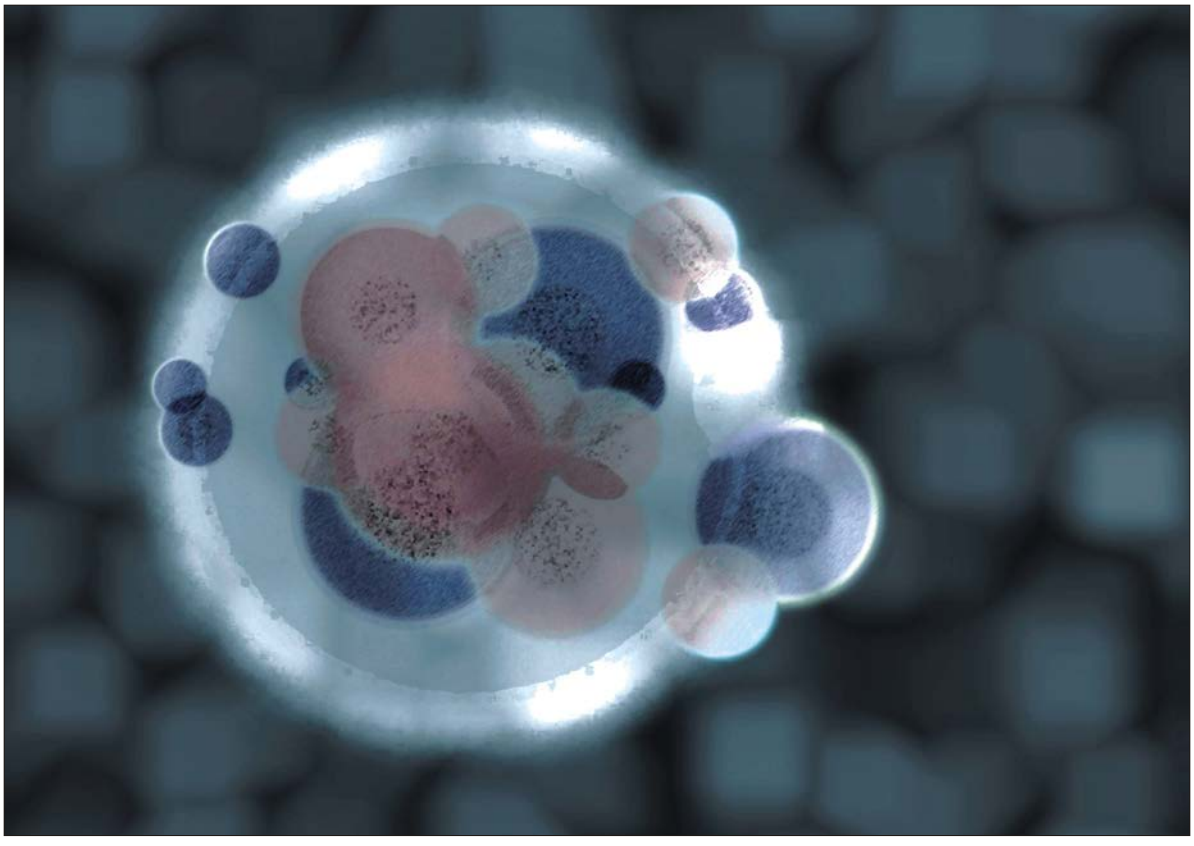

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Screening of *Morus alba*, *Citrus limon* and *Trigonella foenum-graecum* Extracts for Antimicrobial Properties and Phytochemical Compounds

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Abstract: In the present study, the effect of aqueous and methanol extracts of *Trigonella foenum-graecum* seed (fenugreek), *Citrus limon* peel (lemon) and *Morus alba* foliage (mulberry) against two Gram- negative bacteria, *Aeromonas hydrophila* and *Escherichia coli* and two Gram-positive bacteria, *Streptococcus agalactiae* and *Staphylococcus aureus* were investigated and the phytochemical compounds of the tested herbal extracts were determined. The results indicated that the aqueous extracts of *Trigonella foenum-graecum* seed and *Citrus limon* peel revealed weak antibacterial activity against the bacteria. The methanol extracts of all herbs exhibited stronger antimicrobial activities against the tested pathogens. Among the entire methanol extracts, the *Morus alba* had the strongest activities. *Aeromonas hydrophila* was the most sensitive microorganism tested. The phytochemical screening of the plants showed the presence of secondary metabolites such as phenols, volatile oils, tannins, saponins, steroids, flavonoid, terpenoids and alkaloids.

