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LOCAL MEDICINAL PLANTS OF PAKISTAN IN THE FIGHT AGAINST GASTRO-INTESTINAL TRACT PATHOGENIC BACTERIA

Running title: Local Medicinal Plants of Pakistan against Gastrointestinal Tract Bacteria

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ABSTRACT
The increase of drug-resistant pathogens is persistent in the control of pathogens. Several effective drugs have their roots in natural products, which have a traditional use in different diseases. To authenticate the efficacy of these traditional medicines, we selected the gastrointestinal tract infection as a target disease. Two plants *Withania coagulans* and *Ehretia obtusifolia*, were selected on the bases of their usage in folk medicine for the gastrointestinal tract complications. The bacteria selected were *Escherichia coli***,** *Shigella dysenteriae, Salmonella typhi, Pseudomonas aeruginosa, Vibrio cholera* and *Aeromonas* spp. The crude extracts and fractions at three concentrations (15, 30, and 50 mg/ml) were tested for effectiveness against *E. coli*, *S. dysenteriae*, *V. cholrea*, *S. typhi, P. aeruginosa* and *Aeromonas spp*. The results show that the *Ehretia obtusifolia* has good activity against all test bacteria except *S. dysenteriae* while *W. coagulans* had activity against all the test bacteria.

Key words: *Withania coagulans; Ehretia obtusifolia;* Gastro-intestinal tract; Gram negative bacteria

INTRODUCTION
The gastrointestinal (GI) tract is a long hollow tube extending from the oral cavity where food enters the body, via the esophagus, stomach, small intestine, large intestine, rectum, and finally to the anus where undigested food is expelled. The function of the GI system is to process nutrients and energy from food and fluids that we ingest. In case of any complication in the gastrointestinal system these functions of food processing are impaired. These complications may be functional gastrointestinal disorders or may be due to various microbial infections. Patients with infections of the gastrointestinal tract may have various symptoms such as malaise and anorexia to more serious manifestations such as severe diarrhea and sepsis. Most of microbial infections of GI system are bacteriostatic and the most common cause of these infections are the gram-negative bacteria. Only *Escherichia coli* accounts for more than 80% of urinary tract infections [Delzell and Lefevre, 2000]. GI tract infections are widespread; nearly every person has faced the problem occasionally. Only in the USA every year there are 211 million cases per year of diarrhea. At present the only available therapy for GIT infections is the use of antibiotics. Resistance against current antimicrobial drugs has taken the world by storm and the researchers are introducing new antibiotics to cope with the situation [G.O. Adeshina, 2012]. The control of excessive use of antibiotics, understanding the genetic mechanisms undunderlying drug resistance and developing new drugs either synthetic or natural may alleviate this problem [Ahmad and Beg, 2001]. Resistance against antibiotics used for pathogens affecting gut has shown a 5-fold increase [Roshni Barad, 2013]. To overcome the situation, the old therapies are reviewed as a replacement of antibiotics. FDA has been approved Nitazoxanide as treatment of diarrhea caused by *Giardia intestinalis* and *Cryptosporidium paryum* [Bobak, 2006]. Fosfomycin tromethamine, a soluble salt has been approved for the treatment of uncomplicated urinary tract infections (UTIs) caused by *E. coli* and *Enterococcus faecalis* ("Fosfomycin: Use beyond urinary tract and gastrointestinal infections," 2010). The other option is to turn back to the classical traditional medicines which are safe with the minimum side effects. The use of traditional medicines has seen a global expansion recently not only in third world countries but also in many developed countries [Deni, 1996]. Numerous studies report an extensive range of secondary metabolites of medicinal plants, belonging to groups like carbohydrates flavonoids, tannins, sterols, alkaloids, glycosides withanolides and saponins, having anti-microbial potentials [Chevallier, 1996].

Pharmacological effects of *Withania coagulans* are known in the treatment of many disorders like fati-

gue, sleeplessness, weakness, liver disorders, asthma, nausea [Hanberger and Nilsson, 1996; Cowan, 1999]. Apart from these the anti-inflammatory, antimicrobial, hepato-protective, immunosuppressive, antitumor, anti-hypoglycemic and cardiovascular potentials are reported [Hoareau and J. DaSilva, 1999]. The fruits of *W. coagulans* contain volatile oil that are active against *S. aureus* and *V. cholera*. *Ehretia obtusifolia* is useful in infections, itches, cough, dysentery, venereal diseases, diarrhea, fever and syphilis [Iqbal et al., 2004; Iqbal et al., 2005].

The medicinal importance and the usage of the two species in traditional medicine especially in GIT ailments prompted us to investigate and scientifically ascertain the antibacterial potential of these plants against important and common gut pathogenic bacteria.

MATERIALS AND METHODS

Collection and Identification of Plants Material: Aerial parts (leaves and stems) of *Ehretia obtusifolia* were collected from Swat while the *Withania coagulans* was collected from Kohat area of Khyber Pakhtunkhwa, Pakistan. The Plants were identified by Mr. Muhammad Zafar Department of Botany, Kohat University of Science and Technology, Kohat, Pakistan.

