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Original Article

EFFECT OF HEAT AND FILTER STERILIZATION ON THE EFFICIENCY OF *COLEUS AROMATICUS* AS AN ANTIBACTERIAL AGENT AGAINST DIABETIC WOUND PATHOGENS

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ABSTRACT

Objective: The purpose of the present study is to evaluate the effect of sterilization on the efficiency of *Coleus aromaticus* on eradicating diabetic wound pathogens.

Methods: The investigation was explored by deploying disc diffusion, Minimum Inhibitory Concentration (MIC), Minimum Bactericidal Concentration (MBC) and time kill studies on *Escherichia coli*, *Staphylococcus aureus*, *Klebsiella pneumonia*, *Pseudomonas aeruginosa* and *Proteus mirabilis*.

Results: Zone of inhibition was 17.5-27.0 mm for unsterilized extract, 11.0-20.0 mm for heat sterilized extract and 6.0-12.0 mm for filter sterilized extract. MIC ranged from 1.02-2.60 mg/ml for unsterilized extract, 2.60-5.21 mg/ml for heat sterilized extract and 8.33-18.75 mg/ml for filter sterilized extract. MBC ranged from 2.60-8.33 mg/ml for unsterilized extract and much higher values were noted with heat and filter sterilized extract. Time kill assay showed log reduction of more than 3 in 2 x MIC unsterilized extract on microorganisms tested. Both heat and filter sterilized extract didn't show 3 log or more reduction even at 2 x MIC. However, better log reduction was observed in heat sterilized extract compared to filter sterilized extract.

Conclusion: *C. aromaticus* is a potent antibacterial agent and heat sterilization could be used to prepare this agent pharmaceutically. The evaluation on the effect of other sterilization methods on its efficiency as an antimicrobial wound remedy is highly suggested.

Keywords: *Coleus aromaticus,* Wound pathogens, disc diffusion, Minimumm inhibitory concentration, Minimum bactericidal concentration, Time kill assay, Heat sterilization, Filter sterilization.

INTRODUCTION

Diabetes mellitus(DM) is a syndrome characterized by chronic hyperglycemia and relative insulin deficiency, resistance or both [1]. DM has been documented to establish micro and macrovascular complications. Macrovascular complications are far more serious than the micro vascular complications because it affects big arteries causing peripheral arterial diseases such as those that are supplying to heart, brain and feet [2].10-15% of diabetic patients develop foot ulcers at some stage of their lives. Diabetic foot problems are responsible for nearly 50% of all diabetes related hospital admissions [3]. Patients with DM usually exhibit impaired leukocytic function, inadequate migration of neutrophils and macrophages to the site of injury with the reduced level of chemotaxis. All these sequences of actions could increase the risk of wound infection. Infection slows down the wound healing process by deteriorating the design of normal inflammatory phase of wound healing [4].

Diabetic wounds are frequently infected by gram negative bacteria and they comprise at least 76% percent of the 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%) [5]. Infection is one of the major causes of delayed wound healing. So most of the current wound topical agents are actually antibacterial in origin. Topical antimicrobial agents have been produced and manufactured as ointments that contain petrolatum and it is usually works best on the dry wounds, whereas cream is the other type of topical wound healing agent that is used to treat moist wounds and it is easy to clean by simple washing [6]. Although modern antibiotic has an extensive mode of use, it has been noted that despite of its advantages it possesses serious adverse effects which may worsen the condition. Over usage of antibiotics could cause the development of antibiotic resistant microorganisms and the bacteria could become less susceptible to a particular antibiotic [7]. The ethanolic extract of *Coleus aromaticus* shows high impact of antibacterial activity against *E. coli*, *Bacillus sp*, *Pseudomonas sp*, *Staphylococcus* and *Klebsiella sp*[8]. 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 [9]. Ethanolic extract of *C. aromaticus* has also been proven to have an impressive antibacterial activity against *S. aureus* and *P. aeruginosa.* This increases the opportunities for *C. aromaticus* to act as the important source of herbal antibacterial agents [10].