Key words: *Morus alba, Citrus limon, Trigonella foenum-graecum*, bacteria, antimicrobial properties, phytochemical compounds

INTRODUCTION

Drug resistance to wide range of pathogens has been usually reported from all around the world (Mulligan *et al*., 1993; Huebner *et al*., 1998). On the other hand, the condition is alarming in both developing and developed countries because of indiscriminate application of antibacterial medicines. In this situation, it is necessary to look for new antimicrobial agents from other sources such as herbs. The herbs, medicinal types in particular, have been known for their use to cure many kinds of diseases in human since long time ago. Medicinal herbs include different types of secondary metabolites of known therapeutic capacities (Iyengar, 1985; Chopra *et al*., 1992; Harborne and Baxter, 1995). These natural secondary metabolites (phyto-ingredients) can either inhibit the growth of pathogens or kill them and have least toxicity effect (compared to the synthetic counterparts) to host cells that make them good alternatives to develop new antimicrobial medicine (Dassonneville *et al*., 2000; Lisgarten *et al*., 2002; Guittat *et al*., 2003; Nweze *et al*., 2004).

There is, however, no information on the effect of *Morus alba* foliage, *Citrus limon* and *Trigonella foenum-graecum* seed extracts (water and methanol) on the bacterial pathogens. Therefore, the objective of the present study was to investigate the effect of the extracts on two Gram- negative bacteria (*Aeromonas hydrophila, Escherichia coli*) and two Gram-positive bacteria (*Streptococcus agalactiae, Staphylococcus aureus*). Furthermore, the extracts were screened for their phytochemical compounds.

MATERIALS AND METHODS

Herbal collection and extract preparation: Three herbs species were used in this study as shown in Table 1. The fresh *Morus alba* foliage were harvested from the University's field station. *Citrus limon* and *Trigonella foenum-graecum* seed were purchased from a local market. All plants and fruit were botanically and taxonomically identified by department of botany of the UPM.

Preparation of herbal extracts (water and methanol): The collected *Citrus limon* (peel) and *Trigonella foenum-graecum* (seed) and *Morus alba* (foliage) were washed in sterile distilled water. They were

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Table 1: Plants studied against tested bacteria in the present study

Botanical name	Family	Plant part used	Extract
Trigonella foenum-graecum	Fabaceae	Seeds	Met, Wtr
Citrus limon	Rutaceau	Peel	Met, Wtr
Morus alba	Moraceae	Foliage	Met, Wtr

Met, Methanol, Wtr, Water

air dried and powdered using electric blender. The aqueous extracts were obtained using 100 g of each sample dissolved in 1000 mL sterile distilled water. They were tightly covered with aluminum foil, allowed to stand for 7 days at room temperature. Afterward, the extract was filtered using sterile muslin cloth. The filtrate was collected and the solvent was removed by rotary vacuum evaporator. The residue achieved after evaporation, freeze-dried and stored at -20°C until use (Harikrishnan *et al*., 2009).

To prepare methanol extracts, the dried powder of each herb was subjected to maceration by 95% methanol in conical flasks, plugged with cotton wool and kept for 7 days at room temperature. The mixture was then filtered, methanol was evaporated using a rotary vacuum evaporator and the obtained extract was freeze-dried and stored at -20°C until use (Harikrishnan *et al*., 2009).

Bacterial strain and culture conditions: Four bacterial isolates were tested: two Gram- negative bacteria (*Aeromonas hydrophila, Escherichia coli*) and two Gram-positive bacteria (*Streptococcus agalactiae, Staphylococcus aureus*). Pure cultures of the bacteria were provided by Bacteriology laboratory, Faculty of Veterinary Medicine, UPM. Conventional biochemical tests such as Gram-staining and other tests specific to the different bacteria were conducted to identify the bacteria provided. To store the bacteria for short period, the bacterial stock were kept on Muller Hilton Agar (MHA) (Oxoid, UK) slants at 4°C, kept away from any contamination until the start of the experiment. For long period storage, the bacterial cultures were stored in nutrient broth at -80°C (Smail and Jones, 1984).

