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ANTIFUNGAL ACTIVITY AND SYNERGISM OF *Allium sativum*, *Zingiber officinale*, AND *Citrus limon* peel ESSENTIAL OILS AGAINST *Tinea capitis*

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ABSTRACT

The burden of *Tinea* infections, common dermatologic fungal diseases remain high globally, especially in Africa. This is sustained mainly by emergence of resistance to current antifungal drugs in use. Alternative treatment and preventive measures need to be sought in order to eradicate these infections. Antifungal activity and synergism of garlic in combination with ginger and lemon peel essential oils against *Tinea capitis* is assessed using inhibition assays. Ginger, lemons and garlic samples were randomly collected from Nakuru town market in Nakuru County, Kenya. They were identified by a botanist and then extracted and, analyzed. Antifungal activity against *Tinea capitis* and synergism among extracts was assessed by the disk diffusion method and minimum inhibitory concentration (MIC), respectively. The average essential oil yield for ginger, lemon, and garlic was 22%, 20% and 16%, respectively. Ginger, lemon and garlic essential oils depicted a comparable antifungal activity against *Tinea capitis* isolates (P value > 0.05). The combined essential oils formulation of ginger, lemon and garlic showed the highest activity compared to single extracts (P > 0.05). The MIC for the combined essential oils was 6.25 mg/ml. A combination of the three extracts act in synergy against the *Tinea* isolates. The rate of inhibition was directly proportional to the concentration of the extracts. Ginger, lemon and garlic are sources of molecules that act in synergy in controlling the *Tinea spp* infections. They can be leveraged to support the Ministry of health in achieving its universal healthcare goal.

Keywords: ***Tinea capitis, Allium sativum, Zingiber officinale, Citrus limon*, antifungal, synergism.**

INTRODUCTION

Tinea species infections remains a significant public health challenge globally (Leung et al., 2020). The burden of dermatophytosis worldwide ranges between 20% to 25% (Chowdhary et al., 2022). In Africa, the burden of these infections remains largely undetermined, because they are highly neglected and under-reported (Moto et al., 2015). *Tinea capitis* is the most common fungal infection of the hair shaft and follicle of the scalp that is difficult to treat (Leung et al., 2020). Though it can affect all age groups, it is prevalent among school going children aged 4 to 7 years, because their scalp sebum lacks fungistatic fatty acid (Neetha, 2020). The spread of this diseases is aggravated by predisposing factors such as overcrowding, low socioeconomic status, poor hygiene, and sharing of clothes, utensils and furniture (Avire et al., 2021).

Systemic antifungal drugs are preferred over topical ones for treatment of *Tinea capitis*, because the latter does not penetrate the root hair shaft, and also have some adverse effects to the skin (Ali *et al,* 2017; Mayser et. al, 2020). However, combined therapy of the two antifungals is preferred for it increases the cure rates (Sun et al., 2016). The drugs of choice for the management of ringworm are the oral antifungal agents and they include; terbinafine, griseofulvin, itraconazole and fluconazole (Xiao et al., 2021). Griseofulvin is the first-line drug for the treatment of *Tinea capitis.* The duration of treatment of tinea capitis is four to six weeks or beyond (Treat and Levy, 2018). Moreover, an alternative antifungal drug is recommended in case the first-line drug fails (Alkeswani et al., 2019).

Resistance to antifungals as a result of drug pressure, is becoming a bigger problem globally (Revie et al., 2018). Antimicrobial resistance increases the survival and spread of microorganisms through their ability to tolerate drug clearance effects (Nainu et al., 2021). Antimicrobial resistance has made it difficult to treat patients with fungal diseases hence prolonging the suffering and subsequently their death. Additionally, fungal drugs have serious side effects to human tissues when used for a long time (Gnat et al., 2020). Amphotericin B is an antifungal drug that kill fungal cells, but it causes nephrotoxicity in patients (Cavassin et al., 2021). There are few standard antifungal drugs, and fungi may develop resistance against all of them (Arastehfar et al.,2020). Therefore, to keep the war against fungal infections, the discovery of new and safer antifungals is urgently required (Nicola et al., 2019).

