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ANTIMICROBIAL ACTIVITY OF *Echinops kebericho* AGAINST HUMAN PATHOGENIC BACTERIA AND FUNGI

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Abstract

Background: Traditional medicine remains the primary source of health care in developing countries. *Echinops kebericho* Mesfin is a well known endemic medicinal plant in Ethiopia and is traditionally used to treat both infectious and non-infectious diseases. The aim of this study was to evaluate antibacterial and antifungal activities of water, ethanol and methanol based crude extracts of *E. kebericho* Mesfin against selected human pathogenic bacteria and fungi.
Materials and methods: Crude extracts of *E. kebericho* Mesfin were prepared by maceration method. Disc diffusion assay of the extracts was carried out in four different concentrations against three different bacterial species and two clinically isolated fungal species. Agar dilution method was used to determine minimum inhibitory concentration, minimum bactericidal and fungicidal concentrations of the extracts.
Results: Water based extracts exhibited the lowest antimicrobial activity when compared the ethanol and methanol based extracts ($P< 0.05$). Among the tested microorganisms, *S. aureus, C. albicans* and *A. flavus* were the most sensitive to alcohol based extracts. The minimum inhibitory concentration of the alcohol based extract ranged from 3.12 to 25µg/ml while those of the water based extracts were 100µg/ml and above. The lowest bactericidal and fungicidal concentrations of the ethanol and methanol based extracts were observed to be 6.25µg/ml against *S. aureus* and *C. albicans* and 37.5µg/ml against *E. coli.*
Conclusions: The traditional use of *E. kebericho* by local people in treating various types of infectious and non-infectious diseases was supported by this study. Antimicrobial activity of the medicinal plant varied with those of extraction solvents and against tested microorganisms.

Key words: Antimicrobial activity; Crude extracts; *Echinops kebericho* Mesfin

Introduction

Medicinal plants have been used by all cultures throughout history, as an integral part in the advance of modern civilization (Fabricant and Farnsworth, 2001). However, scientific evidence of these medicinal plants including their antimicrobial activities remain unknown; hence the need for more research. People since ancient times search-out medicinal plants based on superstition and assumption (Balandrin et al, 1993).

Echinops kebericho Mesfin (Family *Asteraceae*) is a well known endemic medicinal plant in Ethiopia. Traditionally, this medicinal plant has been used in treating different infectious and non infectious diseases such as fever, headache, stomachache, malaria, and cough (Toma et al, 2015). Small air-dried pieces of *E. kebericho* root were added to fire brands placed on specially prepared earthen or metallic crucible-like materials to repel mosquitoes and snakes as well as getting relief from headache, typhus and spiritual illness of Ethiopian folk belief (Karunamoothi et al, 2009; Teklehaymanot et al, 2007).

Previous studies (Toma et al, 2015; Ashebir and Ashenafi, 1999; Hymete et al, 2007) on extracts and essential oils of the roots of *E. kebericho* reported that, they exhibited antimicrobial, antihelminthic, molluscicidal and *in vivo* antiplasmodial activities. Antileishmanial activity was also reported against promastigote and axenic amastigote stages of *Leshmania aethiopica* and *L. donovani* (Tariku et al, 2011). Phytochemical analysis of root extracts of the plant also showed the presence of many antimicrobial chemical compounds (Ashebir and Ashenafi, 1999). In Guinea pig oral administration of water based root extract of *E. kebericho*, neither significant toxic signs nor death was observed during the period of study (Fisseha and Workineh, 2013).

Medicinal plant preparations are generally popular in developing countries with a long traditional history of using medicinal plants (Calixto, 2000). Even though many plant species have been tested for antimicrobial activities, majority of them have not been adequately evaluated (Gemechu et al, 2015). Furthermore, the occurrence of multi-drug resistance in bacterial and nosocomial fungal infections have raised an increasingly life threatening concern given the nature of these infections and toxicity of many antimicrobial drugs necessitate a continued search for unconventional and novel drug sources (Lopez et al, 2001). Moreover, most of the population of developing countries still lives in rural areas with low access to modern pharmaceuticals. Even when available, some of the modern drugs are prohibitively expensive. Most rural people, thus heavily rely on traditional medicine for the treatment of many human and animal diseases. The aim of current study was thus to assess antibacterial and antifungal activities of water, absolute ethanol and methanol based crude extracts of *E. kebericho* root against selected human pathogenic bacterial and fungal species.