Coleus aromaticus Benth (Lamiaceae) also known as Indian or country Oregano [11]. *C. aromaticus* Benth syn. *Coleus amboinicus* (Lour) Spreng or *Plectranthus amboinicus* is traditionally used to cure wounds [12]. Apart from that,*C. aromaticus* is also used in cephalgia, otalgia, anorexia, dyspepsia, flatulence, colic, diarrhoea, cholera, halitosis, convulsions, epilepsy, cough, chronic asthma, hiccough, bronchitis, renal calculi, vesical calculi, strangury, hepotopathy, malarial fever and spasm. In Malaysia, bruished leaves are applied to burns and their poultice on centipede and scorpion bites. In Jawa, it is used to treat cracks at the corner of the mouth [13].

C. aromaticus had shown enhancement of wound healing by immunostimulation on the diseased giant murrels with lesions and deformed fins at the dorsal side and with reddish spot after five days of paste application[14]. Ten percent ointment of leaves and roots of *C. aromaticus* showed complete epithelialization after 12 days of treatment in albino rats on the excision wound[15]. Phytochemical screening of *C. aromaticus* showed the presence of flavonoids, steroids, terpenoids, tannins, coumarin, saponins, anthraquinones, glycoside and many more[16]. Despite the presence of abundant phytochemicals, *C. aromaticus* can be considered as one of the safest remedy in the area herbal medicine because its toxicity profile reveals no mortality even at 2000 mg/kg of dose[17].

Sterilization of pharmaceutical products involving both traditional and modern pharmaceutical agents is quite important in the current world of medical practice. Several different approaches are taken into consideration to handle the pharmaceutical products with care in predefined environmental conditions and the processes usually involve highly trained personnel. Failure to achieve the given set of requirements may directly affect the safety of the patients that are undergoing treatment [18]. Although there are varieties of methods under the umbrella of sterilization, two of them are quite famous in the area of drug purification and they are the heat (steam or moist) sterilization and filter sterilization method. It was documented earlier by some researchers of Universiti Putra Malaysia that these two types of sterilization methods have some negative effects on the properties of the phytochemical compounds and on the quality of the plant nutritious ingredients [19].

In regards with the fact that traditional pharmaceutical agents need sterilization, this study was focused on determining the action of these two types of sterilization methods on the efficiency of *C. aromaticus* as an antibacterial agent as well as to identify the suitable method for sterilizing the extract for future medical use.

MATERIAL AND METHODS

The ethanolic extract of *C. aromaticus* was prepared according to the method described by Delahaye[20]. 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 extraction 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.

The method of Adeshina [21] was adopted to prepare heat sterilized ethanolic extract of *C. aromaticus*. Part of ethanolic extract was autoclaved at temperature 121°C for 15 minutes. The heat sterilized ethanolic extract of *C. aromaticus* was stored at a temperature of 4°C until future use. The method of Hashemi [19] was adopted to prepare filter sterilized ethanolic extract of *C. aromaticus*. Part of ethanolic extract was filtered using Acrodisc syringe filter membrane of 0.45 µm and the flow-through was captured into sterile dark bottle and was kept at temperature 4°C until future use.

Disc diffusion susceptibility test using Modified Kirby-Bauer technique [22] was used to identify antimicrobial activity of unsterilized and sterilized ethanolic extract of *C. aromaticus*. The disc diffusion tests were directed against the five common diabetic wound pathogens (*E. coli, S. aureus, P. mirabilis, K. pneumonia* and *P. aeruginosa*). Using a sterile wire loop, 3-5 well isolated colonies of similar appearance to the test organism were touched and emulsified in 3-4 ml of sterile physiological saline. Sterile swab was immersed into bacterial suspension. Excess fluid was removed by pressing and rotating the swab against the side of the tube above the level of the suspension. The swab was streaked evenly over the surface of the 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 (not longer than 15 minutes). The prepared unsterilized extract of ethanolic extract (100 µg) was placed into filter paper discs that were about 6 mm in diameter. The filter paper discs were prepared by using sterile cork borer. It was then autoclaved before impregnating it with extract. Using the sterile forceps, 6 extract impregnated discs were placed on the Mueller Hinton agar plates that were swabbed with single bacteria. The discs was about 15 mm from the edge of the plate and not closer than 25 mm from disc to disc. Each disc was lightly pressed down to ensure that it is in contact with the agar and it was not moved once in place. Within 30 minutes of applying the discs, the plate was inverted and incubated aerobically at 35°C for 16-18 hours.

