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Antimicrobial Activity of the Ethanolic Extract of *Coleus aromaticus* against Common Wound Pathogens.

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ABSTRACT

Antibacterial activity of the ethanolic extract of *Coleus aromaticus* leaves and roots were tested against Gram positive and Gram negative wound pathogenic microorganisms using disc diffusion method, time kill assay and the determination of Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC). Disc diffusion test of the ethanolic extract of *C. aromaticus* demonstrated good antibacterial activity against *Escherichia coli*, *Proteus mirabilis* and *Staphylococcus aureus* and moderate activity against *Pseudomonas aeruginosa* and *Klebsiella pneumonia*. The MIC values ranged from 1.04 to 2.60 mg/ml for Gram negative bacteria where as the MIC value for Gram positive bacteria (*S. aureus*) was 1.30 mg/ml. Average log reduction was noted to be more than 3, after 24 hours in 1 x MIC where as the average log reduction in 2 x MIC was more than 3 after 3 hours of incubation. This antibacterial study indicates the crude extract as a bioactive compound that could be useful to develop new antimicrobial agents and it can be used to assist us in reducing the burden of cost and drug resistance.

Keywords: *Coleus aromaticus*, wound pathogens, Minimum Inhibitory Concentration, Minimum Bactericidal Concentration, Time Kill Assay

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INTRODUCTION

Diabetes mellitus is a metabolic syndrome that is associated with high blood sugar level with some establishments of disturbances in the process of glucose metabolism. *D. mellitus* is caused by reduced insulin secretion, insulin resistance or both [1]. Population growth, aging, urbanization, obesity and physical inactivity cause the number of diabetic patients to increase steadily and the estimated number of patients with diabetes would increase from 171,228 cases (2000) to 366,212 cases (2030) worldwide [2]. Diabetic foot ulcer is one of the frequent complications of *D. mellitus* in which 20 percent of total admissions to a hospital are due to the presence of diabetic foot ulcers that may need urgent amputation [3].

Wound healing process consists of four distinct but overlapping stages of hemostasis, inflammation, proliferation and tissue remodeling. Wounds that establish a delay in the process of healing are generally due to the failure of the progression of the wound from one stage to the other. Inflammation as one of the stages of wound healing could be delayed if the wound is contaminated with some microorganisms [4]. Patients with *D. mellitus* usually unveil impaired leukocytic function, inadequate migration of neutrophils and macrophages to the site of injury with the reduced level of chemotaxis that increases the risk of wound infection while slowing down the wound healing process [5].

The polymicrobial nature of the infected diabetic ulcers could result in increasing level of morbidity among diabetic patients. Diabetic wounds are more frequently infected by Gram negative bacteria which are for about 76% percent of total bacterial isolates. *Pseudomonas aeruginosa* (22%) is the most common Gram negative bacteria followed by *Escherichia coli* (19%), *Klebsiella pneumonia* (17%) and *Proteus spp* (11%). While on the other hand, the most common Gram positive bacteria that causes wound infection in diabetic patients is *Staphylococcus aureus* (19%) [6].

Both Gram negative and Gram positive bacteria have evolved to overcome wide range of antibiotics by exhibiting resistance. In Japan, more than 50% percent of clinical bacterial isolates of *S. aureus* established multidrug resistance [7]. While Gram negative bacteria on the other hand, are inherently resistant to various number of antibiotics such as Vancomycin, Fusidic acid and others. Within the Gram negative group, *P. aeruginosa* is highly resistance to many antibiotics and it became one of the serious chemotherapeutic problems [8].

Coleus aromaticus Benth (Lamiaceae) is known as country borage in English [9]. It is large succulent herb with aromatic leaves that are found abundantly in tropical countries [10]. It is a dense shrub with a foetid scent, the flowers are white with the throat barred with red or yellow [11]. Their leaves are thick, succulent and juicy and it emanates pleasant smell upon crushing or squeezing [12].