Processing and Extraction of Plants Material: The plant materials were washed with tap water and shade dried for three weeks, ground the dried plants and then soaked in methanol for 12 days. After every four days methanol was filtered from powder and concentrated on a rotary evaporator and then added fresh solvent into the powder. All the extracts of each plant were combined, dried and weighed. The crude extract yields for *E. obtusifolia* leaves and stems parts were 51g (8.4 %) and 47g (6.3 %), respectively, While *W. coagulans* leaves yielded 56g (8.2 %) and stem parts gave 43g (5.8 %). All the four extracts were re-suspended in distilled water and fractionated in a sequence using hexane, chloroform, ethyl acetate in increasing order of polarity leaving an aqueous residual fraction at the end. Fractions and crude extracts were dissolved in dimethyl sulphoxide (DMSO), and prepared three concentrations (15 mg/mL, 30 mg/mL, and 50 mg/mL) of each fraction for testing against the selected bacteria. DMSO was used as a negative control while amoxicillin (30µg disc) served as a positive control [Khandelwal, 2010].

Bacterial Strains and Antibacterial Assays: The plant extracts were tested against 6 gram-negative gut pathogenic bacteria, *Escherichia coli, Salmonella typhi, Shigella dysenteriae, Pseudomonas aeruginosa, Vibrio cholera* and *Aeromonas spp.* The bacteria strains were kindly provided by the Department of Microbiology, Kohat University of Science and Technology. Nutrient agar medium was used to check the different plant extract against gastrointestinal bacteria and prepared according to the manufacturer procedure. The microorganisms were sub-cultured into the nutrient broth and incubated at 37^0C for18 hours. They were further streaked on the Nutrient agar and incubated at 37^oC for 24 hours. The 24-hour broth culture of the test organism was suspended into the sterile nutrient broth. The organisms were collected in their mid-log growth phase and standardized according to National Committee for Clinical Laboratory Standards (NCCLS, 2002) by gradually adding normal saline to compare its turbidity to McFarland standard of 0.5 which is approximately 1.0 × 106 CFU/mL. The standardized inoculum was spread evenly on the entire surface of Muller Hinton agar plates using a sterile cotton swab. Subsequently, a sterile borer, of 6mm diameter was used and the required numbers of wells were made in the inoculated plates, which were then filled with 100µL of each test solutions (crude extracts, fractions, serial dilutions) along with negative control of DMSO and positive control of amoxicillin. Plates were placed upright in a refrigerator for an hour to allow diffusion of extract into the surrounding media. After that, the plates were incubated overnight at 37 °C in an inverted position. Following incubation the zones of inhibition were measured for all the tests that were conducted in triplicate . An inhibitory zone of <10 mm was considered to be no activity [Palombo and Semple, 2001; G.O. Adeshina, 2012; Djeussi et al., 2013].

Phytochemical analysis: Phytochemical analysis were performed to detect the bioactive compounds in both plants by employing the standard procedures [Khandelwal, 2010; Mathur, 2011].

Test for Carbohydrates (Molish's test): Two drops of α-naphthol and 2 mL of concentrated Sulphuric acid was added into 5mg of plant extract to form a layer below the mixture. A red-violet ring appeared, indicating the presence of carbohydrates.

Test for Alkaloids (Mayer's test): A few drops of Mayer's reagent were added to the crude extract. The formation of a pale-yellow precipitate indicates the presence of alkaloids in the extract

Test for Flavonoids (Lead acetate test): 2 mL of lead acetate solution was added to the plant extract. A yellow precipitate was formed, which confirm the presence of flavonoids.

Test for Proteins (Biuret's test): A few drops of sodium hydroxide solution were added to the test extract and then added two drops of copper sulph-

ate solution. A violet-red color was formed indicating the presence of proteins.

Test for Phenol (Ferric Chloride test): Few drops of ferric chloride solution were added to 5mg extract. A bluish black color was formed indicating the presence of phenolic compounds.

Test for Steroids (Salkowshi test): A few drops of concentrated Sulphuric acid were added to the crude extract a red color appeared in the lower layer indicating the presence of steroids.

Test for Glycosides (killer killani test): A few drops of the mixture of glacial acetic, 5% $FeCl_3$ and conc. H_2SO_4 were added to 4mg extract. A reddish-brown color appeared at the junction of the two liquid layers and the upper layer appears bluish green

Test for Saponins (Foam test): Five mg of the extract was vigorously shaken with water. The existence of persistent foam indicates the presence of saponins

Test for Tannins (Gelatin test): 1% gelatin solution containing sodium chloride was stirred with nearly 10 mg of extract. The formation of a white precipitate indicates the presence of tannins.

Statistical Analysis: Results were expressed as mean value, ± standard error deviation and differences between means were analyzed statistically using an analysis of variance (ANOVA) according to LSD test through SPSS 16.0 software package. Differences were considered significant when $P \leq 0.05$.

RESULTS

The present study revealed the antibacterial activities of various fractions of *E. obtusifolia* and *W. coagulans* using aerial parts of these plants. The phytochemical screening depicted several secondary metabolites. All dilutions were made in dimethyl sulphoxide (DMSO) which was found to have no inhibitory effects on the growth of bacteria. Three concentrations i.e., 15mg/mL, 30mg/ml and 50mg/ml of each extract were tested against six bacterial species (*E. coli, S. typhi, S. dysenteriae, P. aeroginosa, V. cholrae* and *Aeromonas spp.*). All the experiments were repeated in triplicate and results were recorded as mean ± SEM. The results indicated that both plants have good antibacterial activity against these gastrointestinal bacteria.

