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Phytochemical analysis and antimicrobial study of *Cassia tora* leaves extract by using NCCLS antimicrobial susceptibility method

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Abstract
This study focuses on the phytochemical analysis and antimicrobial efficacy of *Cassia tora* leaves extract, a plant known for its medicinal properties. The research aims to identify the bioactive compounds present in the leaves and evaluate their antimicrobial activity using the National Committee for Clinical Laboratory Standards (NCCLS) antimicrobial susceptibility method. The phytochemical screening of the *Cassia tora* leaves extract was conducted, revealing the presence of alkaloids, flavonoids, tannins, saponins, and glycosides. These compounds are recognized for their potential antimicrobial properties. The extract was prepared using methanol as the solvent and subjected to antimicrobial testing against three microbial strains: Gram-positive *Staphylococcus aureus*, Gram-negative *Escherichia coli*, and the fungal strain *Candida albicans*. Antimicrobial susceptibility was assessed by measuring the zone of inhibition around the extract and determining the minimum inhibitory concentration (MIC) following NCCLS guidelines. The results demonstrated that the *Cassia tora* leaves extract exhibited significant antimicrobial activity, particularly against *Staphylococcus aureus* and *Candida albicans*, while showing moderate activity against *Escherichia coli*. The observed antimicrobial effects can be attributed to the synergistic action of the various phytochemicals present in the extract. In conclusion, the findings confirm the traditional medicinal use of *Cassia tora* and suggest its potential as a source of natural antimicrobial agents. Further research is recommended to isolate specific compounds and explore their individual antimicrobial mechanisms.

Keywords: *Cassia tora* leaves, ethanol extraction, phytoanalysis, NCCLS methods, *in-vitro* antibacterial, antifungal

1. Introduction

Cassia tora (Synonym *Senna tora*) is a small plant of the family Leguminosae that spreads as a common weed practically in all Asian countries. The Leguminosae, or legume, peas, or bean family, are one of the largest and most valuable families of flowering plants. That is why legumes can be defined as a type of species in which the seeds develop to grow into a pod. In terms of nutrition, they are a significant source of starch and dietary fiber and moderate in protein and minerals, and hence the legumes belong to the group of healthy food products [1]. *Cassia tora* belongs to the legume and sub-family Caesalpinioideae [2-4]. Its name is also derived from the Sinhala language. This plant is called Tora and is commonly a wild 30-90 cm tall annual herb that grows around wasteland in India, especially during the rainy season. The identifiable functional parts of the *Cassia tora* plant include the leaf, root, and seed. *Cassia tora* is known to contain several active compounds such as anthraquinone, quercetin, chrysophenol, emodin, rhein, etc. Hence, it is scientifically proven that *Cassia tora* has a significant antimutagenic reaction [5-9]. Since anthraquinone is used as a fluorescence sensor or fluorophores, this plant also possesses a sensing nature [10-15]. It comprises Dadhughnavati, which is one of the best-known ayurvedic preparations for antifungal agents [16-19]. The biologically active constituents of *Cassia tora* include flavonoids, which have antioxidant, hepatoprotective, and hypolipidemic activities with regard to antibacterial, antifertility, antimutagenic, antitumor, anti-inflammatory, antifungal, and anthelmintic activities [20-22].

2. Materials and Methods

2.1 Plant sample collection: The fresh leaves of *Casia tora* were properly collected and brought to the laboratory from Dhanvantary Medicinal Graden, Sanjivani Rural Education Society, Kopargaon, Ahmednagar district of Maharashtra, India. After washing the leaves under running water and then allowing them to dry at room temperature. These dried plant samples were crushed into powders and enclosed in sealed plastic bags before further analyses. The plant was subjected to botanical authentication with reference to the APG IV classification for the purpose of an authenticity test.

2.2 Preparation of plant extract

The dried powder was then successively extracted with ethanol. Soxhlet extraction was performed with 160 ml of each ethanol for 6 to 8 hours, with 10 g of the dried and powered plant material at a temperature lower than the solvents boiling point. The crude extract thus obtained was filtered and purified through Whatman No. 1 filter paper and stored at 4 °C in a refrigerator for subsequent use. Concentration was done under vacuum at 40 °C by using a rotary evaporator.

2.3 Phytochemical analysis

The extract of *Casia tora* was subjected to the test to identify the presence of bioactive compounds by employing the following methods [23].

