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Anti-Oxidant, Anti-Inflammatory and Antiproliferative Effect of *Tecoma Stans F*ruit

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Abstract- *Tecoma stans*, member of family Bignoniaceae, is a popular ornamental plant grown for its bright yellow flowers. It is widely used for its medicinal properties in curing diabetes, digestive problems and yeast infections. Almost all parts (leaves, root, flower, seed, fruit, and bark) of the plant is have its medicinal use. In present study fruit extract was prepared and checked for its antioxidant, free radical scavenging ability and antiproliferative activities in cancerous cell line. Ethyl acetate and methanol extracts exhibited high antioxidant activity and free radical scavenging capacity. The expression of the pro-inflammatory gene(s) cox-2, IL1β, TNFα were checked in a cancerous cell line treated with the methanol extract in comparison to the untreated cells. The expression of all three genes was significantly reduced in the treated cancerous cells in comparison to untreated ones. Further when the cancerous cell line was treated with methanol extract it exhibited potent anti-proliferative activity and significantly changed the morphology of the cancerous cell.

Keywords**:** *Tecoma stans*, fruit extract, Antioxidant, anti-inflammatory, Anti-proliferative. Malls

I. INTRODUCTION

Herbs have been used by human for medicinal uses for ages. As per the WHO report around 80% of the population is dependent on medicinal plants for their health care needs [1]. Various active compounds like flavonoids, phenolic compounds, terpenoids, tannis, and alkaloids are found in medicinal plants which impart the medicinal value [2]. Synthetic drugs which are currently used to cure diseases are developed by the chemical process and have many harmful effects on our body in long-term uses this is the need of the hour to identify new bioactive compounds from traditional medicinal plants which are not only effective treatment but also safe to use. Recently there have been many studies on the use of medicinal plants on diseases such as diabetes, cancer, malaria, tuberculosis. *Tecoma stan* is an ornamental shrub found throughout India. *T. stan*s flower and fruit both contain flavonoids, phenolic compound, alkaloids and other bioactive compounds. Approximately all parts of this plant (leaves, root, flower, seed, fruit, bark) are used in the treatment of various disease such as diabetes, digestive problems, yeast infections in traditional medicine. It has powerful diuretic and vermifuge properties[3]. In the present study, we are investigating the bioactive compounds of *T. stan*s and their antioxidant, anti-inflammatory and cytotoxic effect. Given by its medicinal values *T. stans* could be a good source for such compounds.
Oxidative stress is the major cause of many chronic and degenerative diseases via several signaling pathways. The disease like atherosclerosis, ischemic heart disease, aging, diabetes mellitus, cancer, neurodegenerative diseases and chronic inflammation is caused by oxidative stress [4]. Oxidative stress causes immune system suppression. The harmful effect of oxidative stress can be controlled by antioxidant compounds. Antioxidants are chemical compounds which break the free radical generation chain by scavenging them. Many active antioxidants are found in medicinal plants which are helpful in reducing oxidative stress-induced signaling pathways [5]. Oxidative stress is responsible for chronic inflammation which then induces cancer by many biochemical factors [6][7]. Many epidemiological and experimental data proved the link between cancer and inflammation, also the fact that anti-inflammatory therapies show efficacy in cancer prevention and treatment[8]. Medicinal plant extracts or natural compounds have anti-inflammatory properties and hence might serve as a good candidate for cancer treatment. Rest of the paper is organized as follow, section I contains an introduction about *T. stans* and its uses, antioxidant properties of plant extract and their use in cancer treatment

and section II contains methodology of *T. stans* fruit extract preparation and its measurement of its antioxidant capacity also this section deals with methods to study effect of fruit extract on cancer cell proliferation, expression of pro-inflammatory genes.

II. METHODOLOGY

A. ***Plant extract preparation.*** The extract was prepared from *T. stans* fruit. The fruit was dried and ground and then subjected to solvation method by using hexane, ethyl acetate, and methanol in the order of increasing polarity. 100 gm of dried fruit powder was used for extract preparation. The extract was then dried and dissolved in DMSO for further experiments.

B. ***Determination of total phenolic content.*** The total phenolic content of the plant extracts was determined using the Folin-ciocalteu reagent by the method of SVC Rao et al., 2010. The reaction mixture contained: 200 µl of diluted extract, 800 µl of freshly prepared diluted Folin-Ciocalteu reagent and 2 ml of 7.5% sodium carbonate. The final mixture was diluted with deionized water to final volume of 7 ml. The mixture was incubated in dark at room temperature for 2 hours to complete the reaction. The absorbance at 765 nm was measured. Gallic acid was used as standard and the Results were expressed as (GAE)/g extract.