Antibacterial Activity of Stem Fractions of *Heretia obtusifolia:* The five extracts of stem of *E. obtusifolia* presented good antibacterial activity against all the test bacteria except *S. dysenteriae* where no activity was observed.

All the fractions showed excellent activity at 50mg/mL concentration against *Aeromonas spp.*, n-hexane chloroform and ethyl acetate having a zone of inhibition of 18.67mm, 19.67mm, 20.33 mm and 18mm respectively. At 15mg/mL ethyl acetate fraction depicted the lowest activity, while the chloroform fraction showed no activity. Similarly, no activity was recorded for the aqueous fraction (Table 1).

Antibacterial Activity of Leave Fractions of *Ehretia obtusifolia:* The leaves extract of *E. obtusifolia* showed good activity against all gastrointestinal pathogens except for *S. dysenteriae* where the extracts remained inactive. Crude leave extract had maximum activity against *Aeromonas spp.* (21 mm) at 50 mg/ml (Table 1), followed by *E. coli* and *P. aeroginosa* (>18 mm each) *S. typhi* and *V. cholerae* were also better controlled with inhibitions ranging between 15-16 mm.

Table 1: *Ehretia obtusifolia* stem and leaves extract inhibition zones (mean mm ± SEM) against selected Gram-negative bacteria

	Stem extracts			**Leave extracts**			***Test Bacteria***
TC	***15mg/mL***	***30mg/mL***	***50mg/mL***	***15mg/mL***	***30mg/mL***	***50mg/mL***	
C	15 ± 0.4	16.7±0.2	18.7±0.2	12.3±0.3	17±0.4	21±0.4	***Aeromonas spp.***
H	15 ± 0.4	17.7±0.47	19.7±0.2	0	10.7±0.3	13.7±0.3	
Ch	0	16.3±0.2	20.3±0.2	0	12.7±0.4	15±0.4	
EA	10±0.6	12.7±0.6	18±0.4	0	0	14.7±0.4	
A	0	0	0	0	0	11±0.40	
N	0			0			
P	14.67± 0.47			15.3±0.47			
C	13±0.4	17.3±0.2	21±0.4	12±0.40	16.3±0.6	18.3±0.6	***E. coli***
H	15±0.2	18.7±0.2	21±0.4	14±0.8	19.7±0.3	21.7±0.2	
Ch	14±0.40	17.3±0.2	20.3±0.2	0	17±0.4	22±0.4	
EA	0	12±0.4	16±0.4	14±0.81	15.6±0.5	19.6±0.2	
A	0	0	0	0	0	0	
N	0			0			
P	15±0.40			14.3±0.62			
C	10±0.40	11.7±023	15±0.4	14.7±0.2	17.7±0.2	18.7±0.6	***P. aeruginosa***
H	13.3±0.6	16.7±023	18±0.4	15±0.4	20±0.4	23±0.4	
Ch	0	15.67±0.2	18.7±0.2	15±0.4	19±0.4	22.3±0.2	

EA	0	10.7±0.6	16±0.4	0	0	13.3±0.6	
A	0	0	0	0	0	12.7±0.2	
N	0			0			
P	14±0.70			14.7±0.62			
C	13±0.4	16.7±0.2	18±0.4	11±0.4	12.7±0.5	15.7±0.3	***S. typhi***
H	12.7±0.5	13±0.4	17±0.4	11±0.4	14±0.8	15.3±0.2	
Ch	13±0.40	19±0.40	21.7±023	0	16.7±0.2	18.3±0.2	
EA	11±0.40	15.3±023	18.3±023	0	11.7±0.8	14±0.4	
A	0	0	0	0	0	0	
N	0			0			
P	12.33±0.23			13.33±0.40			
C	0	0	0	0	0	0	***S. dysenteriae***
H	0	0	0	0	0	0	
Ch	0	0	0	0	0	0	
EA	0	0	0	0	0	0	
A	0	0	0	0	0	0	
N	0			0			
P	14.1±0.2			14.4±0.02			
C	11.7±023	14±0.40	17.3±0.5	10.2±0.2	13.3±0.2	16±0.40	***V. cholrea***
H	13±0.40	14±0.40	18±0.4	12±0.4	13.7±0.3	16.7±0.3	
Ch	13.3±0.6	16±0.40	18±0.4	13.3±0.3	14.7±0.2	17.7±0.2	
EA	0	0	12±0.40	0	11.3±0.6	13±0.40	
A	0	0	0	0	0	0	
N	0			0			
P	14±0.40			13.33±0.23			

TC = Test concentration; C = Crude; H = hexane; Ch = Chloroform; EA = Ethyl acetate; A = Aqueous; N = Negative control; P = Positive control

Hexane and chloroform fractions were the most effective ones against all the bacterial strains apart from *S. dysenteriae* which showed complete resistance. Hexane fraction showed maximum activity (23mm) against *P. aeroginosa* at 50mg/ml. Chloroform fraction showed highest activity against *E. coli, P. aeroginosa* (22mm) and *Aeromonas spp.* (>15 mm inhibition zones)., while the lowest activity was observed against *E. coli*. The aqueous fraction was marginally active against only two bacterial strains (*Aeromonas spp.* and *P. aeroginosa*). Hexane, chloroform and ethyl acetate fractions showed modest activity within the range of 14-16mm against *S. typhi* and *V. cholera* at high concentration (Table 1).