After overnight incubation, the test plates were examined to ensure the growth is confluent or near confluent. Using a ruler on the underside of the plate, the diameter of each zone of inhibition is measured in mm. The endpoint of inhibition is where the growth starts. The sizes of zones were interpreted. The procedure was replicated to obtain another set of results. These procedures were repeated for heat and filter sterilized extract of *C. aromaticus*. The zone of inhibition that was obtained from the heat sterilized ethanolic extract was then compared with the filter sterilized extract to show the differences of antibacterial efficiency between them as well as to choose the best sterilization method for preparing extract pharmaceutically.

Determination of the Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) for all the five test microorganisms were performed using the method adapted from Dhiman[23]. The tube dilution method was used for determination of MIC and MBC. Unsterilized 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. Another tube containing only nutrient broth 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. These procedures were repeated for heat and filter sterilized extract.

The MBC of the unsterilized plant extract on the clinical 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. These procedures were repeated for the heat and filter sterilized ethanolic extract of *C. aromaticus*. The differences of the MIC and MBC results between the heat and filter sterilized extract were compared to identify the most suitable method of sterilization. The comparison of MIC and MBC results was also done between the sterilized and unsterilized extract to investigate the overall effect of sterilization on the concentration needed to establish bacterial inhibitory and bactericidal activity. Bacterial killing studies were performed by using the method adopted from Mandal[24]. The Bacterial killing studies were carried out using the initial inoculum of approximately 5×10^5 CFU/ml. The fixed concentration of the unsterilized extracts used were ½ × 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 unsterilized 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. The procedures were repeated for heat and filter sterilized ethanolic extract of *C. aromaticus*. 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. The pattern of time killing assay was compared between heat and filter sterilized extract to give a clear picture on the pattern of killing in terms of concentration and the time of exposure. The results that could be obtained from the time kill assay of the sterilized extract can be used to explain the effect of sterilization on the time of action.

RESULTS

The range for zone of inhibition by unsterilized ethanolic extract of *C. aromaticus* (100µg) was noted to be from 17.5 mm to 27.0 mm (Table 1). On the other hand, the range for zone of inhibition of heat sterilized ethanolic extract (100µg) was from 11.0 mm to 20.0 mm whereas the range for zone of inhibition for filter sterilized ethanolic extract of *C. aromaticus* was from 6.0 mm to 12.0 mm in diameter. Unsterilized extract showed highest zone of inhibition with *E. coli*, moderate zone (more than 20 but lesser than 25) of inhibition with *P. mirabilis* and *S. aureus* and lowest zone of inhibition with *P. aeruginosa* and *K. pneumonia*. Heat and filter sterilized extract have almost similar pattern of bacterial inhibition but they were found to establish lesser zone of inhibition. Heat sterilized extract however has better range of inhibition compared to filter sterilized extract.

Table 1: Zone of Inhibition of ethanolic extract of *C. aromaticus*, heat sterilized ethanolic extract of *C. aromaticus* and filter sterilized ethanolic extract of *C. aromaticus*. (Mean ± SD)

Microorganisms	Ethanolic extract *C. aromaticus* (100µg)	Heat sterilized ethanolic extract *C. aromaticus* (100µg)	Filter sterilized ethanolic extract *C. aromaticus* (100µg)
S. aureus	21± 1.4	14.5 ± 0.4	8.5 ± 0.7
E. coli	27 ± 1.4	20 ± 0.7	12 ± 1.4
P. aeruginosa	19.5 ± 0.7	11 ± 0.7	6.5 ± 2.1
K. pneumonia	17.5± 0.7	11.5 ± 0.4	6.0 ± 0
P. mirabilis	22.5 ± 0.7	12 ± 0.7	7.5 ± 2.1

The range of MIC for unsterilized ethanolic extract against these five strains of wound pathogens is from 1.042mg/ml till 2.604mg/ml (Table 2). The MIC range for heat sterilized ethanolic extract of *C. aromaticus* is from 2.604mg/ml till 5.208mg/ml whereby the MIC range for filter sterilized ethanolic extract of *C. aromaticus* is from 8.333mg/ml till 18.750mg/ml.

