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Investigation and quantification of bioactive compounds from leaf extract of *Acacia arabica* as an effective antibacterial agent against *E.coli* associated with diarrheal infections

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Abstract

In this study antibacterial effect of bioactive compounds present in crude methanolic leaf extract of *Acacia arabica* was investigated against four different *E.coli* serogroups associated with diarrheal infections in animals. The serogroups were *E.coli* O22, O11, O89 and O61 along with one standard *E.coli* strain carrying LT and ST enterotoxins. Separation and identification of bioactive compounds was carried out by CC (Column chromatography), Preparative HPLC coupled with LC TOF ESI MS. Determination of antibacterial activity was carried out by well diffusion and broth microdilution method. The main compounds were identified including, methyl 3,4,5 tri hydroxyl benzoate ($C_8H_8O_5$), *p*-coumaroyl glucoside ($C_{15}H_{19}O_7$), *p* -coumaroyl quinic acid ($C_{16}H_{18}O_8$), Ferulic acid ($C_{10}H_{10}O_4$), isoferulic acid, epi catecine-3-gallate ($C_{22}H_{18}O_{10}$), ascorbic acid , quercetine 3-O- (4'-O-acetyl)-rhamnopyranoside ($C_{28}H_{30}O_{16}$), oleic acid (C_{18} $H_{34}O_2$), myristic acid ($C_{14}H_{28}O_2$), Palmitic acid ($C_{16}H_{32}O_2$) and steroidal sapogenin aglycons. The compound *p*-coumaroyl glucoside and *p* -coumaroyl quinic acid was obtained in combined form and largest antibacterial efficacy was observed in this combination. In addition, Ferulic acid and sapogenin aglycon was found to be having antibacterial potential.

Keywords: *Acacia arabica,* Preparative HPLC, LC TOF ESI MS

INTRODUCTION

Diarrheal infections associated with *E.coli* strain in live stocks and cattle in animal husbandry greatly affect dairy farmers. It not only affects the quality of milk but also economically effect dairy farmers annually. In villages most of the farmers rely on medicinal plants for treatment of domesticated animals but in recent years traditional system for treatment of animals using medicinal plants declined. Mubarack *et al*., (2011) reported the effective usage of medicinal plants for ethno veterinary practices in India. However, scientific approach is necessary for selection of medicinal plant associated with particular infection causing microorganisms in animals. The present study deals with antibacterial activity of methanolic leaf extract of *Acacia arabica* against diarrheagenic strains of *E.coli* responsible for infections in cows and buffaloes.

The tree was principally used for long time for treatment of various ailments including tooth ache, itching, diarrhea and dysentery. Crushed leaves are also reported to be effective to cure soar throat, eye infections and can be taken internally with out any side effect and also reported to cure chronic diarrhea & blood dysentery (Asolkar *et al*., 1992, Warrier *et al*., 1994). Gum obtained from plant was reported to be effective to diabetes mellitus (Chopra *et al.,* 1992). Bark extract of Indian variety of *Acacia arabica* was reported to be having potential inhibitory activity against three different fish pathogens *Aeromonas hydrophila, Pseudomona flurescens* and *Edwardenella tarda* responsible for heavy mortality of cultured and wild fishes (Muniruzzanman and Chowdhury, 2004). The present finding deals with antibacterial activity of purified bioactive compounds separated and identified by TOF ESI MS techniques.

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METHODOLOGY

Culture

Four different strains of *E.coli* O22, O11, O89 and O61 were collected from Veterinary Hospital, Supela and environmental samples (drinking water sources from unorganized firms and animal husbandry). Two clinical isolates, strain O22 and O11 were isolated from diarrheagenic calf and buffalo stools while strains O89 and O61 were isolated from environmental samples. In addition one standard strain *E.coli* MTCC 723 was obtained from IMTECH, Chandigarh.

Culture Identification

Serotyping and identification of all strains was performed at National Salmonella and Escherichia Center, CRI, Kasauli (H.P) and ribotyping was done at DBRI, Hyderabad.

Collection of plant materials

Leaf of *Acacia arabica* was collected during November in specimen voucher and thoroughly washed and shade dried. All plant materials were separately pulverized for preparation of extracts.