Ethyl acetate fractions showed excellent antibacterial activity against *E. coli* (>19mm), and good activities against the rest of the test bacteria. However, weak activity was observed for *Aeromonas spp*. The inhibitory zones produced by *E. obtusifolia* against the test bacteria are shown in Fig. 1.

Fig.1.1. *E. obtusifolia* against *Aeromonas spp.*

Fig.1.2. *E. obtusifolia* against *Aeromonas spp.*

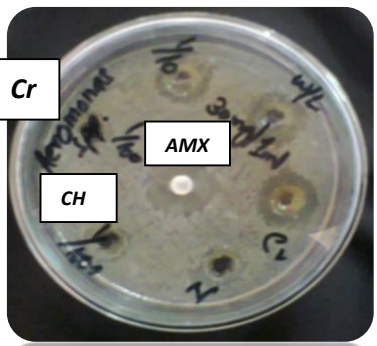

Fig.1.3. *E. obtusifolia* against *S. typhi*

Fig.1.4. *E. obtusifolia* against *Aeromonas spp.*

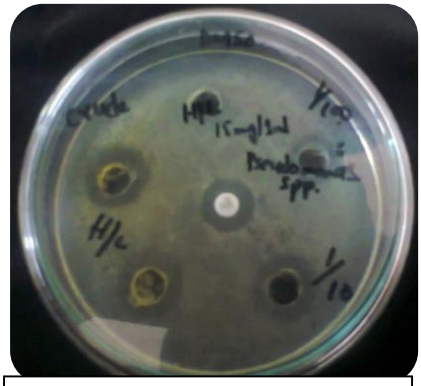

Fig.1.5. *E. obtusifolia* against *S. typhi*

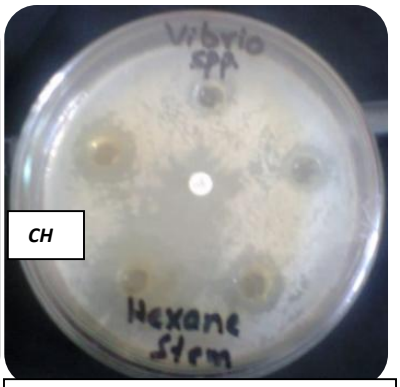

Fig.1.6. *E. obtusifolia* against *V. cholrae*

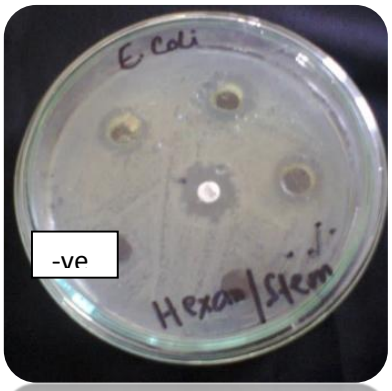

Fig.1.7. *E. obtusifolia* against *E. coli*

Fig.1.8. *E. obtusifolia* against *S. typhi*

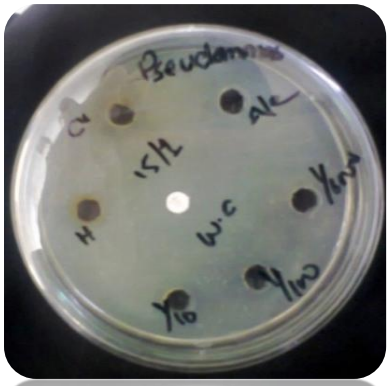

Fig.1.9. *E. obtusifolia* against *P. aeroginosa*

Fig 1: Activities of the *E. obtusifolia* against the selected pathogens

Antibacterial Activity of Stem Fractions of *Withania coagulans:* The stem extracts of *W. coagulans* showed antibacterial activity against all test bacteria. The highest zone of inhibition was shown by hexane extract (Table 2) at a conc. of 50mg/mL against *Aeromonas spp.* (>17 mm), while at concentration of 30 mg/ml it inhibited *E. coli* (>10 mm), *S. dysenteriae* (>12 mm), *V. cholrea* (>10 m m), and *Aeromonas spp.* (>15 mm).

