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Research Paper

Antibacterial activity and modulation of antibiotic resistance by *Crataegus azarolus* extracts

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ABSTRACT

Antibiotic resistance among bacterial pathogens is a serious problem for human and veterinary medicine, which necessitates the development of novel therapeutics and antimicrobial strategies. The aim of the present study was to investigate the antibacterial activity of *Crataegus azarolus* leaves extracts against both Gram-positive and Gram-negative bacteria and against four multidrug-resistant strains of *E. coli*. Minimum inhibitory concentration (MIC) values of tested extracts as well as of some antibiotics were determined by the standard broth microdilution method. All extracts exhibited antibacterial effect against reference strains; *Staphylococcus aureus*, *Enterococcus faecalis*, *Salmonella enteritidis* and *Salmonella typhimurium*. It appears that ethyl acetate and TOF (Total oligomer flavonoids) enriched extracts have the greatest antibacterial activity against these reference strains. Besides, as these two active extracts revealed the best antibacterial effect against multiresistant strains of *E. coli*, we decided to test the effect of each, combined to the antibiotic against which the strains were resistant. In the interaction study, the tested extracts acted in synergy to lower the susceptibility of two strains of multidrug-resistant *E. coli* (EC 6574 and EC 6228), to amoxicillin and ofloxacin with TOF enriched extract and to ofloxacin and cefotaxim with ethyl acetate extract, but no synergy was observed for the remaining strains. These results indicate that extracts from *C. azarolus* could be used as a source of natural products when administered in combination with beta-lactam antibiotics to combat bacterial infections caused by resistant *E. coli*.

Keywords: *Crataegus azarolus*; Antibacterial activity; Antibiotic resistance; Synergism.

INTRODUCTION

Escherichia coli are one of the principal causes of infectious diseases in humans. These bacteria are known to produce enterotoxins whose properties and role in diarrheal disease have been widely investigated. The activity of cytotoxins and their

role in human infection have been identified, mainly in infections of the urinary tract (Hughes, et al., 1982; Scotland, et al., 1980). In relation to pathogenic bacteria, a growing and worrisome problem is the increase in bacterial resistance to antibiotics (Georgopapadakou, 2005; Nostro, et al., 2004). Acquired multi drug resistance to antimicrobial agents creates an extensive trouble in case of the management of intra and extra intestinal infections caused by *E. coli*, which are a major source of illness, death, and increased healthcare costs (Gupta, et al., 2001). According to the development of antimicrobial resistance, a considerable scientific research has focused on the antibacterial properties of plant products. In fact, medicinal plants have been the source of many medications that are now applied in clinical practice. The use of extracts as antimicrobial agents shows a low risk of increasing resistance to their action, because they are complex mixtures, making microbial adaptability very difficult (Daferera, et al., 2003; Gibbons, 2004).

Hawthorn (*Crataegus oxyacantha* and *Crataegus laevigata*) leaves, flowers and berries are recognize for along for their effects on heart health (treatment of chronic heart failure, high blood pressure, and arrhythmia). However, little information is available on their antimicrobial effect. Indeed, Kostic, et al. (2012) evaluated antimicrobial activity of *Crataegus oxyacantha* against selected test microorganisms: *Bacillus subtilis, Staphylococcus aureus, Escherichia coli, Pseudomonas aeruginosa*, and *Salmonella abony* and revealed that tested extracts from hawthorn fruit exhibited good antimicrobial activity. But Tadic et al. (2008), showed that hawthorn berries ethanol extract revealed a moderate bactericidal activity, especially against gram-positive bacteria: *Micrococcus flavus, Bacillus subtilis, and Lysteria monocytogenes*.

Based on the foregoing, it can be said that *C. azarolus* was not studied for such activity. And to our knowledge, we investigated for the first time the effectiveness of extracts from *C. azarolus* leaves against multidrug-resistant strains of *E. coli* as well as their modulating effect on bacterial resistance to the target antibiotics.

