			
		AGS	EGS	AcGS	EaGS
1.	Alkaloids	+	-	-	+
2.	Carbohydrate	+	+	+	-
3.	Glycosides	+	-	+	-
4.	Triterpenoids	+	+	-	-
5.	Steroids	-	-	-	-
6.	Saponins	-	-	-	-
7.	Proteins	+	-	+	+
8.	Phenols	+	+	+	+
9.	Flavonoids	+	+	+	-

'+' indicates the presence of particular compound; '-' indicates the absence of particular compound; AGS = *G. sylvestre* aqueous extract; EGS = *G. sylvestre* ethanol extract; AcGS = *G. sylvestre* acetone extract; EaGS = *G. sylvestre* ethyl acetate extract

Table 2: Total phenol, flavonoid content and total antioxidant capacity of AGS. #

Sample	Total Phenols (mg GAE/g) ^A	Total Flavonoids (mg RE/g) ^B	Total antioxidant capacity (mg AAE/g) ^C
AGS	3.57±0.28	7.41±0.10	23.07±0.63

#Values are expressed as mean ± SD (n = 3). AGS = Aqueous extract of *G. sylvestre*; ^AGAE = gallic acid equivalents; ^BRE = rutin equivalents; ^CAAE = Ascorbic acid equivalents.

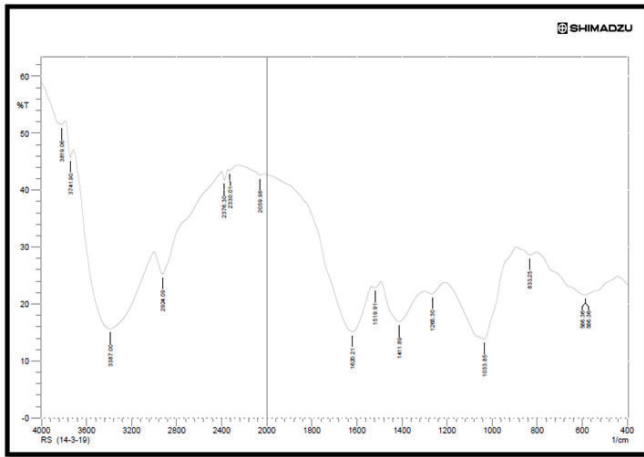


Figure 1: FT-IR of AGS.

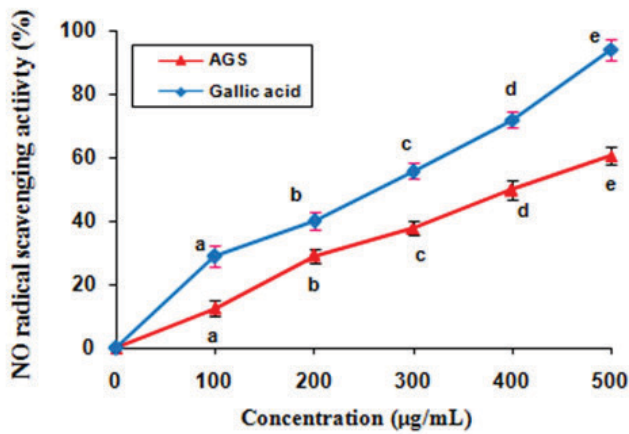


Figure 2: Nitric oxide radical scavenging activity of AGS [Values are expressed as mean±SD (n = 3). Different letters (a-e) indicate a significant difference between the concentrations of the same extract (P <0.05, ANOVA, DMRT)].

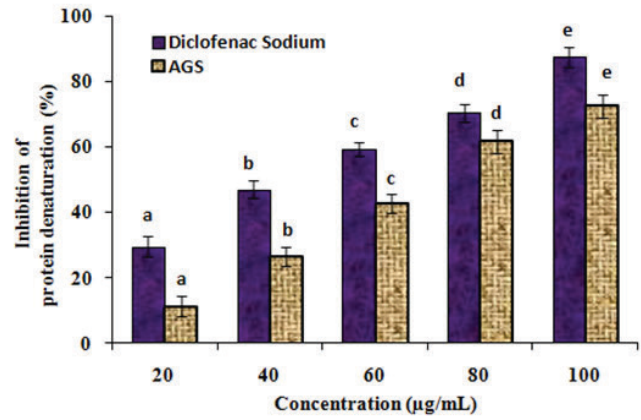


Figure 3: Anti-inflammatory effect of AGS (Inhibition of BSA denaturation).

[Values are expressed as mean±SD (n = 3). Different letters (a-e) indicate a significant difference between the concentrations of the same extract (P <0.05, ANOVA, DMRT)].

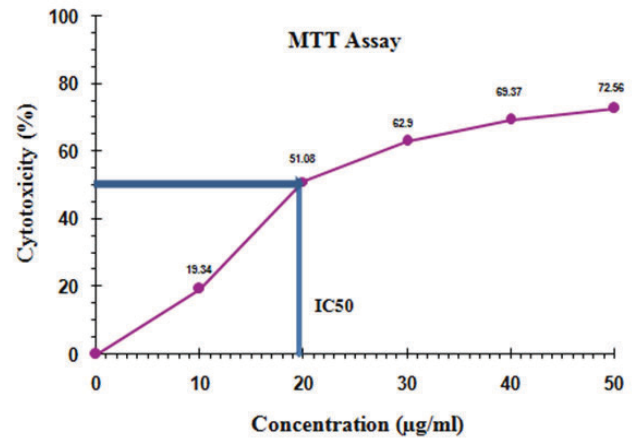


Figure 4: Anticancer effect of AGS on MG63 cell line.