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Research Article

**ASSESSMENT OF *IN VITRO* ANTIBACTERIAL, ANTI-
INFLAMMATORY, ANTIOXIDANT AND ANTI-
PROLIFERATIVE ACTIVITY OF *CASSIA FISTULA* LINN.
METHANOLIC EXTRACT****R. Santhi^{1,*}, V.Saravanan²**

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Abstract:

Cassia fistula Linn. belonging to the family Caesalpiniaceae, is one of the long-established medicinal plant used in our traditional system for healing various diseases. In the present investigation methanolic extract of *Cassia fistula* was tested against different Gram positive and Gram negative pathogens revealing a good potential antibacterial activity. The methanolic extracts were also screened for in vitro anti-inflammatory activity by membrane stabilization assay and it was found to be inhibiting RBC lysis upto 100 µg/ml concentration. In addition, the free radical scavenging activity of the extracts was analyzed using 2,2-diphenyl- 1-picrylhydrazyl (DPPH). The maximum antioxidant activity was observed at 80 µg/ml concentration of plant extract. The MTT assay revealed that the *C. fistula* extract displayed highest anti-proliferative activity against HeLa cells ($IC_{50} = 67.17$ µg/ml) when compared to Vero cells ($IC_{50} = 137.9$ µg/ml). The *C. fistula* leaf extract was found to be possessing excellent antibacterial, anti-inflammatory, antioxidant and anti-proliferative activity and could be further explored for the development of drugs against various disorders.

Keywords: *Cassia fistula*, methanol, anti-inflammatory, antioxidant

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INTRODUCTION

In developing countries, the usage of medicinal plants offers a major source of antibacterial as well as antioxidant agents with potential medicinal applications. It is estimated that more than 25% of the modern medicines used for various diseases are derived either directly or indirectly from plants [1]. India is one of the countries rich in plant biodiversity with a vast array of medicinal plants. These medicinal plants are considered as the source of various pharmacologically active compounds used for multiple ailments [1]. Traditional uses of such medicinal plants led researchers to search for significant number of remedial properties without any side effect [2,3].

Due to the persistent usage of antibiotics, most of the bacteria develop resistance towards conventional antibiotics; therefore there is a need to discover new antimicrobial agents with diverse structure and novel mode of action against these bacteria [4]. Reactive oxygen species (ROS) are a class of highly reactive molecules and their imbalance with antioxidants results in the occurrence of free radicals. The rapid production/accumulation of free radicals may lead to degenerative disorders, cancer, neural disorders etc. [5]. As the available synthetic antioxidants have many side effects, there is a need for more potent but less toxic antioxidants. Most of the medicinal plants possess large amount of antioxidants such as polyphenols, Vitamin C, Vitamin E, etc. [6].

The various parts of *Cassia fistula* tree have been extensively used in Ayurvedic medicine for various ailments. It is widely used in Indian traditional medical practice and possesses hepatoprotective, anti-inflammatory, antimicrobial and wound healing properties [4]. As these properties were incurred mainly due to its active compounds, the extraction and pharmaceutical evaluation of such active constituents served as prime objectives for many researchers [3,7]. Hence, the present study deals with the extraction of active metabolites from *Cassia fistula* leaves for the evaluation of antibacterial, anti-inflammatory and antioxidant activity. The cytotoxicity profile of the *C. fistula* against HeLa cell lines was also evaluated.

MAERIALS AND METHODS

Chemicals

The chemicals, minimal essential medium (MEM), fetal bovine serum, antibiotic solution (PSN), 1, 1-Diphenyl-2-picryl-hydrazil (DPPH), ABTS were procured from Himedia Laboratories, India and all the other specified chemicals were purchased from Merck Specialties Pvt. Ltd. (Mumbai, India).

Plant Materials

The leaf samples of *Cassia fistula* were collected from Centre for Advanced Studies in Botany, University of Madras, Guindy campus, Chennai. The leaf samples were brought to laboratory in sealed sample storage covers and processed immediately.