MATERIALS AND METHODS

Bacterial material: Five bacterial strains were used as reference, Gram-positive bacteria (*Staphylococcus aureus* ATCC 25923 and *Enterococcus faecalis* ATCC 29212), and the Gram-negative bacteria (*Escherichia coli* ATCC 25922, *Salmonella enteritidis* ATCC 13076 and *Salmonella typhimurium* NRRLB 4420). Synergetic activities of *C. azarolus* leaf extracts were tested against four multidrug-resistant strains of *E. coli* with the resistance profile described in Table 1. All these strains were kindly provided from the clinical bacterial collection of CHU Fattouma Bourguiba, Monastir-Tunisia. The antibiotics used are the Cefotaxim, the Amoxicilin, the Piperacilin, the Ofloxacin and the tetracycline (Sigma-Aldrich, Canada Ltd., Oakville, ON).

Preparation of plant extracts: *C. azarolus*'s leaves were collected from Oued Hatem in Sousse, a region situated in the coast center of Tunisia, in September 2011. Identification was carried out by Prof. Harzallah Skhiri Fethia (Higher Biotechnology Institute of Monastir, University of Monastir, Tunisia) according to the Flora of Tunisia (Pottier-Alapetite, 1979). A voucher specimen (C. a-09-11) has been kept in our laboratory for future reference.

The fresh leaves of *C. azarolus*, were dried at room temperature and reduced to coarse powder. The powdered leaves were extracted by boiling water for 15 to 20min. The crude extract obtained was filtered and lyophilized (aqueous extract). The

residue was dissolved in water. In order to obtain an extract enriched with Total Oligomer Flavonoids (TOF), powder was macerated in water/acetone mixture (1v/2v), for 24h with continuous stirring. The extract was filtered and the acetone was evaporated under low pressure in order to obtain an aqueous phase. Tannins in the aqueous phase were precipitated with an excess of NaCl for 24h at 5°C. The mixture was then filtered and the filtrate solution was recovered. This latter was extracted with ethyl acetate, concentrated and precipitated with an excess of chloroform. The precipitate was separated and yielded TOF extract (Ghedira, et al., 1991). Hexane (Hex), chloroform (Chl), ethyl acetate (EA), and butanol (BuOH) extracts were obtained by liquid-liquid extraction with solvents increasing polarity after ten days maceration in methanol. These four types of extracts, with different polarities, were concentrated to dryness and each residue was kept at 4°C. Then each extract was resuspended in the adequate solvent. The same solvent was used in the corresponding negative control.

Quantitative and qualitative analysis of extracts

Determination of total polyphenol and flavnoid contents: The polyphenol content of *C. azarolus* leaves extracts was quantified by the Folin–Ciocalteau reagent (Kumar and Chattopadhyay, 2007). Aliquots of test samples (100µl) were mixed with 2ml of 2% Na_2CO_3 and incubated at room temperature for 2min. After addition of 100µl 50% Folin–Ciocalteau phenol reagent, the reaction tube was further incubated for 30min at room temperature, and finally absorbance was read at 720nm. Gallic acid was used as a standard. A known volume of each extract was placed in a 10 ml volumetric flask to estimate flavonoid content according to the modified method of Zhishen, et al., (1999). After addition of 75µl of $NaNO_2$ (5%), 150µl of freshly prepared $AlCl_3$ (10%), and 500µl of NaOH (1N) solutions, the volume was adjusted with distilled water until 2.5ml. After 5 min incubation, the total absorbance was measured at 510nm. Quercetin was used as a standard for constructing a calibration curve.