Materials and methods

Study design

In-vitro experimental study of antibacterial and antifungal activity of *E. kebericho* root crude extracts was carried out using disc diffusion and agar dilution methods to determine minimum inhibitory concentration (MIC), minimum bactericidal concentrations (MBC) and minimum fungicidal concentrations (MFC). Positive and negative controls were used to monitor the antimicrobial activities of the extracts in all the assays. All measurements were repeated three times and mean ± SD was used to describe the measurements.

Collection and extraction of Plant Materials

Roots of *E. kebericho* Mesfin were collected from Hawassa market, southern Ethiopia. The identity of the plant was ascertained by a plant taxonomist and specimen was deposited at the National Herbarium, Department of Biology, Addis Ababa University Herbarium with voucher number of GA001/15. The roots of the medicinal plant were cut into smaller pieces followed by thorough washing under running tap water. The root pieces were finally rinsed twice with sterile distilled water. The root pieces were then dried in an oven at 40^{o}C. The dried root pieces were ground into fine powder using electric grinder. About 20 gm of fine powder was suspended in 200 mL of each of the three solvents Ethanol absolute (AVONDALE LABORATORIES Ltd., England), Methanol absolute (LOBA CHMIE Pvt. Ltd., India) and distilled water separately in sterilized screw capped 500 mL glass bottles. The suspensions were shaken using orbital shaker for 12 hours at room temperature. The suspensions were filtered using sterilized WHATMAN NO. 1 filter paper (**Figure 1**). The crude extracts were then dried by evaporation under vacuum in rotary evaporator kept at 40 ºC. The powder of the crude extracts obtained using the three extraction solvents (Water, ethanol and methanol) were weighed and dissolved in distilled water to prepare a stock solution of 200µg/ml and stored at a temperature of -20 ºC until further use (Handa et al, 2008; Parekh et al, 2005).

Figure 1: Root of *Echinops kebericho* Mesfin grinding, after grinding to fine powder and filtered extract (From left to right)

Determination of disc diffusion assay

In all the assay methods, *S. aureus* (*ATCC*-25923), *E. fecalis* (*ATCC*-29212) and *E. coli* (*ATCC*-25922), and clinical isolates of *C. albicans* and *A. flavus* were used to screen antimicrobial activities of the crude root extracts. Mueller Hinton agar medium and Sabouraud's dextrose agar (SDA) were used to carry out disc diffusion assay of antibacterial and antifungal activities, respectively.

For the purpose of conducting disc diffusion assay, crude extracts of root obtained using ethanol, methanol and water as extraction solvents were prepared at concentrations of 10, 20, 40 and 80 µg/ml. Diffusion discs of approximately 6mm diameter were prepared from WHATMAN NO. 1 filter paper and sterilized by autoclaving followed by drying in an oven. Ten microliter of each concentration of crude extracts was impregnated on separate sterile disc using sterile micropipette tips and stored at 4^{o}C in separate sterile containers. Then, disc diffusion assay was carried out according to (Jorgensen et al, 1999; Salie et al, 1996) methods. Agar plates with Gentamycin (10 µg/disk) (HI-MEDIA, India) and Ketoconazole (10 µg/disk) (HI-MEDIA, India) disc were placed were used as positive control for bacteria and fungi, respectively. Agar plates on which blank discs impregnated only with the respective solvents were placed were used as negative control. All the plates were prepared in triplicates and incubated at 27 ºC for 48 hrs and 37 ºC for 24 hrs for fungi and bacteria, respectively. At the end of the incubation periods the diameter of the zone of inhibition in each of the inoculated plates was measured to evaluate the antifungal and antibacterial activity of all the plant extracts.

Determination of MIC

Agar dilution method was used to determine the MIC of the root extracts. Equal amount of the stock solution of 200 µg/ml was added into a double strength sterilized molten Mueller Hinton agar and SDA after cooling to 45^{o}C in water bath followed by two fold serial dilution to obtain 100, 50, 25, 12.50, 6.25, 3.125 and 1.56 µg/ml. Each of the diluted mixture of the crude root extract and agar was aseptically poured to sterile 90 mm Petri dish and let to solidify. Then, the plates were aseptically inoculated with a 3 mm loopful of 0.5 McFarland standard diluted suspension of each test microorganism. The plates were then incubated at 37 ^{o}C for 24 hrs and at 27^{o}C for 48hrs for bacteria and fungi, respectively. The minimum dilution of the root extracts that completely inhibited the growth of each test organism was taken as the MIC. Control treatments comprised of agar plates prepared by pouring sterilized Mueller Hinton agar and SDA with and without the extraction solvents inoculated with the test organisms.