The ethanolic extract of *C. aromaticus* shows high impact of antibacterial activity against *E.coli*, *Bacillus sp*, *Pseudomonas sp*, *Staphylococcus* and *Klebsiella sp* [13]. Various kinds of solvent extract of *C.aromaticus* as well as its essential oils have demonstrated high

antimicrobial activity on both Gram positive and Gram negative bacteria. It is also found to be quite effective against drug resistant microorganisms as well as the phytopathogenic microorganisms [14]. The isolated oil of *C. aromaticus* showed great antibacterial activity against *S. aureus*, *E. coli*, and *K. pneumonia* with mild activity shown against *P. aeruginosa* [15]. Ethanolic extract has been proven to have impressive antibacterial activity against *S. aureus* and *P. aeruginosa* which increase the opportunities for *C. aromaticus* to act as an important source of herbal antibacterial agents [16].

This study will help to evaluate the antimicrobial activity of *C. aromaticus* against *S. aureus*, *P. mirabilis*, *K. pneumonia*, *P. aeruginosa* and *E.coli* by using the ethanolic extract of *C. aromaticus.*

METHODS

Ethanolic extract of *C. aromaticus* was prepared according to the method described by Delahaye [17]. The leaves and roots of *C. aromaticus* were washed and dried at room temperature for fourteen days. Then it was crushed into coarse powder using a mortar and pestle. The powder was used for the ethanol extractions at 67°C. 20 g of powder was added to Soxhlet extractor for 18 hours. The extract was then placed on rotary evaporators at 67 and 92 °C respectively to remove the ethanol and water. A sample of 0.1 g of the dried leaf extract was dissolved in 10 ml of sterile water and two fold serial dilutions were made, to give 10 extract concentrations which are 100, 50, 25, 12.5, 6.25, 3.125, 1.563, 0.781, 0.390 and 0.195 mg/ml.

Disc diffusion susceptibility test using Modified Kirby-Bauer technique [18] was used to identify antimicrobial activity of *C. aromaticus*. Using a sterile wire loop, 3-5 well isolated colonies of similar appearance of a test organism was touched and emulsified in 3-4 ml of sterile physiological saline. Sterile swab was dipped into bacterial suspension. The swab was streaked evenly over the surface of the Mueller Hinton agar medium in three directions, rotating the plate approximately 60° to ensure even distribution. With the Petri dish lid in place, the surface of agar was allowed to dry for 3-5 minutes. The steps were repeated for the rest of four bacteria. 60 µL of ethanolic extract with the concentration of 100mg/ml was placed into filter paper discs that were about 6mm in diameter. Using the sterile forceps, 6 extract impregnated discs were placed on all the agar plates that were swabbed with single bacteria. Within 30 minutes of applying the discs, the plates were inverted and incubated aerobically at 35°C for 16-18 hours. Positive control Mueller Hinton plates were prepared with each and every test microorganisms using Ampicillin 10µg, Chloramphenicol 30µg and Streptomycin 10µg respectively. After incubation, the diameter of the zone of inhibition was measured in mm.

Determination of the MIC and MBC for all the five test microorganisms were performed using the method adapted from Dhiman [19].The tube dilution method was used for determination of MIC and MBC. Extract was serially diluted to give a concentration of 100, 50, 25, 12.5, 6.25, 3.125, 1.563, 0.781, 0.390, 0.195 *mg*/ml in test tubes containing 1 ml sterile nutrient broth. Then, the tubes were inoculated with 100 μL of bacterial suspension .To serve as a positive control, Chloramphenicol was serially diluted to give 100, 50, 25, 12.5, 6.25, 3.125,

1.563, 0.781, 0.390, 0.195 µg/ml in test tubes containing 1 ml sterile nutrient broth which were then inoculated with 100 µL of bacterial suspension. Another tube containing nutrient broth only was seeded with the test organism to serve as negative control. All the tubes were then incubated at 37 °C for 24 hours and then examined for growth by observing its turbidity. The MBC of the plant extract on the bacterial isolates was carried out by pipetting 0.1 ml bacterial culture from the mixture obtained in the determination of MIC tubes which did not show any growth and subcultured on to nutrient media and incubated at 37 °C for 24 h. After incubation, the concentration at which there was no single colony of bacteria was taken as MBC.