Determination of tannin content: According to Nwabueze (2007), quantification of tannins in the samples was achieved by dissolving 5g of extract in 50ml of distilled water in a conical flask, allowing the mixture to stand 30min with shaking the flask at 10min intervals and then centrifuging it at 5000xg to obtain a supernatant (tannin extract). The extract was diluted to 100ml in a standard flask using distilled water. Five milliliters of the diluted extract and 5ml of standard tannic acid (0.01g/L) were measured into different 50ml volumetric flasks. One milliliter of Folin-Denis reagent was added to each flask, followed by 2.5ml of saturated sodium carbonate solution. The solutions were made up to the 50ml mark with distilled water and incubated at room temperature (20–30°C) for 90min. The absorption of each solution was measured against that of the reagent blank (containing 5ml of distilled water in place of extract or standard tannic acid solution) in a Genesys 10 UV scanning spectrophotometer at a 760nm wavelength. Tannin content (tannic acid equivalents) was calculated in triplicate, using the following formula:

$$\text{Tannin content} = \frac{\text{Sample reading} - \text{blank}}{\text{Standard reading} - \text{blank}}$$

Determination of proanthocyanidins content: The proanthocyanidins were determined by UV spectrophotometry method based on acid hydrolysis and colour formation. The HCl/butan-1-ol assay was used to quantify the total proanthocyanidins (Porter, et al., 1986). One milligram of each extract was dissolved in 1ml of methanol. 0.25ml of this solution was added 3ml of a 95% solution of n-butanol/HCl (95/5; v/v)

in stoppered test tubes followed by addition of 0.1ml of a solution of NH_4 Fe $(SO_4)_2$, 12 H_2O in HCl [2M] (0.2%; w/v). The tubes were incubated for 40min at 95°C. For a control sample, 0.25ml of methanol was used. After incubation, the samples were cooled and analyzed by measuring absorbance at 540nm. Cyanidin chloride was used as a standard.

***High Performance Liquid Chromatography "HPLC" conditions*:** HPLC analyses were performed with Shimadzu HPLC system which consisted of valve injector, SCL-10A VP model system controller, SPD-10A model UV/VIS detector and LC-10AS model pump. The chromatographic column was Novapak C18 (4µm, 150 × 4mm i.d.) (Waters). For the analysis of triterpenic acids, eluent A and B were acetonitrile/H_2O (20:80; v/v) and 100% acetonitrile, respectively. The gradient elution program was as follows: 0min = 75% B, 15min = 85% B. Before injecting the next sample, the column was equilibrated with the initial mobile phase for 10min. The flow rate was constant at 1ml/min. For the analysis of flavonoids, eluent A and B were acetic acid 0.5% in water and 100% acetonitrile, respectively. Gradient elution was carried out according to the following program: solvent B was increased from 17 to 25% in the first 30min, then increased more from 25 to 50% from 30 to 60min and then returned to 17% in 5min. The flow rate was constant at 1ml/min in both two analyses and injection volume was 20µl. UV detection was performed at 254 and 360nm. Concentrations of injected solutions were 5mg/ml for crude extract and 1mg/1ml for pure standard in methanol.

Determination of the minimum inhibitory concentration (MIC): MIC values were determined by standard broth microdilution method (Clinical and Laboratory Standards Institute (CLSI), 2002; Palanippan and Holley, 2010). Nutrient broth (Pronadisa, Hispanlab, S.A, Spain) was used for bacterial growth. Antibiotic solutions and natural antimicrobials were prepared at different concentrations for each bacteria and serially diluted in the microwell plates. Test inoculums were prepared from the fresh bacterial cultures by serial dilutions to yield 5×10^5cfu/ml in each well (CLSI 2002) and incubated for 16h at 37°C in a 96 wells plate. The MIC was defined as the lowest antibiotic or antimicrobial concentration which prevented visible growth (Palanippan and Holley, 2010). 40µl of 2-[4-iodophenyl]-3-[4-dinitrophenyl]-5-phenyltetrazolium chloride (INT) (Sigma, St. Louis, MO, USA) dissolved in ethanol (0.2mg/ml) was added to each well, and plates were incubated for 1 to 2h at 37°C to allow detection of cell viability (Palanippan and Holley, 2010). The electron transport system of respiring organisms reduces INT to INT-formazan and absorbance was measured directly at 450nm (Grare, et al., 2008). Absence of microbial growth is translated by the absence of culture medium coloring, when incubated with INT. The determination of MIC values was made in triplicate (CLSI, 2002).