Preparation of crude extracts

Crude methanolic leaf extract was prepared by the method

recommanded by Johnson *et al.,* (2008).

Bioassay guided fractionation of crude extracts
Column chromatography of leaf extract

Crude methanolic leaf extract was subjected to sephadex LH-20 column. Elution was carried out using polar solvent mixture of methanol; water (1:1), methanol: water (8:2), acetone: water (1:1) and acetone: water (8:2) as per method recommended by Saleem *et al.* (2009) with minor modifications. Successive fractions were collected separately and dried under vacuum using rotary evaporator for further investigation of antibacterial activity against *E.coli* by well diffusion and broth microdilution method.

Preparative HPLC of leaf extract of *Acacia arabica*

All the four biologically active fractions were subjected to Preparative HPLC (LC-18, Shimadzu, Japan) for separation of bioactive compounds. About 10 µl of each sample was injected to C18 column with pore size of 5µM using different wavelengths 280nm, 294nm and 289nm. The mobile phase used in the process was acetonitrile: water (7:3) with flow rate of 10mL/min. Fractions collected for each peak were concentrated and used for LC-MS analysis.

Spectral analysis of bioactive components using LC-MS analysis

Each peak collected separately was lyophilized and redissolved in known volume of 100% MeOH for further using LC-MS. For LC analysis Perkin Elmer Series (Japan) was used with ESI- TOF and LC conditions were same as used in RP-HPLC with methanol flow rate of 1 ml min^{-1}. Both positive and negative ESI was used for detection of mass of every individual peaks *i.e* sub- fractions. Positive ESI was used for detection of saponins and aldehyde groups and negative ESI was used for estimation of organic acids and OH containing organic bioactive components. For negative ESI mass ranges from 200 to 900 *m/z,* scan speed 60-700 *m/z* (cycle time : 330 m sec), dry gas N_2, dry gas temperature 360° , capillary voltage 4000 V (Theerasin and Baker, 2009). For positive ESI mass ranges from 400 to 1000 *m/z,* scan speed 400-1122 *m/z* (cycle time dry gas N_2, dry gas temperature 230, capillary voltage 4500 V. Data acquisition was performed from the company supplied software with the instrument. The injection volume was 1 µl. The data was collected and compared with mass spectra of data from literatures and softwares. The fragmentation was used to detect and identify few of the compounds present in the extracts

Investigation of antibacterial activity of fractions and sub fractions

The effects of fractions (Column Chromatography derived) and sub fractions (Preparative HPLC derived) was investigated by well diffusion and broth microdilution technique with some minor modifications (Cock, 2008, Kyung *et al.,* 2007).

Data analysis

All experiments were carried out in triplicates and the results were present as mean SEM (Standard Error of Mean). The data was subjected to one way ANOVA ($p<0.05$ and 0.01)

RESULTS AND DISCUSSION

Plants and plant derived compounds always act as safest agent to cure various ailments including diarrheal infections. Tang *et al.,* 2010 strongly advocated the use of medicinal plants as safest alternative to cure infections. The method used to determine antibacterial activity of four different fractions obtained from CC was well diffusion method and broth micro dilution method. Among both method broth micro dilution method using INT dye was found to be most reliable and accurate over well diffusion method due to its feasibility of handling large number of samples at the same time as well as very limited amount of extract fractions were required to obtain visible results.

Table 1. Antibacterial activity of fractions obtained from column chromatography IZD± SEM (mm)

E.coli	Fraction 1 methanol: water fraction (1:1)	Fraction 2 methanol:water fraction (8:2)	Fraction 3 acetone : water fraction (1:1)	Fraction 4 acetone : water fraction (8:2)
O22	10 ±1.1	10 ±1.1	6.6± 0.6	0
O11	2± 1.1	6.6 ± 0.6	2.6± 2.6	0
O89	3.3 ± 1.76	9.3± 1.73	1.3 ±1.33	0
O61	11.3 ±1.76	8.6± 0.6	8 ±1.1	0
MTCC 723	12.6± 1.1	19.3± 0.6	19.3± 0.6	19± 0.4

Table 1 represents antibacterial activity of four different sub fractions against five different *E.coli* strains on the basis of inhibitory zone diameter. IZD observed in fraction 1 , 2 and 3 ranged between 2 to 12.6 mm. 6.6 to 19.3 mm and 2.6 to 19.3 mm respectively with $p<0.01$.