Extraction of Active Metabolites From *Cassia fistula* Leaves

In the laboratory, the leaf samples of *Cassia fistula* were shade dried at $(25 \pm 2 \text{ }^\circ\text{C})$ and ground to fine powder using mixer grinder. The active metabolites from the *Cassia fistula* were extracted by Soxhlet extraction using the solvent, methanol. After extraction, the solvent was recovered using rotary vacuum evaporator and the crude extract was concentrated and stored at 4°C till further use.

Phytochemical Profile of *Cassia fistula* Leaf Extract

The freshly prepared methanolic leaf extract of *Cassia fistula* was qualitatively analyzed for the presence of phytochemical constituents such as phenolic compounds (FeCl_3 test), alkaloids (Meyer's test), flavones, saponins (foam test), terpenoids and tannins by standard methods [8,9].

Antibacterial Activity Profile of *Cassia fistula* Leaf Extract Bacterial Strains

For the antibacterial study, the cultures of Gram positive bacteria such as *Bacillus subtilis* (MTCC 121), *Staphylococcus aureus* (MTCC 7443) and *Staphylococcus epidermidis* (MTCC 435), and Gram negative bacteria such as *Escherichia coli* (MTCC 7410), *Klebsiella pneumoniae* (MTCC 7407) and *Pseudomonas aeruginosa* (MTCC 7903) were procured from MTCC, Chandigarh, India.

Well Diffusion Assay

The antibacterial activity of the methanolic extract of *Cassia fistula* leaves was performed using well diffusion method against different pathogenic microbes as mentioned above [10]. The overnight bacterial cultures were used for the preparation of lawn over the Muller-Hinton agar medium. Using sterile cork borer, wells of 4 mm in diameter were punctured, 40 μL of methanolic plant extract (1 mg/mL), 30 μL of methanol solvent and 30 μL streptomycin as an antibiotic control was added into the respective wells. The plates were then incubated at 37°C for 24 h and antibacterial activity was determined by measuring the diameter of zone of inhibition around the wells.

Determination of Minimum Inhibitory Concentration (MIC)

The minimum inhibitory concentration of the *C. fistula* extract was determined using macrodilution method. Different concentration (1000, 500, 250, 125, 62.5, 31.25, 15.6, 7.8, 3, and 1.95 µg/ml) of the extract was prepared by diluting (two fold dilution) the methanolic extracts in test tubes containing 1ml sterile nutrient broth. Then 1 ml of bacterial suspension was added to each tube and incubated for 24 h at 37 °C. Appropriate controls were maintained and at the end of incubation time, the bacterial growth was observed based on the appearance of turbidity in the test tubes. The MIC of the *C. fistula* leaf extract was calculated based on the visible turbidity in the test tubes which was further confirmed by inoculating a loopful of culture from each test tube on to sterile nutrient agar for the appearance of colony after 24 h.

Anti-inflammatory Profile of *Cassia fistula* Leaf Extract

The anti-inflammatory activity of the *Cassia fistula* leaf extract was determined via membrane stabilization assay described by Amin *et al.* [11]. For the purpose, 5 ml of blood was collected from healthy volunteers and mixed with equal volume of Alsever solution (2% dextrose, 0.8% sodium citrate, 0.05% citric acid, 0.42% sodium chloride in distilled water). The suspension was centrifuged at 3000 rpm for 5 min. Different concentration of extracts (60, 80 and 100 µg/ml) was prepared and mixed with 1mL of phosphate buffer, 2 mL of hyposaline and 0.5 mL of HRBC cell suspension. The preparation was incubated for 30 min at 37 °C and then centrifuged for 20 min at 3000 rpm. Finally the hemoglobin content present in the supernatant was determined spectrophotometrically at 560 nm and the percentage of membrane protection was calculated using following formula:

$$\text{Percent of membrane protection} = 100 - \left(\frac{\text{Absorbance of the test}}{\text{Absorbance of the control}} \times 100 \right)$$

