INTRODUCTION

Since the speculation of "magic bullet" by Paul Ehrlich, the development of chemotherapy had enabled humans to combat pathogenic microorganisms with the shield of antibiotic agents. The overexploitation of these antimicrobial agents have fueled the evolution of microorganisms into one of the most pathogenic ones leading to the development of antibiotic resistance which are now a potential threat to life on Earth [24]. These bottlenecks had forced researchers globally to focus on herbal medicines, towards Ayurveda [8].

One of the most well documented plant in Indian Ayurvedic system of medicine is C. fistula Linn [9 and 19]. It is commonly known as Amultas or Indian Laburnum or Golden shower tree, is a native plant of Indian subcontinent, its flower is the state flower of Kerela and it is the national tree of Thailand [8]. It is also widely cultivated as an ornamental tree and is relatively drought, salt tolerant. It is distributed across the India subcontinent as common plant of deciduous forest, occupying an area from plains to 1400 m altitude within the Himalayas [11].

In Indian literature this plant has been described to be useful against skin diseases, liver troubles, tuberculosis and has been recommended for its potential use in the treatment of rheumatism, haematemesis, pruritus, leucoderma and diabetes [2, 3 and 16]. It has been advocated to have several antimicrobial and antitumor pharmacological properties [14]. The leaves, flowers, and stems of the plant are used to prepare decoction, infusions, and powders, and have been found to be effective against a variety of infections [15].

One of the well documented plant in Indian Ayurvedic system is C. fistula, known as Amultas or Indian Laburnum or Golden shower tree. It is a native plant of the Indian subcontinent, its flower is the state flower of Kerala and it is the national tree of Thailand. It is also widely cultivated as an ornamental tree and is relatively drought, salt tolerant. It is distributed across the India subcontinent as a common plant of deciduous forest, occupying an area from plains to 1400 m altitude within the Himalayas.

In Indian literature, this plant has been described to be useful against skin diseases, liver troubles, tuberculosis, and has been recommended for its potential use in the treatment of rheumatism, haematemesis, pruritus, leucoderma, and diabetes. It has been advocated to have several antimicrobial and antitumor pharmacological properties.
medicinal properties such as antipyretic, analgesic, antioxidant, anti-inflammatory, antifertility, antimicrobial, anti-urothelic and anti diabetic [5, 6, 13 and 15]. Thus the present study was taken to scientifically validate the folklore use of this plant by performing qualitative phytochemical analysis and determining the antimicrobial potential of its HAEP, HAELE & HMLE extracts against well known pathogens: Staphylococcus aureus, E. coli, Pasteurella multocida and Candida albicans.

MATERIALS AND METHODS

Collection of plant material
Fresh pods and leaves of Cassia fistula L. (Family: Fabaceae) were collected during the month of May to June 2009 from Mathura and its adjoining areas. Plant was identified & authenticated at the facility of Botany, B.S.A. College, Mathura headed by Dr A.K. Agrawal (Professor and head). Fresh pods and leaves were separated from the plant and thoroughly washed with fresh, distilled water twice, kept for shard dry at ambient temperature to get rid of excess moisture until analyzed.

Preparation of extract
Dried pods and leaves of C. fistula were powered with electric grinder, at room temperature and 70 gm of the powered pods, leaves were extracted with 750 ml of solvent in the form of triple distilled water and hydromethanol (7:3, v/v) using Soxhlet apparatus. The extracts obtained within the round bottom flask were evaporated to dryness at 45°C using hot air oven. The percentage yield was determined.

Phytochemical Screening
Preliminary phytochemicals screening was performed for the secondary metabolites using the standard protocols [12]. Mayer’s test, Hager’s test and Dragendorff’s test were performed for Alkaloids. Legal’s test was performed for identifying glycosides; Ferric chloride test was followed to determine the presence of tannins and polyphenolic compounds. Flavonoids were tested through Alkaline test. Ninyhydrin and Biuret test were used to detect proteins. Steroids were identified through Salkowski test while carbohydrates presence was tested through Biuret and fehling’s test.

Preparation of samples for HPLC
10 mg of powdered plant extracts of C. fistula was dissolved in 10 ml of methanol to get final concentration of 1mg/ml subsequently the solution was filtered using 0.45μm syringe filter (millipore) for sterilization. 1 mg of each standard was dissolved individually in 1ml of methanol and sterile filtered through 0.45 μm syringe filter (millipore) before subjecting to HPLC analysis.