Natural products provide many opportunities for development of new antimicrobial drugs because of their wide matchless chemical diversities, hence they are gaining attention by researchers (Orchard et al., 2019). World Health Organization recommends medicinal plants as lead compounds for synthesis and semi synthesis of chemical drugs (Anand et al., 2019). Despite the frequent recommendations on the use of essential oil combinations against fungal infections, minimal research has been done on essential oil combinations against fungal infections (Cassella et al, 2002). Essential oils are widely used in management of superficial fungal infections (Abd Rashed et al., 2021; Orchard et al., 2019). Garlic essential oils (diallyl disulfide, allyl methyltrisulfide, trisulfide, di-2 propenyl, and daillyl sulfide), ginger (geranial, neral, ar-curcumene, camphene, zingiberene, and β-bisabolene) and lemon essential oils (eugenol, iso-eugenol, caryophyllene and furfural) have shown activity against other fungal strains (Moghaddam, M., & Mehdizadeh, 2016, Simonetti et al., 2018, Munda et al., 2018, Dehariya et al., 2021). However, the information on the antifungal activity of the three extracts essential oils against *Tinea capitis* remains scanty. This study isolated essential oils from garlic, ginger, and lemon peel extracts using steam distillation method. Formulated a lotion from the three extracts, and assessed the antifungal activity alongside the synergistic antimicrobial effects of the essential oils against *Tinea capitis.*

MATERIALS AND METHODS

Sample Collection, Identification of the Plant Samples and ethical statement

Study Samples (ginger, lemon and garlic) were randomly collected from Nakuru market in Nakuru County, Kenya. All the samples were taken to the pharmacognosy lab and authenticated by the school's botanist. The samples were stored at room temperature awaiting extraction. *Tinea capitis* isolates were obtained through the kind donation from Kenya Medical Research Institute (KEMRI). This study was approved by the Kabarak University Research Ethics Committee (KUREC-180322) and National Commission of Science, Technology and Innovation NACOSTI (NACOSTI/P/22/17467), respectively.

Preparation and Extraction of the Plant Extracts by Steam Distillation

Fresh samples of ginger, lemon peel and garlic were crushed separately using a knife. Each of the crushed material was transferred into a 2-liter round bottom flask and then 200 mL of distilled water was added to it and stirred to mix. The mixture was subjected to steam distillation for 2 hours as described (Mahindra et al., 2014). A mixture of essential oils and distilled water was obtained in the collecting flask for each of the three samples. The mixture was then separated using a separating funnel to obtain essential oils which were finally stored in a refrigerator at 4°C for further analyses.

Formulation of ginger, lemon and garlic lotion

About 30 mL of cocoa butter and 30 mL of coconut oil were mixed in the blender machine for two minutes. Thereafter, 2 mL of each of the three essential oils were added to the blender machine, followed by mixing for two minutes to form a lotion. The resulting lotion was tested for pH and viscosity. Additionally, the organoleptic properties of the lotion were also noted.

Culturing of Tinea isolates

About 6.5 grams of the Sabouraud agar power was suspended in 100 mL of distilled water. It was then heated with frequent agitation to dissolve the medium and finally sterilized through autoclaving at 121°C for 15 minutes. The media was then poured into the petri dish and allowed to solidify for some time. *Tinea* isolate was therefore inoculated in the medium using a sterile loop and was incubated at 25°C in an inverted position for one week for fungal growth.

Nutrient Broth Preparation

About 1.3 grams of nutrient broth powder was measured and added to the conical flask of 500 mL capacity. 100 mL of distilled water was added to the flask and placed on the stir plate to dissolve the powder. After sometime, the flask was removed from stir plate and its mouth was plugged with cotton wool and covered with Aluminum foil. The flask was then placed in the autoclave for sterilization at 121°C for 15 minutes.

Antimicrobial Susceptibility Testing and Inoculation

The colony of *Tinea capitis* was picked from cultured petri dish and was inoculated in the Sabouraud Dextrose Agar petri dish by spreading the colony in the petri dish evenly. A sterile filter circular disc was punched from Whatman filter paper and immersed in extracts concentration of 100%, 50%, 25% and 12.5% for twenty-four hours. The inoculated agar dish was divided into three, where discs with varying concentrations of extracts were placed. The petri dishes were placed upside down in an incubator for 24 hours at 25°C. The zones of inhibition diameter were measured using a Vernier caliper. The method above was also carried out for a combination of all the three essential oils. The synergism in activity of the three extracts was determined by the extent of diameter of inhibition.

Determination of Minimum Inhibitory Concentration of Combined Extracts

Nutrient broth was made and sterilized in an autoclave. A stock solution containing 100 mg/mL of the three combined extracts was also prepared. The three extracts were serially diluted as follows; 1 mL of broth was placed into three test tubes using a syringe. Thereafter, 2 mL of the stock solution was placed into the first tube. Subsequently, 1 mL of the solution was serially transferred from the first tube to the second one. This procedure was repeated for the third and fourth tube, 1 mL of the solution was discarded from the fourth tube. *Tinea capitis* isolates were prepared in sterile nutrient broth as follows; about 1 mL of the nutrient broth was added to *Tinea capitis* inoculum then transferred into each of the four tubes. The final concentration of the combined extract in each of the four test tubes was 100, 50, 25, 12.5 mg/mL, respectively. The tubes were incubated at 25°C for 24 hrs and checked for growth.

