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ORIGINAL ARTICLE

Phytochemicals Screening and Analysis using HPLC to determine the Antimicrobial efficacy of *Cassia fistula* extract

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ABSTRACT
In the quest to achieve success in fighting infections, innumerous antibiotics have been overexploited leaded to drug resistance, forcing researchers to focus on ayurvedic medicines. C. fistula, a native of Indian subcontinent is distributed throughout South-East Asia, is used as a medicinal plant for treatment of pruritus, leucoderma, diabetes, haematemesis, etc in India. The study was aimed to qualitatively analyze aqueous, methanolic extract of pods & aqueous extract of leaves of C. fistula using phytochemical, HPLC analysis. Antimicrobial activity of extracts was tested using disk diffusion method. Results confirmed the presence of biologically active substances within hot aqueous, methanolic extract of Pods (HAEP/HMEP), hot aqueous extract of leaves (HAEL). HPLC analysis revealed the presence of quercetin dihydrates among all extracts, kaempferon within HAEL and sinapic acid, galic acid within HMEL. Different extracts displayed different antimicrobial potential against pathogens i.e. Staphylococcus aureus, Past. Multocida. But only leaves extract was found effective against C. albicans. Study concluded that different extracts of pods & leaves of C. fistula contained active phytochemicals while the antimicrobial properties is credited to the presence of quercetin dihydrate, kaempferol in plant extract providing a scientific validation to the folklore use of plant.
Keywords: *Antibiotics, Phytochemicals, HPLC, Quercetin dihydrate, Kaempferol.*

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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

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 up taken to scientifically validate the folklore use of this plant by performing qualitative phytochemical analysis and determining the antimicrobial potential of its HAEP, HAEL & HMEL extracts against well known pathogens: *Staphylococcus aureus, E. coli, Past. 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 45ºC 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. Ninhydrin and Biuret test were used to detect proteins. Steroids were identified through Salkowaski 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 the 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 identity the compounds, several standards of flavonoids (kaempferol, quercetin dihydrate) and phenolic acids (o-coumaric acid, p-coumaric acid sinapic acid, caffeic 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[7]. 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/HMEL 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

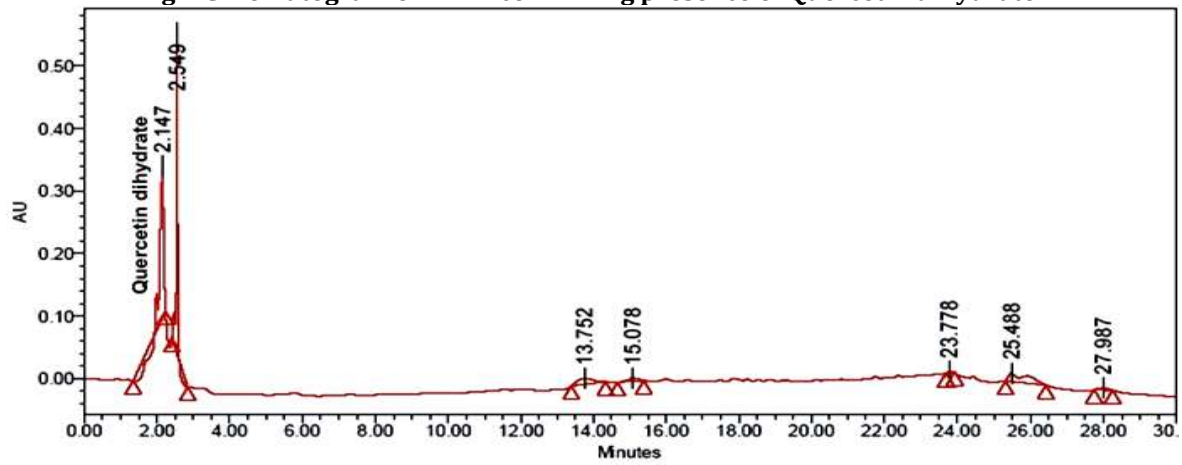
The percentage yield of HAEP, HAEL and HMEP through soxhlet extraction method was found to be 14-15%, 12-13% and 16-17%. All these extract were subjected to phytochemical screening, HPLC analysis and antimicrobial activity. The result of the phytochemical screening are represented within Table 1, which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

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

Extract	Alkaloids			Glycosides	Tannins & Phenolics	Flavonoids	Proteins		Steroids	Terpenoide	Carbohydrate	
	Mayer's	Hager's	Dragendorff's	Legal	Ferric chloride	Alkaline	Ninhydrin	Biuret	Salkowaski	Salkowaski	Biuret	Fehilling
HAEP	–	–	–	+	–	+	–	+	–	+	+	+
HAEL	–	+	+	+	+	+	–	+	–	+	+	–
HMEP	+	–	–	+	+	–	–	–	+	-	+	+

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/HAEL and HMEP has been represented within figure 1, 2 & 3.