C. ***Total flavonoid content.*** Total flavonoid content was determined using aluminum chloride ($AlCl_3$) according to a previously established method using Quercetin as a standard. The plant extract (0.1ml) was added to 0.3 ml distilled water followed by 5%$NaNO_2$ (0.03ml). After 5 min at 25 °C, $AlCl_3$ (0.03 ml, 10%) was added. After a further 5 min, the reaction mixture was treated with 0.2 ml of 1mM NaOH. Finally, the reaction mixture was diluted to a final volume of 1ml with water and the absorbance was measured at 510nm. The results were expressed as mg quercetin (QE)/g extract.

D. ***Total antioxidant capacity.*** The total antioxidant capacity was determined by the method followed by SVC Rao et al., (2010). For total antioxidant assay, various concentrations of ascorbic acid were mixed with 1 ml of the reagent solution (0.6 M sulfuric acid, 28mM sodium phosphate, and 4mM ammonium molybdate). The reaction mixture was incubated in a water bath at 95°C for 90 min. After cooling to room temperature, the absorbance was measured at 695 nm. A standard graph was prepared by using data obtained for ascorbic acid.

$$\textbf{Viability (\%)} = (ABS_T / ABS_C) \times 100$$

The absorbance for the same reaction was taken by using plant extract (100 and 200 µg/mL) in place of Ascorbic acid. The total antioxidant capacity was determined in gram equivalent of ascorbic acid from the standard graph.

E. **Total Reducing power.** The reducing power of all plant extracts was determined according to a method followed by Srinivas et al., 2005. Different concentrations of Extracts (100-500 µg/mL) which were dissolved with 1 mL of distilled water were mixed with phosphate buffer (2.5 mL, 0.2 M, pH 6.6) and potassium ferricyanide [$K_3Fe(CN)_6$] (2.5 mL1%). The mixture was incubated at 50°C for 20 min. A portion (2.5 mL) of trichloroacetic acid (10%) was added to the mixture, which was then centrifuged at 3000 rpm for 10 min. The upper layer of the solution (2.5 mL) was mixed with distilled water (2.5 mL) and $FeCl_3$ (0.5 mL, 0.1%) and then absorbance was measured at 700 nm. Increased absorbance of the reaction mixture indicated increased reducing power. Ascorbic acid was used as the positive control.

F. **Free radical scavenging assay (DPPH Assay).** α-Diphenyl-β-picryl- hydrazyl (DPPH) assay for the determination of the free radical scavenging ability of plant extracts was done. The DPPH is a stable free radical and is widely used to assess the radical scavenging activity of antioxidant compounds. The reaction mixture (3.0 ml) consisted of 1.0 ml of DPPH in methanol (0.3 mM), 1.0 mL of extract and 1.0 mL of methanol. Reaction mixtures were incubated for 10 minutes in dark, then the absorbance was measured at 517 nm. Ascorbic acid was used as a positive control.

G. **Microculture tetrazolium test (MTT assay).** Microculture tetrazolium test (MTT assay) is a colorimetric assay based on the ability of viable cells to reduce soluble yellow tetrazolium salt into insoluble blue formazan crystal by mitochondrial reductase. MTT assay was done to check the proliferative effect of the plant extracts in Hep G2 cancer cell line. Exponentially growing cells were plated onto 96-well plates containing 5000 cell/ well in 200 µL DMEM medium for 24 h. The medium was then replaced with either control medium or medium containing methanol extract at 0.01, 0.05, 0.1, 0.5, 1.0, 10, 50, and 100 µg/mL for 24 hour . 20 µL of 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide stock solution (5 mg/mL) was added into each well and cells were further incubated at 37°C for 2 h. The supernatant was replaced with 200 µL of DMSO to dissolve formazan production. Absorbance was measured at 517nm. The percentage of viability was calculated using the equation, Where ABS_T and ABS_C are the absorbances of treated and control cultures, respectively.

H. **Effect of extract on the expression of the pro-inflammatory gene.** HaCat cells were cultured in 6-well plates (4×10^6 cells/well) with or without LPS (1µg/mL) and in the presence or absence of methanol extract at various concentration 0.01,0.1,1.0,10.0 µg/mL in DMEM medium supplemented with 10% serum.