Table 2: Comparison of Minimum Inhibitory Concentration (mg/ml) of ethanolic extract of *C. aromaticus* with heat sterilized ethanolic extract and filter sterilized ethanolic extract (Mean ± SD)

Microorganism	Ethanolic extract of *C. aromaticus*	Heat sterilized extract of *C. aromaticus*	Filter sterilized extract of *C. aromaticus*
S. aureus	1.302 ± 0.37	4.688 ± 2.21	18.750 ± 8.84
E. coli	1.042 ± 0.37	2.604 ± 0.74	8.333 ± 2.95
P. aeruginosa	2.084 ± 0.74	5.208 ± 1.47	12.500 ± 0
K. pneumonia	2.604 ± 0.74	4.688 ± 2.21	10.417 ±2.95
P. mirabilis	1.042 ± 0.37	2.604 ± 0.74	9.375 ±4.42

The range of MBC is from 2.604mg/ml till 8.333mg/ml for ethanolic extract of *C. aromaticus* (Table 3). The heat sterilized ethanolic extract of *C. aromaticus* showed MBC from the range of 4.167 mg/ml till 20.833mg/ml. Filter sterilized ethanolic extract of *C. aromaticus* shows greater MBC compared to the heat sterilized extract which ranged from 20.833mg/ml till 41.667mg/ml.

Table 3: Comparison of Minimum Bactericidal Concentration (mg/ml) of ethanolic extract of *C. aromaticus* with heat sterilized ethanolic extract and filter sterilized ethanolic extract (Mean ± SD)

Microorganism	Ethanolic extract of *C. aromaticus*	Heat sterilized extract of *C. aromaticus*	Filter sterilized extract of *C. aromaticus*
S. aureus	3.646 ± 1.95	9.375 ± 4.42	37.5 ± 17.68
E. coli	3.125 ± 2.21	4.167 ± 1.47	20.833 ±5.89
P. aeruginosa	5.208 ± 1.47	16.667 ± 5.89	41.667 ± 11.79
K. pneumonia	8.333 ± 2.95	20.833 ± 5.89	41.667 ± 11.79
P. mirabilis	2.604 ± 0.74	5.208 ± 1.47	37.5 ± 17.68

Average log reduction in viable cell count in time kill assay for unsterilized ethanolic extract of *C. aromaticus* ranged between 0.603 to 3.685 $\log_{10}$CFU/ml after 3 hours of interaction and between 1.433 to 7.607 $\log_{10}$CFU/ml after 6 hours of interaction in 1xMIC and 2xMIC concentration of extract (Table 4). On the other hand, the average log reduction in viable cell count in time kill assay for unsterilized ethanolic extract of *C. aromaticus* ranged between 3.767 to 8.027 $\log_{10}$CFU/ml after 24 hours of interaction in 1xMIC and 2xMIC concentration of the extract.

Average log reduction in viable cell count in the time kill assay for heat sterilized ethanolic extract of *C. aromaticus* ranged between 0.555 to 0.643 $\log_{10}$CFU/ml after 3 hours of interaction and between 1.224 to 1.335 $\log_{10}$CFU/ml after 6 hours of interaction in both 1xMIC and 2xMIC of heat sterilized ethanolic extract of *C. aromaticus*. The average log reduction in viable cell count for heat sterilized ethanolic extract of *C. aromaticus* in time kill assay ranged between 1.808 to 2.041 $\log_{10}$CFU/ml after 24 hours of interaction in 1xMIC and 2xMIC (Table 5).