Bacterial killing studies were performed by using the method adopted from Mandal [20]. The bacterial killing studies were carried out using the initial inoculum of approximately 5 × 10^5 cfu/ml. The fixed concentration of the extracts used were ½ x MIC, 1 x MIC and 2 x MIC for each bacteria, and the viable cell counts were determined at 0, 3, 6 and 24 hours. The effect of varied concentration of the extracts on bacterial density (cfu/ml) was determined after incubating the bacterial suspension (5 × 10^5 cfu/ml) in fresh Mueller-Hinton broth for 24 hours at 37°C. After incubating at 37°C for 24 hours, emergent bacterial colonies were counted, cfu/mL calculated, and compared with the count of the culture control without the extract. Graph Log_{10} cfu/ml against time was plotted for ½ × MIC, 1 x MIC and 2 x MIC respectively for each and every bacteria and the average log reduction was tabulated in a table.

RESULTS

Disc diffusion

The zone of inhibition by the ethanolic extract of *C. aromaticus* (100µg) ranged from 17.5 mm to 27.0 mm in diameter (Table 1). Ethanolic extract showed its least inhibition zone with *K .pneumonia* and its highest inhibition zone with *E. coli.* When the comparison of the zone of inhibition is done between the ethanolic extract of *C.aromaticus* and the modern antibiotics, it is pretty interesting to say that the ethanolic extract acts almost equal to Chloramphenicol in terms of bacterial growth inhibition and its action can be considered as to be superior to the action of both Ampicillin and Streptomycin. (Figure 1)

Table 1: Zone of Inhibition (mm) of the ethanolic extract of *C. aromaticus* and some modern antibiotics tested with five common wound pathogens.

Microorganism	Ethanolic extract *C. aromaticus* (100µg) (Mean with SD)	Ampicillin (10µg) (Mean with SD)	Chloramphenicol (30µg) (Mean with SD)	Streptomycin (10µg) (Mean with SD)
S. aureus	21.0± 1.4	20.5 ± 2.1	21.5 ± 0.7	11.5 ± 0.7
E. coli	27.0 ± 1.4	18.5 ± 0.7	20.5 ± 2.1	15.5 ± 0.7
P. aeruginosa	19.5 ± 0.7	18.0 ± 1.4	19.5 ± 2.1	19.0 ± 1.4
K. pneumonia	17.5± 0.7	21.0 ± 1.4	24.0 ± 1.4	14.5 ± 0.7
P. mirabilis	22.5 ± 0.7	20.0 ± 1.4	20.5 ± 2.1	18.5 ± 0.7

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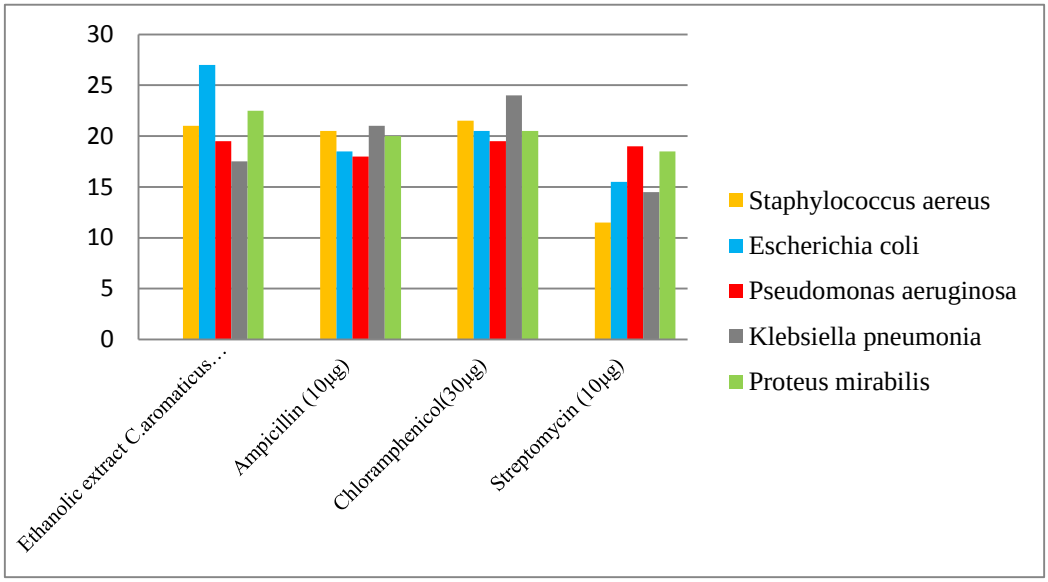