***Synergy testing*:** The checkerboard method, which is commonly used for measurement of interactive inhibition (Pillai, et al., 2005; Vigil, et al., 2005), was used for the determination of synergy between the antibiotics and natural antimicrobials. This method is often combined with calculation of a fractional inhibitory concentration (FIC) index, to test the antimicrobial potencies of drugs in medical laboratories (Pei, et al., 2009). Synergistic interactions involving the natural antimicrobials (e.g. drug A) and the antibiotics (drug B) to which the bacteria were normally resistant, were tested. The concentrations of the used agents were started from their MIC value and were serially diluted in two-fold steps. The effects of combinations were evaluated by calculating the FIC index for each combination using

the following formula: FIC of drug A = MIC of drug A in combination with natural antimicrobials/ MIC of drug A alone; FIC of drug B = MIC of drug B in combination with natural antimicrobials/ MIC of drug B alone; FIC index = FIC of drug A + FIC of drug B (Pillai, et al., 2005; Vigil, et al., 2005). When FIC index ≤ 0.5 we talk about synergistic effect between drug and antibiotics. When the FIC index fell between 0.5 and 4.0 it indicated 'no interaction' between the agents. A FIC index > 4.0 would indicate an antagonism effect between the two agents (Palanippan and Holley, 2010).

Statistical analysis: Data were analyzed with student's t-test and presented as mean value±SEM of three independent measurements in separate experiments.

RESULTS

This study is designed to evaluate the antimicrobial activity of *C. azarolus* leaf extracts and to determine their potentiation of the antibiotic activity against multidrug-resistant strains of *E. coli*. The metabolite contents of extracts as well as their HPLC analysis were also determined.

***Metabolite content and HPLC analysis*:** The metabolite contents of the tested extracts are presented in Table 2. TOF enriched extract exhibited the most important quantity of flavonoids, proanthocyanidins and total polyphenolic compounds, followed by ethyl acetate, methanolic, butanolic and chloroform extracts. The highest content of tannin was recorded in the butanolic extract with 62.64mg/100g.

When compared with authentic standards (chlorogenic acid, epicatechin, catechin, apigenin, naringenin, eriodictyol, ferulic acid, quercetin, isoquercetin, hyperoside, rutin, procyanidin B2, procyanidin C1, vitexin, vitexin-2"-*O*-rhamnoside, vitexin-4"-*O*-glucosid, ursolic acid, oleanolic acid, betulin) and based in the comparison of their retention times and peak area when injected alone or coinjected with the standard, the HPLC chromatograms of TOF and ethyl acetate extracts indicated the presence of chlorogenic acid, epicatechin, rutin, hyperoside, vitexin-2"-*O*-rhamnoside, procyanidin B2, procyanidin C1 and ursolic acid in both extracts. However, oleanolic acid is present only in TOF extract.

***Antimicrobial activity*:** The antimicrobial activity of *C. azarolus* extracts was evaluated against five reference bacteria. It appears that *C. azarolus* leaves extracts exhibited various levels of antibacterial effect against these strains. MIC values varied from 0.3mg/ml to over 5mg/ml (Table 3). Ethyl acetate and TOF enriched extracts displayed strong activity against both Gram-negative and Gram-positive bacteria.

S. typhimurium was the most susceptible bacterial species to TOF enriched extract, followed by *S. enteritidis*, and *S. aureus*, with MICs of respectively 0.3, 0.6 and 1.25mg/ml. In comparison ethyl acetate extract exhibits significant inhibitory effect against *S. enteritidis*, *S. faecalis* and *E. coli* with MICs of respectively 0.6, 0.6 and 1.25mg/ml. Methanolic extract was active on *S. aureus* and *E. coli* with the same MIC of 1.25mg/ml. *S. enteritidis* was the most susceptible bacteria to chloroform extract followed by *S. typhimurium* with MICs of respectively 0.6 and 1.25mg/ml. Whereas butanolic extract exhibited the lowest antibacterial activity.