The average log reduction in viable cell count in time kill assay for filter sterilized ethanolic extract ranged between 0.478 to 0.644 $\log_{10}$CFU/ml after 3 hours of interaction and between 1.038 to 1.246 $\log_{10}$CFU/ml after 6 hours of interaction in 1xMIC and 2xMIC concentrations of filter sterilized ethanolic extract. After 24 hours of interaction, the average log reduction ranged between 1.574 to 1.991 log10CFU/ml in 1xMIC and 2xMIC concentrations of filter sterilized ethanolic extract of *C. aromaticus* (Table 6).

Table 4: Log Reduction for Ethanolic Extract of *C. aromaticus*

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	0.378	0.883	1.741	2.592	0.265	0.888	1.889	3.767	0.303	3.174	7.604	8.012
K. pneumonia	0.162	0.664	1.289	2.127	0.231	0.799	1.815	4.121	0.272	3.685	7.573	8.014
P. aeruginosa	0.143	0.592	1.249	1.977	0.164	0.703	1.433	3.783	0.280	3.529	4.585	8.027
S. aureus	0.119	0.576	1.205	2.175	0.181	0.603	1.452	3.801	0.363	3.652	7.580	8.026
P. mirabilis	0.069	0.579	1.204	1.881	0.134	0.737	1.785	4.541	0.302	3.183	7.608	8.018

DISCUSSION

The average zone of inhibition for unsterilized extract is about 21.5 mm whereas the average of zone of inhibition for heat sterilized extract is about 13.8 mm.

It is just about35.8% of the difference between them and it can be improved with the increased concentration. The average for the range of zone of inhibition by filter sterilized extract would be 8.1

mm. When this is compared with the unsterilized ethanolic extract, the difference was for about 62.3%. There is a drastic difference of average between the unsterilized and filter sterilized extract. Filter sterilized extract is not even half equivalent to unsterilized extract in terms of bacterial inhibition. It is less than that. At least the strength of heat sterilized extract is almost half equivalent to the unsterilized ethanolic extract because the percentage of difference between the heat sterilized and unsterilized extract is still below 50%. So, definitely heat sterilized extract is better than the filter sterilized extract.

Table 5: Log Reduction for Heat Sterilized Ethanolic Extract of *C. aromaticus*

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	0.005	0.494	1.116	1.746	0.027	0.572	1.259	1.850	0.046	0.628	1.323	1.999
K. pneumonia	0.028	0.477	1.104	1.777	0.041	0.584	1.254	1.883	0.476	0.643	1.304	2.015
P. aeruginosa	0.049	0.463	1.111	1.753	0.023	0.555	1.256	1.836	0.144	0.313	1.010	1.672
S. aureus	0.067	0.444	1.080	1.726	0.062	0.573	1.225	1.884	0.471	0.631	1.335	2.042
P. mirabilis	0.049	0.459	1.108	1.623	0.052	0.583	1.255	1.808	0.477	0.617	1.319	1.921

Table 1: Log Reduction for Filter Sterilized Ethanolic Extract of *C. aromaticus*

Susceptible Isolates	log10Kill (1/2 x MIC)				log10Kill (1 x MIC)				log10Kill (2 x MIC)			
	0 hours	3 hours	6 hours	24 hours	0 hours	3 hours	6 hours	24 hours	0 hours	3 hours	6 hours	24 hours
E. coli	0.011	0.454	1.045	1.505	0.021	0.487	1.094	1.591	0.036	0.567	1.239	1.824
K. pneumonia	0.020	0.483	1.016	1.525	0.022	0.478	1.037	1.574	0.065	0.574	1.220	1.991
P. aeruginosa	0.055	0.468	1.029	1.508	0.011	0.480	1.080	1.576	0.061	0.644	1.227	1.864
S. aureus	0.037	0.458	1.013	1.531	0.061	0.522	1.098	1.610	0.058	0.557	1.203	1.990
P. mirabilis	0.027	0.499	1.065	1.512	0.040	0.499	1.087	1.592	0.059	0.586	1.246	1.892

MIC is for about 2 to 4 times higher with heat sterilised extract compared to the one that was unsterilized. Filter sterilized extract on the other hand, needs 8 to 9 times higher concentration than the unsterilized extract. It can justified that heat sterilized extract is far more effective than the filter sterilized extract because for any pharmaceutical agents that function to eradicate signs and symptoms, it is quite important for it to establish its functional property at much lower concentration. This is because the side effects or the adverse effects of any medicine tend to show up only when the concentration surges high in the blood.