Bacterial cell suspension preparation: Bacterial cell suspension was prepared to conduct antimicrobial test. Bacterial isolates were activated by introducing 0.05 mL of frozen stock bacteria into 10 mL Tryptic Soy broth (TSB) (Oxoid, UK) and incubated overnight at 37°C. Afterward, the content of the broth was streaked onto TSA petri dishes and incubated for 24 h at 37°C. Inoculums were provided using one loopful of bacterial colony from TSA petri dishes in to 10 mL of TSB and incubated in the TSB liquid in an orbital shaker for 24 h at 37°C. After that, 1 mL of the latest bacterial culture was transferred to a newly made TSB. The bacterial suspensions were adjusted till turbidity was the same as a ca 0.5 Mc Farland Standard, obtaining a final cell concentration 10^8 CFU mL^{-1} (Sagdic and Ozcan, 2003).

Disk diffusion method to determine antimicrobial sensitivity: To examine antimicrobial characteristics of each extracts against tested bacteria in the present study the paper disk diffusion method was used (Bauer *et al*., 1966). The bacterial culture (10^8 CFU mL^{-1}) was spread on BHI agar petri dish before putting the paper disks on impregnated petri dishes. Sterile 6.0 mm diameter filter paper disk (Whatman No.1) seeded with three dilution of each of herbal extracts as follow: 50, 75 and 100 mg mL^{-1} solvent. A 20 μL of the extracts was used per disks and the disks were allowed to dry before locating onto petri dishes. Each petri dish contained five extract-impregnated paper disks. Oxytetracycline (commercial antibiotic, 20 μg mL^{-1}) (Oxoid, UK) was served as a positive control whereas sterile distilled water and methanol were employed as a negative control. Thereafter, petri dishes were incubated at 37°C for 24 h. After that antibacterial activity was determined by measuring the diameter of the inhibition zone formed around the disk. The inhibition zone bigger than 15 mm was considered as strong activity, the zone from 10 to 15 mm was considered as moderate activity and the zone smaller than 10 mm as weak activity.

Phytochemical compounds determination: Phytochemical screening tests were conducted to determine the presence of phyto-compounds in the extracts. The phyto-compounds include volatile oils, glycosides, terpenoids, phenols, tannins, flavonoids, alkaloids, saponins and steroids that determined based on the methods reported by Trease and Evans (1989), Sofowora (1993), Evans (1996), Houghton and Raman (1998) Harborne (1998), Dahiru *et al*. (2006) and Aiyegoro and Okoh (2010) with some modifications as follows:

Tannin: One gram of herbal extract was dissolved in 10 mL of distilled water and filtered using No. 1 filter paper (Whatman). A blue color resulting from the addition of ferric chloride reagent to the filtrate showed the existence of tannins in the extract (Harborne, 1998).

Steroid: Approximately 0.5 g of the plant extract was dissolved in 3 mL of chloroform and filtered. Concentrated H_2SO_4 was cautiously supplemented to the filtrate to make lower layer. A reddish brown color at the interface was considered as indicative for steroid ring (Aiyegoro and Okoh, 2010).

Flavonoid: Approximately 0.2 g of the extract was dissolved in 2 mL of methanol and heated. A chip of magnesium metal was served to the mixture and thereafter a few drops of HCl were added into it. The existence of a pink-tomato red or orange color displayed the presence of flavonoids (Harborne, 1998).

Saponin: Test of Froth was used to indicate the presence of saponin. Five gram of the herbal extract was treated with 15 mL methanol. After evaporation, the residue was shaken strongly with ethyl ether and 5 mL HCL 2N. Turbidity showed the presence of saponin (Aiyegoro and Okoh, 2010).

Volatile oils: One gram fresh plant sample was boiled in 10 mL petroleum ether and filtered. Thereafter 2.0 mL of extract solution was shaken with 0.1 mL sodium hydroxide and a little amount of hydrochloric acid. A white turbidity exhibited the existence of volatile oils (Dahiru *et al*., 2006).

Alkaloid: Half gram of the herbal extracts was dissolved in 5 mL of 1% HCl on steam bath. One milliliter of the filtrate was supplemented with few drops of Dragendorff's reagent. Creamish precipitate was considered as an indicator for the presence of alkaloid (Trease and Evans, 1989).

Phenolic: To determine the presence of phenolic compounds in the plants, 2 mL of the herbal extract were supplemented with 2 mL of ferric chloride. A dark bluish green color that appeared was an indicator of phenols (Evans, 1996; Houghton and Raman, 1998).

Terpenoids: Two hundred mg plant powders was boiled in ten mL chloroform and mixed thoroughly. The obtained residue was filtered and 2 mL filtrate mixture was introduced in to 2 mL acetic anhydride and sulfuric acid (H_2SO_4). Red colour is the indicator of the presence of terpenoids (Houghton and Raman, 1998).

