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ORIGINAL ARTICLE

Evaluation of the Methanol Extract and Fractions of the Aerial Parts of *Emilia coccinea* Against Selected Fungal Isolates

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ABSTRACT

Background: *Emilia coccinea* (EC) commonly known as scarlet tassel flower and a fast-growing annual herb is used ethno-medicinally for treating different types of fungal infections. Selected species of *Candida, Aspergillus, Microsporum, Rhodotorula* and *Trichophyton* genera are known pathogenic fungi that could cause skin and life-threatening infections, especially in immune-compromised patients.

Objective: This study aims to evaluate the antifungal property of the aerial parts of EC extract and fractions against selected clinical isolate of fungi.

Methodology: The aerial parts of the plant were pulverized, extracted with methanol and portioned into n-hexane, dichloromethane, ethylacetate and aqueous fractions. Modified agar well diffusion method was used to evaluate the antifungal potential of the methanol extracts and fractions. This activity was achieved by increasing the concentration of the methanol extract and fractions from 40 mg/ml to 100 mg/ml. The selected isolates are *Candida albicans, Aspergillus fumigatus, Microsporum audounii, Rhodotorula glutinis* and *Trichophyton rubrum*.

Results: *Trichophyton rubrum* and *Rhodotorula glutinis* showed susceptibility to the methanol extract of EC at 40 mg/ml for the former and between 60 mg/ml to 100 mg/ml for the later. *Candida albicans* displayed susceptibility to the n-hexane, ethylacetate and dichloromethane fractions, *Trichophyton rubrum* further showed susceptibility to the aqueous fraction. *Microsporum audounii* susceptibility was observed in the dichloromethane fraction at 100 mg/ml. The lowest MIC value was observed with the dichloromethane fraction for the different clinical isolate study.

Conclusion: The methanol extract, n-hexane, dichloromethane and aqueous fractions of EC possessed antifungal activity against *Candida albicans, Microsporum audounii* and *Trichophyton rubrum*. However, the dichloromethane fraction was found more effective against *Candida albicans*.

Keywords
Antifungal Activity, Aspergillus Fumigatus, Aandida Albicans, Emilia Coccinea, Microsporum Audounii, Rhodotorula Glutinis, Trichophyton Rubrum, Zones of Inhibition.

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Article info.
Received: October 30, 2022
Accepted: April 03, 2023

Cite this article: *Odion EE, Onwubiko CN, Junior AM, Ugwu FC, Dowe E. Evaluation of the Methanol Extract and Fractions of the Aerial Parts of Emilia coccinea Against Selected Fungal Isolates. RADS J Biol Res Appl Sci. 2023; 14(1):29-38.*

INTRODUCTION

Over a million species of fungi have been estimated to exist, they include the yeasts, rusts, smuts, mildews, molds and mushrooms[1,2]. Selected species from *Candida, Aspergillus, Microsporum, Rhodotorula* and *Trichophyton* are known pathogens of human[3]. *Aspergillus fumigatus* is one of the utmost ubiquitous saprophytic fungus that plays an important function in the recycling of carbon and nitrogen, it is also known to cause illness called aspergillosis[4,5]. *Microsporum* and *Trichophyton* species are involved in *Tinea capitis* infecting the skin, normally seen in children. It presents with hair loss, often with scale, patchy areas of broken hairs covered by white scales resembling seborrheic dermatitis[6]. *Rhodotorula glutinis* is an opportunistic ubiquitous saprophytic yeast, less frequently isolated from natural environments and some of the localized infections. It causes, meningeal, skin, ocular, peritoneal, and prosthetic joint infections[7].

Emilia coccinea (EC) commonly known as scarlet tassel flower belongs to the family of the sun flower (Asteraceae). It is a fast-growing annual herb that colonizes fallow land and cultivated farms[8]. It thrives well in properly drained soil with slight alkaline to neutral pH and an environmental temperature range of 15-22°C[9]. It is native to Africa but has been found to naturalize in North and South America, Asia and some Pacific Islands[10]. Ethno-medicinally, it is used for treating different types of illness, which include fungal infections[11].