Table 2. MIC of Fractions (in µg)

E.coli	Fraction 1 methanol: water fraction (1:1)	Fraction 2 methanol : water fraction (8:2)	Fraction 3 Acetone : water fraction (1:1)	Fraction 4 Acetone : water fraction (8:2)
O22	1.25	1.25	0.6	0.3
O11	1.25	"	"	"
O89	1.25	"	"	"
O61	0.6	"	"	"
MTCC 723	1.25	"	"	"

Minimum inhibitory concentration (MIC) as detected by broth micro dilution method of four different sub fractions were observed to be 1.25 µg for both fraction 1 and 2 , 0.6 µg for fraction 3 and 0.3 µg for fraction 4.

All the four biologically active fractions were subjected to Preparative HPLC for proper and accurate separation of bioactive compounds present in sub fractions. Since the bioactive compounds are found in complex form, step wise separation using Preparative HPLC act as powerful tool for obtaining the sub fractions in proper way. Further these sub fractions was detected using LCMS in order to identify the compound of interest.

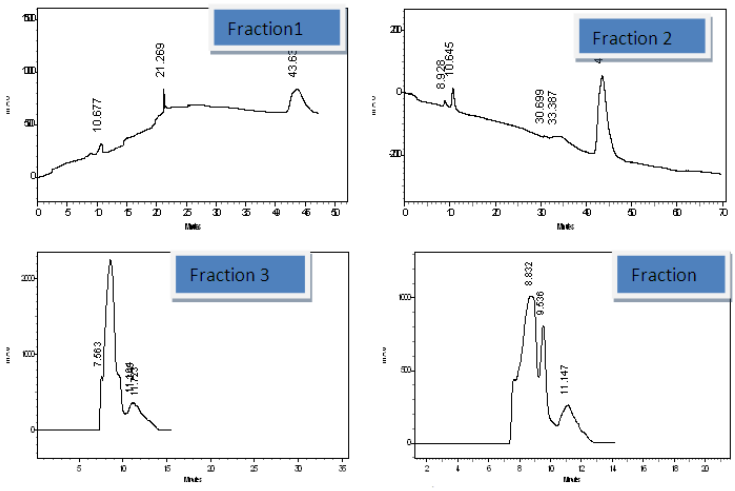
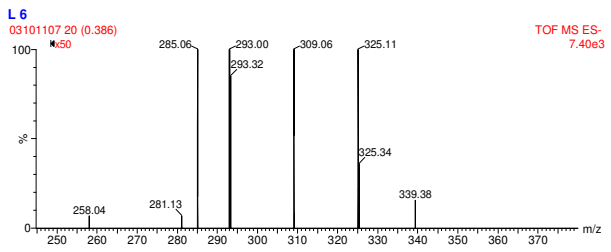


Fig 1. Preparative HPLC Chromatogram of four different fractions of leaf extract of *Acacia arabica*



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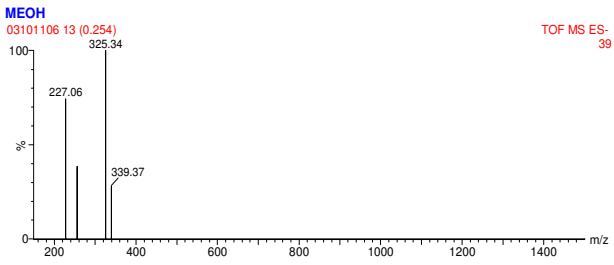

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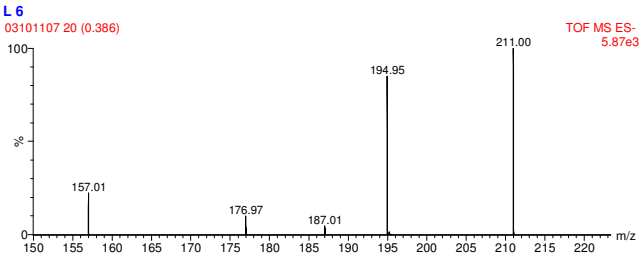

