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Original Research

Phytochemical composition and *In-vitro* activity of ethanolic extract of *Eucalyptus globulus* leaves against multidrug resistant poultry pathogens

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Abstract: Aim of the present study was to determine the *In-vitro* antibacterial activity of ethanolic extract of *E. globulus* leaves against common multidrug resistant poultry pathogens. Phytochemical analysis through HPLC revealed that kaempeferol (7.315min) followed by querecetin (6.655min) and myrecetin (3.655min). Percent area of kaempeferol (6826.88%) was highest, followed by myrecetin (5516.22%) and querecetin (163.748%). Phytochemical investigation of ethanolic extract of *E. globulus* leaves through GCMS showed highest retention time (min) α-pinene (20.43) and α-terpineol (20.15) accompanied by spathulenol (11.97), piperitone (11.04). The ethanolic extracts of *E. globulus* leaves showed a highest zone of inhibition against *S. pullorum* SP6; 20.64± 2.08, *E. coli* SE 12; 19.75± 2.83, *C. perfringens* type *A* (CPM38-01); 19.46± 2.02. The highest level of MIC of *E. globulus* noted were against *S. gallinarum* S22; 133.37±53.294, *S. gallinarum* S1; 130.20±45.10, *S. gallinarum* S4; 129.47±24.182, *S. gallinarum* S3; 126.83±72.392. In conclusion, the study confirmed that the ethanolic extract of *E. globulus* is composed of active ingredients having antibacterial activity and can be referred as an alternate to antibiotics.

Key words: *Eucalyptus globulus*; *Escherichia coli; Salmonella pullorum; Salmonella gallinarum; Clostridium perfringens* type A.

Introduction

The poultry sector retains a valuable contribution to the agriculture sector and Pakistan's economy (1). The current investment in poultry industry is more than Rs 700 billion. Pakistan is the 11th largest poultry producing country and has 1,163 million broilers annually published in Pakistan Economic Survey 2018-19. Poultry sector has developed rapidly in Pakistan. So far, there are many challenges to the poultry industry in the country. Among these challenges, bacterial infectious diseases cause high commercial losses to the poultry sector. The most prevalent pathogens include *E. coli*, *Salmonella* species and *Clostridium perfringens* (2). *Salmonella* species produce food borne illness. *Clostridium perfringens* type *A* cause necrotic enteritis by producing enterotoxin at the moment of sporulation in broiler birds (3). Therefore, it is the most important barrier in the export of poultry meat.

To treat the bacterial infection at sub clinical level, a list of antibiotics is added as a growth promoter in poultry feed. The bacterial infections add up pharmaceutical expenses and production losses that place a tremendous burden on a per kg cost of broiler meat. Due to the enormous spending of antibiotics both for at therapeutic and nominal degree at feed formulation contributes to antibiotic resistance in micro-organisms (4).

The antibiotic residues in bird's meat introduced into human food that prompts to antibiotic resistance in the treatment of human diseases (5). To handle, antibiotic resistance issues in both animal and human treatment, the researchers are in search to find an alternative to antibiotics used in poultry feed. It may curtail a number of antibiotics entering into the individual food chain and food industries (6).

Bacterial pathogens have a genetic ability to achieve and transmit resistance to drugs. Extensive use of antibiotics in veterinary medicines affecting public health and led to emergence of antibiotic resistance (7). The consumers are demanding for new antibiotics against resistant pathogens. It is known that plant extract and essential oil has good antiseptic role. Plant extract have antioxidant, antibacterial, antifungal, antiparasitic, insecticidal and antiviral effects (8).

Human and health services encourage plant extract as safe for health and some plants have medicinal ingredients. Research studies have shown that plant extract has antibacterial activity and also endeavors against other pathogens. Therefore, it is used in food industry. It improves and maintains the quality and hygiene of food (9).