Statistical analysis: The experimental results were subjected to analysis of variance by the GLM procedure of SAS (SAS Institute, 1990). All differences among means were declared significant at $p<0.05$.

RESULTS

Antimicrobial screening of extracts: Antibacterial activity of various concentrations of aqueous and methanol extracts of tested plants against all the bacteria in the present study are shown in Table 2 and 3. Aqueous extracts of tested plants showed weak antibacterial properties against the bacteria. Aqueous extracts of *Trigonella foenum-graecum* seed and *Citrus limon* peel were not active or had weak activity against all the microorganisms investigated, while all the tested bacteria were sensitive to the aqueous extract of *Morus alba* foliage but slightly.

Table 2: Antibacterial activity of different concentration of aqueous extracts of tested plants against the bacteria after 24 h incubation

		Microorganism			
Herbs	Concentration	*S. aureus*	*A. hydrophila*	*E. coli*	*S. agalactiae*
Trigonella foenum-graecum	50 mg mL^{-1}	-	-	-	-
	75 mg mL^{-1}	-	-	-	-
	100 mg mL^{-1}	-	-	-	-
Citrus limon	50 mg mL^{-1}	-	-	-	-
	75 mg mL^{-1}	-	+	+	+
	100 mg mL^{-1}	+	+	+	+
Morus alba	50 mg mL^{-1}	-	-	-	-
	75 mg mL^{-1}	+	+	-	+
	100 mg mL^{-1}	+	+	+	
OTC (positive control)	20 μg mL^{-1}	+++	+++	+++	+++
Water (negative control)	20 μL	-	-	-	-

IZ: Inhibition zone (mm), -: Lack of IZ, +: IZ >10, ++: IZ >10<15, +++: IZ >15, OTC: Oxytetracycline

Table 3: Antibacterial activity of different concentration of methanol extracts of tested plants against tested bacteria after 24 h incubation

		Microorganism			
Herbs	Concentration	*S. aureus*	*A. hydrophila*	*E. coli*	*S. agalactiae*
Trigonella foenum-graecum	50 mg mL^{-1}	-	-	-	-
	75 mg mL^{-1}	-	-	-	-
	100 mg mL^{-1}	+	+	-	+
Citrus limon	50 mg mL^{-1}	-	-	-	-
	75 mg mL^{-1}	+	+	+	+
	100 mg mL^{-1}	++	+	++	++
Morus alba	50 mg mL^{-1}	+	+	+	+
	75 mg mL^{-1}	+	++	++	++
	100 mg mL^{-1}	++	+++	++	+++
OTC (positive control)	20 μg mL^{-1}	+++	+++	+++	+++
Methanol (negative control)	20 μL	++	++	++	++

IZ: Inhibition zone (mm), -: Lack of IZ, +: IZ>10, ++: IZ>10<15, +++: IZ>15, OTC: Oxytetracycline

All the microorganisms served in the present study were affected by the methanol extracts of the plants studied. *Trigonella foenum-graecum* seed and *Citrus limon* peel extracts (50 and 75 mg mL^{-1}) failed to inhibit the growth of the bacteria or showed weak activities while 100 mg mL^{-1} of those extracts slightly inhibited the growth of the bacteria. Methanol extracts of *Morus alba* foliage (75 and 100 mg mL^{-1}) exhibited

moderate and strong activities against all the microbes whereas 50 mg mL^{-1} of the extracts showed weak inhibition of the growth of all the bacterial strains.

According to the inhibition zone the aqueous extract of *Trigonella foenum-graecum* at the concentration of 50, 75 and 100 mg mL^{-1} exhibited weak antibacterial activity. The *Citrus limon* (75 and 100 mg mL^{-1}) water extracts exhibited weak activity against the bacteria. As compared to the water extract, the methanol extracts of all herbs especially *Morus alba* foliage exhibited stronger antimicrobial activity against the bacteria tested in this study.

Antimicrobial activity (inhibition zones of different concentration of *Morus alba* foliage) of methanol extracts was presented in Table 4. Among all, the *Morus alba* foliage methanol extract at the concentration of 100 mg mL^{-1} had the strongest activities, while *Trigonella foenum-graecum* and *Citrus limon* peel methanol extract at the concentration of 100 mg mL^{-1} showed weak and moderate activities, respectively. The largest inhibition zones were obtained for the methanol extract *Morus alba* foliage at the concentration of 100 mg mL^{-1}. According to the results, among the three plants' extracts tested in the present study methanol extracts of *Morus alba* foliage exhibited significant ($p<0.05$) growth-inhibiting capacity against the bacteria studied. Moreover, the results indicated that *Aeromonas hydrophila* was the most sensitive microorganism tested, with the highest inhibition zone in the presence of the methanol extracts obtained from *Morus alba* foliage.