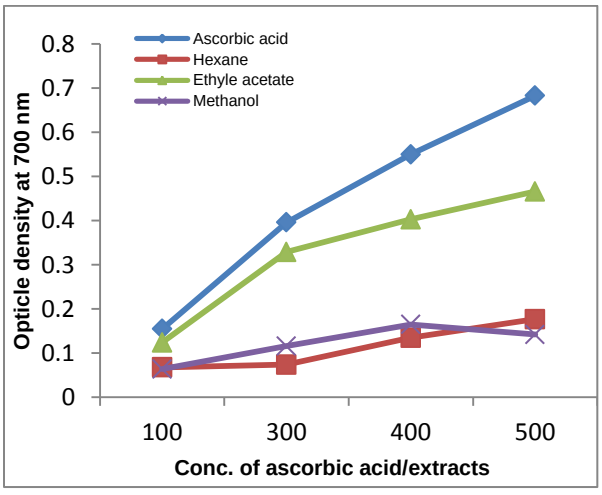
I. **RNA preparation and reverse transcriptase polymer ase chain reaction (RT-PCR).** Total cellular RNA was extracted from both the control and treated cells in a 6-well plate by using an RNA isolation kit (Sigma), according to the manufacturer's instruction. From each sample, 1µg of RNA used to make cDNA using cDNA synthesis kit (Agilent) according to the manufacturer's instruction. Real-time PCR reaction was performed in an ABI 7500 Fast sequence detection system (Applied Biosystems, Life Technologies, USA). The following cycles were used: pre-incubation at 95 °C for 5 min, denaturation 94 °C for 10 sec (4.8 C/s), annealing at 60 °C for 10 sec (2.5 °C/s), extension at 72 °C for 10 sec (4.8 °C/s), 40 cycles of amplification and final extension at 72 °C for 3 min. The Ct values were automatically calculated, the transcript levels were normalized against 18s rRNA expression. The fold change was calculated based on the non-treated control. Two-step Real time-PCR protocol was used in different conditions. The Fold Change values were calculated using the expression, where $^{\Delta\Delta}$CT represents the $^{\Delta}$CT condition of interest gene $^{\Delta}$CT control gene. The fold expression was calculated according to the $2^{-\Delta\Delta CT}$ method mentioned elsewhere.

III. RESULT

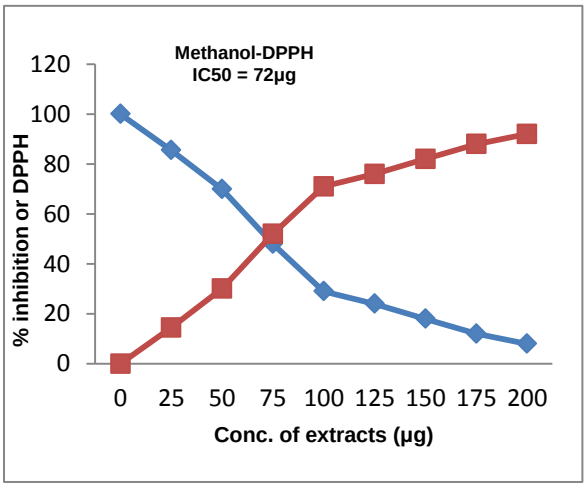
Total phenolic, flavonoids, antioxidant and free radical scavenging ability of the *T. stans* extracts: All the extracts were subjected to many assays in order to find out their phenolic, flavonoids and anti-oxidant content and free radical scavenging ability. The total phenolic content was 5 mg, 32 mg and 54 mg of hexane, ethyl acetate and methanol extracts respectively. The flavonoid content was 245mg in methanol extract which was highest and 108mg in ethyl acetate extract by comparing with the quercetin as standard. Methanol extract showed good antioxidant capacity of 720 mg followed by ethyl acetate extract 320 mg by comparing with ascorbic acid (Table 1). Total free radical scavenging ability of the extracts was analyzed by comparing them with the ascorbic acid, the highest free radical scavenging ability is found in methanol extract with IC50 of 72µg/ml (Table 2). The free radical scavenging ability of methanol extract was increased by an increase in the concentration of extract. All the extracts have equally good reducing power capacity (50 to 250 µg/ml of extracts equivalent to 10 to 50 µg/ml of ascorbic acid) (figure: 1A). As methanol extract exhibited good anti-oxidant and free radical scavenging abilities, it was taken for further experiments.

Table: 1: Total phenolic content (equivalent to Gallic acid), flavonoid content (equivalent to Quercetin), anti-oxidant capacity (equivalent to Ascorbic acid) and IC50 value of free radical scavenging capacity by DPPH assay in the fruit of *T. stans*.