World Health Organization is in view that medicinal plants can be the best source to replace antibiotics used at different therapeutic and sub therapeutic levels. Therefore, there is need to investigate such medicinal plants which safe to use. These plants that have antimicrobial properties can be of high significance to be used as medicine or therapeutic use. Plants can be natural op-

portunities for antibiotics. Currently, plant extracts and essential oils of plants are utilized as a particular source of substantial antimicrobial agents (10). The presence of particular bioactive compounds such as fatty acid, flavonoids and phenolic compoinds in plant extracts and essential oils may counter with pathogenic microorganisms and suppress microbial production (11). The plant extract achieved more attention of scientists due to its pharmacological active compounds, minor toxicity and its economic importance. Different studies revealed that plant extract have antimicrobial and antioxidant effects on food items (12)

Among medicinal plants, *E. globulus* is one of the medicinal plants generally raised at the roadside of the country. It has historic medicinal properties. It can be applied as a nominal-grade antibiotic replacement employed in poultry feed. *Eucalyptus* belongs to the *Myrtaceae* family which includes more than 140 genera and about 3800 species disperse in tropical and subtropical areas of the world. *Eucalyptus* is one of the world's significant and most extensive planted genera (13).

Research has proved that *E. globulus* has antioxidant activity. It is used in traditional and modern medicine. The leaf extract of *E. globulus* was found to have antibacterial, antifungal, antiviral, anti-inflammatory and anti-helminthic active components. *E. globulus* has high active ingredients having phenolic acid, flavonoids and tannins. It is used in medicines, perfumes and the food sector due to its diverse biological activities (14).

In conclusion, the widely escalate of drugs resistant microbial pathogens is one of the most genuine threats to effective treatment of chronic and infectious diseases. *E. coli*, *Salmonella* are the two pathogens that cause severe and life-threatening infections in humans, animals, and poultry. The gram-negative bacterium *E. coli* is present in the poultry intestine and causes different infections such as coleocystis or septicemia. Several studies have documented increasing resistance rates in *S. pullorum* and *E. coli* to antibiotics (15).

Therefore, the present study aimed to investigate the phytochemicals composition and antimicrobial activity of *E. globulus* against poultry pathogens such as *E. coli, S. pullorum, C. perfringens* type *A* and *S. gallinarum*.

Materials and Methods

Collection and identification of plant leaves

The fresh mature leaves of *E. globulus* were collected from Multan road, Bhaiphero, and Manga Mandi, Lahore, Pakistan. The leaves were washed with fresh and clean water and placed under the shade at room temperature to make it dry. Plant leaves were identified and confirmed as *Eucalyptus globulus Labill* from the Department of Botany, Government college university (GC), Lahore. A wide voucher specimen number was issued i.e. (GC. Herb. Bot. 3437).

Enriched extraction of *Eucalyptus globulus* leaves through Soxhlet apparatus

The under shade-dried *Eucalyptus globulus* leaves were crushed into powder by using an efficient grinding machine having a sieve size of 2 mm. Then 100g of powder of plant were rolled placed in a thimble of Soxhlet Apparatus against 600mL of absolute ethanol in a bottom flask. The temperature was kept up to 50ċ and water inflow was continued, to settle down the water vapors. After 10 to 12 cycles of solvent, the maximum extracts were collected in a bottom flask. After, 8-12 cycles, the extract was collected.

Phytochemical analysis of an ethanolic extract of *E. globulus* leaves through HPLC and GCMS

Column chromatography using silica gel was performed for the fractions of most potent *Eucalyptus globulus* extract on increasing solvent polarity. Flavonols were determined by using high-performance liquid chromatography (Model LC-110A) and CTO-10A column oven, SPD-10A UV–vis detector, and data acquisition class LC-10 software was used. A 20 µL volume of the filtered sample was injected into an analytical Supelco (Supelco Inc., Supelco Park, Bellefonte, PA, USA) ODS reverse phase (C18) column (250 4.6 mm; 5 lm particle size). The solvent system used was A: contained 3% trifluoroacetic acid and B: contained acetonitrile and methanol (80:20 v/v). The isocratic elution of the mobile phase was used for chromatographic separation and filtered under vacuum through a 0.45 µm membrane before use) at a flow rate of 1.0 mL min at 30 C. Wavelength of 360 nm was used for detection. The flavanols (kaempeferol, quercetin, myricetin) were identified by comparing their retention times with those of authentic standards (Sigma Chemicals Co., St Louis, MO, USA). Calibration curves of standards was used for quantification. The detection limit for kaempeferol, quercetin and myricetin was 0.04, 0.05, 0.05 mg L, respectively (16)

The ethanolic extract of *E. globulus* collected was settled to make bubble of Nitrogen gas into the solution. Both polar and non-polar phytochemicals were used for GC-MS (Sermakkani and Thangapandian, 2012). Gas chromatography-mass spectrometry (GCMS) analysis of *E. globulus* extract was analyzed on a Hewlett–Packard GC split injector at 280 Ċ connected to a Hewlett–Packard, having electron voltage 70 eV, temperature provision source 230 Ċ, temperature of quad 150 Ċ with multiplier voltage of 2,000 V, temperature of interface was 310 Ċ. HP pc chemstation computer-controlled the acquisition in a full scan mode. 1µl of the sample (dcm) was injected through an HP autosampler set on split mode. On a fused silica capillary column separation was performed. The temperature of GC was settled from 40 to 300 Ċ at 4 Ċ /min and was kept at the final temperature for 01 min in the presence of Helium which was considered as the carrier gas.