Table 4: Antimicrobial activity (inhibition zone [mm] of different concentrations of *Morus alba* foliage) of methanol extract after 24 h incubation

Microorganisms	Positive control (OTC)	Negative control (MeOH)	50 mg mL^{-1}	75 mg mL^{-1}	100 mg mL^{-1}
Staphylococcus aureus	24.6±2.3^{a}	11.1±1.1^{b}	5.1±0.4^{e}	6.7±0.8^{e}	13.1±1.1^{b}
Aeromonas hydrophila	24.7±3.5^{a}	10.2±0.7^{c}	6.2±0.3^{d}	14.1±0.9^{b}	21.6±2.9^{a}
Escherichia coli	23.9±2.7^{a}	10.5±1.5^{c}	8.5±0.4^{e}	13.9±1.3^{b}	14.1±1.6^{b}
Streptococcus agalactiae	24.4±2.9^{a}	11.7±1.2^{c}	6.7±0.5^{d}	14.5±1.4^{c}	20.1±3.4^{b}

Means within the same row with different superscript letters are significantly different ($p<0.05$), OTC: Oxytetracycline, MeOH: Methanol, Values are the Mean±SE

Phytochemical screening of extracts: Phytochemical screening test revealed the presence of various active bio-compounds including tannins, steroids, flavonoids, saponins, volatile oils, alkaloids, phenolics and terpenoids in the plants extracts (aqueous and methanol) tested in the present study (Table 5 and 6). Water extracts of *Trigonella foenum-graecum* contains flavonoids, saponins, volatile oils and phenolics. *Citrus limon* peel has tannins, alkaloids, steroids, flavonoids, phenolics and terpenoids. *Morus alba* contains tannins, flavonoids, alkaloids, saponins, phenolics and terpenoids. While methanol extracts of *Trigonella foenum-graecum* includes flavonoids, saponins, alkaloids, phenolics and volatile oils. *Citrus limon* peel contains tannins, flavonoids, volatile oils, alkaloids, phenolics and terpenoids. *Morus alba* contains tannins, flavonoids, alkaloids, saponins, volatile oils, phenolics and terpenoids.

DISCUSSION

In the present study, significant differences were detected between the effects of aqueous and methanol extracts of plants against the bacteria tested. Viewing the magnitude of inhibitory zone, the methanol and aqueous extracts of *Trigonella foenum-graecum* seed and *Citrus limon* peel failed to show activity against the bacteria or showed weak activities. The aqueous extracts of *Morus alba* foliage was not strong bacterial growth inhibitor. Only methanol extract of *Morus alba* foliage was found pronouncedly effective against the four

Table 5: Phytochemical screening of aqueous extracts of *Trigonella-foenum-graecum*, *Citrus limon* and *Morus alba*

	Phytochemical compounds							
Herbs	Tannins	Alkaloids	Flavonoids	Saponins	Volatileoils	Steroids	Phenolic	Terpenoids
Trigonella-foenum-graecum	-	-	+	+	+	-	+	-
Citrus limon	+	+	+	-	+	+	+	+
Morus alba	+	+	+	+	-	-	+	+

-: Lack of phytochemical compounds, +: Presence of phytochemical compounds

Table 6: Phytochemical screening of methanol extracts of *Trigonella-foenum-graecum*, *Citrus limon* and *Morus alba*

	Phytochemical compounds							
Herbs	Tannins	Alkaloids	Flavonoids	Saponins	Volatile oils	Steroids	Phenolic	Terpenoids
Trigonella-foenum-graecum	-	+	+	+	+	-	+	-
Citrus limon	+	+	+	-	+	-	+	+
Morus alba	+	+	+	+	+	-	+	+

-: Lack of phytochemical compounds, +: Presence of phytochemical compounds

microorganisms tested. Our findings is in agreement with postulation that herbal plant extracts using organic solvents provide more consistent antimicrobial activity compared to the extraction with water (Parekh *et al*., 2005). This could be because of the better solubility of the active compounds in organic solvent (De Boer *et al*., 2004). This observation rationalizes in terms of the polarity of the compounds being extracted by each solvent.