Figure 1: Comparison between the zone of inhibition of ethanolic extract of *C. aromaticus* with three common antibiotics against five common wound pathogens

Minimum Inhibitory Concentration

The range of MIC values for the ethanolic extract of *C. aromaticus* is from 1.04 till 2.60 mg/ml with the highest value noted with *K. pneumonia* and the lowest value noted with *E.coli* and *P. mirabilis* (Table 2). Higher dose of extract for about 1 mg/ml more is needed to combat the growth of both *K. pneumonia* and *P. aeruginosa.* However, *S. aureus* on the other hand, needs slightly lower concentration to be restricted from growth compared to *K. pneumonia* and *P. aeruginosa.* Chloramphenicol, the modern antibacterial agent has lower MIC values ranged from 0.01 mg/ml till 0.02 mg/ml.

Table 2: Minimum Inhibitory Concentration of ethanolic extract of *C. aromaticus* and the modern antibiotic chloramphenicol with five common wound pathogens

	Ethanolic extract of *C. aromaticus*	Chloramphenicol
Microorganism	(mg/ml) (Mean with SD)	(mg/ml) (Mean with SD)
S. aureus	1.30 ± 0.37	0.02 ± 0.01
E. coli	1.04 ± 0.37	0.02 ± 0.01
P. aeruginosa	2.08± 0.74	0.01 ± 0
K. pneumonia	2.60 ± 0.74	0.02 ± 0.01
P. mirabilis	1.04 ± 0.37	0.02 ± 0.01

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Minimum Bactericidal Concentration

The range of MBC is from 2.60 mg/ml to 8.33 mg/ml for ethanolic extract of *C. aromaticus* with the highest value noted in *K. pneumonia* (Table 3). MBC however did not exceed fourfold of MIC value with the range of just between 2 to 4 times higher than the MIC. On the other hand, Chloramphenicol showed MBC values that ranged between 0.03 mg/ml till 0.04mg/ml.

Table 3: Minimum Bactericidal Concentration of ethanolic extract of *C. aromaticus* and the modern antibiotic chloramphenicol with five common wound pathogens

	Ethanolic extract of *C. aromaticus*	Chloramphenicol
Microorganism	(mg/ml) (Mean with SD)	(mg/ml) (Mean with SD)
S. aureus	3.65 ± 1.95	0.04 ± 0.01
E. coli	3.13 ± 2.21	0.04 ± 0.01
P. aeruginosa	5.21 ± 1.47	0.03 ± 0.01
K. pneumonia	8.33 ± 2.95	0.04 ± 0.01
P. mirabilis	2.60 ± 0.74	0.04 ± 0.01

Time Kill Assay

The result of time kill assay was constructed by looking into the average log reduction and is tabulated in Table 4. Average log reduction in viable cell count for ethanolic extract of *C. aromaticus* ranged between 0.60 to 3.69 $\log_{10}$ CFU/ml after 3 hours of interaction and between 1.43 to 7.61 log10CFU/ml after 6 hours of interaction in 1 x MIC and 2 x MIC concentration of extract (Table 4). On the other hand, the average log reduction in the viable cell count for ethanolic extract of *C. aromaticus* ranged between 3.77 to 8.03 $\log_{10}$ CFU/ml after 24 hours of interaction in 1 x MIC and 2 x MIC concentration of the extract.