Collection of Indicator microbes, Agar well diffusion assay and Minimum inhibitory concentration

Already identified poultry pathogens including *Escherichia coli* (SE12, SE13, SE14), *Salmonella pullorum* (SP5, SP6, SP11)*, Salmonella gallinarum* (S1, S22, S3, S4) and *Clostridium perfringens* type *A* (CPM36-02) were procured from the Department of microbiology, UVAS, Lahore.

Antimicrobial testing of ethanolic extract of *E. globulus* leaves against *E. coli, S. pullorum*, *S. gallinarum* and *C. perfringens* type *A* was done through Agar well diffusion and Minimum inhibitory concentration. The pathogenic *E. coli*, *S. pullorum, S. gallinarum* and *C. perfringens* type *A* isolates were subjected to antibacterial susceptibility to ethanolic extract of the

plant using Kirby Bauer agar-well diffusion method. For that purpose, 100mg *Eucalyptus globulus* ethanolic extract was dissolved in dimethylsulphoxide 0.5% (DMSO). The 8 mm diameter wells were cut from the agar, filled its bottom with specific agar for each bacterium, then plant extract was added into each well up to the surface. The zone of inhibition of all plates was examined after incubation for 24 h at 30 C. Minimum inhibitory concentration of *E. globulus* against all four bacteria was determined. Optimal density of cultured 96 wells plate was taken at zero hours and after 24 hours and readings were noted. Nutrient broth media was prepared and 100uL of it was put in 1-12 wells of a microtiter plate (96 well). 10mg-9.75µg plant extract was dissolved in 1mL DMSO (Dimethyl sulfoxide). 100uL of that dissolved plant extract was put in 1st well of 96.

Results

Eucalyptus leaves were collected and dried under shade. Ethanolic extract was prepared by two different methods i.e. Soxhlet apparatus method and soaking method. The result of the percent yield of ethanolic extract of *Eucalyptus globulus* by Soxhlet apparatus (20±3.37). Phytochemical analysis of ethanolic extract was performed with High-performance liquid chromatography. The ethanolic extract obtained from the *E. globulus* leaves was screened for various phytochemicals. *E. globulus* phytochemical analysis of an ethanolic extract of leaves is shown in table 1 and in figure 1.

The results show the highest retention time for Kaempferol (7.315min) followed by Quercetin (6.655min) and Myrecetin (3.655min). Percent area of Kaempferol (6826.88%) was highest, followed by Myrecetin (5516.22%) and Quercetin (163.748%), respectively. The GCMS chromatograph curves peaks of active ingredients of *E. globulus* given in figure 1. The various phytochemicals compounds which contribute to the medicinal activities of the plant were shown in Table 2. The mass spectra of all the phytochemicals identified in the plant leaves ethanolic extract of *E. globulus* of the thirteen compounds identified, the most prevailing compounds were an a-pinene compound (19.36%), Piperitone (17.77%), 1-8 cineol (17.62%) and the lowest prevalent compound was detected as Sabinol (2.57%). Antimicrobial testing of ethanolic extract of *E. globulus* leaves against different poultry origin pathogens such as *E. coli, S. pullorum*, *S. gallinarum,* and *C. perfringens* type *A* using Agar well diffusion method and Minimum inhibitory concentration (17).