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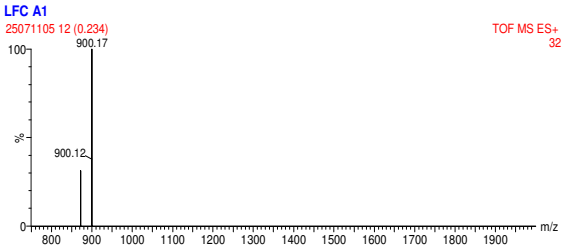

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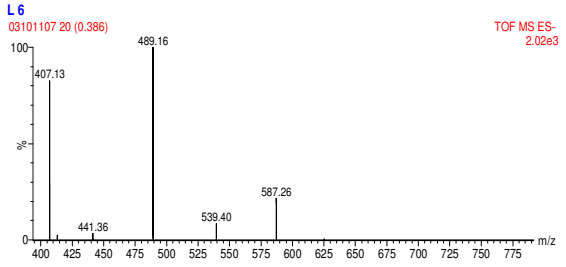

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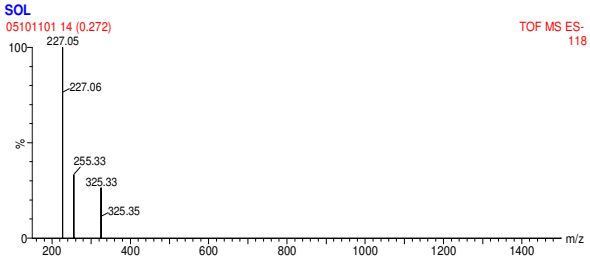

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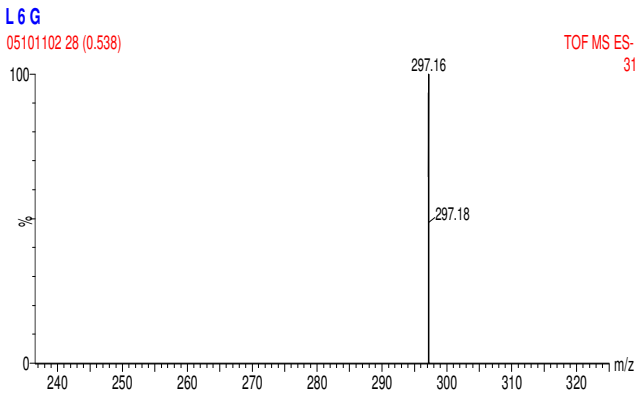

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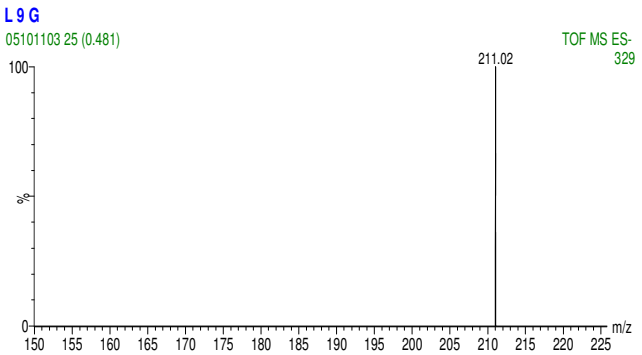

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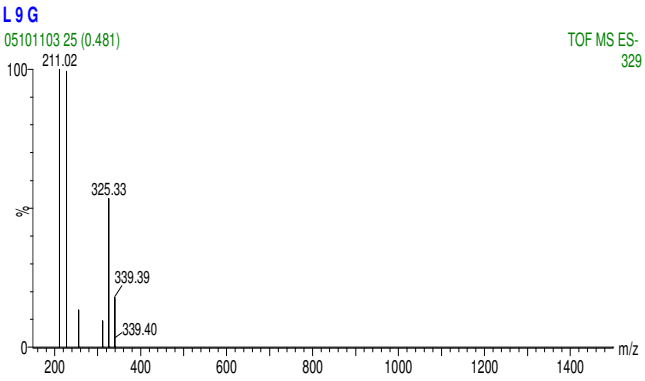