2.3.1 Test for Alkaloids

a. Mayer's test

Coating the sides of the test tubes, 2 mL of Mayer's reagent was added to 1 mL of plant sample extract. The presence of white creamy precipitate gives a confirmation of the presence of alkaloids.

b. Wagner's test

Wagner's reagent was added along the sides of the test tube to a 1 mL of plant sample extract in a similar manner as previous. If positive, the precipitate formed is reddish-brown in colour.

c. Hager's test

In this test they added 3ml of Hager's reagent to 1ml of extract and yellow precipitate formed is an indication of a positive result.

2.3.2 Test for Steroids

a. Libermann-Burchard's test

The extract was then dissolved in of 2 mL acetic anhydride. For this, one or two drops of concentrated sulphuric acid was diluted and then was slowly poured from the side of the test tube. A spectrum of colours change indicates the existence of steroid.

b. Salkowaski test

Concentrated sulphuric acid was added to 1ml of extract, chloroform and two layers were formed. Represents a positive test result characterized by the bluish red colour turning to cherry red in the chloroform layer and green fluorescence in the acid layer.

2.3.3 Test for Flavonoids

a. Lead acetate test: In an experiment, 1 mL of plant extract was taken and then gradually few droplets of 10% Lead acetate solution were added. In case of a positive result, the solution forms a yellow precipitate.

2.3.4 Test for Glycosides

a. Keller kilani test

To 1 mL of extract, there was added 1 mL of acetic acid with traces of ferric chloride which was then transferred to a test tube containing concentrated sulphuric acid. This function has positive results when colour changes from reddish brown to blue at function of two phase.

3. *In-vitro* Antimicrobial Activity

Preparation of test Sample

Solid extract was dissolved in the appropriate solvent to produce a concentration in the range of 1-4 mg/ml for the antibacterial studies of the leaves.

Preparation of bacteria for the experiment

Bacterial species used in the current study were obtained from microbial type culture collection, Institute of Microbial Technology, Chandigarh. The specimens were originally isolated from animals during disease investigation and kept lyophilized.

a. Preparation of stock culture

From the cultures, which were maintained on nutrient agar slants, one loopful of the respective organisms were taken and aseptically transferred to 100ml of sterile nutrient broth in a flask, which was shaken thoroughly and incubated at 37oC for 24hrs. Purity and viability of the organisms was checked by plating, gram staining, by conducting primary and secondary biochemical tests. The test bacteria were suspended into sterile universal bottles containing nutrient broth separately and incubated at 37 °C for 18 h. Normal saline was added gradually to adjust the culture turbidity to that of McFarland turbidity standard, which corresponds to approximately (107 CFU/ml).

The antibacterial activity was done by antimicrobial susceptibility tests, NCCLS 1993, Approved standard M2-A5.

Antimicrobial susceptibility test [24]

A. Preparation of broth medium: 1.3 gm nutrient broth was dissolved in 100 ml of distilled water sterilized at 100 °C for 20 min in autoclave. After cooling the medium pour it in 96 well plates in quantity of 100 µl in each well.

B. Inoculum Preparation

Growth Method

1. At least three to five well-isolated colonies of the same morphological type are selected from an agar plate culture. The top of each colony is touched with a loop, and the growth is transferred into a tube containing 4 to 5 ml of a suitable broth medium, such as tryptic soy broth.
2. The broth culture is incubated at 35 °C until it achieves or exceeds the turbidity of the 0.5 McFarland standards (Usually 2 to 6 hours).
3. The turbidity of the actively growing broth culture is adjusted with sterile saline or broth to obtain turbidity optically comparable to that of the 0.5 McFarland standards. This results in a suspension containing approximately 1 to $2x10^8$ CFU/ml for *E. coli* ATCC 25922. To perform this step properly, either a

photometric device can be used or, if done visually, adequate light is needed to visually compare the inoculum tube and the 0.5 McFarland standard against a card with a white background and contrasting black lines.

C. Preparation of samples: Stock solutions of the plant extracts and the positive control drug streptomycin were prepared in dimethyl sulphoxide (DMSO) at the concentrations of 100 mg/ml.