Data analysis

Susceptibility data was expressed as median inhibition with interquartile range (IQR) and proportions. Differences in inhibition between extracts were compared using the Kruskal-Wallis (H-test). Proportions were examined using the Chi-square test (χ^2) and Fisher exact test. All statistical tests were carried out in GraphPad version 8.0 (GraphPad Software, Inc., San Diego, CA, USA). Two-sided P value < 0.05 was considered statistically significant.

RESULTS AND DISCUSSION

The yield of the essential oils obtained from the three plant extracts was calculated as follows:

Yield of extract

$$Percentage\ Yield = \frac{volume\ of\ essential\ oil\ (V1)}{weight\ of\ the\ plant\ extract\ (W1)} \times 100$$

Ginger essential oil yield was found to be 22% while lemon essential yield was 20% and lastly garlic essential oil yield was found to be 16%. This yield was higher compared to what was reported in earlier studies using the same method of extraction (Bar et al., 2022, Khan et al., 2017, Fitriady et al., 2017, Sikdar et al., 2017). The difference in essential oil yield can be attributed to different temperature, time, pressure, size of sample used, and territorial origin of the samples as argued previously by Bar and colleagues (Bar et al., 2022).

Table 1:

Mean of inhibition zones of different concentration of various extracts, their combinations relative to controls against Tinea capitis isolates in mm

Extract/ Control	100%	50%	25%	12.5%	2%	99%
Lemon	7.20	6.76	6.42	6.08		
Garlic	8.18	7.78	6.72	6.38		
Ginger	11.15	10.78	7.26	6.59		
Combined Extract	12.90	11.05	7.97	6.83		
Positive control (Ketoconazole)					11.21	
Negative control (DMSO)						5.58

This study screened the antifungal activity of ginger, garlic and lemon peel essential oil against *Tinea capitis* isolates. From the findings, all the three extracts demonstrated different antifungal activities against *Tinea capitis*. Ginger showed the greatest antifungal activity against *Tinea capitis* isolates, followed by garlic and lastly lemon essential oils respectively as shown in (Tables 1), although this was not statistically significant (*P* > 0.05) meaning there was no difference in activity of the three extracts. The median (interquartile range) of the antifungal activity of the three extracts essential oils were as follows; 9.02 (6.76-11.06mm), 7.1 (6.47-8.01mm) and 6.59 (6.17-7.09mm), respectively for ginger, garlic and lemon.

These results suggest that *Tinea capitis* was sensitive to the three plant extracts namely; garlic, ginger and lemon peel and can therefore be used as a remedy for *Tinea capitis* infection. These results are consistent with what Costa et al., and Delgado *et al* reported, where lemon peel essential oils had antifungal activity against *Candida albicans* strain (Costa et al., 2014; Delgado *et al*., 2020). Garlic essential oils has demonstrated good antifungal activity against dermatophytes as ketoconazole in earlier studies (Gebreselema & Mebrahtu, 2013; Mahboubi & Kazempour, 2015), this further supports our findings. Ginger showed the greatest antifungal activity as compared to the other two extracts. This is consistent with what Macwan *et a*l., reported (Macwan *et al*., 2016; Nguyen et al., 2022).

The combined essential oils inhibited *Tinea capitis* growth with zones of inhibition ranging from 6.83 to 12.90 mm. It was shown that the diameters of zone of inhibition produced by the three extracts combined was larger than those produced by the individual extracts at same concentrations (Table 1). However, the variation in activity for the single and combined extracts when compared using Kruskal Wallis test, was not statistically significant ($P > 0.05$). The results were comparable with results of standard drug, ketoconazole which was used as the positive control (Table 1). The diameters of zone of inhibition produced by combined extract against *Tinea capitis* exhibited larger inhibition zones than those produced by the individual extracts at same concentration, this suggests a synergistic effect against *Tinea capitis* of the three extracts.

Ketoconazole (positive control) inhibited *Tinea capitis* growth with a zone of 11.21 mm. The diameter of zone of inhibition produced by ketoconazole against *Tinea capitis* exhibited larger inhibition than those of individual extracts (Table 1). The combined extract at a concentration of 100% showed a comparable zone of inhibition as ketoconazole (reference antifungal drug) (Table 1). DMSO solvent (Negative control) showed no antifungal activity against *Tinea capitis.* This depicts that the solvent did not contribute to or affect the antifungal activity of the extracts.