Procedure
The prepared samples of extracts and standards were used for HPLC. Binary system (Waters) equipped with PDA detector connected to system processor was used for analysis. The system used Empower software with standard certification for analysis of the results. A maximum pressure of 2500 psi and minimum of 1500 psi was maintained. The HPLC of solvents was run at 200 nm to 600 nm wavelength using reverse phase C-18 column. During the run, a flow rate 1ml/min was maintained using binary mode of gradient system. Various combinations of the solvents 20:80, 80:20, 60:20, 50:50 of methanol and water were used respectively. Ultimately for achieving best resolution of peaks the experiment were performed at 50:50 ratio of the solvent (methanol and water). In order to identify the compounds, several standards of flavonoids (kaempferol, quercetin dihydrate) and phenolic acids (o-coumaric acid, p-coumaric acid sinapic acid, caffiec acid, chlorogenic acid, cinnamic acid, ferulic acid and gallic acid) were used. The peaks were identified by comparing the retention time (RT) of the standard compounds with that of different peaks obtained in HPLC analysis of extracts.

Antimicrobial Study of extracts

Bacterial Isolates
The test organisms used includes Escherichia coli, Staphylococcus aureus and C. albicans, were obtained from the Department of Microbiology and Immunology, DUVASU, Mathura. Culture of Pasteurella multocida was obtained from Central Institute of research on Goats (CIRG), Farah, Mathura. Prior to use these isolates were characterized on the basis of morphological, cultural and biochemical characteristics. These organisms are commonly known animal pathogens which have the ability to cause several diseases from simple boils to food poisoning and toxic shock syndrome, etc (S. aureus), intestinal and urinary tract infection (E. coli), fungal infection of alimentary canal, etc (C. albicans).

Preparation of Culture media & innocula

Media
Nutrient agar media was used for S. aureus, Muller Hinton Agar (MHA, Himedia) for E.Coli, Sabouraud’s dextrose agar media (Himedia) for C. albicans.

Preparation of inoculums
Small amount of microbial culture from the stock was taken, inoculated into 5 ml of nutrient broth and incubated for 6 hrs at 37°C. The broth culture was centrifuged at 3000 rpm for 10 minutes to collect the bacterial pellet that was then washed twice with normal saline. After washing, the pellet was suspended in 5 ml of normal saline. Density of microbial suspension was adjusted equal to that of 5x10^{6} CFU/ml by using 0.5 McFarland nephelometer.

**Antibacterial activity by disc diffusion method**

Discs of 6 mm diameter were prepared from what man’s filter paper no.-1, sterilized at 160°C for 90 min in hot air oven, dipped in solution of different conc., of HAEP/HAEL/HMEP of *C. fistula*. Discs were then allowed to dry & used as herbal discs [4]. Discs containing 0.625, 1.25, 2.5, 5 and 10 mg of HAEP/HAEL/HMEP were used to study the antimicrobial activity against *S. aureus* while disc containing 1.25, 2.5, 5, 10 and 20 mg of HAEP/HAEL/HMEP were used to study *E. coli, P. multocida* and *C. albicans*. 0.5 ml of respective bacterial culture containing approximately 5x10^{6} CFU/ml was swabbed on the top of the Muller Hinton Agar (MHA) medium for bacterial culture and fungal culture on SDA medium [4]. Disc containing different concentrations 0.625, 1.25, 2.5, 5, 10 and 20 mg of HAEP/HAEL/HMEP were placed at even distances on test culture seeded plates. Discs dipped in triple distilled water were used as negative control while Vancomycin, Kanamycin and Streptomycin were used as a positive control. The culture plates with disc were incubated at 37°C for 24-48 hrs. The antimicrobial efficacy of extract, marked by the zone of inhibition around the disc, was measured at 24 hrs and 48 hrs intervals. Each experiment was repeated thrice.

**RESULTS**

The percentage yield of HAEP, HAEI and HMEP through soxhlet extraction method was found to be 14-15%, 12-13% and 16-17%. All these extract were subjected to phytochemical screening, HPLC analysis and antimicrobial activity. The result of the phytochemical screening are represented within Table 1, which shows the presence of different phytochemicals within HAEP, HAEI & HMEP of *C. fistula*.