Table 4: Log reduction for ethanolic extract of C. aromaticus at ½ x MIC, 1 x MIC and 2 x MIC

LOG REDUCTION FOR ETHANOLIC EXTRACT												
SUSCEPTIBLE ISOLATES	$\log_{10}$Kill (1/2 x MIC)				$\log_{10}$Kill (1 x MIC)				$\log_{10}$Kill (2 x MIC)			
	0 hour	3 hours	6 hours	24 hours	0 hour	3 hours	6 hours	24 hours	0 hour	3 hours	6 hours	24 hours
E.coli	0.38	0.88	1.74	2.59	0.26	0.89	1.89	3.77	0.30	3.17	7.60	8.01
K. pneumonia	0.16	0.66	1.29	2.13	0.23	0.80	1.82	4.12	0.27	3.69	7.57	8.01
P. aeruginosa	0.14	0.59	1.25	1.98	0.16	0.70	1.43	3.78	0.28	3.53	4.58	8.03
S. aureus	0.12	0.58	1.21	2.17	0.18	0.60	1.45	3.80	0.36	3.65	7.58	8.03
P. mirabilis	0.07	0.58	1.20	1.88	0.13	0.74	1.78	4.54	0.30	3.18	7.61	8.02

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Log reduction in viable cell count in time kill assay for ethanolic extract is more than 3 for 2 x MIC of ethanolic extract of *C.aromaticus* against all the five microorganisms tested after 3 hours. However, with 1 x MIC concentration of ethanolic extract the reduction which is more than 3 was only observed after 24 hours of interaction. The bacterial colonies were almost wiped out after incubating for 6 hours with 2 x MIC of ethanolic extract of *C .aromaticus* (Figure 1). With 1 x MIC concentration on the other hand, greater reduction was observed after 6 hours and it steadily decreased until it reaches 24 hours (Figure 2). On contrary, there was less reduction in all the test isolates when it was subjected to ½ x MIC of concentration of ethanolic extract (Figure 3).

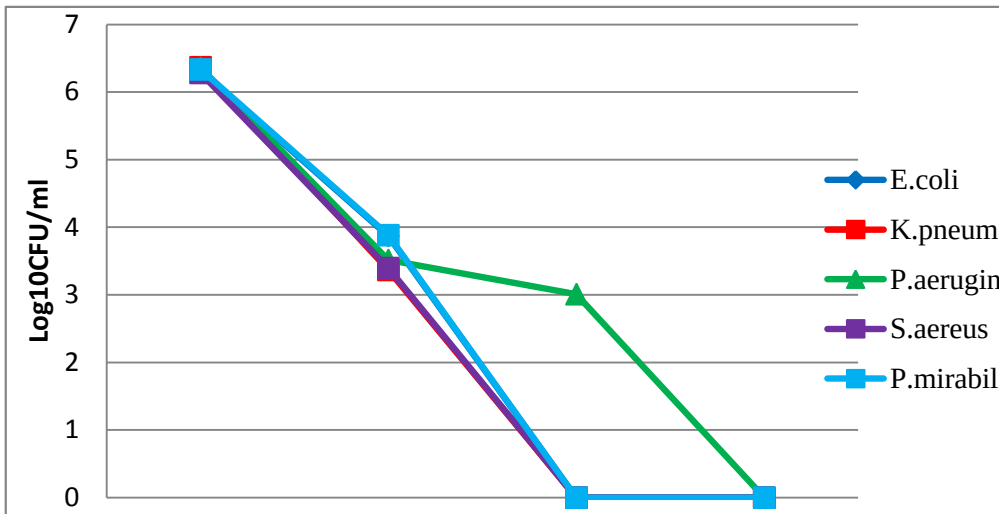

Figure 2: Time Kill Assay for 2 x MIC of ethanolic extract of *C. aromaticus* against wound pathogens