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Determination of minimum bactericidal and fungicidal concentration

Concentration of the crude root extract determined as MIC, the concentration preceding the MIC and one more concentration between the MIC and the preceding concentration were used to determine the MBC and MFC. Then, concentrations of the root extracts were adjusted in nutrient broth and Sabouraud's dextrose broth in 10 mL sterilized test tubes followed by inoculation with the test microorganisms by transferring one loopful of the suspension of each microorganism. The inoculated test tubes were then incubated at 37 ºC for 24 hrs for bacteria; and for 48hrs at 27 ºC for fungi.

After incubation, to confirm the absence of microorganisms in each of the cultures, each liquid culture was subcultured by transferring a loopful of the culture to sterile Mueller-Hinton agar and SDA plates. The inoculated plates were incubated at the same temperatures used for incubation of the liquid cultures. A control comprised the test organisms grown on agar plates poured with sterilized agar medium with and without the respective extraction solvents (water, ethanol and methanol) (CLSI, 2009).

Table 1: Antimicrobial activity of *E. kebericho* extracts against pathogenic bacteria and fungi using disc diffusion methods.

• Extraction solvents	• Extract concentration (µg/ml)	• Zone of Inhibition (mm)				
		• Bacterial species			Fungal Species	
		S. aureus	*E. coli*	*E. faecalis*	*A. flavus*	*C. albicans*
Water	10	-	-	-	-	-
	20	-	-	-	-	-
	40	-	-	-	-	-
	80	8.33 ± 0.57	-	-	-	-
Ethanol	10	12.66 ± 0.57	-	-	10.33 ± 2.08	11.33 ± 1.14
	20	14.00 ± 1.00	-	-	12.33 ± 0.57	12.66 ± 1.52
	40	16.33 ± 0.57	-	8.33 ± 0.57	14.00 ± 1.73	16.33 ± 0.57
	80	19.33 ± 1.15	9.66 ± 0.57	11.66 ± 0.57	17.33 ± 1.52	18.66 ± 1.52
Methanol	10	11.33 ± 1.15	-	-	11.00 ± 1.00	11.00 ± 1.00
	20	13.00 ± 1.00	-	-	13.33 ± 1.52	13.00 ± 0.00
	40	15.66 ± 0.57	-	11.33 ± 1.52	16.00 ± 1.00	16.66 ± 2.08
	80	18.00 ± 1.00	8.66 ± 0.57	14.00 ± 1.00	18.66 ± 0.57	20.33 ± 057
Positive control		22.33 ± 0.57	20 ±0.00	17.00 ± 1.00	22.33±0.57	23.66±0.57
Negative control		-	-	-	-	-

(-) = No activity; Values are mean of inhibition zone (mm) ± S.D of three replicates

Statistical analysis

For disc diffusion assay, all the measurements were replicated three times and the results were presented as mean ± SD. To compare the impacts of the extraction solvents and the difference in sensitivity of the test microorganisms, one-way analysis of variance was used. The statistical analysis was carried out by using SPSS version 16; and P-values < 0.05 were considered statistically significant.

Results

The MIC of the medicinal plant ranged from 3.12 to 25µg/ml in alcohol based extracts but it was higher in the water based extracts. The MIC of water based extracts of *E. kebericho*, against *S. aureus* was 100µg/ml. At this concentration, the water based extracts did not inhibit the rest of the tested microorganisms. On the other hand, for the ethanol and methanol based extracts, the MIC were 3.125µg/ml, 6.25µg/ml and 25µg/ml in the case of *S. aureus*, *C. albicans* and *E. coli*, respectively (Table 2)

In the disc diffusion assay, the ethanol and methanol crude extracts of the roots of *E. kebericho* showed significant higher antimicrobial activities ($P < 0.05$) as compared to water based extracts (Table 1). The water based extracts exhibited inhibitory effects only against *S. aureus* at the concentration of 80 µg/ml. Ethanol and methanol based extracts exhibited high antimicrobial activities against *S. aureus, C. albicans* and *A. flavus* while lower antimicrobial activities were observed against *E. coli. Entrococcus faecalis* was inhibited by ethanol and methanol based root extracts at concentrations of 40 and 80µg/ml, respectively.

In general, there was no significant difference ($P> 0.05$) between antimicrobial activities of methanol and ethanol based extracts against the tested microorganisms. The widest zone of inhibition (20.33 ± 0.57mm) was observed in the case of methanol based extracts against *C. albicans* while the narrowest was observed in the case of *E. coli*. Antimicrobial activity of the root extract showed significant difference ($P< 0.05$) between *S. aureus* and the other tested bacterial species (*E. coli* and *E. faecalis*). In contrast, there was no significant difference between *A. flavus* and *C. albicans* with regards to their sensitivity towards the root extracts of *E. kebericho* (Table 1).