### Table 1: Qualitative analysis of phytochemicals in HAEP and HAEI HMEP of *C. fistula*.

<table>
<thead>
<tr>
<th>Extract</th>
<th>Alkaloids</th>
<th>Glycosides</th>
<th>Tannins &amp; Phenolics</th>
<th>Flavonoids</th>
<th>Proteins</th>
<th>Steroids</th>
<th>Terpenoids</th>
<th>Carbohydrates</th>
</tr>
</thead>
<tbody>
<tr>
<td>HAEP</td>
<td>–</td>
<td>–</td>
<td>+</td>
<td>–</td>
<td>+</td>
<td>–</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>HAEI</td>
<td>–</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>–</td>
<td>–</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>HMEP</td>
<td>+</td>
<td>–</td>
<td>–</td>
<td>+</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>+</td>
</tr>
</tbody>
</table>

Results of the biochemical analysis were further illustrated and tested through HPLC analysis to confirm the presence of different phytochemicals and secondary metabolites within these extract. The HPLC analysis of HAEP/HAEI and HMEP has been represented within figure 1, 2 & 3.

**Fig.1 Chromatogram of HAEP confirming presence of Quercetin dihydrate.**
HPLC analysis of different extracts confirmed the presence of Quercetin dihydrate within HAEP, HAEI and HMEP of *C. fistula*. The presence of Sinapic acid and Gallic acid could only be detected within HMEP while Kaempferol presence was restricted to HAEI. Antimicrobial activity of *C. fistula* aqueous extract of pods, leaves and methanolic extract of pods were screened against selected human pathogens *S. aureus*, *E. coli*, *Pasteurella multocida*, *Candida albicans* (both bacterial and fungal strains). The results of different antimicrobial assays have been summarized within table no. 2 & 3. HAEP, HAEI and HMEP showed dose dependant antimicrobial activity against *Staphylococcus aureus*. Lower most concentration (0.625 mg/disc) could not display inhibitory effect against *S. aureus* while higher concentration (10 mg/disc) showed max zone of inhibition against the test organism (Fig.4). Against *E. coli* none extract (HAEP/HAEI/HMEP) in any given concentration showed inhibitory effect. Even higher concentration (20 mg/disc) also failed to show any effect on the multiplication of *E. coli*. All extracts even exhibited dose dependant anti microbial activity against *Past. multocida* with max zone of inhibition at a conc. of 20 mg/disk while no zone of inhibition was observed below a conc. of 5 mg/disk (Fig.5). Only HAEI displayed antifungal activity against *Candida albicans*. HAEI displayed best antifungal activity at a conc. of 10-20 mg/disk (Fig 6). It was further noted in all the above antimicrobial tests that on prolonged incubation, zone of inhibition was narrowed down.

### Table 2: Effect of HAEP, HAEI and HMEP of *C. fistula* against *Staphylococcus aureus*

<table>
<thead>
<tr>
<th>S.No</th>
<th>Extract (mg/disc)</th>
<th>Zone of inhibition (mm) <em>Staphylococcus aureus</em></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>24 hr</td>
</tr>
<tr>
<td></td>
<td></td>
<td>HAEP</td>
</tr>
<tr>
<td>1.</td>
<td>10.0</td>
<td>15.2+0.2</td>
</tr>
<tr>
<td>2.</td>
<td>5.0</td>
<td>11+0.2</td>
</tr>
<tr>
<td>3.</td>
<td>2.5</td>
<td>9+0.2</td>
</tr>
<tr>
<td>4.</td>
<td>1.25</td>
<td>8+0.2</td>
</tr>
<tr>
<td>5.</td>
<td>0.625</td>
<td>Nil</td>
</tr>
<tr>
<td>6.</td>
<td>Negative Control</td>
<td>Nil</td>
</tr>
</tbody>
</table>
Table 3: Effect of HAEP, HAEL & HMEP of C. fistula against E. coli, P. multocida & Candida albicans