D. Preparation of plates: The minimum inhibitory concentration (MIC) of the extracts for each test organism was determined by a modification of the broth dilution method performed in96 well micro-trays (NCCLS, 1993). A stock solution of extract was made in di methyl sulphoxide (DMSO). Two-fold serial dilutions of the reconstituted extract (100 µl) of each sample were made in sterile broth (nutrient broth) to achieve a concentration range of 1.95-1000 µg/ml. The same amount of test organisms (100 µl) was added to each dilution to give a final volume of 200 µl. After incubation at 37 °C for 18-24 h the plates were examined for growth of the organisms. The lowest concentration that inhibited growth was recorded as the MIC. Growth was determined as the difference in absorbance at 595nm between reading taken before and after incubation of the plates. Readings less than 20% of the positive control were recorded as inhibition. Absorbance was read in a plate reader. Four replicates were performed for extract against each of the test organisms. The Positive (100 µl cells + 100 µl medium) and negative (200 µl medium) controls were performed with each experiment. Each plate also included a solvent control in which 100 µl of the appropriate diluted solvent was added to 100 µl of the test organism [25-26].

E. Data analysis: The data analysis was accomplished using Graph Pad InStat version 3.00 for Windows 95, GraphPad Software Inc., San Diego California USA. IC_{50} values were obtained from regression lines with coefficient factors between R^2 = 0.52 and 0.99 at absorbance value at 595 nm. There were two reading were taken for comparision of turbidity before and after incubation of the plates.

4. *In-vitro* Antifungal activity

The fungus strains used for the study were obtained from the Microbial Type Culture
Collection (MTCC, India) for the study purpose. The fungus strains used in the study were *Candida albicans* (MTCC No. 183) and *Aspergillus Niger* (MTCC No.514).

Preparation of sample and standard solution

a. Preparation of plant extracts

- One hundred grams of fresh leaves of *M. oleifera* Lam. were shade dried at room temperature (32-35 °C) to constant weight over a period of 5 days.
- The dried leaves were ground into powdered using a mortar and pestle.
- 100 g of the powdered leaves were extracted in 500ml conical flasks with 30 ml of deionised distilled water and 70% ethanol.
- The conical flasks were plugged with rubber corks, then shaken at 120 rpm for 30 min and allowed to stand at room temperature for 5 days with occasional manual agitation of the flask using a sterile glass rod at every 24 hour.
- The extracts were separately filtered using sterile Whatman no. 1 filter paper.
- The resulting filtrates were then concentrated in a rotary evaporator and subsequently lyophilized to dryness.

b. Dilution of plant extracts

1: 2 dilution of test compound was done for NCCLS assay (antifungal screening assay).
Well 10 containing 1000 µg/ml test compound and according to serial dilution a final dilution of test compound was. 05 µg/ml.

c. Reference substance

For Antifungal screening method Amphotericine B was taken as the reference standard substance in the assay and I was taken 1: 2 dilutions.
Antifungal susceptibility test using standard method of NCCLS (The National Committee for Clinical Laboratory Standards).

a. Preparation of broth medium: 10.4 gms of RPMI-1640 medium supplemented with glutamine and phenol red, without bicarbonate and 34.53g 3-(N-morpholino) propanesulfonic acid (MOPS) was dissolved in 400 ml of distilled water. pH was adjusted to 7.0 at 25 °C with 1 mol/L sodium hydroxide. The volume was made up to 0.5 L with water and was filtered, sterilized and was stored at 4 °C until required.

b. Preparation of inocula: Fungal strains were sub-cultured on to their respective growth medium and incubated for 48 hrs at 25-30 °C. From these plates, several colonies were transferred to 5 ml of sterile distilled water. The suspensions were mixed for 15 s to ensure homogeneity and were subsequently diluted to match the turbidity of a 0.5 McFarland standard (i.e. OD = 0.12-0.15 at k = 530 nm, corresponding to 1–5* 10^6CFU/ml). Then the working suspensions were prepared by a 1 in 30 further dilution of the stock suspension in sterile distilled water to yield 1-5 * 10^3 CFU/ml. 0.1 ml sterilized solution of resazurin (20 mg/ml in water) was supplemented to the working suspension [25].

c. Preparation of samples: Stock solutions of the plant extracts and the positive control drug amphotericin B were prepared in dimethyl sulphoxide (DMSO) at the concentrations of 100 mg/ml. Further it was diluted to 1:50 in broth.