Antioxidant Activity Profile - DPPH assay

The free radical scavenging activity of the methanolic extract of *Cassia fistula* leaves was determined using 1,1-Diphenyl-2-picryl-hydrazil (DPPH) [12]. Various concentrations of the extract via. 10, 20, 40, 60, 80 and 100 µg/ml was prepared by dissolving it in methanol. To 2 ml of each extract solution, 1 ml of 0.2 mM DPPH in methanol was added and the mixture was shaken vigorously and incubated in dark for 30 mins. After incubation, the colour change was read spectrophotometrically at 517 nm. From the absorbance, the free radical scavenging activity/percentage reduction was calculated using the

formula:

$$\text{Percentage reduction (\%)} = \frac{\text{Absorbance of the control} - \text{Absorbance of the test}}{\text{Absorbance of the control}} \times 100$$

The radical scavenging activity of the extracts was compared with a standard curve of prepared gallic acid.

Cytotoxic Activity Using MTT Assay

The Vero and HeLa cell lines were procured from National Centre for Cell Science (NCCS), Pune (India). Both the cell lines were grown in minimal essential medium supplemented with 1mM sodium pyruvate, 2mM L-glutamine, 1.5g per liter sodium bicarbonate, 1% penicillin-streptomycin-neomycin (PSN) and 10% fetal bovine serum (FBS) and incubated at 37 °C in a 5% CO₂ atmosphere. The cells were separately seeded (1 × 10⁴ cells per well) in 96 well plates and incubated for 24h. The cells were treated with different concentrations of methanolic extracts (10, 20, 30, 40, 50, 60, 70, 80, 90, and 100 µg/mL) and incubated for 24 h at 37 °C in 5% CO₂ atmosphere. After incubation, 20µl (5mg/ml in PBS) of 3-(4, 5-dimethyl-2-thiazolyl)-2,5-diphenyl-tetrazolium bromide (MTT) was added in each wells prior to wash with phosphate-buffer saline (PBS, 7.5) and incubated for 4 hours. Then 100 µl of dimethyl sulfoxide (DMSO) was added and absorbance was recorded at 450 nm using microplate reader. The readings were used for the calculation of cell viability in percentage [13,14]. The percentage of growth was calculated using the following formula:

$$\text{Cell viability (\%)} = \left(\frac{\text{Absorbance of treated cells}}{\text{Absorbance of the control cells}} \times 100 \right)$$

Statistical Analysis

All the experiments were performed in triplicates and the data are shown as mean ± standard deviation. IC₅₀ values were calculated using the software tools of GraphPad PRISM 6.0 software.

RESULT AND DISCUSSION

The *Cassia fistula* leaves were soxhlet extracted with methanol and concentrated using rotary vacuum evaporator. A highest yield of 9.2% was obtained with methanolic extraction which is a promising good yield. From the phytochemical analysis, the methanolic extracts of *Cassia fistula* leaves showed the presence of high phenolic compounds. Plant phenolic compounds contribute significantly to their antioxidant potential. The presence of aromatic rings bearing hydroxyl groups in phenolic compounds will allow them to quench free radicals and will transform into resonance-stabilized phenoxyl radical [15]. Phytochemical analysis of the *Cassia fistula* leaf

extract also revealed the presence of flavonoids and terpenoids (Table 1). The obtained results were sufficient for further studies involving the applications of the active compounds to evaluate the possible synergism for their antioxidant, anti-inflammatory and antimicrobial activities [2, 16].