Plant extracts have been evaluated for different pharmacological activities with resounding success[12]. These successes could be attributed to the presence of the phytochemicals with different chemical structure. They include alkaloid, phenol, tannins, triterpenoids, glycosides and flavonoids[13]. Some of these phytochemicals have been associated with antimicrobial properties-antibacterial and antifungal[14]. The effect of these phytochemicals against microbial pathogens will depend on their ability to inhibit growth or cause microbial cell death.

Antifungal agents are classified based on their site of activity into three namely: Azoles, Polyenes and 5-fluorocytosine. Azoles inhibit C14α demethylation of lanosterol in fungi, and thus alter the synthesis of ergosterol in the fungal cells[15]. Polyenes interact physically and chemically with the sterols in the fungal membrane, resulting in the production of aqueous pores on the cell walls of the fungi. While 5-fluorocytosine inhibit the synthesis of the fungal macromolecule such as 5-fluorodeoxyuridinemonophosphate, thus preventing DNA synthesis[16]. However, resistance to these agents have been reported as a result of changes in drug target and sterols biosynthesis, reduction in intercellular concentration of target enzymes and over-expression of antifungal drug target[17]. These observed resistance to already existing drugs, increased incidence of fungal infection and unbearable side effects associated with some of these synthetic medicines. Thus, there is need to search for novel antifungal agents from plants which is an under explored drug source. Apart from the unexplored nature of many plant, it is also belief that herbs do not have side effects and they are ready available. A thorough search of the literature, show lack of information on the antifungal potential of EC against *Aspergillus fumigatus, Trichophyton rubrum, Candida albicans, Microsporum audounii* and *Rhodotorula glutinis.* Thus, this study aims to evaluate the antifungal potential of the methanol extract of EC and its fractions by using modified agar well diffusion method. And also determination of the minimum inhibitory concentration (MIC) of both the methanol extarct and fractions.

MATERIALS AND METHODS

Plant Collection, Extraction and Fractionation

The whole plant was collected within the vicinity of the University of Benin, Ugbowo Campus, in the month of August and identified as EC by Dr. Akinnibosun H.A. of the Department of Plant Biology and Biotechnology, University of Benin. Voucher specimen number (UBH-E363) was issued and the plant specimen was deposited in the herbarium.

Aerial part of the plants were air-dried under shade following the detachment of the root by the use of a stainless-steel knife. This was done away from direct sunlight for three weeks and then transferred into an air-drier oven for 12 hours before been pulverized in an electrical milling machine into fine powder.

Six hundred grams of the powdered plant was then macerated with 2.5 L of methanol (99 %) with intermittent shaking every 30 minutes for 2 hours and allowed to stand for three days (72 hours) after which the solvent was decanted and passed through filter paper (size 1). The filtrate was then concentrated using rotary evaporator at 60°C and the concentrated extract obtained were stored in a refrigerator at a temperature of 4°C until used for activity.

Fifty grams of the crude (methanol) extract was dissolved in 200 ml of methanol and 50 ml of distilled water was then added to the mixture and stirred properly. This was then poured into a separating funnel before the addition of 200 ml of n-hexane, the mixture was swirled and then allowed to stand for 30 minutes for proper separation of the polar solvent from the non-polar solvent. The n-hexane fraction was decanted and collected in a beaker. This process was repeated until a clear solution was obtained for the n-hexane fraction. All the n-hexane fractions were bulk together and concentrated using rotary evaporator at 60°C and semi-solid concentrate obtained was stored in a refrigerator at a temperature of 4°C. The same procedure was repeated for dichloromethane and ethylacetate fractionation. The residue from the separating funnel was also concentrated and the weight noted (aqueous fraction).