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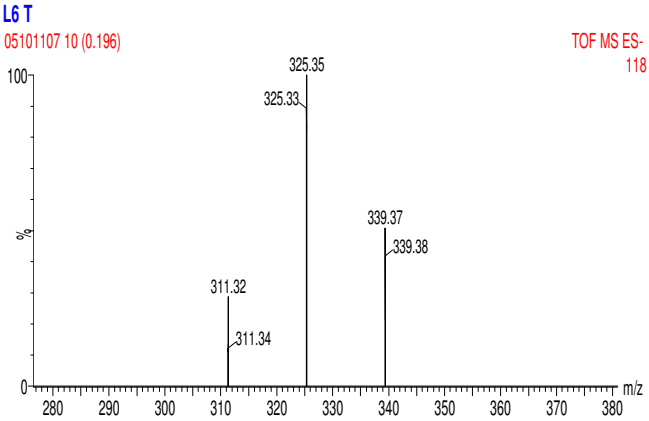

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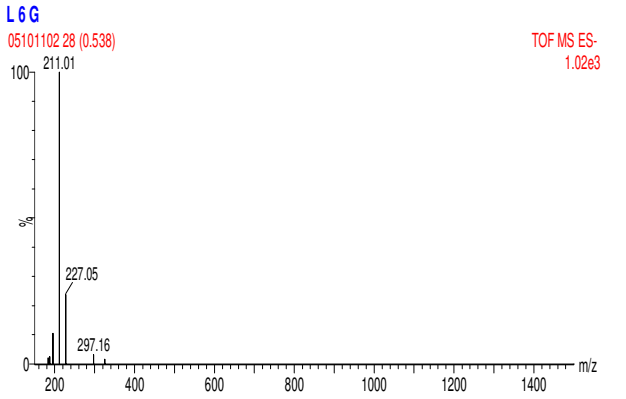

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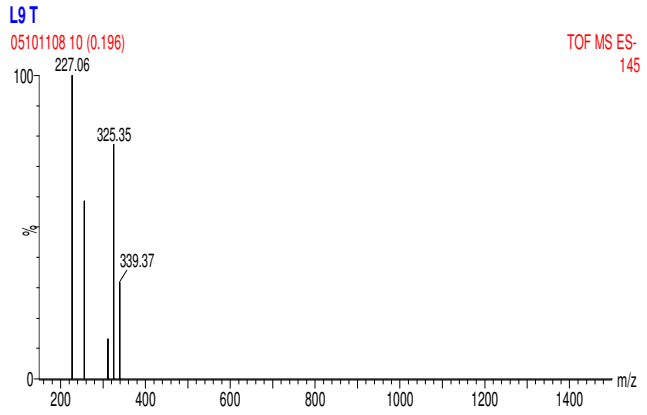

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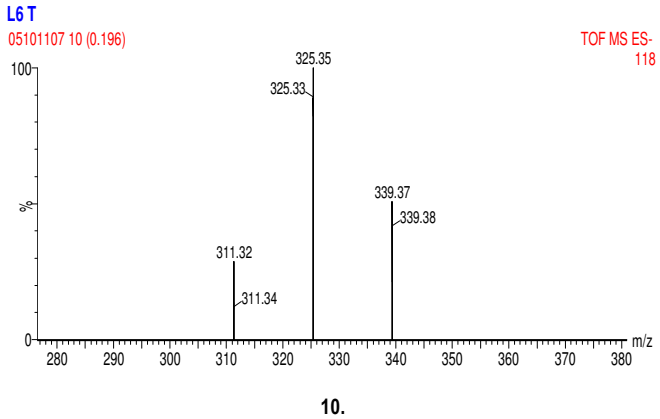