Antibacterial activity of the *Cassia fistula* leaf extract was evaluated by agar dilution method against *Bacillus subtilis* (MTCC 121), *Staphylococcus aureus* (MTCC 7443), *Staphylococcus epidermidis* (MTCC 435), *Escherichia coli* (MTCC 7410), *Klebsiella pneumoniae* (MTCC 7407) and *Pseudomonas aeruginosa* (MTCC 7903). The antibacterial activity of the extract was quantitatively assessed by the inhibition zone developed around it. The results of the antibacterial activity are shown in

table 2. The plant extract showed significant antibacterial activity against the tested bacterial strains. Antibacterial activity of methanolic extract of *Cassia fistula* against the bacterial strains was found in the following decreasing order *Bacillus subtilis* > *Staphylococcus aureus* > *Staphylococcus epidermidis* > *Pseudomonas aeruginosa* > *Klebsiella pneumoniae* > *Escherichia coli*. Rizvi *et al.* [2] also observed the antibacterial activity of *Cassia* sp. and claimed that the activity was attributed due to the presence of flavonoids and polysaccharides like compounds. Abo *et al.* [17] and Vasudevan *et al.* [18] also found that methanolic extraction of plant parts of *Cassia* sp. will incur considerable antimicrobial as well as antioxidant activity.

Table 1: Phytochemical Profile of Methanolic Extract of *Cassia Fistula*

Phytochemical	Test	Observation
Phenolic compounds	FeCl ₃ test	Positive
Alkaloids	Meyer's test	Positive
Flavanoids	With aqueous NaOH solution	Positive
Saponins	foam test	Negative
Terpenoids	Salkowski Test	Positive
Tannins	FeCl ₃ test	Negative

Table 2: Antibacterial Activity of *Cassia fistula* Leaf Extract

Pathogenic strains	Methanolic extracts (1 mg/ml)	Methanol solvent	Streptomycin (1 mg/ml)
<i>B. subtilis</i>	19.33 ± 0.15	-	21.37 ± 0.15
<i>S. aureus</i>	18.47 ± 0.25	-	23.90 ± 0.31
<i>S. epidermidis</i>	17.43 ± 0.15	-	22.57 ± 0.21
<i>E. coli</i>	12.57 ± 0.25	-	19.47 ± 0.12
<i>K. pneumoniae</i>	14.37 ± 0.31	-	18.47 ± 0.21
<i>P. aeruginosa</i>	15.53 ± 0.12	-	18.43 ± 0.15

The MIC of *Cassia fistula* leaf extract against different pathogenic bacteria varied depending on the bacteria tested. Among the bacteria tested, *E. coli* and *K. pneumoniae* were found to be inhibited from the concentration of 15.6 $\mu\text{g/ml}$. The *S. aureus*, *P. aeruginosa*, *B. subtilis* and *S. epidermidis* were found to be grown at the concentrations above 7.8 $\mu\text{g/ml}$ (Fig. 1). The antibacterial activity may be attributed to the type and amount of phenolic content. In general, Gram-negative bacteria are more resistant than Gram-positive bacteria towards the *Cassia fistula* leaf extracts. The higher resistance of Gram-negative bacteria might be attributed due to the composition of the outer membrane [4].

The anti-inflammatory activity of the *Cassia fistula* leaf extracts was evaluated *in vitro* by membrane stabilization assay. The prevention/inhibition of hypotonic membrane lysis was taken as a measure of anti-inflammatory activity of the extract. In the hypotonic solution, the percent of lysis was reduced

by *Cassia fistula* extract between the concentrations ranging between 60 and 80 $\mu\text{g/ml}$. At 80 $\mu\text{g/ml}$ concentration, the *Cassia fistula* leaf extract inhibited 28.43% of RBC lysis as compared with 37.92% by Aspirin at the same concentration (Fig. 2). The human red blood cell membranes are selected for the purpose, as they are similar to lysosomal membrane components. The anti-inflammatory assay results demonstrated that methanolic leaf extract of *Cassia fistula* can significantly inhibit RBC hemolysis. The investigation suggests that, even at the higher concentration (100 $\mu\text{g/ml}$), the extract was able to prevent RBC lysis of 31.06% which was yet 6.86% less than that of Aspirin. The methanolic extract of the *Cassia fistula* leaves has good membrane stability and also possess good anti-inflammatory activities. Apart from the leaves, the root and barks of *Cassia fistula* also possess an excellent membrane protective properties or anti-inflammatory properties [19].