d. Preparation of plates: Microdilution susceptibility testing was performed in flat bottom
96-well clear plates containing broth medium (50 µl) in each well. Sample solutions (50 µl) were subsequently serially diluted two-fold in the plates with the broth, starting with the final concentration of 5000 mg/L. The working inoculum suspension (50 µl) was added to give a final inoculum concentration of 0.5–2.5 * 10^3 CFU/ml. Amphotericin B was used as the standard antifungal drug. Sterility and growth controls in the presence of DMSO were also included. The plates were then incubated at 37 °C for 48 h. The amount of growth was measured using plate reader at λ=492 nm [NCCLS document M27-A2].

Percentage inhibition of the extract against all cell line was calculated using the following formula.

% Cell survival = (AT-AB) / (AC-AB) X 100

Where
AT=Absorbance of treated cells (drug)
AB=Absorbance of blank (without cell)
AC=Absorbance of control (untreated)
There by

% Cell growth inhibition = 100- % cell survival

The effects of extracts were expressed by IC_{50} values calculated from dose response curves.

5. Results and Discussion

Phytochemical Screening

Phytochemical screening of the drug is a crucial step in standardization as well as quality control. Depending on environmental conditions and storage facilities, the phytochemical constituents can vary qualitatively and quantitatively, even within samples of the same species. The results are presented in Table 1.

Table 1: Qualitative phytochemical analysis of ethanolic and water extracts of *Cassia tora* leaves extract

Sr.	Phytoconstituents	Ethanolic extract	Water extract
1.	Alkaloids	+	-
2.	Flavonoids	+	+
3.	Terpenoids	+	+
4.	Saponin	+	+
5.	Tannin	+	+
6.	Carbohydrates	-	-
7.	Phenols	-	+
8.	Proteins	+	+
9.	Anthraquinones	-	+
10.	Glycosides	+	+
11.	Steroids	-	+
12.	Gums	-	-
A) Alkaoids		**Test Result**	
Wagner's test		+ve	
Mayer's test		+ve	
Hager's test		+ve	
B) Steroids			
Salkowaski test		+ve	
C) Flavonoids			
Lead acetate test		+ve	
D) Glycosides			
Keller kilani test		+ve	

5.2 *In-vitro* Antifungal activity

Table 2: Percentage growth inhibition of Hydro-Ethanolic extracts (Test) of *Cassia tora* against fungal strains and Amphotericin- B (Std)

Conc (µg/ml)	Extracts		Standard	
	Candida albican	*Aspergillus niger*	*Candida albican*	*Aspergillus niger*
0.05	16.93	4.19	22.75	5.68
0.15	22.61	7.56	30.81	10.64
0.46	28.52	11.67	35.45	14.47
1.37	37.45	16.44	43.18	18.63
4.12	43.83	30.17	50.36	32.87
12.35	53.33	39.38	57.76	49.58
37.04	63.3	56.72	65.27	65.65
111.11	75.13	66.01	72.45	68.61
333.33	89.62	85.82	80.73	87.09
1000.00	98.67	98.64	95.97	99.08
IC50	22.05	27.52	13.68	14.39
R^2	0.9522	0.9679	0.9207	0.9685

Amphotericin-B and Hydro-Ethanolic extracts were tested against two different fungal strains C*andida albicans* and *Aspergillus niger*, in dose dependent manner. End point was determined spectrophotometrically by ELISA plate reader. NCCLS test replicates for anti-fungal activity were analyzed every 24 hr for two days to determine the percentage growth inhibition in the presence of the extracts or drug. Hydro-Ethanolic extract was serially diluted in concentration range of 1000 to 1.95 µg/ml in plates with the different fungal strains. The working inoculums suspension was added to give final inoculums concentration of 0.5–2.5 X 10^3CFU/ml. The plates were incubated at 37 °C for 48 h. The amount of growth was measured using plate reader at λ= 450 nm. Data were analyzed using Graph pad Prism with use of Dose response curve equation. Data were normalized and curve fitting was opted with 95% confidence limit. IC_{50} was derived from curve of % Inhibition V/s Log dose of compound. This assay was also performed for Amphotericin B.