Table 2: *Withania coagulans* stem and leaves extract inhibition zones (mean mm ± SEM) against selected Gram-negative bacter*ia*

	Stem extracts			Leave extracts			Test Bacteria
TC	***15mg/mL***	***30mg/mL***	***50mg/mL***	***15mg/mL***	***30mg/mL***	***50mg/mL***	
C	16.3±0.2	18.7±0.2	22±0.40	16±0.40	16.7±0.2	20.3±0.2	***Aeromonas spp.***
H	11.6±0.3	15±0.4	17.3±0.3	11±0.4	17±0.4	20.3±0.8	
Ch	0	15±0.4	20±0.4	0	0	15.3±0.8	
EA	0	12.3±0.6	17.6±0.6	0	12±0.4	15±1.0	
A	14±0.4	15±0.4	19±0.4	0	0	0	
N	0			0			
P	14.3±0.2			15.3±0.2			
C	11±0.40	16.3±0.6	21.7±0.6	14±0.4	15.7±0.2	16.6±0.3	***E. coli***
H	0	10±0.81	15.3±0.6	11±0.4	14.7±0.6	17±0.4	
Ch	0	0	11.3±0.6	10±0.4	14±0.4	19±0.4	
EA	0	0	12.6±0.6	0	11.7±0.6	15.3±0.8	
A	0	0	15.3±0.4	0	12.3±0.6	17±0.4	
N	0			0			
P	10.3±0.6			11±0.4			
C	10.7±0.4	15.3±0.2	16.3±0.6	0	11.6±0.3	16.6±0.2	***P. aeruoginosa***
H	0	0	12±0.40	0	10.66±0.5	15 ±0.40	
Ch	11.3±0.6	16±0.4	19.7±0.2	10±0.4	15±0.4	18± 0.4	

EA	0	12±0.4	15±0.4	0	12±0.4	16 ±0.4	
A	0	14±0.4	15.3±0.3	0	12±0.40	16.7±0.6	
N	0			0			
P	14.7±0.2			14.3±0.2			
C	0	14±0.4	17.6±0.2	11±0.4	12.3±0.4	15±0.4	***S. typhi***
H	0	0	0	0	11.3±0.6	14±0.4	
Ch	12.6±0.2	17.6±0.6	19.6±0.2	0	12±0.4	15±0.4	
EA	0	0	14.6±062	0	10.7±0.2	14±0.4	
A	0	11.3±0.6	14±0.4	0	10.7±0.2	14±0.4	
N	0			0			
P	13±0.4			12.7±0.62			
C	0	10.6±0.2	13.7±0.2	0	11.3±0.2	13.6±0.2	***S. dysenteriae***
H	0	12±0.4	13.6±0.6	0	11.3±023	14.7±0.3	
Ch	12.3±0.3	14.3±0.2	15±0.4	0	11.3±0.3	14.3±0.2	
EA	0	10±0.4	13.7±0.2	0	12.3±023	13.7±0.8	
A	0	0	0	0	13±0.4	15.3±0.3	
N	0			0			
P	12±0.4			13±0.4			
C	0	12.3±0.5	14.3±0.2	9.7±0.4	11.4±0.2	14±0.2	***V. cholrea***
H	0	10.3±0.2	12.7±0.2	10±0.3	12.7±0.2	15.7±0.40	
Ch	0	11.7±0.2	14±0.4	0	10.7±0.2	13.7±0.3	
EA	0	0	13.3±0.5	0	11.3±0.2	14.3±0.5	
A	0	10.7±0.2	12.330.6	0	11±0.4	14.3±0.2	
N	0			0			
P	12.3±0.2			12.3±0.2			

TC = Test concentration; C = Crude; H = hexane; Ch = Chloroform; EA = Ethyl acetate; A = Aqueous; N = Negative control; P = Positive control

Chloroform extract at a conc. of 50mg/mL inhibited very well the test *Aeromonas spp.* (>20mm). However, at 30mg/ml concentration *S. dysenteriae, S. typhi, V. cholrea, P. aeruginosa* and *Aeromonas spp* showed moderate inhibitory zones. Ethyl acetate extract at a conc. of 50mg/mL was inhibitory to *E. coli, S. dysenteriae, S. typhi, V. cholrea, P. aeruginosa* and *Aeromonas spp*. (Table 2). The aqueous extract showed inhibition of *Aeromonas spp.* and *P. aeruginosa* at 50mg/mL test concentration, but at lower concentrations the aqueous extract did not present a promising activity.

For crude extract (Table 2), the zone of inhibition at a conc. of 50mg/mL was, *E. coli* (>21 mm), *S. dysenteriae* (>13 mm), *S. typhi* (>17 mm) *V. cholrea* (>14 mm) *P. aeruoginosa* (>16 mm) and *A. spp.* (>22 mm). Crude extract also exhibited activities at a concentration of 30mg/ml against *E. coli* (>16 mm), *S. typhi* (>14 mm) *V. cholrea* (>12 mm), *P. aeruoginosa* (>15 mm), and *Aeromonas spp.* (>18 mm).

Antibacterial Activity of leaf Fractions of *Withania coagulans:* The antibacterial activity of leaves extract of *W. coagulans* also exhibited good inhibitions against test bacteria. The stem extracts showed better inhibitions compared to leaves extracts. The zone of inhibition for leaf crude extract at 50mg/mL concentration was, *A. spp.* (>20 mm), *E. coli* (>16 mm) and *P. aeruoginosa.* However, at the test concentration of 30 mg/ml moderate zones of inhibition were observed against all tested gastrointestinal pathogens (Table 2).