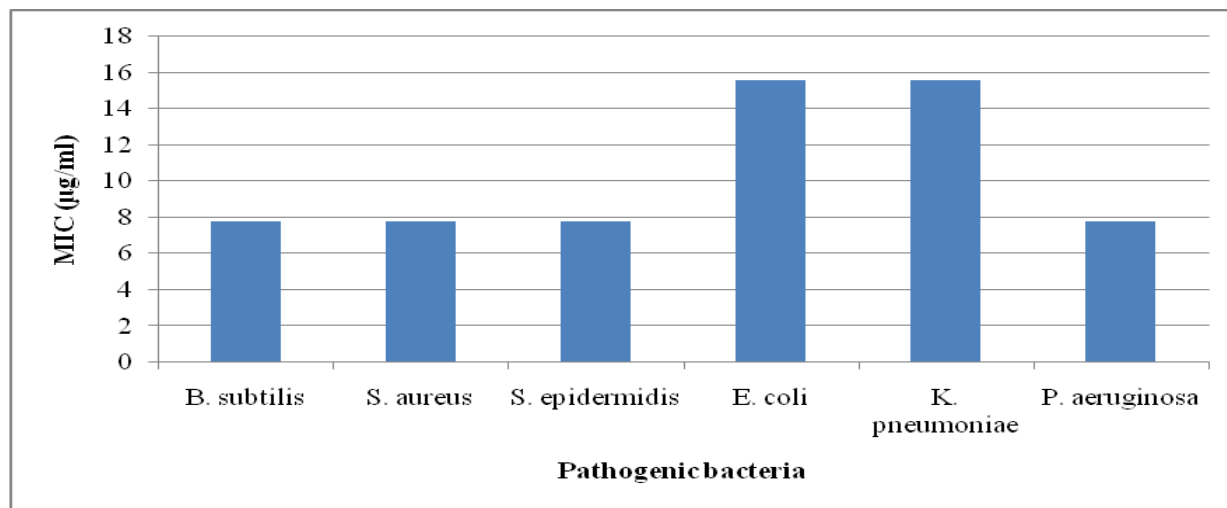


Fig. 1: Minimal Inhibitory Concentration of *Cassia fistula* Leaf Extract Against Bacteria