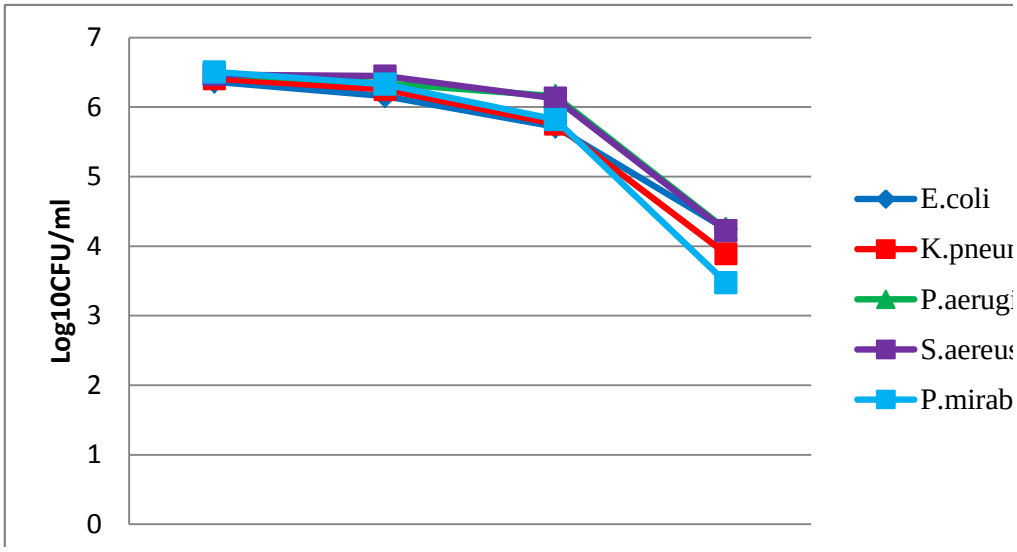

Figure 3: Time Kill Assay for 1 x MIC of ethanolic extract of C. aromaticus against wound pathogens

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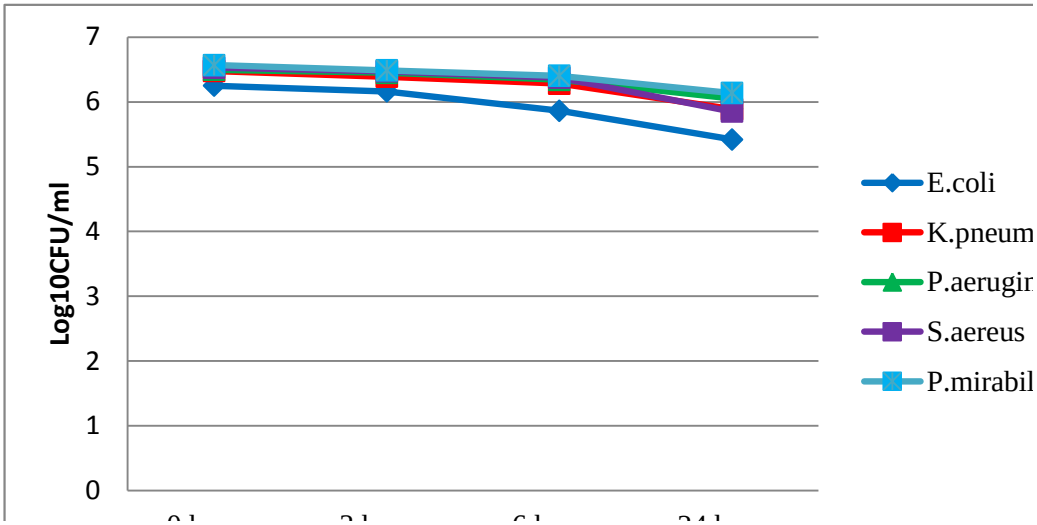

Figure 4: Time Kill Assay for ½ x MIC of ethanolic extract of *C. aromaticus* against wound pathogens

DISCUSSION

Antimicrobial susceptibility testing was carried out by utilizing four different approaches which were disc diffusion, MIC, MBC and time kill assay. Inspite of limitations, such as formation of concentration gradient in solid phase assays and the delay for about two to three days for obtaining results, the Kirby Bauer disc diffusion method is the only widely used clinical method [21]. Ethanolic extract of *C.aromaticus* showed potent antibacterial efficiency on all five microorganisms tested with maximum zone of inhibition in *E.coli*. This shows *E.coli* is very susceptible to ethanolic extract of *C.aromaticus*. Since, ethanolic extract is effective against not only *E. coli* but against *S. aureus* and *P. mirabilis* equally strong, so it can be used to eliminate both Gram positive and Gram negative skin pathogens.