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Table 2: Average minimum inhibitory concentrations, minimum bactericidal and fungicidal concentrations of *E. kebericho* in three different extraction solvents

Assay methods	Extract solvent	MIC, MBC and MFC (µg/ ml)				
		Bacterial species			Fungal species	
		S. aureus	*E. coli*	*E. faecalis*	*A. flavus*	*C. albicans*
MIC	Water	100.00	*	*	*	*
	Ethanol	3.12	25.00	12.50	12.50	6.25
	Methanol	3.12	25.00	12.50	6.25	3.12
MBC/MFC	Water	*	*	*	*	*
	Ethanol	6.25	37.5	18.75	22.92	12.50
	Methanol	9.37	37.5	18.75	12.50	6.25

(*) = No inhibitory or bactericidal/fungicidal activity at 100µg/ml, MIC=Minimum inhibition concentration, MBC=Minimum bactericidal concentration, MFC= Minimum fungicidal concentration

Discussion

In this study, variation was observed based on the extraction solvent used, the concentration of the extracts used and the species of the test microorganisms. Alcohol extracts of *E. kebericho* at 80µg/ml showed comparable antimicrobial activity with the positive control against *S. aureus A. flavus* and *C. albicans*. Conversely, water based extracts showed weak antimicrobial activity. Methanol and ethanol are more effective solvents in extracting phenolic compounds (Flavonoids anthocyanins, and phenolic acids) than water. Different studies (Das et al, 2010; Kim et al, 2005) emphasized the importance of these phenolic compounds in the antimicrobial activity of plant extracts. Therefore, the higher polyphenols present in alcohol based extracts of *E. kebericho* may have contributed to stronger antimicrobial activity of these extracts when compared with the water based extract. Alcohols are also good preservatives which provide a particularly effective way of maximizing the bioavailability of the actives extracted from the plant. Besides, alcohols are effective in extracting slightly non-polar (used for both polar and non polar) compounds. Polyphenol oxidase that inactivates polyphenol is not inactivated when water is used as extraction solvent, which may also be another possible reason why antimicrobial activities of water based extracts were weaker than those of the alcohol based ones (Shan et al, 2007; Lapornik et al, 2001).

E. coli was the most resistant microorganism while *S. aureus* was the most sensitive to the extracts of the medicinal plant. Antimicrobial studies also showed that Gram-negative bacteria show a higher resistance to plant extracts than Gram-positive bacteria. This may be due to the variation in the cell wall structures of Gram-positive and Gram-negative bacteria (Kim et al, 2005). Presence of potential efflux pumps inhibitors in gram negative bacteria may also be the possible cause of resistance (Hsieh et al, 1998).

The highest sensitivity of *S. aureus* to the crude root extracts of *E. kebericho* could be due to its thick peptidoglycan layer which is more receptive to antibiotics than the Gram-negative bacteria (Hsieh et al, 1998). Despite being gram positive, *E. faecalis* has both intrinsic and acquired antibiotic resistance as well as the presence of several virulence determinants and its ability to survive in adverse environments may have contributed to its resistance to the extracts of the medicinal plant [Franz et al, 2001; Reid et al, 2001).

The MIC, MBC and MFC assays strengthened the assertion that water is a poor solvent for extraction of phytochemicals from *E. kebericho*. However, the fact that higher concentrations of the water based extracts produced antimicrobial activities against *S. aureus* conforms to the traditional practice of treating several diseases caused by this microorganism with the various preparations from the roots of *E. kebericho*.

Even though people have been using this medicinal plant for a long period of time, the effective compounds which act as antibacterial and antifungal have not been characterized according to the rules of thumb for defining anti-infective potential in natural products (Cos et al, 2006). Therefore, further research is needed to investigate and understand the relationship between antimicrobial activity and chemical structure of each bioactive compound in the tested crude root extracts.

Conclusion

This study observed that, extraction solvents played a crucial role in the extraction of antimicrobial compounds and different microorganisms had different levels of sensitivity against the extracts. In the current study, *Staphylococcus aureus* was the most sensitive microorganism followed by *C. albicans*and *A. flavus*.The extensive use of *E. kebericho*by the local people in treating various types of infectious disease was supported by the findings of the current study as well.

Abbreviations

ATCC: American type culture collection; MBC: Minimum bactericidal concentration; MFC: Minimum fungicidal concentration; MIC: Minimum inhibitory concentration; SD: Standard deviation; SDA: Sabouraud's dextrose agar

Competing interests

The authors declare that we have no any competing interests.

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