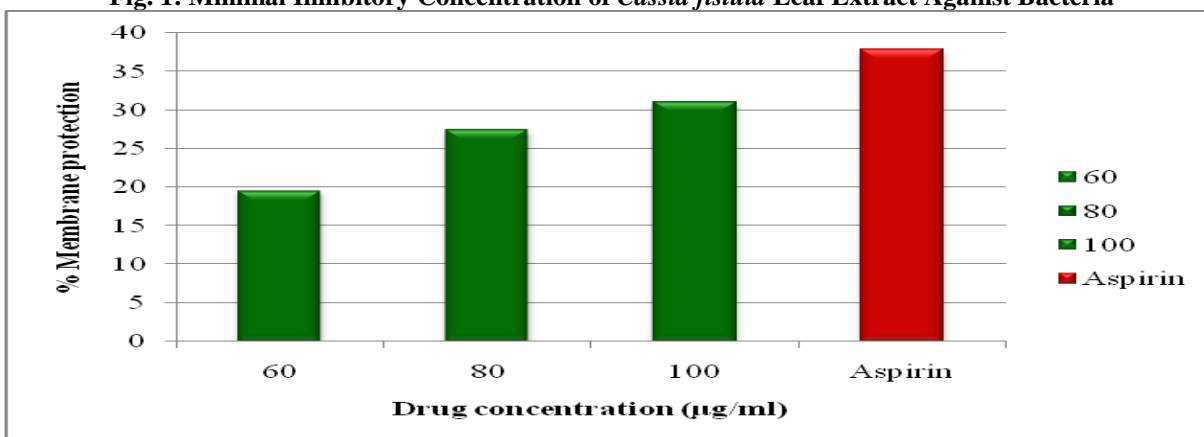


Fig. 2: Anti-inflammatory Activity of *Cassia fistula* Leaf Extract

For evaluating the antioxidant property of the *Cassia fistula* leaf extract, DPPH radical was employed. DPPH was found to be a widely used model for the determination of antioxidant activity by many researchers [6,12]. DPPH is a violet coloured free radical with nitrogen core, which turns yellow upon reduction. Antioxidants/radical scavengers are referred as the substance that facilitates the reduction of such free radical [15]. The radical scavenging activity of different concentrations of *Cassia fistula* leaf extracts and gallic acid is shown at fig. 3 and 4. The results revealed that the antioxidant activity was

dose dependent and the highest radical scavenging activity/percent inhibition was observed at the concentration of 100 $\mu\text{g/ml}$. The DPPH scavenging activity of the *Cassia fistula* leaf extract in the present study was 69.4% at 100 $\mu\text{g/ml}$, which was at par with that of DPPH scavenging activity of *Momordica charantia* leaves at similar concentration [20]. The antioxidant property of the *Cassia fistula* was also evidenced by Raju and co-workers, who have worked on bark extracts of *Cassia fistula* to determine their antioxidant profile [21].