Source of Test Microorganisms

The microbial cultures used were obtained from stock cultures of clinical isolates cases of *Tinea capitis* (ringworm of the scalp) maintained in Sabouraud Dextrose Agar (SDA) slant from State University Teaching Hospital in Oghara, Delta State, Nigeria. The reference fungal cultures include *Aspergillus fumigatus, Trichophyton rubrum, Candida albicans, Microsporum audounii* and *Rhodotorula glutinis.*

Antifungal Assay of the Extract and Fractions

Modified agar well diffusion method described by Cheesbrough,[18] and Dowe and co-workers[19] was used to determine the antifungal susceptibility test of the methanol extract and fractions (n-hexane and dichloromethane, ethylacetate and aqueous) of EC. Wells (6 mm diameter) were made into plates of previously seeded Potato Dextrose Agar (PDA) utilizing sterile cork borer. Before seeding, isolated colonies/spores stored in Sabouraud Dextrose Agar slants were sub-cultured. These were vigorously shaken and diluted (1:100) to achieve 0.5 MacFarland turbidity standard (approximately 10^6 spores/mL). Sterile swab sticks were dipped into the microbial suspension and gently spread over (seeding) the surface of the agar plates in even strokes to obtain a uniform growth pattern across the entire surface of the plate. This was achieved by rotating the plate 90 degrees followed by 45 degrees with continuous streaking, and finally by streaking round the diameter of the agar. The 6 mm wells were filled with equal volumes (100 µL) of the crude extract and fractions corresponding to 100, 80, 60 and 40 mg/mL concentrations. The same quantity of 10% Tween-80 and 10 µg/mL of Ketoconazole served as negative and positive controls respectively. The plates were incubated at ambient temperature (27±2˚C) for 72 hours in an upright position to allow proper diffusion of extracts and the experiments were done in triplicates. After incubation, the absence or presence of growth was observed on the plates and the diameter of clear zones were measured using a millimetre (mm) calibrated ruler and the mean Inhibition Zones Diameters (IZDs) calculated and recorded.

Minimum Inhibitory Concentration Determination of the Extract and Fractions

Agar dilution technique was used to decide the MICs of the leaf extracts and fractions of MC. Stock concentrations of 200 mg/ml of extract and fractions were prepared and two-fold serial dilutions were made to get concentrations of extract and fraction solutions ranging from 10.00 mg/ml to 36.00 mg/ml. Isolates of the fungal that grew on the plates were diluted to 10^6 CFU/ml and 0.025 ml volume was placed on marked areas that contained different concentrations of the extract tested. Incubation of the plates was at a temperature of 25°C for a period of 48 hours for species of Candida and a period of 72 hours for the isolates of the other fungal. The lowest concentration at which there was no noticeable fungal growth was recorded as the MICs[20].

Data Presentation and Statistical Analysis

The experimental data were expressed as Mean± Standard Deviation (SD). Student's t-test was used in evaluating the statistical difference between two groups with $p<0.05$ considered statistically significant. Statistical analysis was performed using Statistical Package for Social Science software version 2010.

RESULTS

The weight of the crude (methanol) extract and fractions are provided in Table **1**. Following extraction, 170.98 g of methanol extract was obtained, from which 50 g of the crude was partitioned into non polar solvent (n-hexane), semi-polar solvent (dichloromethane) and polar solvent (ethylacetate). N-hexane fraction (21.55 g) was more in quantity of all the fractions while dichloromethane fractions (3.46 g) was less in quantity. This implies that EC is rich in non-polar compounds such as sterols, this partitioned into the n-hexane fraction. Semi-polar fraction-dichloromethane, though the yield may be poor but could contain important phytochemical such as alkaloid.