<table>
<thead>
<tr>
<th>S.No</th>
<th>Extract (mg/disc)</th>
<th>Inhibition Zone (mm) against E. coli</th>
<th>Inhibition Zone (mm) against Pasteurella multocida</th>
<th>Inhibition Zone (mm) against Candida albicans</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>HAEP/HAEL/HMEP</td>
<td>HAEP</td>
<td>HAEL</td>
<td>HMEP</td>
</tr>
<tr>
<td></td>
<td>24hr-48 hr</td>
<td>24 hr</td>
<td>48 hr</td>
<td>24hr-48 hr</td>
</tr>
<tr>
<td>1</td>
<td>20</td>
<td>12±0.2</td>
<td>11±0.2</td>
<td>Nil</td>
</tr>
<tr>
<td>2</td>
<td>10</td>
<td>9±0.2</td>
<td>7±0.2</td>
<td>9±0.2</td>
</tr>
<tr>
<td>3</td>
<td>5</td>
<td>6±0.2</td>
<td>5±0.2</td>
<td>6±0.2</td>
</tr>
<tr>
<td>4</td>
<td>2.5</td>
<td>Nil</td>
<td>Nil</td>
<td>Nil</td>
</tr>
<tr>
<td>5</td>
<td>1.25</td>
<td>Nil</td>
<td>Nil</td>
<td>Nil</td>
</tr>
</tbody>
</table>

Table 4: Av. zone of Inhibition (mm) of antibiotics against test organisms (Positive Control).

<table>
<thead>
<tr>
<th>S.No</th>
<th>Antibiotics</th>
<th>Staphylococcus aureus</th>
<th>Escherichia coli</th>
<th>Pasteurella multocida</th>
<th>Candida albicans</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Vancomycin</td>
<td>18±0.2</td>
<td>16±0.2</td>
<td>15±0.2</td>
<td>12±0.2</td>
</tr>
<tr>
<td>2</td>
<td>Kanamycin</td>
<td>20±0.2</td>
<td>22±0.2</td>
<td>17±0.2</td>
<td>15±0.2</td>
</tr>
<tr>
<td>3</td>
<td>Streptomycin</td>
<td>21±0.2</td>
<td>19±0.2</td>
<td>20±0.2</td>
<td>17±0.2</td>
</tr>
</tbody>
</table>

Fig. 4: In Vitro antimicrobial effect of HAEP, HAEL & HMEP against Staphylococcus aureus

Fig. 5: In Vitro antimicrobial effect of HAEP, HAE and HMEP against Pasteurella multocida
DISCUSSION
Qualitative analysis of HAEP, HAE and HMEP of C. fistula revealed the presence of different phytochemicals like tannins, phenolic compounds, flavonoids, terpenoids, etc, and these active compounds are well known for their pharmacological activities [21]. Tannins, flavonoids and terpenoids are well known for their antimicrobial properties [22 and 23]. HPLC based comparison showed that there is a variation with regard to retention times within different extracts indicating the presence of different phytochemicals. However this variation has also been reported in literature [17 and 20]. All extracts exhibited dose dependant antimicrobial activity against S. aureus and Past. multocida and Candida albicans in different manner except for E.coli. Various workers showed reported good antimicrobial activity of different extracts of different parts of C. fistula against S. aureus [1, 14 and 24]. Other research workers have also shown similar results with no activity of extracts of pods and leaves of C. fistula against E. coli [18 and 24]. In contrast to our results obtained some research workers have reported no inhibitory effect of extract of C. fistula against Past. multocida while in our study all extracts demonstrated effective antibacterial activity against Past. Multocida [24]. As on prolonged incubation bacterial colonies reappeared within the zone of inhibition this indicating bacteriostatic effect. It was also noted that there was a variation in the degree of the antimicrobial activity of HAEP, HAE and HMEP which could be due to the variation of different phytochemicals present within these extracts. The antibacterial activity displayed by different extracts may be advocated due to the presence of Quercetin dihydrate among them [25]. Only HAE showed antifungal activity against Candida albicans which can be attributed to the presence of Kaempferol within HAE [10].

CONCLUSION
The study concludes that HAEP, HAE and HMEP of C. fistula contained bioactive compounds that may be responsible for the antimicrobial properties observed and also supports the folklore use of both pods and leaves of C. fistula in various diseases. The study confirmed the presence of Quercetin dihydrate within HAEP, HAE and HMEP of C. fistula, presence of Sinapic acid and Gallic acid within HMEP while Kaempferol presence was restricted to HAE. However, detailed study regarding mineral, vitamins, digestibility, hepatic toxicity testing are needed to be carried out before standardizing the use of these antimicrobial agents as a effective medicine.

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REFERENCES
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