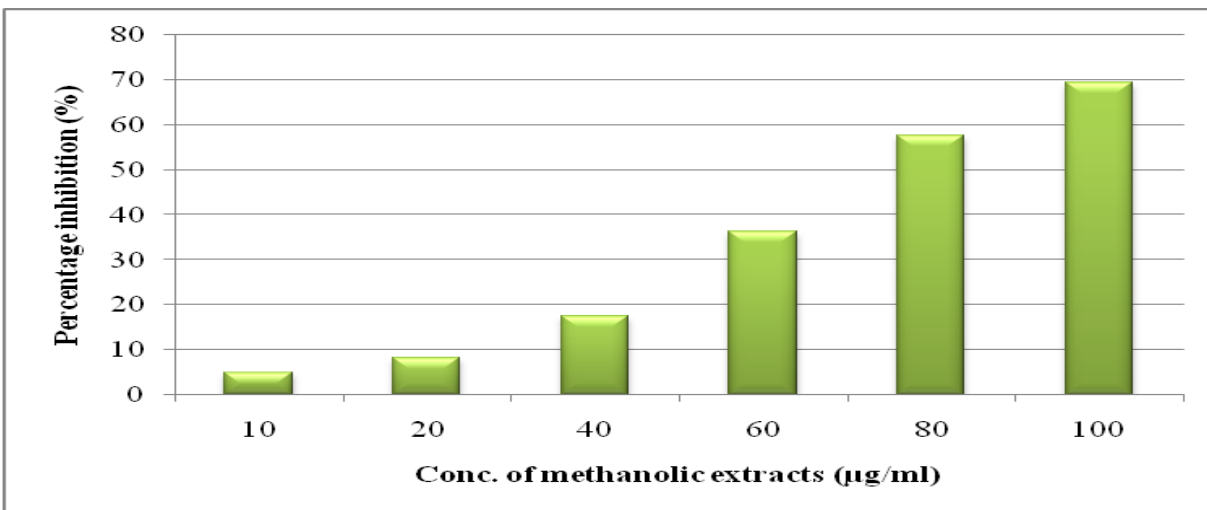


Fig. 3: DPPH Radical Scavenging Activity of *Cassia fistula* Leaf Extract

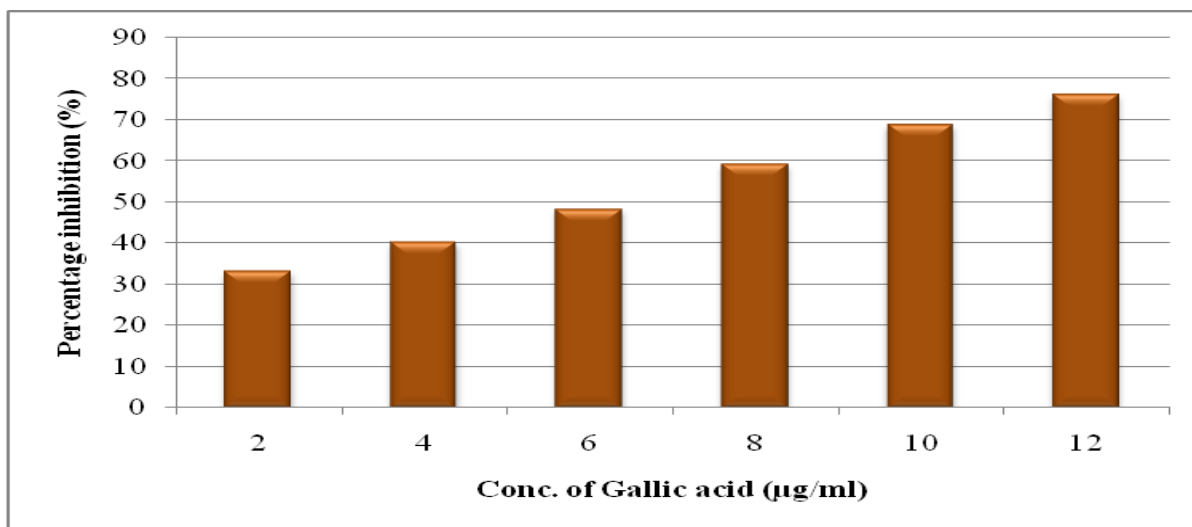


Fig. 4: DPPH Radical Scavenging Activity of Standard - Gallic acid

As a next part to the antioxidant assay, the extracts were tested on cultured human normal and cancer cell lines to determine the cytotoxic potential of methanolic extract of *C. fistula*. The cytotoxicity study of methanolic extracts of *C. fistula* was tested against Vero and HeLa cell line showing an IC_{50} of 137.9 and 67.17 $\mu\text{g/ml}$ (Fig. 5). The above results reveal that methanolic extracts shows significant anti

proliferation activity against HeLa cell lines compared to the normal cells, Vero (Fig. 6a and b). The anti-proliferative and cytotoxicity activity profile of different plant extracts against cancer cell lines were reported by various researchers [14, 15, 20].

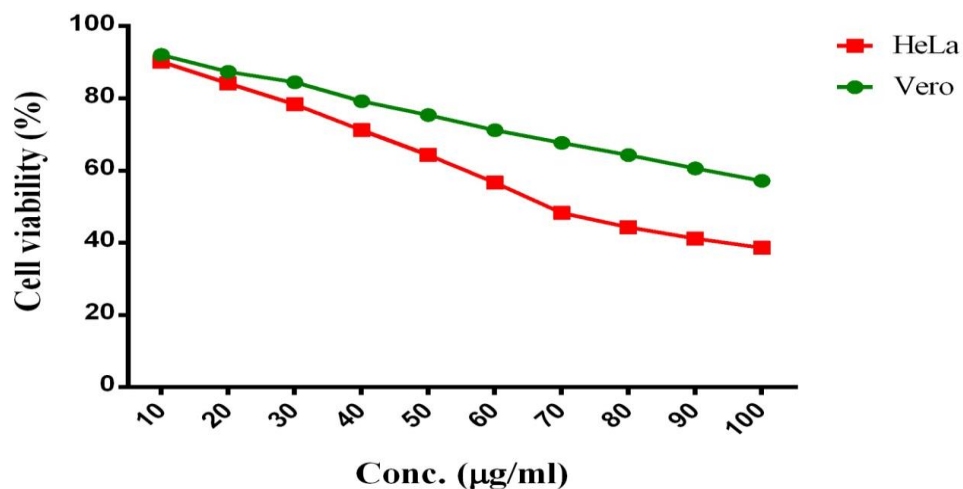


Fig. 5: *in vitro* Cytotoxicity of *Cassia fistula* Methanolic Extract against HeLa and Vero Cell Lines