All the bacteria were susceptible to *Morus alba* foliage extracts though to varying degrees, however *Aeromonas hydrophila* was the most sensitive microorganism tested in the present study, with the highest inhibition zone in the presence of the methanol extracts obtained from *Morus alba* foliage. Karou *et al*. (2006) demonstrated that the susceptibility of bacteria to herbal extracts, with respect to inhibition zone diameters varied based on strains and species, similar to the results obtained in the present work.

Some *in vitro* studies conducted with *Morus alba* foliage revealed strong activity against some Gram-negative and Gram-positive bacteria (Nomura, 2001; Sohn *et al*., 2004). Also, Kim *et al*. (1993) found that the chloroform extracts of *Morus alba* foliage has strong antimicrobial activity against *Bacilllus subtilis* and the extract with acetic acid is active against *Staphylococcus aureus*, *B. subtilis* and *Escherichia coli* (*In vitro*). Elsewhere, Ahmad and Beg (2001) in an *in vitro* study found out that *Morus alba*'s leaves alcohol extract revealed strong antimicrobial activity against *Staphylococcus aureus*, *Salmonella paratyphi* and *Shigella dysenteriae*.

The efficiency of the methanol extract of *Morus alba* foliage can be due to the presence of some phytochemical compounds that play remarkable role as antimicrobial agents (Harborne and Baxter, 1995; Hernandez-Medel *et al*., 1998; Dassonneville *et al*., 2000; Guittat *et al*., 2003). Phytochemical screening test revealed the presence of various active bio-compounds including tannins, flavonoids, alkaloids, saponins, volatile oils, phenolics and terpenoids in the *Morus alba* foliage extracts tested in the present study. According to several reports (Kusano *et al*., 2002; Enkhmaa *et al*., 2005; Kang *et al*., 2006) *Morus alba* L. includes flavonoids, tannins, triterpenes, anthocyanins, anthroquinones, phytosterols, sitosterols, benzofuran derivatives, morusimic acid, oleanolic acid, alkoloids, steroids, saponins and phenolic compounds.

There are extensive investigations which attribute the observed antimicrobial activities of herbal extracts to the presence of the phytochemical ingredients (Dassonneville *et al*., 2000; Lisgarten *et al*., 2002; Guittat *et al*., 2003; Nweze *et al*., 2004). An *in vitro* investigation conducted by Rauha *et al*. (2000) revealed the effective growth-inhibiting activity of flavones, flavonols (flavonoids) and naringenin on *Staphylococcus aureus*. Also, the activity of these compounds against *Staphylococcus aureus* was demonstrated by Tsuchiya *et al*. (1996).

Other study (*in vitro*) indicated the antibacterial effects of the flavonoids on *Aeromonas* spp. such as *A. hydrophila* and *A. salmonicida* (Rattanachaikunsopon and Phumkhachorn, 2007) most probably because of their ability to complex with extracellular and soluble proteins and to complex with bacterial cell wall. Tannins are also known to have excellent antimicrobial property *in vitro* (Rauha *et al*., 2000). They are strongly effective against some bacteria (Tona *et al*., 2000) and viruses (Freiburghausa *et al*., 1996). Tannins present in the cells of plants and known as potent inhibitors of numerous hydrolytic enzymes such as pectolytic macerating enzymes used by plant pathogens (Aboaba and Efuwape, 2001). Moreover, tannins act by iron deprivation, hydrogen binding or non-specific interactions with the important proteins like enzymes (Scalbert, 1991). Saponins have been reported to show antimicrobial effect on some microorganisms (Ojo *et al*., 2006). They form complexes with sterols present in the membrane of microorganisms which causes destruction of the cell membrane (Morrissey and Osbourn, 1999) however saponins are shown unable to inhibit the growth of the Gram-negative bacteria and the fungus (Aboaba *et al*., 2006).

Volatile oils and polyphenols are able to inhibit bacterial growth (Karou *et al*., 2006). Oils due to their hydrophobicity are able to partition the lipids of the bacterial cell membrane and mitochondria, disturbing the cell structures and rendering them more permeable (Knobloch *et al*., 1986; Sikkema *et al*., 1994). Extensive leakage from bacterial cells or the exit of critical molecules and ions will lead to death (Denyer and Hugo, 1991).

CONCLUSION

In the present study, *Morus alba* foliage methanol extract was active against all the bacteria tested particularly *Aeromonas hydrophila*, however, no marked activity for aqueous extracts of those plants were noted. Phytochemical screening test also revealed the presence of various active bio-compounds including tannins, flavonoids, alkaloids, saponins, volatile oils, phenolics, steroids and terpenoids in the extracts.

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