Table 2 showed IC_{50} and R^2 values of Hydro-Ethanolic extract of leaves of *Moringa oleifera* against *candida albicans* and *Aspergillus niger*. It indicates that the high IC_{50} value of Hydro-Ethanolic extract was found against *Aspergillus niger* having (IC_{50}: 27.52 µg/ml). Comparative lower IC_{50} value was found against *candida albicans* (IC_{50}: 22.05 µg/ml).

The test extract show the dose-effect co-relation with maximum linearity in cases of *Aspergillus niger* at value being 0.9679 and comparatively lower linearity in case of *candida albicans* at value being 0.9522. Similarly, The Amphotericine B shows the dose effect co-relation with maximum linearity in cases of *Aspergillus niger* at value being 0.9685 and comparatively lower linearity in case of *candida albicans* at value being 0.9207.

Table 2 showed IC_{50} and R^2 values of Amphotericine B against *candida albicans* and *Aspergillus niger*. It indicates that the high IC_{50} value of Amphotericin B was found against *Aspergillus niger* having (IC_{50}: 14.33 µg/ml). Comparative lower IC_{50} value was found against C*andida albicans* (IC_{50}: 13.68 µg/ml).

After screening with the C*andida albicans* and *Aspergillus niger*, it indicate that the Hydro-Ethanolic extract of leaves of *Cassia tora* gives good antifungal activity, and comparison with the Amphotericin B, the difference of IC_{50} value is more than 20 µg/ml, it indicate that the Hydro-Ethanolic extract doesn't give potent effect against presented fungal strain.

Antibacterial Activity

Table 3: Percentage cell inhibition by Hydro-Ethanolic extract of leaves against various Bacterial strains

Conc. (µg/ml)	% Growth Inhibition			
	E. coli	*Pseudomonas aeruginosa*	*Propionibacterium Acne*	*Staphylococcus epidermidis*
0.05	8.97	12.35	4.93	1.55
0.15	18.83	16.67	9.85	5.67
0.46	33.63	24.07	15.76	11.60
1.37	51.12	31.48	21.67	21.39
4.12	64.57	35.80	39.41	33.76
12.35	69.96	51.85	50.25	40.72
37.04	84.75	74.07	62.56	54.90
111.11	103.14	83.33	70.44	64.18
333.33	121.08	90.74	88.67	78.09
1000.00	135.87	96.30	103.45	92.53
IC50	62.2	71.23	86.01	87.48
R^2	0.9608	0.9931	0.9806	0.9812