The combined extract of the concentration of 100% showed a comparable zone of inhibition as the reference antifungal drug ketoconazole which is the positive control (Macwan *et al*., 2016; Nguyen et al., 2022). This suggests that a combination of the three extracts can be used for treatment of *Tinea capitis*, and this will help to alleviate the burden of antifungal resistance which is on the rise. The findings of this study showed that the combined extracts of garlic, ginger and lemon essential oils have antifungal activity and hence a potential reservoir of new drug against fungal infections. There was no statistically significant variation in the activity of the herbal extracts and ketoconazole ($P > 0.05$) and hence the formulated extracts of these essential oils can be used as an alternative to ketoconazole drug in treatment of *Tinea capitis.*

The extent of the zone of inhibition of *Tinea capitis* isolates growth was directly proportional to the concentration of the extract essential oils and the combination of the three extracts. The concentration of 100% had the largest zone of inhibition compared to 50%, 25% and 12.5% concentrations (Table 1). This is similar to the previous findings of (Mercy et al., 2014) who demonstrated that the zone of inhibition increased with increase in concentration of the extract.

Minimum Inhibitory Concentration

The minimum inhibitory concentration (MIC) of a combination of the three extracts was established. The MIC was found to be 6.25 mg/mL. The minimum inhibitory concentration was determined for combined extracts only because of the highest zone of inhibition exhibited by them at various concentrations as compared to the individual extracts. The minimum inhibitory concentration of the combined extracts that was found to fully inhibit any visible microorganism growth after 24 hours was 6.25%. These results are in agreement with what Macwan *et al*., and Nguyen et al., reported earlier (Macwan *et al*., 2016; Nguyen et al., 2022). This suggests that the method of MIC determination was accurate and it can be adapted in our laboratories.

The antifungal activity of ginger, garlic and lemon peel extracts could be attributed to essential oils. According to Layman aromatherapeutic literature, essential oils are widely used in management of superficial fungal infections with about 38% to 40% of volatile oils used in dermatology (Orchard et al., 2019). This further supports the use of garlic, lemon and ginger lotion as a therapy against dermatophytes.

Lotion

In this study we successfully formulated a lotion and the essential oils were mixed in the ratio of 2:2:2. The concentration of the essential oils in lotion was calculated and found to be 9.09 mg/mL which is above the MIC of the combined extracts. The formulated lotion depicted the following properties; It showed a slightly acidic pH of 5.77 which was between the normal range of a skin preparation (4.5 to 7). The lotion was yellow in color, it had a smooth texture, good pourability and a garlic odor. The viscosity of the lotion was found to be 93.3 m. Pas. The formulated lotion was found to have good physical and organoleptic properties. It depicted a good pH, texture, pourability as well as spreadability as recommended by Gyawali et al., 2016. This implies that a lotion can be successfully formulated from the three extracts. However, the viscosity of the lotion was found to be low as compared to the average normal range of 424.40 mPa-s. In order to make the viscosity of the lotion normal, it is recommended that glycerol should be added to it (Mast, 2018).

CONCLUSION AND RECOMMENDATIONS

In conclusion, our study findings showed that the extract of garlic, ginger and lemon essential oil have good antifungal activity against *Tinea capitis* isolates. A combination of the three extracts act in synergy against the isolates. The herbal extracts inhibited *Tinea capitis* isolates as good as ketoconazole and hence these extracts are a promising source of drugs for treatment of *Tinea capitis*. However, it is further recommended that more studies of this kind should be done using other synthetic antifungal drugs in the market other than ketoconazole. The essential oil of the three extracts may also contain specific active components that may enhance effective antifungal activities. Thus, isolation of compounds from these oils is important for further drug discovery. We also recommended that laboratory and clinical studies of these essential oils should be performed in order to better understand its antifungal activity and toxicity. Lastly, the formulated lotion of the three extracts should be tested against other fungal infections.

Consent to publish

Consent to publish was obtained from KEMRI and Kabarak University.

Abbreviations

MIC: minimum inhibitory concentration

NACOSTI: National Commision for Science, Technology and Innovation

KUREC: Kabarak University Research Ethics Committee

DMSO: Dimethyl Sulfoxide

Competing interests

The authors declare that there are no competing interests.

Authors' Contributions: A. W. K; conceptualization of the project, data curation, formal analysis, preparation of the original draft, visualization D.M.W; conceptualization of the project, visualization, preparation of the original draft, data interpretation, writing-review and editing, M.L.K; conceptualization of the project, methodology, preparation of the original draft, supervision, data interpretation J.J.O; visualization, preparation of the original draft, supervision, data interpretation. All authors have read and approved the final manuscript.

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