Agar well diffusion of *E. globulus* against *E. coli, S. pullorum*, *S. gallinarum* and *C. perfringens* type *A* was analyzed through the Kirby Bauer agar method. Ethanolic extract of *E. globulus* leaves 100 mg/mL dissolved in DMSO (0.5%). The diameter of the Zone of inhibition for each bacterium was measured (mm) given in figure 3. The Zone of inhibition against all these antibiotic resistance pathogens is given in Table 3. The Ethanolic extracts of the plant had excellent antibacterial activity against all pathogens listed in the table

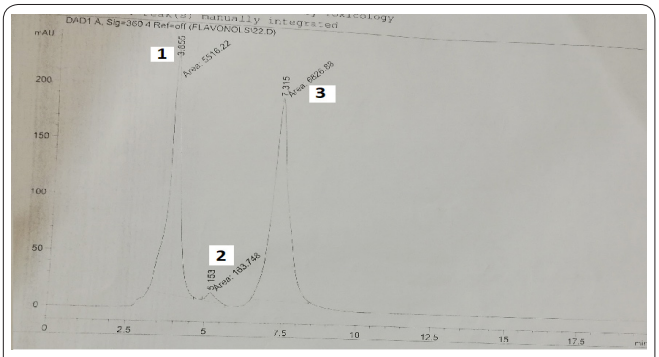

Figure 1. HPLC (Sample weight 1gram ethanolic extract of *Eucalyptus globulus* leaves) A) Graph shows flavonoids content through HPLC (Agilent 1100 series). 1) Myrecetin 2) Querecetin 3) Kaempeferol.

Table 1. Phytochemical analysis of ethanolic extract of *E. globulus* leaves by HPLC.

S. No	RT(min)	Phytochemical constituents	Percent Area
1	3.655	Myrecetin	5516.22%
2	6.655	Querecetin	163.748%
3	7.315	Kaempeferol	6826.88%

HPLC;high performance liquid chromatography. RT;retention time.

Table 2. Chemical constituent of ethanolic extract of Eucalyptus globulus leaves obtained through GC-MS

S. No	RT(Min)	Compound	Percent area
1	6.210	1-8 cineol	17.62
2	6.431	B-Myrecene	1.73
3	6.599	Globulol	4.20
4	6.877	Gamma-terpenene	2.74
5	6.927	Methane 1,2,3 triol	3.79
6	6.273	P-Cymene	5.22
7	9.142	Sabinol	2.57
8	9.325	Cryptone	6.28
9	9.697	Limonene	10.13
10	11.048	Piperitone	17.77
11	11.974	Spathulenol	4.06
12	20.159	a-terpineol	4.53
13	20.433	a-pinene	19.36

RT-retention time (min), relative area percentage.

3. The activity of plant ethanolic extract was checked against all the pathogens. The highest Zone of inhibition was observed against *E. coli* (19.75± 2.83b) and the lowest Zone of inhibition was observed again *S. gallinarum* (16.83±2.48a). The ethanolic extracts of *E. globulus* leaves showed a highest Zone of inhibition against *Salmonella pullorum* SP6; 20.64± 2.08, *E. coli* SE 12; 19.75± 2.83, *Clostridium perfringens* type *A* (CPM38-01); 19.46± 2.02. Lowest zone of inhibition were found against *Salmonella gallinarum* S3; 14.68±1.62, *Salmonella gallinarum* S1; 16.83±2.48, *Salmonella gallinarum* S4; 16.94±2.40, *Salmonella gallinarum* S22; 15.36± 1.86 and Control positive; 16.20± 1.50 as mentioned in table 3.

The highest level of MIC of *E. globulus* noted were against *Salmonella gallinarum* S22; 133.37±53.294, *Salmonella gallinarum* S1; 130.20±45.10, *Salmonella gallinarum* S4; 129.47±24.182, *Salmonella gallinarum* S3; 126.83±72.392 and the lowest MIC of *E. globulus* were *Clostridium perfringens* type *A* (CPM38-01); 89.325±35.27, *Clostridium perfringens* type *A* (CPM36-02); 91.145±49.66, *Salmonella pullorum* SP5; 104.160±45.10, *E. coli* SE 12; 104.166±45.105, Control positive; 105.60±28.30, and *Salmonella pullorum* SP6; 105.951±82.548 as presented in Table 3.

Discussion

Continuous use of antibiotics in the poultry industry as a growth promoter for poultry production leads to the development of high resistance, drug toxicity and residual effects in poultry as well as in humans (18, 19). Concerns about food safety, general health risks, and environmental contamination have pushed the European Union and World Health Organization banned the prophylactic usage of antibiotics in poultry feed formulation which leads to search out alternate of antibiotics.