Hexane extract gave the highest inhibitory zones at a conc. of 50mg/mL against *Aeromonas spp.* and *E. coli* (>20 mm and 17 mm, respectively). In contrast moderate inhibitions were observed for *P. aeruoginosa, S. typhi, S. dysenteriae* and *Vibrio cholrea* (Table 2). At a conc. of 30mg/mL the activities were further reduced. Chloroform extract exhibited highest inhibitions against *E. coli* (>19 mm) and *P. aeruginosa* (>18 mm), while moderate inhibitions were observed against *Aeromonas spp., S. typhi, S. dysenteriae and V. cholera* (Table 2). The inhibitory zones are depicted in Fig. 2 against the test bacteria.

Phytochemical Analysis: The preliminary phytochemical investigations of *E. obtusifolia* and *W. coagulans* were performed, which shows the presence of glycosides, alkaloids, carbohydrates, flavonoids, steroids, saponins, protein, starch, phenol with anolides and tannins as main secondary metabolites.

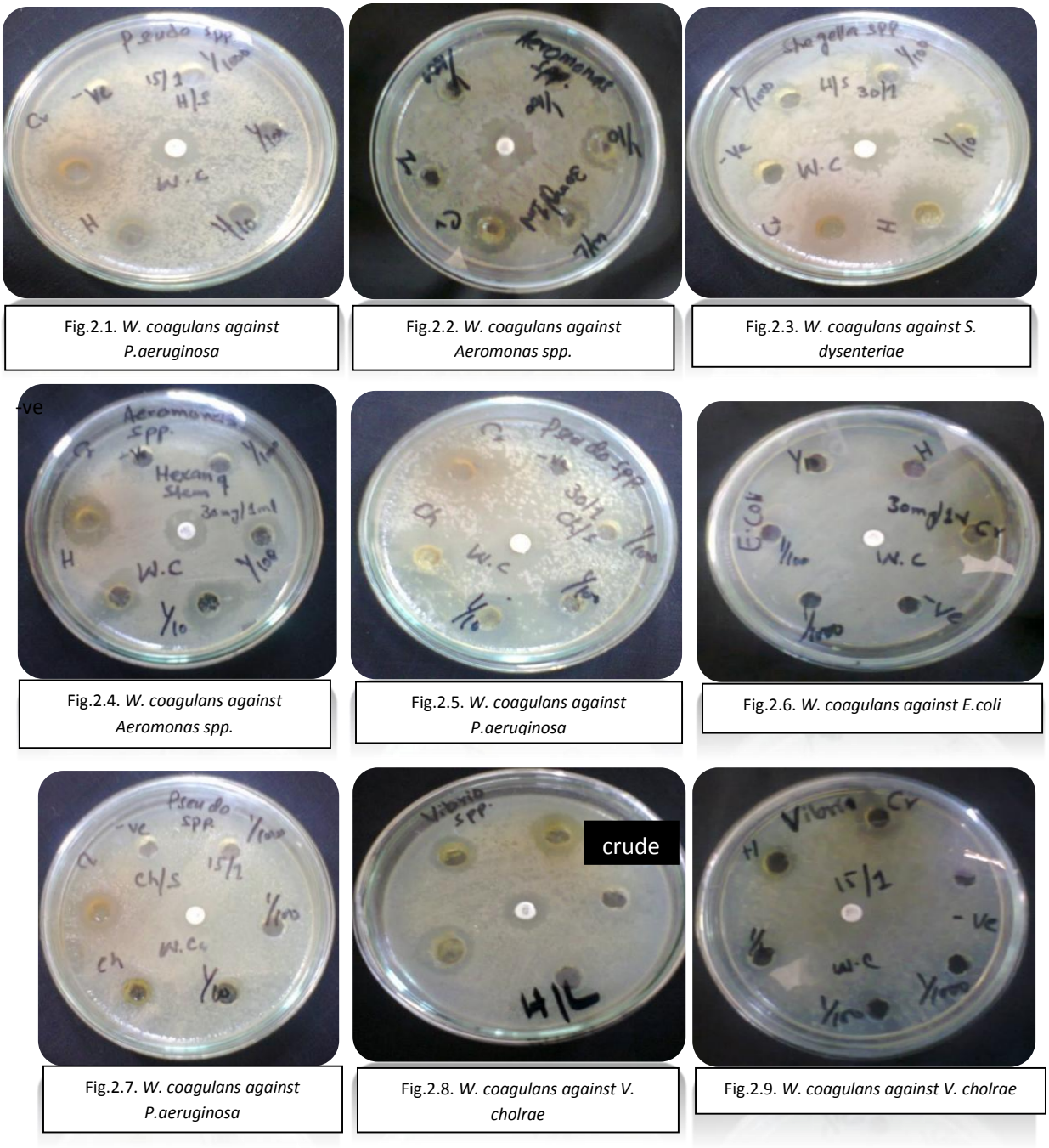

Fig.2.1. *W. coagulans against P.aeruginosa*

Fig.2.2. *W. coagulans against Aeromonas spp.*

Fig.2.3. *W. coagulans against S. dysenteriae*

Fig.2.4. *W. coagulans against Aeromonas spp.*

Fig.2.5. *W. coagulans against P.aeruginosa*

Fig.2.6. *W. coagulans against E.coli*

Fig.2.7. *W. coagulans against P.aeruginosa*

Fig.2.8. *W. coagulans against V. cholrae*

Fig.2.9. *W. coagulans against V. cholrae*

Fig 2: Activities of the *W. coagulans* against the selected pathogens

Phytochemical screening showed that alkaloids, steroids, saponins and flavonoids were present in higher amounts in stem of *E. obtusifolia* (Table 3), while carbohydrates, starch and protein were absent. Tannins were present in moderate amount. Glycosides and phenol were present in very low amount in crude stem extract. In leaves, steroids and tannins were present in higher amounts while, flavonoids, alkaloids and glycosides were present in moderate amounts. However, protein and starch were absent.