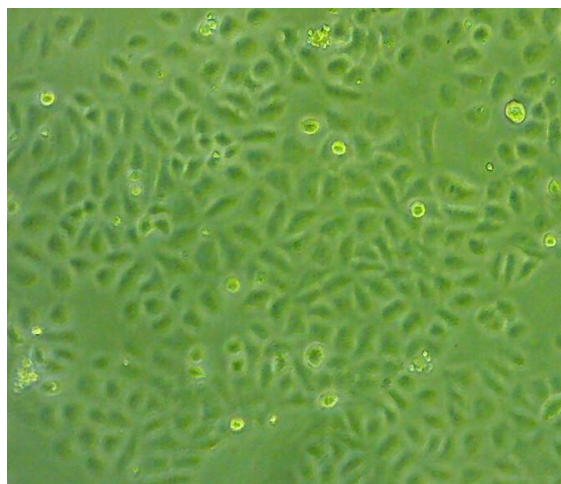


Fig. 6a: HeLa Cells Grown on MEM Medium (Control)

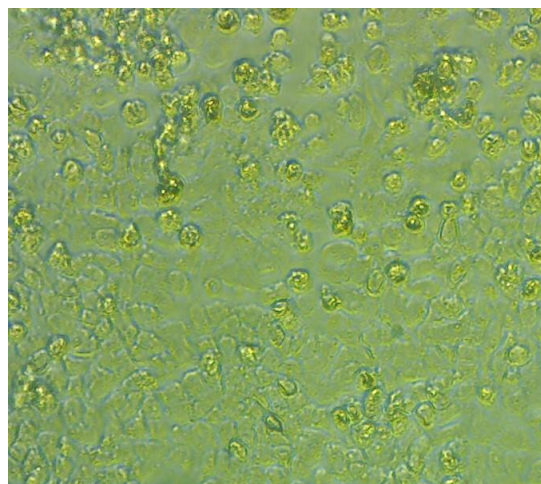


Fig. 6b: HeLa Cells Grown on MEM Medium Treated with *Cassia fistula* Methanolic Extract (Test)

In search of anticancer drugs from plant origin, evaluating cytotoxic activities against human cancer cell lines would be considered as one of the reliable source of information. Since different cancer cells exhibit different sensitivity profile, the assay of specific cell lines towards cytotoxic compound is therefore considered necessary in the detection of specific cytotoxic compounds [23]. Duraipandiyan *et al.* [10] evaluated anticancer activity profile of Rhein, a phytochemical isolated from *Cassia fistula* flower extract against colon cancer cell lines, COLO 320 DM. The compound Rhein exhibited minimal cytotoxic effect against Vero cells when compared to COLO 320 DM cells.

CONCLUSION

In conclusion, the present study showed that the active metabolites from *Cassia fistula* can be extracted with methanol. The methanolic extract showed higher antibacterial activity. The extract also showed highest anti-inflammatory activity and good antioxidant activity. The cytotoxicity studies also prove the significant anti-proliferative activity of methanolic extracts against HeLa cells. These properties observed in the methanol extract do provide opportunities to explore their potential applications in the treatment of innumerable health disorders including neurodegenerative disorders, cancer, cardiovascular diseases, etc. Further studies are necessary to evaluate the above properties of the active compounds from *Cassia fistula* at molecular level.

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