Several medicinal plants can be used an alternative source of *antibiotics* in the poultry environment. One of the most significant medicinal plants is known as E. globulus leaves extract has been used as an alternate to antibiotics (20). The plant extract of *E. globulus* obtained with Soxhlet apparatus was 23%. Earlier, the extraction obtained by (21) was similar to our results.

The main constituent found through GCMS were a-pinene and carvacrol and 1,8-cineole revealed by (22). Active ingredients in plants varies with change in geography. Other compounds found in Eucalyptus are limonene and a-phellandrene (23). The active compounds found in *Eucalyptus globulus* through HPLC were Quercetin, flavanol, phenol, gallic acid and ellagic acid (24). The activity of ethanolic extracts was checked against all the pathogens. The highest Zone of inhibition was observed against E. coli (19.75±2.83). According to a previous study, E. globulus has strong antibacterial activity against gram-positive as well as gram-negative bacteria. Our findings were similar to the finding of (25, 26) the zone of inhibition he found against E. coli was in the range of 10-26 mm which is quite similar to our results. Our results were also similar to the findings of (Chhetri, 2008) i.e. 18mm Zone of inhibition of *E. glo-*

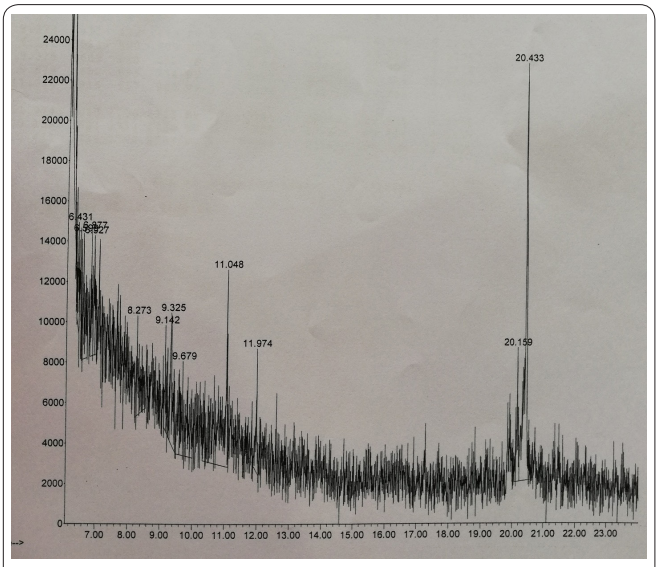

Figure 2. Vertical column- absorbance (2000-3200nm), Horizontal column- time (7-23 min) GC-MS ion chromatogram of ethanolic extract of *Eucalyptus globulus* leaves.

Table 3. *In-vitro* activity of *Eucalyptus globulus* determined by Agar well diffusion and Minimum inhibitory concentration.

Indicator bacteria	Activity	
	Well diffussion assay Mean ZOI (mm)	Mean MIC(µg/mL)
Control negative	----	-----
Control positive	16.20± 1.50^{c}	105.60±28.30^{b}
E. coli SE 12	19.75± 2.83^{b}	104.166±45.105^{a}
E. coli SE 13	18.37± 1.84^{b}	108.124±36.102^{b}
E. coli SE 14	17.8± 1.96^{a}	106.210±27.385^{b}
Salmonella pullorum SP5	17.08±2.36^{a}	104.160±45.10^{a}
Salmonella pullorum SP6	20.64± 2.08^{c}	105.951±82.548^{a}
Salmonella pullorum SP11	18.04±1.60^{b}	107.47±34.514^{b}
Salmonella gallinarum S1	16.83±2.48^{c}	130.20±45.10^{b}
Salmonella gallinarum S22	15.36± 1.86^{b}	133.37±53.294^{c}
Salmonella gallinarum S3	14.68±1.62^{a}	126.83±72.392^{a}
Salmonella gallinarum S4	16.94±2.40^{c}	129.47±24.182^{b}
Clostridium perfringens type *A* (CPM36-02)	18.16±1.34^{ab}	91.145±49.66^{a}
Clostridium perfringens type *A* (CPM38-01)	19.46± 2.02^{a}	89.325±35.27^{ab}

^{a,b,c}Different superscript in different rows of same column show statistically significant difference at $p \leq 0.05$.