Fig.1 Chromatogram of HAEP confirming presence of Quercetin dihydrate.



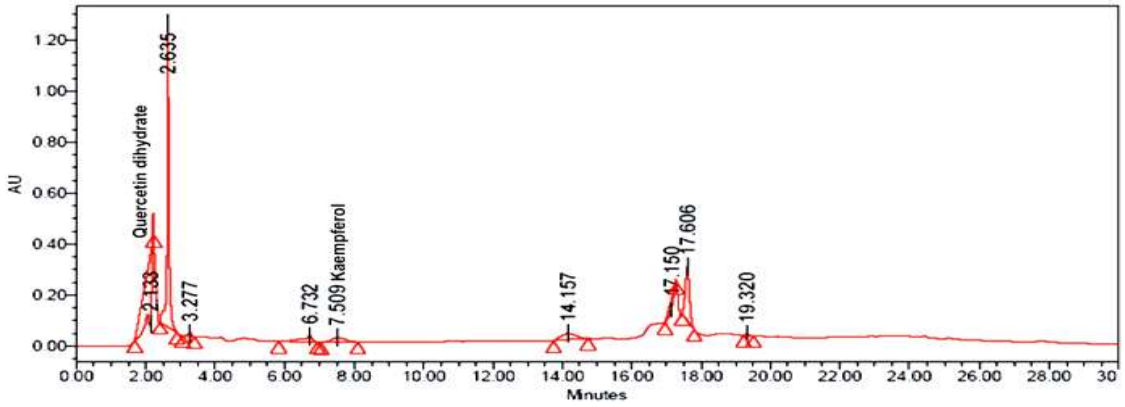


Fig.2 Chromatogram of HAEL confirming presence of Quercetin dihydrate, Kaempferol.

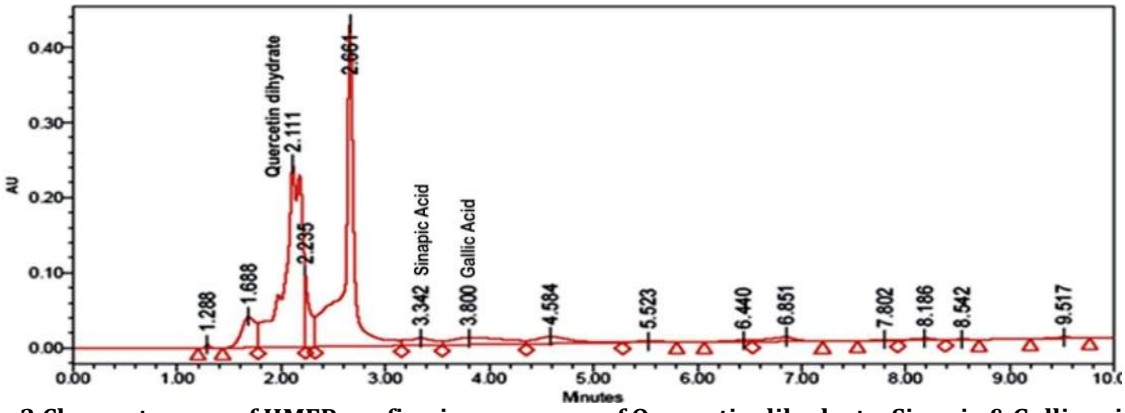


Fig.3 Chromatogram of HMEP confirming presence of Quercetin dihydrate, Sinapic & Gallic acid.