Table 3: Phytochemical Analysis of Extracts of *Ehretia obtusifolia* and *Withania coagulans*

S. No	Selected plants		*Ehretia obtusifolia*		*Withania coagulans*	
	Phytochemical	**Tests**	**Stem crude**	**Leaves crude**	**Stem crude**	**Leaves crude**
1.	Alkaloids	Mayer's	+++	++	-	-
2.	Carbohydrates	Molish's	_	+	++	+
3.	Flavanoids	Lead acetate	+++	++	+	+++
4.	Steroids	Salkowshi	+++	+++	_	_
5.	Glycosides	killer killani	+	++	+	++

6.	Saponins	Foam	+++	+	+	++
7.	Phenol	Ferric Chloride	+	+	+	++
8.	Tannins	Gelatin	++	+++	+	++
9.	Protein	Biuret's	_	_	+	++
10.	Starch		_	_	_	_
11.	Withanolides		-	-	+++	+++

*(+) Less amount, (++) moderate amount, (+++) higher amount, (-) absence of compound

The phytochemical analyses of *W. coagulans* indicate the presence of with anolides in higher content in both crude extracts. At the same time, steroids and starch were totally absent in crude extracts of leaves and stem. Proteins, glycosides, flavonoids, tannins and phenol were present in moderate quantities in stem extract while were found in low quantities in leaves crude extract, carbohydrates on the other hand were found in adequate quantities in leaves compared to stem extract (Table 3).

DISCUSSION

In the present study two medicinal plants *E. obtusifolia* and *W. coagulans* were screened for their activities against gastrointestinal bacteria. The crude extract was prepared in methanol and then fractionated into hexane, chloroform, ethyl acetate and water-soluble fractions. These plant extracts were tested in three different concentrations against six gastrointestinal pathogens, including *Aeromonas* spp. *E. coli, P. aeruginosa, S. dysenteriae, S. typhi* and *V. cholrae*.

The study demonstrates the potential of both the plants against selected pathogens and prospects of these plants for future use against the common gastrointestinal diseases. The stem extract of *E. obtusifolia* showed significant inhibitions against *Aeromonas* spp., *E. coli* and *S. typhi*. Hexane, chloroform and crude extracts of stem showed high zones of inhibition against *E. coli* and *Aeromonas* spp. while in case of *S. typhi* chloroform, ethyl acetate and crude extracts were found more effective. In case of *P. aeruginosa* chloroform and hexane showed the best activity while ethyl acetate and crude extract showed moderate zones of inhibition. The present study revealed that ethyl acetate exhibited moderate activity against *A.* spp. *E. coli* and *S. typhi*. The aqueous extract of *E. obtusifolia* stem part showed no activity against the selected pathogens in all test concentrations. In case of *S. dysenteriae* neither the stem nor leaves showed any inhibitory activity. Pharmacological screening of ethyl acetate soluble fraction revealed pronounced lipoxygenase inhibitory activity. Compounds methyl rosmarinate and rosmarinic acid are reported from the plant which has previously been reported to exhibit antihistamine, anti-inflamatory and lipoxygenase inhibitory activity [Mishra et al., 2013]. The presence of these phenolic compounds can be attributed to the antibacterial activities of the test fractions.

The crude leaves extract of *E. obtusifolia* was found most effective against *E. coli* and *P. aeruginosa*, while moderate activity was observed against *Aeromonas* spp. and *S. typhi*. All the sub fractions of the extracts showed significant activity against *E. coli, P. aeruginosa* and *V. cholrea*, while chloroform extract showed high inhibition against *S. typhi* and *Aeromonas spp*. These activities may be due to the presence of tannin, flavones and other phenolic components which are chloroform and ethyl acetate soluble. The leaves aqueous extract showed marginal activity against *Aeromonas spp*. and *P. aeruginosa*, and no activity were noted against *E. coli, V. cholrea* and *S. typhi* at any of the measured concentrations, which may be due to reason that all the phenolic and other non-polar and less polar compounds have been extracted in the organic solvents. The extracts of both stem and leaves gave almost similar results for *V. cholrae*. The chloroform extract of stem and leaves had stronger inhibitions compared to hexane, crude and ethyl acetate extracts. The results of the study suggest that the crude, chloroform extracts are more efficient against all the selected bacterial pathogens, followed by the hexane extract. Aqueous extract of leaves showed low inhibition or no activity against *these pathogens*.