Extracts	Amount of extracts(g)	Phenolic content eqv. to Gallic acid (mg)	Flavonoid content eqv. to Quercetin(mg)	Anti-oxidant capacity eqv. to Ascorbic acid (mg)	Free radical scavenging capacity(in µg/ml)
Hexane	1	2	--	59	368
Ethyl acetate	1	32	108	178	97
Methanol	1	54	254	220	72



(A)



(B)

Figure: 1 Free radical scavenging capacity and total reducing power of different extracts (A) Free radical scavenging capacity of ethyl acetate, hexane, methanol extract compared to standard (A) ascorbic acid. IC50 values of methanol extract for DPPH radical scavenging was 72 µg/ml. (B) Estimation of total reducing power of different extracts at different concentrations by utilizing potassium ferricyanide in comparison to ascorbic acid.

Expression analysis of pro-inflammatory gene. The expression of pro oncogenes was checked in methanol extract treated and untreated cell line. It is found that expression of pro oncogene cox-2 IL-12 and TNF have gone down when treated with methanol extract of *T. stans* in a dose-dependent manner. The expression reduction indicated that the methanol extract has the capacity to alter the expression profile of pro oncogenes and thus could help in the prevention of cancer.

Methanol extract showed antiproliferative activity. In order to check the cytotoxic effect of *T. stans* extracts on cell viability assay was performed using Hep G2 cancer cell line with different concentrations of Methanol extract. Cells treated with methanol extract showed 35% inhibition in proliferation at 1 µg/ml and 74% inhibition for 10 µg/ml concentration in comparison to untreated cells. The morphology of the cells was also changed for treated cells in comparison to untreated cells. There were many abnormal cells observed indicating cell death (Figure 4).

IV. CONCLUSION

Plants contain many chemical compounds to defend themselves from predators which are mostly secondary metabolite. Most of the secondary metabolite found in plants are phenolic and flavonoids