HPLC analysis of different extracts confirmed the presence of Quercetin dihydrate within HAEP, HAEL 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 HAEL. 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, HAEL 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/HAEL/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 HAEL displayed antifungal activity against *Candida albicans*. HAEL 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, HAEL and HMEP of *C. fistula* against *Staphylococcus aureus*

S.No	Extract (mg/disc)	Zone of inhibition (mm) *Staphylococcus aureus*					
		24 hr			48 hr		
		HAEP	HAEL	HMEP	HAEP	HAEL	HMEP
1.	10.0	15.2+0.2	15+0.2	14.5+0.2	14+0.2	13+0.2	13+0.2
2.	5.0	11+0.2	10+0.2	10.5+0.2	9+0.2	8+0.2	9+0.2
3.	2.5	9+0.2	9+0.2	8.5+0.2	8+0.2	8+0.2	7+0.2
4.	1.25	8+0.2	8+0.2	7.5+0.2	7+0.2	7+0.2	6+0.2
5.	0.625	Nil	Nil	Nil	Nil	Nil	Nil
6.	Negative Control	Nil	Nil	Nil	Nil	Nil	Nil

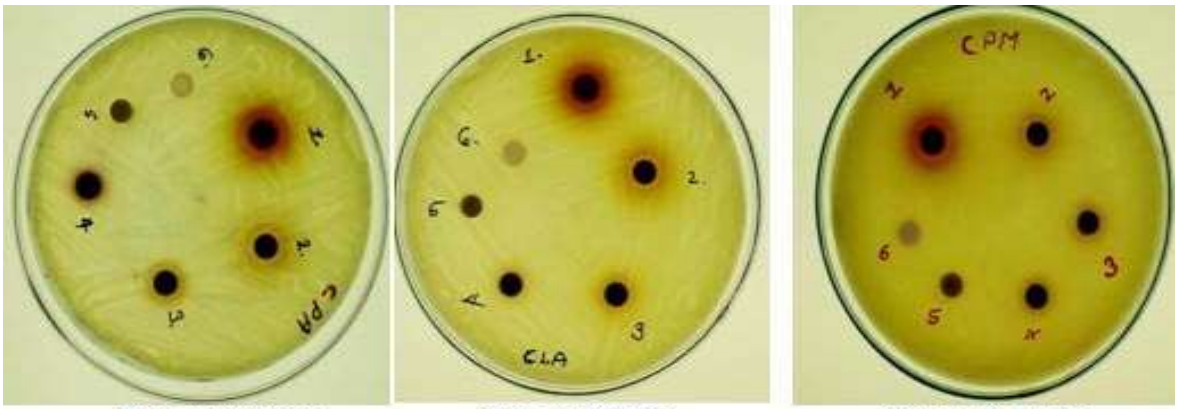
Table 3: Effect of HAEP, HAEL & HMEP of *C. fistula* against *E. coli, P. multocida* & *Candida albicans*

S.No.	Extract (mg/disc)	Inhibition Zone (mm) against *E. coli*	Inhibition Zone (mm) against *Pasteurella multocida*						Inhibition Zone (mm) against *Candida albicans*			
		24hr -48 hr	24 hr			48 hr			24hr-48 hr		24 hr	48hr
		HAEP/ HAEL/ HMEP	HAEP	HAEL	HMEP	HAEP	HAEL	HMEP	HAEP/HMEP	HAEP/ HMEP	HAEL	HAEL
1.	20	Nil	12±0.2	12±0.2	14±0.2	11±0.2	11±0.2	12±0.2	Nil	Nil	13±0.2	11.5±0.2
2.	10	Nil	10±0.2	11±0.2	10±0.2	9±0.2	9.5±0.2	9±0.2	Nil	Nil	11.5±0.2	10±0.2
3.	5	Nil	9±0.2	8±0.2	6±0.2	7±0.2	7±0.2	5±0.2	Nil	Nil	9±0.2	9±0.2
4.	2.5	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil	6±0.2	6±0.2
5.	1.25	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil

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

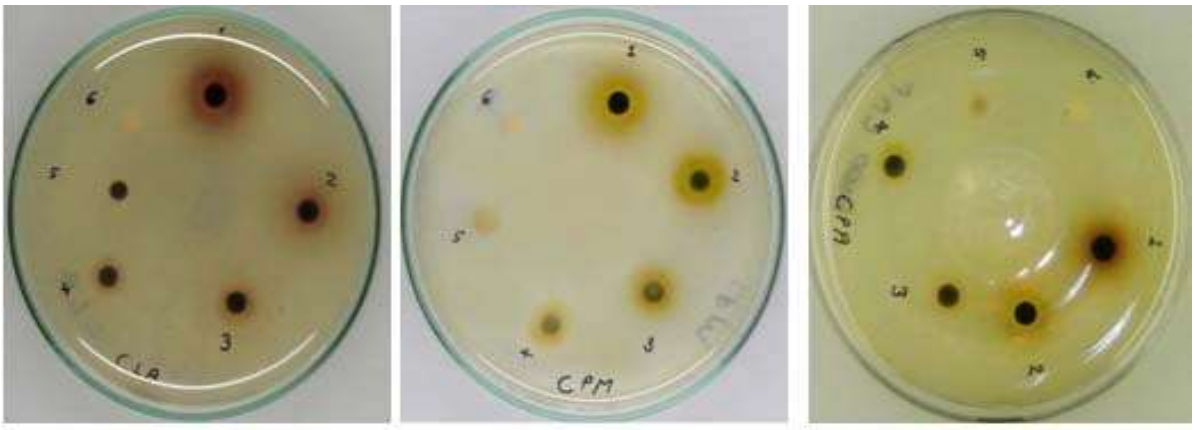
S.No	Antibiotics	*Stahypholoccus aureus*	*Escherichia coli*	*Pasteurella multocida*	*Candida albicans*
1.	**Vancomycin**	18+0.2	16+0.2	15+0.2	12+0.2
2.	**Kanamycin**	20+0.2	22+0.2	17+0.2	15+0.2
3.	**Streptomycin**	21+0.2	19+0.2	20+0.2	17+0.2

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



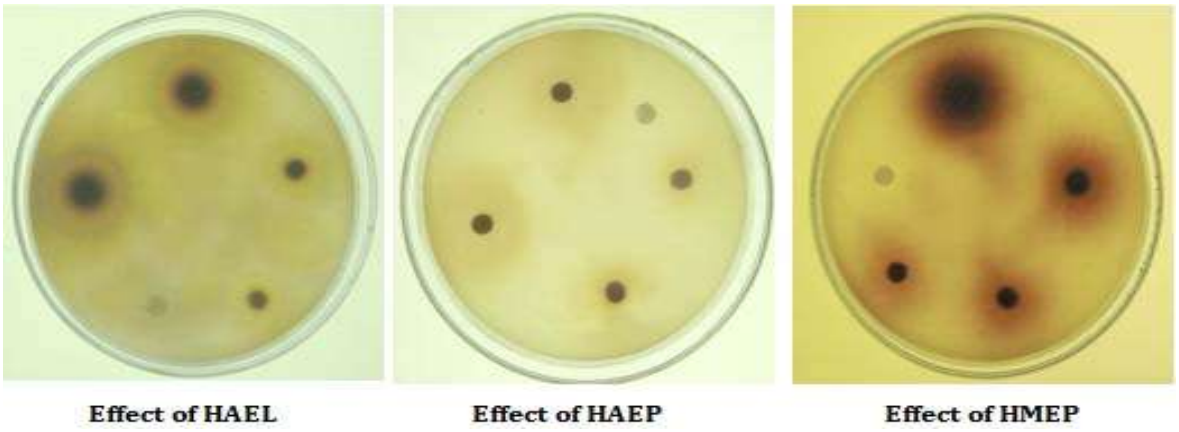
Effect of HAEP Effect of HAEL Effect of HMEP

Fig. 5: In Vitro antimicrobial effect of HAEP, HAEL and HMEP against *Pasteurella multocida*



Effect of HAEP Effect of HAEL Effect of HMEP

Fig. 6: In Vitro antimicrobial effect of HAEP, HAEL and HMEP against *Candida albicans*



DISCUSSION

Qualitative analysis of HAEP, HAEL 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 contract 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, HAEL 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 HAEL showed antifungal activity against *Candida albicans* which can be attributed to the presence of Kaempferol within HAEL [10].

CONCLUSION

The study concludes that HAEP, HAEL 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, HAEL and HMEP of *C. fistula,* presence of Sinapic acid and Gallic acid within HMEP while Kaempferol presence was restricted to HAEL. 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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