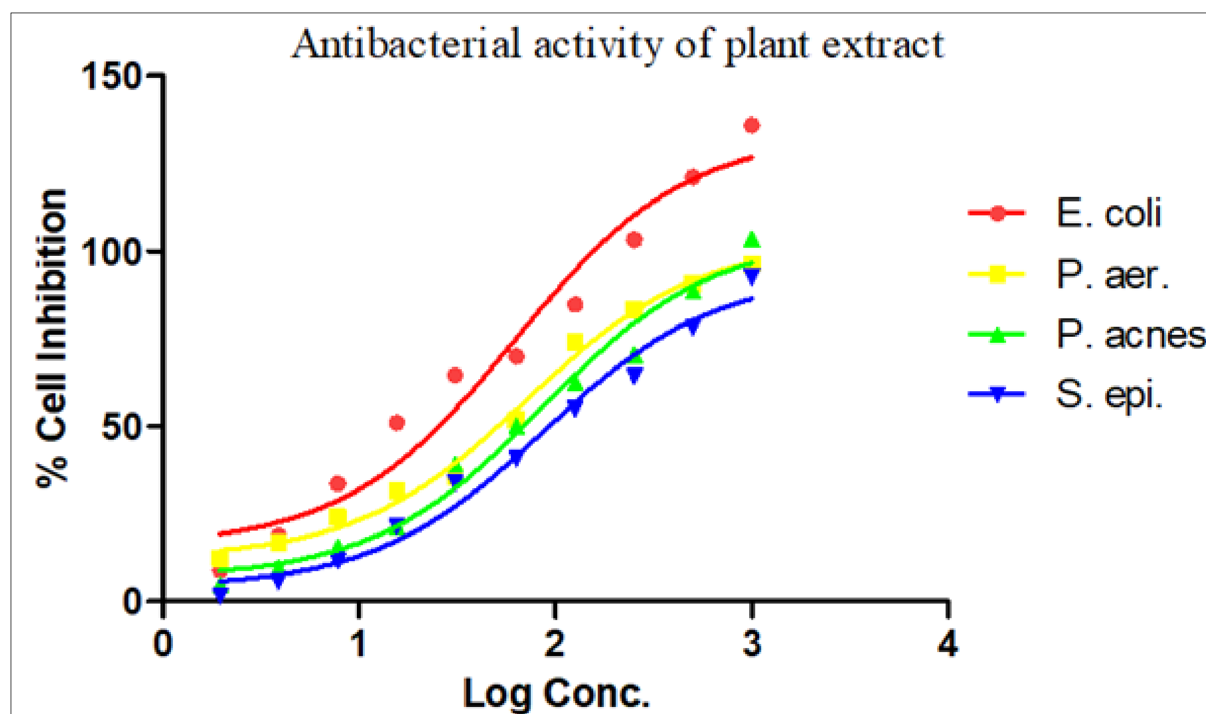

Fig 1: Plot of log conc. (µg/ml) v/s % cell inhibition of test extract against different Bacterial strains

Table 4: Percentage cell inhibition by Streptomycin against various Bacterial strains

Conc. (µg/ml)	% Cell Inhibition			
	E. coli	*Pseudomonas aeruginosa*	*Propionibacterium Acne*	*Staphylococcus epidermidis*
0.05	3.57	1.78	1.87	6.72
0.15	6.7	10.06	7.48	15.5
0.46	12.5	17.75	12.62	21.71
1.37	19.64	26.04	22.9	27.39
4.12	28.13	34.91	32.24	37.73
12.35	37.95	43.79	37.85	44.7
37.04	53.13	63.31	54.67	61.24
111.11	66.07	77.51	68.22	70.54
333.33	76.34	94.67	88.79	83.98
1000.00	91.52	97.04	97.66	97.16
IC50	23.47	19.53	31.3	21.13
R^2	0.9688	0.9686	0.9598	0.9542