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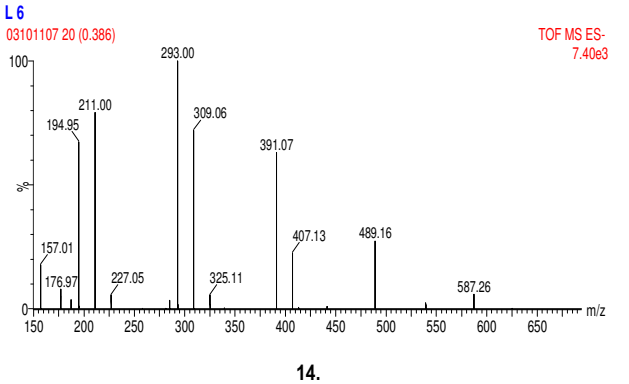

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Fig 2. TOF ESI MS of sub fractions of four different fractions No 1-14 represents m/z values of individual peaks obtained and separated by Preparative HPLC (1-3) fraction 1, (4-8) fraction 2, (9-11) fraction 3 and (12-14) of fraction 4.

In our present study Preparative HPLC separation of fraction 1 show presence of three different peaks with retention time of 10.677, 21.269 and 43.637 min whereas fraction 2 shows five different peaks with retention time of 8.928, 10.645, 30.699, 33.387 and 43.573 min respectively. Three peaks were observed in fraction 3 & 4with retention time of 0.181, 11.243, 11.723 min and 8.832, 9.536 and 11.147 min respectively. Structural identification of individual peak compounds present in fractions was performed by LC TOF ESI MS. Each *m/z* value was matched with molecular weight of available literatures as mentioned in Table: 3

Table 3. Identified compounds on basis of *m/z* values

Tentative ID of compounds	Molecular weight	*m/z* value	Wave length	Ion (+/-)	References
oleic acid $C_{18}H_{34}O_2$	282	281.13	289	$[M-H]^-$	Houjou *et al.,* (2007)
myrestic acid $C_{14}H_{28}O_2$	228	227.6	294	[M-H]-	Houjou *et al.,* (2007)
Palmetic acid $C_{16}H_{32}O_2$	256	255.33	289	[M-H]-	Houjou *et al.,* (2007)
Ferulic acid $C_{10}H_{10}O_4$	194	194.95	294, 289	$[M]^+$, $[M+H]^+$	Giusti *et al*., (1999)
p-coumaroyl glucoside $C_{15}H_{19}O_7$	326	325.11, 325.33, 325.34,325.35	289, 294	[M-H]-	Seeram *et al*., (2006), Aaby *et al.,* (2007)
p -coumaroyl quinic acid $C_{16}H_{18}O_8$	340	339.37,339.38, 339.39, 339.40	289, 294	[M-H]-	Seeram *et al*., (2006), Aaby *et al.,* (2007)
ascorbic acid $C_6H_8O_6$	176	176.97	294	$[M]^+$	Aaby *et al.,* (2007)
epi catecine-3-gallate $C_{22}H_{18}O_{10}$	442.37	441.36	289	[M-H]-	Ma *et al*., (2004)
quercetine 3-O- (4'-O-acetyl)-rhamnopyranoside $C_{28}H_{30}O_{16}$	490	489.16,	289	[M-H]-	Laponen *et al.,* (2001)
Methyl 3,4,5 tri hydroxyl benzoate $C_8H_8O_5$	184.5	183.07	294	[M-H]-	Mahajan and Pai, (2010)
Steroidal sapogenin	700-1500	900.15	289,294	$[M+H]^+$	Berhow *et al*., (2002)

The majority of compounds detected on the basis of fragmentation patterns were gallic acid and tannic acid derivatives; In addition fatty acids, flavonoids and saponins were also detected and matched with available literatures. The gallic acid derivatives observed were epi catecine-3-gallate (*m/z* of 441.36) and methyl 3, 4, 5 tri hydroxyl benzoate (*m/z* of 183.07), flavonid (quercetine 3-O-(4'-O-acetyl)-rhamnopyranoside) fragmented ions of three different fatty acids: oleic acid , myrestic acid and Palmitic acid were also observed as major components. In addition, peaks detected during LC/MS analysis with *m/z* of 194.95 was recognized to be ferulic acid in combination with monogaloyl aglycon unit (*m/z* of 211) and ascorbic acid (*m/z* of 176.97). The major compound present in leaf extract fraction was *p*-coumaroyl glucoside and *p* -coumaroyl quinic acid and suggested to be having antibacterial effect. In addition, fraction was rich in Steroidal sapogenin (*m/z* of 900.15) and its detection was carried out by matching *m/z* value of standard saponin. Sasidharan *et al.,* (2011) documented that Preparative HPLC coupled with LC/MS is one of the most powerful technique for accurate identification of compounds present in botanical extracts.