The effect of a herbal ethanolic extract can be categorized as not active, moderately active or highly active [12]. So, in that sense ethanolic extract of *C.aromaticus* can be said as to be highly active against *E .coli*, *S .aureus* and *P. mirabilis* and moderately active against *K. pneumonia* and *P. aeruginosa*. Ethanolic extract of *C.aromaticus* (100µg) has almost equal antimicrobial activity with modern antibiotics like Ampicillin (10 µg), Chloramphenicol (30µg) and Streptomycin (10 µg). The mass percentage of ethanolic extract as antibacterial agent is obviously higher than the modern antibiotics but dose is often related to its side effects and toxicity on consumers. In comparison with modern antibiotics, ethanolic extract of *C.aromaticus* can be considered as to be safe with less toxic effect even with the dosage of 2000 mg/kg of body weight [9].

But such a high dose could not be possibly tolerant or applicable when it comes to modern antibiotics. Since the ethanolic extract has almost equal efficiency in its activity when it is compared with modern antibiotics, it can be obviously used as the alternative agent to replace the modern antibiotics that are connected with various degrees of side effects and toxicities.

MIC is the lowest concentration of antimicrobial agent that will inhibit visible growth of bacteria after overnight incubation. MBC on the other hand, is the lowest concentration of antimicrobial agent that will prevent the growth of bacteria after subculturing on to antibiotic free media [22]. Bacteriostatic antimicrobial agents are the agents that prevent the growth of bacteria which keeps them in the stationary phase of growth where as the bactericidal antimicrobial agents are the agents that kill bacteria [23].

For bactericidal drugs, the MBC is usually the same as the MIC and generally not more than fourfold higher than their MIC. In contrast, the MBC of bacteriostatic drugs are many fold higher than their MIC [24]. Generally, the MBC for ethanolic extract is noted to be higher than its MIC but still it is less than fourfold. So, it can be said that the ethanolic extract of *C. aromaticus* are quite good at demonstrating its bactericidal activity rather than being bacteriostatic.

In vitro time kill curve are commonly used to characterize the pharmacodynamic interaction between bacteria and antibacterial agent and it is easy to perform [25]. Time kill assay assist in the process of determining the antibacterial efficiency by using log reduction technique analysis. The conventional bactericidal activity standard is 3 log reductions in the viable colony count [26]. There is significant reduction in bacterial population in the presence of ethanolic extract of *C.aromaticus* after 6 hours and it was almost destroyed after 24 hours of incubation. The strength of bactericidal efficiency is higher with the higher concentration of ethanolic extract and this shows that the bactericidal activity of this particular extract is almost proportional to its dose and the time of exposure.

The global emergence of multidrug resistant (MDR) bacteria is increasingly limiting the effectiveness of current drugs and significantly causing a failure in treatment procedures [27]. In general, bacteria have specific genetic ability in which it is capable of acquiring antibiotic resistance and new infection could increase the rate of mortality among hospital admissions [28]. The use of plant derivatives as drugs and dietary supplements has been accelerated with the current use of 25 to 50% of plant pharmaceutical products [29].

The plant extracts are basically safer than the synthetic antibacterial compounds which offer profound therapeutic benefit with more affordable treatment [30]. In regards with that, the ethanolic extract of *C.aromaticus* can always serve as the ultimate antibacterial agents that are safe, effective and affordable in the area of pharmacy and pharmaceutical science.

CONCLUSION

The present investigation suggests that ethanolic extract of *C.aromaticus* is effective against the common diabetic pathogens. The efficiency of ethanolic extract against wound pathogens are dose and time dependent since greater activity was noted with the higher dose and prolonged time of exposure. Since the search for new antibacterial agents is quite intensive in most of the countries, *C.aromaticus* can be used as one of the tool to eradicate pathogenic

bacteria that are being resistant to most of the synthetic antimicrobial agents that are present in the hospital setting.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interests regarding the publication of this paper.

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