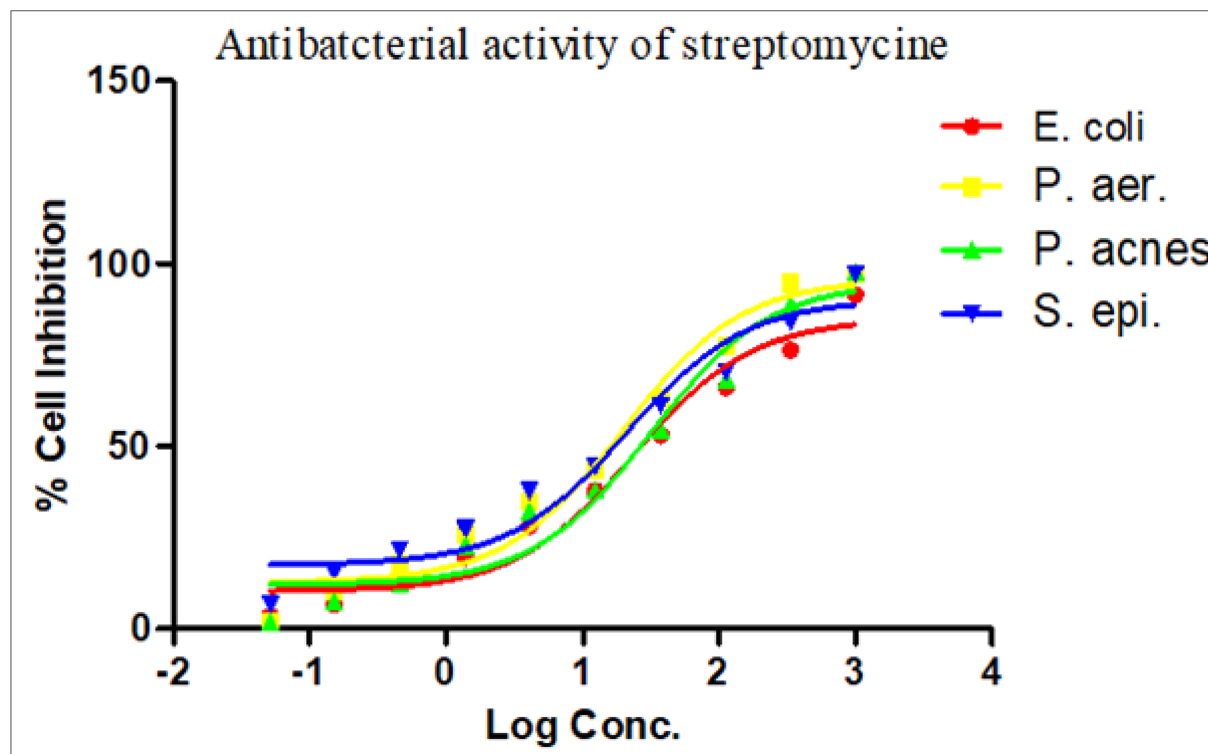

Fig 2: Plot of log conc. (µM) v/s % cell inhibition of Streptomycine against different Bacterial strains.

The antibacterial activity performed by antimicrobial susceptibility tests, NCCLS 1993, Approved standard: M2-A5. The hydro-ethanolic extract of *Cassia tora* leaves taken for antibacterial activity and *E. coli*, *P. aeruginosa*, *P. acnes* and *S. epidermidis* bacterial strains were used for the antibacterial activity. Two-fold serial dilutions of the extract (100 µl) of each sample were made in sterile broth (nutrient broth). The same amount of test organisms (100 µl) was added to each dilution to give a final volume of 200 µl. After incubation at 37 °C for 18–24 h the plates were examined for growth of the organisms. Absorbance was read in a plate reader [27-28].

The data analysis was accomplished using Graph Pad Prism version 5.00, Graph Pad Software Inc., San Diego California USA. IC_{50} values were obtained from regression lines with coefficient factors between R^2 = 0.52 and 0.99. The Absorbance at 595nm reading taken before and after incubation of the plates was taken for calculation of inhibitory values.

Table 3 showed IC_{50} and R^2 values of hydro-ethanolic extract of leaves of *Cassia tora* leaves against *E. coli*, *P. aeruginosa*, *S. epidermidis* and *P. acne*. It indicates that the high IC_{50} value of hydroethanolic extract was found against *S. epidermidis* having (IC_{50}: 87.48 µg/ml). Comparative lowest IC_{50} value was found against *E. coli* (IC_{50}: 62.2 µg/ml).

The figure 1 showed the plot of log concentration vs. % cell inhibition of test extract against *E. coli*, *P. aeruginosa*, *S. epidermidis* and *P. acne*. Test extract show the dose-effect co-relation with maximum linearity in cases of *P. aeruginosa* at value being 0.9608 and comparatively lowest linearity in case of *P. acne* at value being 0.9806 [28-29].

Table 4 showed IC_{50} and R^2 values of Streptomycin against *E. coli*, *P. aeruginosa*, *S. epidermidis* and *P. acne*. It indicates that the high IC_{50} value of streptomycin was found against *P. acne* having (IC_{50}: 31.3 µg/ml). Comparative lower IC_{50} value was found against *P. aeruginosa* (IC_{50}: 19.53 µg/ml).

The figure showed the plot of log concentration vs. % cell inhibition of streptomycin against *E. coli*, *P. aeruginosa*, *S. epidermidis* and *P. acne*. Streptomycin shows the dose effect correlation with maximum linearity in cases of *E. coli* at value being 0.9688 and comparatively lower linearity in case of *S. epidermidis* at value being 0.9542. From the

above results, compare with the reference standard, it is observed that the hydro-ethanolic extract of *Cassia tora leaves* showed good antibacterial activity on *Pseudomonas aeruginosa* with the IC_{50} value of 19.53 µg/ml.

6. Conclusion

The study on the antimicrobial and antifungal activities of *Cassia tora* leaves has provided strong evidence of their potential as natural antimicrobial agents. The phytochemical analysis of *Cassia tora* leaves revealed the presence of several bioactive compounds, including flavonoids, saponins, tannins, and alkaloids, which are known to contribute to their antimicrobial properties. The *in-vitro* assays demonstrated that extracts of *Cassia tora* leaves exhibit significant inhibitory effects against a broad spectrum of bacterial and fungal pathogens. The zones of inhibition observed in these tests were comparable to those of standard antibiotics and antifungal agents, indicating the potential of *Cassia tora* as a natural alternative for managing microbial infections.

7. Conflict of Interest

There is no any conflict of interest for this research work.

8. Acknowledgement

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