The susceptibility test determination through the measurement of the zones of inhibition produced by methanol extract of EC as result of the challenges against the fungi, is highlighted in Figure **1**. *Candida albicans, Aspergillus fumigatus, Trichophyton rubrum, Rhodotorula glutinis,* and *Microsporum audounii* show susceptility to the methanol extract. The sensitivity vary as the concentration of the methanol extract changes. At 40 mg/ml *Trichophyton rubrum* showed the highest sensitivity with Zones of Inhibition- 9.35±0.18 mm. As the concentration of methanol extract of EC increases from 60 to 100 mg/ml, *Rhodotorula glutinis* display increasing sensitivity (Zones of Inhibition at 60 mg/ml: 15±0.35 mm, 80 mg/ml: 18±2.12 mm and 100 mg/ml: 21.25±0.78 mm). These increased sensitivity in *Rhodotorula glutinis* was significance with P= 0.0022 when the difference in zones of inhibition produced by the methanol extract was compared between 60 to 100 mg/ml. Though when compared with the positive control (ketoconazole), the observed zones of inhibition were lowered. At 40 mg/ml, 60 mg/ml and 100 mg/ml of the methanol extract, *Microsporum audounii* showed the least sensitivity of 4.00±0.71 mm, 8.75±0.18 mm and 13.50±0.35 mm respectively, while *Aspergillus fumigatus* (12.50±0.35 mm) was least sensitive at 80 mg/ml. *Trichophyton rubrum* showed zones of inhibition of 0.35+0.18 mm at a concentration of 40 mg/ml.

Table 1. Weight of the methanol extract and fractions of EC.

Extract/Fractions	Weight (g)
Methanol	170.98
n-hexane	21.55
Ethylacetate	10.67
Aqueous	10.19
Dichloromethane	3.46

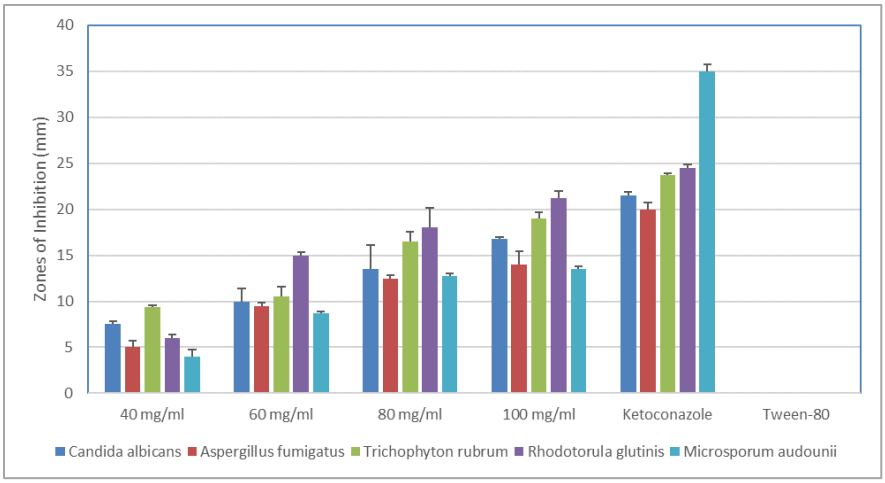

Figure 1. Zones of inhibition in mm of varying concentrations of the methanol extract of EC.

Zones of inhibition of the clinical isolates at different concentrations of n-hexane fraction was provided in Figure **2**. As concentration of the n-hexane fraction increased from 40 mg/ml to 100 mg/ml, the zones of inhibition also increased in *Candida albicans*, *Trichophyton rubrum* and *Rhodotorula glutinis.* However, the n-hexane fraction produced no effect against *Aspergillus fumigatus* and *Microsporum audounii*, indicating resistance to this fraction. An average change of 4.00 mm in zones of inhibition was noticed as the concentration vary from 40 to 100 mg/ml in *Candida albicans* and *Trichophyton rubrum*, while less than 2.00 mm change in zones of inhibition for *Rhodotorula glutinis* was recorded.