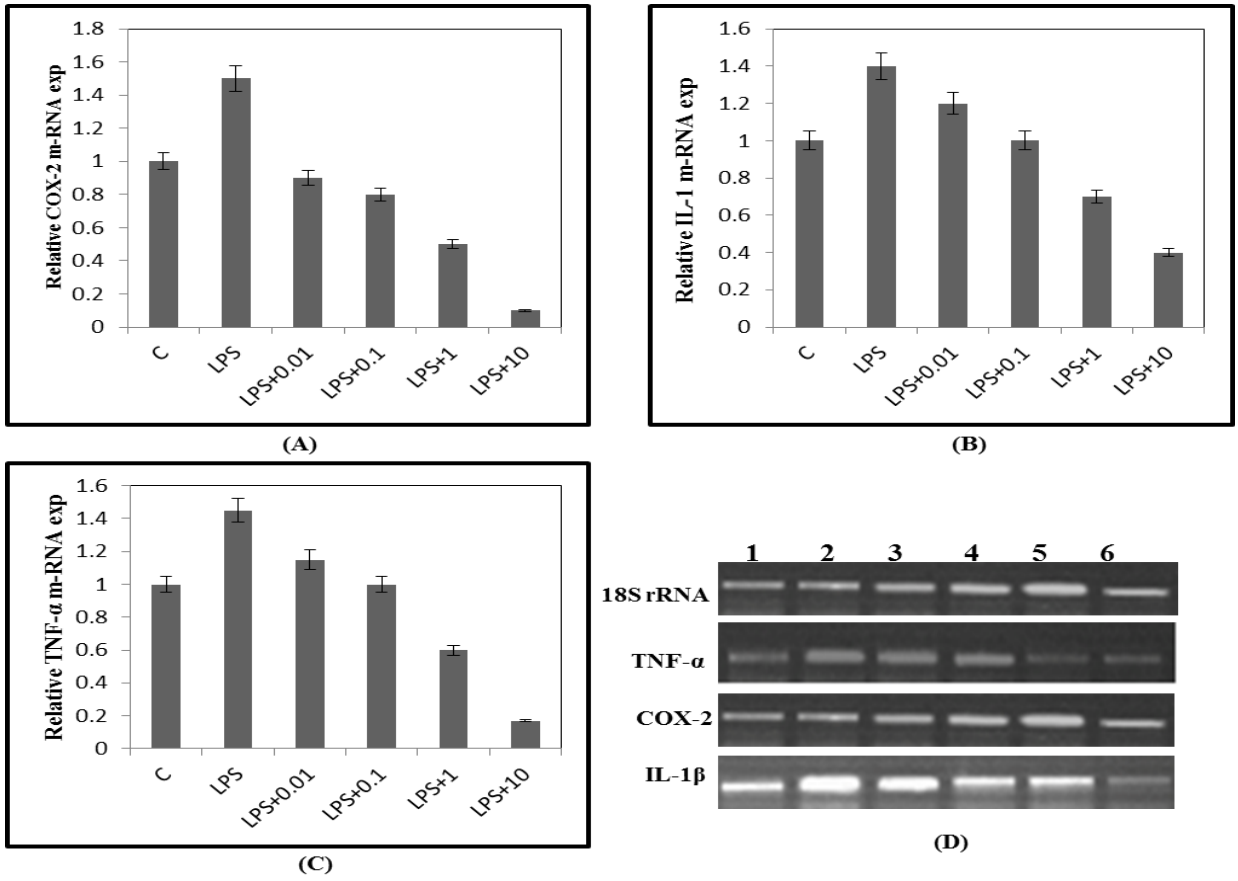


Figure 3: Effect of methanol extract on the expression of the pro-inflammatory gene. Real-time PCR quantitative analysis of (A) COX-2; (B) IL-1β mRNA and (C) TNF-α expression in HaCat cells relative to 18S rRNA expression at different concentrations of methanol extract given along with or without LPS (1- Control, 2- LPS (1 µg/ml), 3- LPS + 0.01 µg/ml, 4- LPS + 0.1 µg/ml, 5- LPS + 1 µg/ml, 6- LPS +10 µg/ml) by qRT-PCR is reported as fold change relative to untreated control calculated using $2^{-\Delta\Delta CT}$. The x-axis represents different treatment conditions. (D) The expression pattern was semiquantitatively observed.

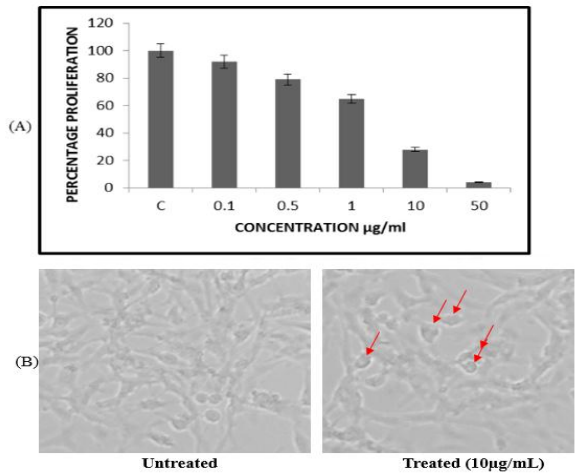


Figure 4: Cell proliferation assay on Hep G2 cell line using Methanol extract. (A) The extract showed potent anti-proliferative property in a concentration-dependent manner and, (B) phase contrast micrographs showing abnormal cells present in the treated cell line in comparison to untreated cells.

and their oxygen-substituted derivatives [9]. *T. stans* fruit contains many phenolic and flavonoid compounds [10]. Fruits and flower of *T. stans* are used for the treatment of diabetes, digestive problems. Roots are reported to be diuretic, tonic, anti-syphilitic and vermifuge while the whole plant is used in the treatment of diabetes (Singh et al, 2013). During inflammation production of many inflammatory cytokines takes place such as TNFα, IL-1β [4]. The induced production of cytokines helps in proliferation and recruitment of cells involved in inflammation. Long exposure of microbial derivatives, like LPS, increases the production of cytokines, increasing inflammation leading to the skin disorder. In the present study, the methanol extract showed a significant decrease in the expression of pro-inflammatory genes (TNFα, IL-1β) in LPS induced skin cells (HaCat cell). Expression of cox-2 also got reduced in treated cells in comparison to non-treated cells which shows that *T. stans* extract have the ability to counter the LPS-mediated skin inflammation. Chronic inflammation leads to cancer susceptibility. Increase production of cytokines play an important role in tumor progression thus the inflammation is considered as the hallmark of cancer [11]. In the present study, the anti-proliferative on a cancerous cell line was evaluated, methanol extract exhibited the cytotoxicity on cancerous Hep G2 on treatment. Compounds of the extracts exhibited anti-proliferative properties and showed their effect on the progression of the cell cycle which block the entry of cells into a particular stage of cell cycle resulting in cell death. Treatment of cells in the present study increased the number of the cells in a particular stage of the cell cycle which can be observed by deformed cells thus the methanol extract of *T. stans* showed a potent antiproliferative capacity.

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