The antimicrobial activity of the tested *C. azarolus* extracts was also evaluated against 4 multidrug-resistant strains of *E. coli* with the resistance profile described in Table 1. It was found that ethyl acetate and TOF enriched extracts displayed strong activity against multidrug-resistant strains of *E. coli* in comparison with others extracts (Table 3). TOF enriched extract was effective against the four multidrug-resistant strains of *E. coli* with the same CMI of 0.6mg/ml, whereas, ethyl acetate

extract exhibited the same effect as TOF extract towards the four tested strains, except against *E.coli* 6574 which revealed a MIC value of 1.25mg/ml; that's why we selected these two extracts to follow their potential synergistic activity with antibiotics.

***Antibiotic resistance*:** The resistance patterns of the bacterial strains to the tested antibiotics are shown in Table 4. Two antibiotics have been tested for each bacterium that is resistant. MICs values varied from 0.12mg/ml to 1.25mg/ml (Table 4).

***Effect of natural antimicrobial and antibiotic combinations*:** When tested with *E. coli* 6574, ethyl acetate extract at 1.25mg/ml, revealed a synergistic interaction with ofloxacin and cefotaxim; in fact the MICs value of antibiotics was reduced from 0.25mg/ml to 0.0625mg/ml as assessed by the FIC value index. Whereas TOF enriched extract at 0.6mg/ml revealed a synergistic effect only with ofloxacin but no interaction with the other tested antibiotic "cefotaxim".

Similarly synergistic interaction was observed between TOF enriched extract and amoxicillin against *E. coli* 6228. In fact, TOF enriched extract at 0.6mg/ml reduced the MIC value of amoxicillin from 1.25mg/ml to 0.3125mg/ml.

No interaction was found between tested extracts either tested antibiotics, against *E. coli* 6708 & 6234, as FIC index value was higher than 0.5 (Table 5).

DISCUSSION

Here, we evaluated the antimicrobial effect of *C. azarolus* leaves extracts against both Gram-positive and Gram-negative bacteria and it was shown that ethyl acetate and TOF enriched extracts were the most active extracts (Table 3). Some studies suggest that the polyphenolic content of plant often determines the pharmacological properties of plants (Orhan, et al., 2007a). In fact, ethyl acetate and TOF enriched extract exhibited the most important quantity of flavonoids, proanthocyanidins and total polyphenolic compounds (Table 2) compared with others extracts. The antibacterial activity of flavonoids against both Gram-positive and Gram-negative bacteria has been reported (Djipa, et al., 2000). Activity against Gram-positive bacteria (*S. aureus*) was demonstrated mainly by compounds that contained hydroxyl groups in ring B. The most active were the 3', 4', 5'-trihydroxyflavonoids, e.g. myricetin-3,5,7, 3',4',5'-hexahydroxyflavone. Flavanone aglycones (naringenin, pinocembrin) and their C-6 or C-8 prenylated derivatives also turned out to be active. Flavones such as apigenin and its C- and O- glycosides were not active. Activity against Gram-negative bacilli (*E. coli*) was demonstrated by the flavones apigenin, vitexin, and saponarin, while flavonoid compounds having two or three hydroxyl groups in rings A or B were active against Gram-positive bacteria (Djipa, et al., 2000).

The antimicrobial activity of the tested *C. azarolus* extracts against four multidrug-resistant strains of *E. coli*, and it was found that ethyl acetate and TOF enriched extracts displayed a strong activity against multidrug-resistant strains of *E. coli* in comparison with others extracts (Table 3). These results may be ascribed to the active components identified in these extracts such as flavonoids and triterpenic acids. In fact, ethyl acetate and TOF enriched extract revealed by HPLC, the presence of hyperoside, vitexin-2"-*O*-rhamnoside, epicatechin and ursolic acid as major compounds. Many reports have established the potent antibacterial activities of terpenes, particularly ursolic acid (Fontanay, et al., 2008; Horiuchi, et al., 2007), and flavonoids such as epicatechin (Park, et al., 2004), hyperoside and vitexin-2"-*O*-rhamnoside (Orhan, et al., 2007b).