The range of MBC values for unsterilized ethanolic extract is not more than fourfold higher than the MIC. Thus, unsterilized ethanolic extract can be considered as to bactericidal rather than being a bacteriostatic agent. The MBC of heat sterilized extract is less than fourfold of its MIC in all the microbes tested except with the *K. pneumonia*. In other way, it can be said that heat sterilized extract shows good bactericidal action only upon *S. aureus*, *E. coli*, *P. aeruginosa* and *P. mirabilis* but not upon *K. pneumonia*. The action against *K. pneumonia*is however bacteriostatic in nature. The MBC for filter sterilized extract show signs of bactericidal activity against three microbes (*S. aureus*, *E. coli* and *P. aeruginosa)* and bacteriostatic activity against two microbes (*K. pneumonia* and *P. mirabilis*). Results from MBC assay however supported the data obtained via disc diffusion and MIC. In general, these 3 different extracts established good bactericidal activity upon the 5 skin wound pathogens. But although sterilized extract is inferior to the unsterilized extract, heat sterilized extract is considered better than the filter sterilized ethanolic extract.

The greatest reduction in the cell count was observed with unsterilized ethanolic extract of *C. aromaticus* followed by heat sterilized ethanolic extract of *C. aromaticus* and the least reduction was observed in filter sterilized ethanolic extract of *C. aromaticus*. Log reduction in viable cell count in time kill assay for ethanolic extract is more than 3 for 2xMIC of unsterilized ethanolic extract of *C. aromaticus* for all the five microorganisms tested after 3 hours and after 24 hours with 1xMIC concentration of ethanolic extract. Although the bacterial colonies were not wiped out even after incubating with 2 x MIC concentration of heat sterilized extract, it showed some stabilization on the growth of bacteria and it had prevented the excessive growth of bacteria. Although heat sterilized extract does not show log reduction which is more than 3 even with prolonged exposure, it had given a smoother pattern of killing compared to filter sterilized extract. The average log reduction for heat sterilized extract is quite intensive at 2 x MIC for the period of 24 hours. This means heat sterilized extract taken at least a step to approach 3 log reduction with the approximate value of 2.042 $\log_{10}$CFU/ml after 24 hours of exposure. Situation is slightly different with the filter sterilized extract that showed very slow approach towards 3 log reduction and it was only about 1.991 $\log_{10}$CFU/ml even after 24 hours of exposure. Ethanolic extract of *C. aromaticus* showed good antibacterial activity. Although it seemed like both sterilization methods (filter and heat sterilization) had negative effects upon the antibacterial efficiency of the extract and it was unable to inhibit the bacterial growth completely but heat sterilized extract preserved its function better than the filter sterilized extract. Heat sterilization might cause only a little damage on the extract. High temperature causes some sort of structural damage and partial or complete loss of the quality of the plant. Whereas the filtration is a mechanical process in which the particulate matter is trapped through the sieve and thus it could lead to the failure of certain phytochemical compounds to pass through the filter causing them to be lacking in the filtrate [19].

High temperature can be one of the important factors that affect the decomposition of herbal preparations. In fact, an increase of temperature can also lead to an increase in the rate of reaction in all active products. This increased kinetics of reaction rate can cause deterioration in the thermolabile products that can only withstand certain range of temperature. But heat sterilization also had proven to show good effect on the activity of a plant extract. Extracts may contain microorganisms that may alter the antimicrobial functions of the extract. This is because foreign microorganisms can produce functionally active proteins and enzymes that can inactivate the antimicrobial agent in a particular plant extract. So, one should use autoclave heat sterilization technique to block these microorganisms which can cause permanent damage on the extract after prolonged storage [21]. Although, the results obtained from the heat sterilized extract is much lower than the unsterilized extract, we can always look for the benefit of heat sterilization on the plant extract like what mentioned here. Although heat sterilization uses autoclave (high temperature for about 121°C), some active components of the plant extract may survive this heat trauma due to their