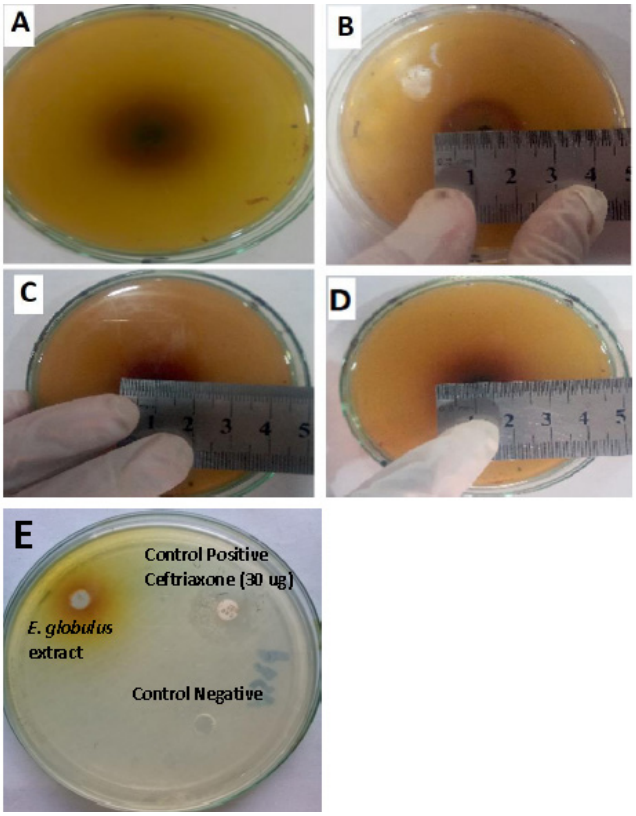

Figure 23. Agar well diffusion A) *Escherichia coli* B) *Salmonella pullorum* C) *Salmonella gallinarum* D) *Clostridium perfringens* type *A* E) Control group.

bulus against *E. coli.* Another study reported antimicrobial activity of E. globulus leaves, and reported that the antimicrobial activities of the extracts against nine different microorganisms varied considerably among Eucalyptus spp. of the twenty-six species examined, the extracts of E. globulus and E. maculata significantly stop the growth of gram-positive bacteria as well as gram-negative bacteria (27). According to (28) the E. globulus extracts have potent antimicrobial activity against both gram-negative and gram-positive bacteria as well as the fungus. The finding was in similarity to our results. E. globulus is composed of important phytochemicals that show good antibacterial, antifungal and anticancer activity. The compounds α-pinene and globulol have antibacterial properties and to citronellal has an important anticancer activity.

Eucalyptus has more antimicrobial activity against gram positive bacteria as compare to gram negative bacteria (29). The active ingredients of *Eucalyptus* having antibacterial activity are phenols, aldehyde and alcohols (30). Due to presence of lipopolysaccharide in gram negative bacteria, they are found more resistant to plant extract then gram negative bacteria (31). Plant extract is composed of many active ingredients and there is found no specific cellular target. It has effect on distortion of membrane permeability resultantly ions exchange differed, membrane potential lost, dysfunction of proton pump and ATP pool deletion occurred. The depreciation of cell wall and membrane lead to lysis of pathogen cell (32). Another study reported that disintegrate by1,8-cineole, p-cymene and Carvacrol can disintegrate outer membrane of bacteria and disrupt the permeability of cytoplasmic membrane (33).

The E. globulus have shown well inhibitory activity against gram-positive bacteria and the MIC values ranged from 0.06 to 1mg/mL. S. pyogenes was the most sensitive strain inhibited by E. globulus. The author also added that *E. globulus* has little or less effect on gram-negative bacteria include against P. aeruginosa, K. pneumonia and E. coli but it had inhibited the growth of A. baumanii with MIC value 1mg/mL. The E. globulus exhibited antibacterial activity against antibiotic-resistant bacteria having MIC value of 0.25-1mg/mL. The globulol inhibited streptococci, E. faecalis and A. baumani with MIC value varies from 1-4mg/mL (17).

In conclusion Antibiotics are extensively operated all over the world to treat infections in humans and animals. The massive service of antibiotics has evolved resistance in microorganisms. That's why the pathogens have produced resistant to antibiotics. Researchers are in the perceiving that there should be a decrease in the practice of antibiotics. To turn up a solution to this issue, the experts are in a search to adopt alternatives to antibiotics both in humans and animals. Hence, overcoming antibiotic resistance plants is a valuable opportunity to replace antibiotics.

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Interest conflict

There is no conflict of interest to declare.

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