Table 4. Mean IZD± SEM (in mm) of sub fractions of leaf extracts

E.coli	*p*-coumaroyl glucoside + *p* -coumaroyl quinic acid	Ferulic acid	Steroidal sapogenin
O22	23.3 ±0.6	14.6± 2.4	8.6±0.6
MTCC 723	24 ± 1.1	16± 0	6

On purification of12 different identified compounds, visible antibacterial activity was recorded only in three major compounds. The largest inhibitory zone (Table: 4) was observed in *p*-coumaroyl glucoside + *p* -coumaroyl quinic acid followed by ferulic acid and Steroidal sapogenin with $p<0.01$

Table 5. MIC of bioactive compounds

E.coli	*p*-coumaroyl glucoside + *p* -coumaroyl quinic acid	Ferulic acid	Steroidal sapogenin	Chloramphencol
O22	1.70 ± 0.4	4.9± 1.2	8.3 ±1.6	3.3±0.8
MTCC 723	2.13± 0.4	3.7 ± 0	6.6 ± 0	1.6±0.41

MIC results obtained from these three identified compounds also show similarity in findings of more antibacterial effectiveness of *p*-coumaroyl glucoside + *p*–coumaroyl quinic acid over rest two compounds and positive Chloramphenicol control (Table: 5). Lowest MIC represents more antibacterial effectiveness of compound over tested organism. The findings on antibacterial efficacy of coumarins is in agreement with the findings of Esterhuizen *et al.*, (2006) who reported antibacterial effectiveness of coumarins derived from

Coleonema album against *E.coli.* The study on antibacterial effectiveness of ferulic acid and sapogenin aglycon justifies the findings of Merkl *et al*., (2010) and Arabski *et al.,* (2012)

Therefore, it can be concluded that the use of plant extract and its derived bioactive compounds act as safest alternative to cure *E.coli* associated diarrheal infections in animals.

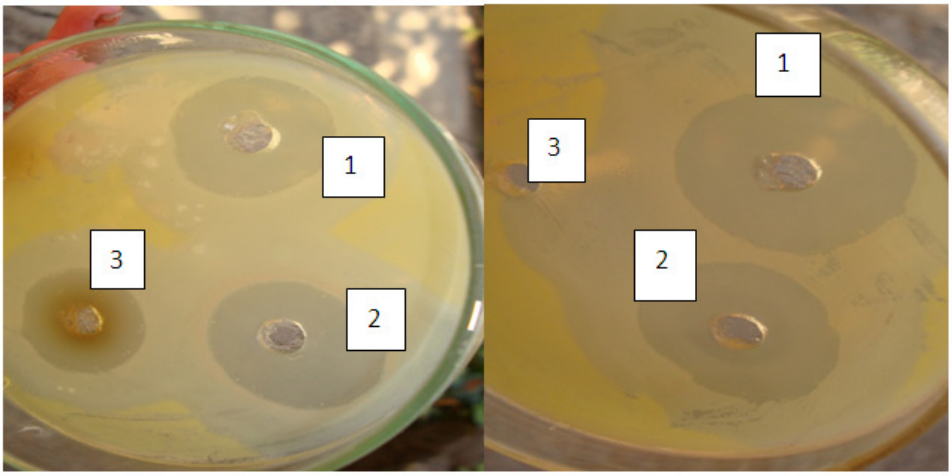

Plate 1 effect of bioactive compounds on E.coli O22 a) Plate 2 b) E.coli MTCC 723
1. *p*-coumaroyl glucoside + *p* -coumaroyl quinic acid, 2. Ferulic acid 3. Steroidal sapogenin

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