W. coagulans have been reported for a number of pharmacological activities, found to exist in fruit, laves and roots [Nascimento et al., 2000]. In the present report, different extracts of *W. coagulans* stem part exhibited greater zones of inhibition against *Aeromonas spp*. Larger zone of inhibition was observed for crude extract of stem followed by chloroform, hexane, ethyl acetate and aqueous extracts. Moderate activity was noted against *E. coli* and *P. aeruginosa*. Crude extracts showed the highest zone of inhibitions against *Aeromonas spp*. and *E. coli*. The aqueous extract of the stem part showed inhibitions against all the pathogens, but the highest activity was recorded against *Aeromonas spp*, which may be due to the presence of some glycoside, while for the rest of test bacteria activity was moderate to low. Chloroform extract demonstrated strong activities against *Aeromonas spp. P. aeruginosa and S. typhi*. Our results are in agree-

ment with earlier finding that reported concentration of 15mg/mL *W. coagulans* had no activity against *S. typhi* [Maurya, 2010]. In the present study, stem's hexane extract showed no activity at any test concentration against *S. typhi*, while for *P. aeruginosa* weak inhibition was observed. However, moderate activities were observed for the rest of test bacteria. In case of ethyl acetate extract, moderate activity against all test pathogens was noted.

Similarly extracts from leaves of *W. coagulans* showed the highest zones of inhibition against *E. coli* and *P. aeruginosa* while moderated activities were observed against remaining test bacteria. The chloroform extract from leaves showed the highest activity against all the selected pathogens. The crude extract from leaves as well as hexane, chloroform, ethyl acetate, and aqueous fractions showed moderate zones of inhibition against *P. aeruginosa, S. dysenteriae, V. cholrae* and *S. typhi*. In case of crude and hexane extracts, highest activity was noted against *Aeromonas spp.*, however, no activity was seen in aqueous extract. The other fractions such as ethyl acetate and chloroform showed moderate activities. Chloroform showed highest activity against *E. coli* while rest of the fractions showed moderate zones of inhibition against the pathogen.

The earlier studies suggest that methanolic crude extract of *W. coagulans* exhibited moderate activity against *E. coli* and *P. aeruginosa* [Hoareau and J. DaSilva, 1999], which are in confirmation with our findings [Khandelwal, 2010]. In present study we also observed that ethyl acetate extract of *W. coagulans* has a good activity against *P. aeroginosa*. As far as concentration- effect was concerned, low activity was recorded at 15mg/ml concentration against all selected pathogens that got maximized at 50mg/mL concentration activity.

The qualitative phytochemical screening of the stem and leaves crude extracts of selected medicinal plants indicated the presence of various bioactive components. The results showed that in crude stem extract of *E. obtusifolia* alkaloids, flavonoids, steroids and saponins were present in high amount followed by tannins which were present in moderate amount. Phenol and glycosides were present in low quantities. In case of leaves crude extract steroids and tannins were present in high amount followed by flavonoids, alkaloids and glycosides that were present in a moderate amount. Similar results are already reported about the presence of alkaloids, flavonoids, tannins and phenols in all the extracts. Steroids were present in hexane and methanol extracts of *Ehretia laevis*, which is specie of *Ehretia* genus [Ullah et al., 2013]. While Carbohydrates, saponins and phenols were present in meager amount while proteins were absent in stem and leaves crude extracts. Carbohydrates were absent in stem crude extract. The presence of triterpenes, naphthoquinones, pyrrolizidine alkaloids, nitrile glycosides, phenolic glucosides, lignans and quinonoid xanthones are reported from some species of *Ehretia*, which supports our phytochemical screening results [Mishra et al., 2013].

In stem extracts of *W. coagulans* with anolides were present in a high amount while carbohydrates were found in moderate amount followed by flavonoids, tannins, glycosides, phenols, proteins and saponins which were present in meager quantities. The results of the presence of phytochemicals are in agreements to the previous reports about the presence of steroids, saponin, phenol, flavonoids, glucoside and tannins in various extracts of *W. coagulans* [Sudhanshu et al., 2012]. In crude leaves extracts withanolides and flavonoids were present in high amounts while saponins, tannins, glycosides, phenol, and proteins were found to be in moderate amounts. Our results are in confirmation to Mathur *et al.*, findings who reported the presence of saponin in extracts of *W. coagulans* [Sivasankari et al., 2013]. Carbohydrates were in trace amounts while steroids were absent in stem and leaves of *W. coagulans*, both of these observations are in agreement with earlier reports [Sivasankari et al., 2013].

CONCLUSION

The results showed that *Withania coagulans* as well as *Ehretia obtusifolia* have promising antibacterial potentials against notable gastrointestinal tract pathogens. *W. coagulans* showed the best activity against all test pathogens, while *E. obtusifolia* showed varying activities against all the test bacteria except *S. dysenteriae.* Phytochemical analysis also showed the presence of bioactive secondary components in both of these plants. This preliminary screening has opened the possibilities of the use of these plants in drug development for important pathogens of the gastrointestinal tract. Since the results of the phytochemical screening have shown that these plants are rich in alkaloids, tannin, with anolides, saponin, flavonoid, glycoside and phenols, further studies on isolation and characterization of the specific constituents are recommended.

Declaration of conflict of interest

None.

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