The potentiating effect of ethyl acetate and TOF enriched extracts in combination with antibiotics, against multidrug-resistant strains of *E. coli*. We found

that the combination ethyl acetate extract-antibiotic was more effective against the strain *E. coli* 6574, but when antibiotics were combined with TOF enriched extract both *E. coli* 6574 and *E. coli* strains demonstrated an enhancement of sensitivity of antibiotics (Table 5). This finding can be due to the presence of compounds with known modulation of antibiotic resistance activity in bacterial pathogens. In fact, it has been reported that ursolic acid and oleanolic acid may be useful when administered in combination with beta-lactam antibiotics to combat bacterial infections caused by some Gram-positive pathogens (Kurek, et al., 2012). Reminded that ursolic acid was found in both ethyl acetate and TOF enriched extract whereas oleanolic acid was found only in TOF enriched extract which explain the founded results. Indeed, Zhao, et al. (2002) reported that epigallocatechin gallate (EGCG) from green tea inhibited the activity of penicillinase produced by *S. aureus* and it restored the activity of penicillin. Similar results were reported by Hu, et al. (2002), who showed that EGCG synergistically enhanced the activity of carbapenems against methicillin-resistant *S. aureus* (MRSA).

Study conducted by Kostic, et al. (2012), showed that ethanolic extract from *C. oxyacantha* fruits exhibited antimicrobial activity against tested microorganisms. This activity was attributed to the presence of flavonoids which their antibacterial activity against Gram-positive and Gram-negative bacteria has been reported (Bylka, et al., 2004; Djipa, et al., 2000; Orhan, et al., 2007b; Park, et al., 2004).

The exact mechanism of decreasing antibiotic resistance by natural antimicrobials is unknown but is likely due to some structural change in the resistant bacteria. For example, the natural antimicrobials may have facilitated penetration of the drug through the outer layers of the bacterial cell wall or acted by blocking the inhibitory effects of protective enzymes, or interfered with single or multiple metabolic targets of the antibiotic (Palanippan and Holley, 2010).

CONCLUSION

Thus ethyl acetate and TOF enriched extract from *C. azarolus* leaves were able to substantially decrease the MIC of antibiotics in a diverse group of bacteria containing genetic elements responsible for drug resistance.

Declaration of interest: The authors declare no conflicts of interest. The authors alone are responsible for the content of this manuscript.

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Table- 1: Antibiotic resistance profile of *E. coli* strains.

Bacetria	Strains	Antibiotic resistance
E. coli	**6574** : beta lactam overproduction of cephalosporin	Amox, Ticar, Cefta, Tic+Clavu, Pipera, Cefo, Cefl, Amox+Clavu, Cefd, Cefxt, Rifam, Oflo, Tetra, Amik
	6228 : beta lactam penicillinase high level	Amox, Ticar, Pipera, Clavu-Amox, Tic+Clavu.
	6708 : beta lactam penicillinase low level	Amox, Ticar, Tetra, Mecl.
	6234 : beta lactam broad spectrum	Amox, Ticar, Cefta, Pipera, Cefo, Cefp, Cefl, Cefd, Genta, Tobra, Tetra, Cipro, Oflo, Norflo, Rifam, Furan, Naldx Ac.

- **Amox**: Amoxicillin, **Amik**: Amikacine, **Ticar**: Ticarcillin, **Cefta**: Ceftazidime, **Pipera**: Piperacillin, **Cefo**: Cefotaxim, **Cefp**: cefpirom, **Cefl**: Cefalotin, **Cefd**: Cefamandol, **Cefxt**: Cefoxitin, **Genta**: Gentamicin, **Tobra**: Tobramycin, **Tetra**: Tetracyclin, **Cipro**: Ciprofloxacin, **Oflo**: Ofloxacin, **Norflo**: Norfloxacin, **Rifam**: Rifampicin, **Naldx Ac**.: Nalidixic acid, **Mecl**: Mecillinam, **Clavu**: Clavulin, **Tic**: Ticarcilline.