thermostability. Some of these compounds such as tannins, alkaloids, anthraquinones, saponin, glycosides and flavonoids are documented to be hydrolysable either to active or non active compounds. This can explain why heat sterilized extract preserved its pharmacological action even though it was treated with high temperature. On the other hand, extract that is sterilized by the passage through a membrane filter that is bacteria proof (filter sterilization) might not show impressive results compared to heat sterilized extract. Most of the antimicrobial components are not water soluble, so when filters are used, it tends to result in the removal of the particles that are bigger than the size of the pore. This eventually causes reduction to the active components which are present in the extract. The lowering down of these active components can thus reduce the potency of any particular extract to work against test microorganisms [25].

Steam sterilization is well studied and validated so it is easy for pharmaceutical manufacturers to qualify it for parametric release. So, it is considered as a best approach of sterilization. Steam can be considered as an excellent carrier of thermal energy and it is capable of penetrating the protective biofilms [26]. Biofilms are formed when bacteria attaches itself to a surface in an aqueous environment with the subsequent formation of slimy, glue like substance that anchors them into anything that they come in contact including human tissues. Initially the bonds between the bacteria and the surface are just weak van der Waals forces and these bonds are replaced with permanent bonds causing cell adhesion. This biofilm covers the bacteria giving a shield like protection against antibiotics and it promotes bacterial growth [27]. The destruction of these protective biofilms is the key of steam sterilization. Filter sterilization on the other hand, acts differently. It does not kill the microbes but it removes them out of the solution. Since filtration only involves exclusion of microorganisms, it carries high risk of contamination. In regards with this, the U S Food and Drug Administration only allows filter sterilization if other alternative methods are not available [26].

Microporous membrane filters are used for sterile filtration because it is capable of trapping microorganisms. However, there were two main disadvantages of using microporous filtration. The first is the nature of the filter membrane itself. Membranes have surface retention mechanism and it decreases the capability of these filters to handle dirt especially when the particles have almost approximate size equal to the size of the pores. Dirt particles are then trapped at the pores and they tend to prevent the flow of liquid. The second is the size of the particles that are coming in contact with the membrane surface. Not all the particles which are smaller than the pores pass through. Some of them stay at the surface itself and situation can become worst if they form tortuous capillaries within themselves. These two factors decrease the quality of sterilized filtrate [28]. The efficiency of unsterilized and sterilized ethanolic extract against wound pathogens are dose and time dependent since greater activity was noted with the higher dose and with 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 a tool to eradicate pathogenic bacteria that are being resistant to most of the synthetic antimicrobial agents that are present in the hospital setting. The preparation of pharmaceutical products is usually accompanied with sterilization to ensure safety during administration to patient. Hence, a proper sterilization technique has to be chosen. Although there are varieties of sterilization procedures, a pharmaceutical manufacturing expert has to choose the best sterilization method.

CONCLUSION

The present investigation obviously suggests that unsterilized ethanolic extract of *C. aromaticus* works best against *E. coli*, *S. aureus*, *P. mirabilis*, *P. aeruginosa* and *K. pneumonia.* Selection of the best sterilization procedure depends on several factors. The method chosen has to be cost effective, easy to handle and it has to be reliable. Reliability however depends on the efficiency of a drug performing its duty deliberately. This study has proven that heat sterilization is more efficient than the filter sterilization in maintaining the efficiency of the extract. So, one can choose heat sterilization instead of filter sterilization to sterilize *C. aromaticus* extract. In future, the effect of other sterilization methods such as ultraviolet radiation, electron beam radiation, gamma radiation and gaseous sterilization on the antimicrobial efficiency of *C. aromaticus* extract can be tested. Thus it can expand the choice of sterilization and limit the disadvantages that a particular sterilization can cause to an extract.

CONFLICT OF INTEREST

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

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