Table- 2: Quantitative phytochemical screening of extracts from *C. azarolus*.

Metabolites	Extracts				
	Methanol extract	**Chloroform extract**	**Ethyl acetate extract**	**Butanol extract**	**TOF extract**
Polyphenols (Gallic acid equivalents)	228.72±0.01	218.02±0.01	378.44±0.03	195.8±0.01	537.33±0.04
Flavonoids (Quercetin equivalents)	1673.85±0.00	-	1228.14±0.00	977.42±0.07	2265.28±0.01
Tannins (mg/100g)	24.49±0.00	57.53±0.1	44.39±0.03	62.64±0.15	57.39±0.07
Proanthocyanidin (Cyanidin chloride equivalents)	49.75±0.03	18±0.004	38.16±0.00	62.58±0.03	114.33±0.01

- Not detectable.

Table- 3: Antimicrobial activity of *C. azarolus* extracts, expressed as MIC[a].

	References strains					*E. coli* multiresistant strains			
	Gram-positive		Gram-negative						
	S. aureus	*E. faecalis*	*E. coli*	*S. enteritidis*	*S. typhimurium*	*6574*	*6228*	*6708*	*6234*
Met	1.25	2.5	1.25	2.5	2.5	1.25	1.25	1.25	>5
Chl	2.5	2.5	2.5	0.6	1.25	1.25	2.5	2.5	>5
EtA	2.5	0.6	1.25	0.6	2.5	1.25	0.6	0.6	0.6
But	5	>5	2.5	2.5	2.5	5	1.25	2.5	5
TOF	1.25	2.5	2.5	0.6	0.3	0.6	0.6	0.6	0.6

- [a]Values in mg/ml, means of three experiments, **Met**: Methanol extract, **Chl**: Chloroform extract, **EtA**: Ethyl acetate extract, **But**: Butanol extract, **TOF**: Total oligomer flavonoids enriched extracts. **6574**: *E. coli* beta lactam overproduction of cephalosporin, **6228**: *E. coli* beta lactam penicillinase high level, **6708**: *E. coli* beta lactam penicillinase low level, **6234**: *E. coli* beta lactam broad spectrum.

Table- 4: Resistance pattern of bacteria against different antibiotics expressed in MIC.

	Amox	Cefo	Oflo	Pipera	Tetra
EC 6574	-	0.25	0.25	-	-
EC 6228	1.25	-	-	0.25	-
EC 6708	1.25	-	-	-	0.12
EC 6234	-	1.25	0.12	-	-

- **Amox**:Amoxicillin, **Cefo**: Cefotaxim, **Oflo**: Ofloxacin, **Pipera**: Piperacillin, **Tetra**: Tetracyclin. **EC 6574**: *E.coli* beta lactam overproduction of cephalosporin, **EC 6228**: *E.coli* beta lactam penicillinase high level, **EC 6708**: *E.coli* beta lactam penicillinase low level, **EC 6234**: *E.coli* beta lactam broad spectrum.
- [a]Values in mg/ml, Means of three experiments.
- - Not tested

Table- 5: Response of antibiotic resistant bacteria to the combined effects of an antibiotic and a natural antimicrobial expressed as the FIC index [a]

Antibiotics	Natural antimicrobial FIC	
	Ethyl acetate extract	**TOF extract**
EC 6574: *E. coli* beta lactam overproduction of cephalosporin		
Ofloxacin	0.5	0.5
Cefotaxim	0.5	1
EC 6228: *E. coli* beta lactam penicillinase high level		
Amoxicillin	1	0.5
Piperacillin	1	1
EC 6708: *E. coli* beta lactam penicillinase low level		
Amoxicillin	2	2
Tetracyclin	2	2
EC 6234: *E. coli* beta lactam broad spectrum		
Ofloxacin	1	1
Cefotaxim	1	1

- [a]Synergy = an FIC index $\leq$ 0.5. An FIC index between 0.5 and 4.0 indicated 'no interaction'. An FIC > 4.0 indicated antagonism between the two agents.