Figure **3** showed the zones of inhibition at different concentrations of dichloromethane fraction of EC. At 40 mg/ml *Rhodotorula glutinis* showed the lowest Zones of inhibition of 7.00±0.35 mm, while at concentrations of 60 mg/ml, 80 mg/ml and 100 mg/ml, *Aspergillus fumigatus* showed the least Zones of inhibition of 9.00±1.41 mm, 9.75±0.18 mm and 13.00±1.41 mm respectively. *Candida albicans* was most sensitive to dichloromethane extract of *EC* at 40 mg/ml, 60 mg/ml and 80 mg/ml with Zones of inhibition measuring 15.75±1.24 mm, 19.25±0.18 mm and 19.75±0.18 mm respectively. Between 80 mg/mL and 100 mg/mL, the Zones of inhibition decrease from 19.75±0.18 mm to 19.50±1.06 mm, this decrease could be that the fraction may have saturated the site of its action. At 100 mg/mL, *Microsporum audounii* showed the highest sensitive of 21.00±0.71 mm.

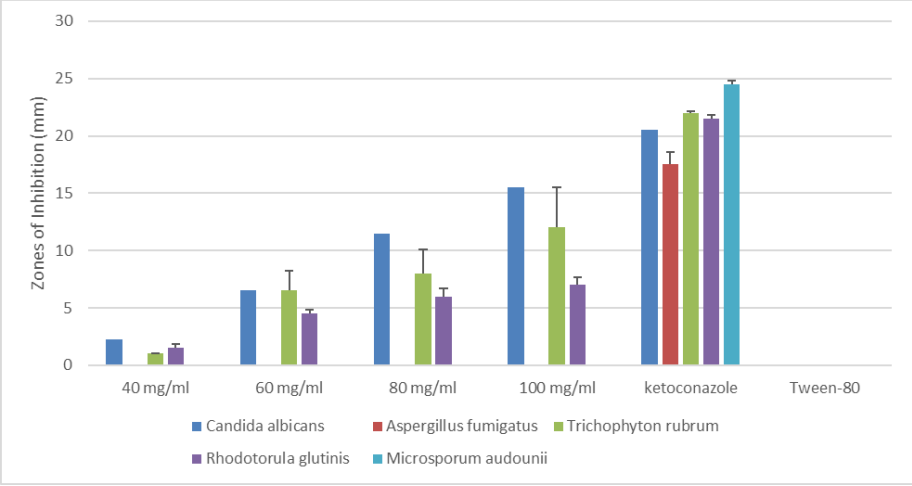

Figure 2. Zones of inhibition in mm of varying concentrations of the n-hexane fraction of EC.

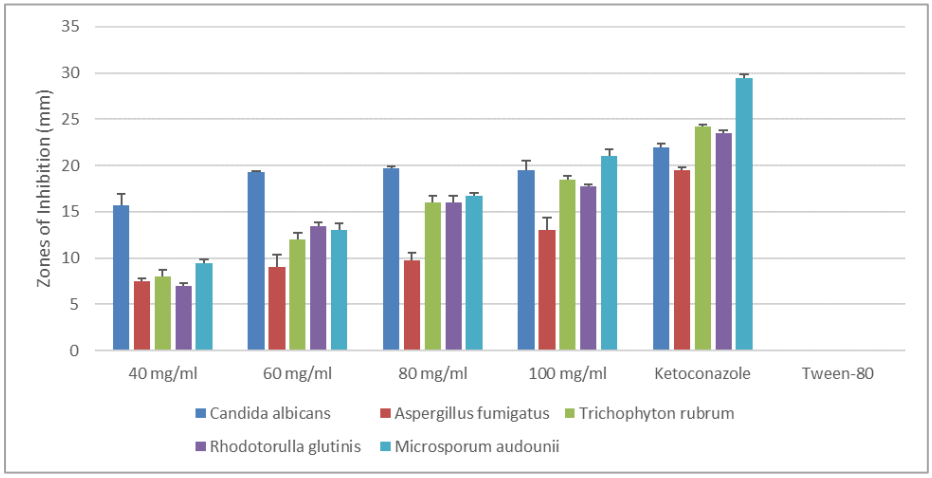

Figure 3. Zones of inhibition in mm of different concentrations of dichloromethane fraction of EC.

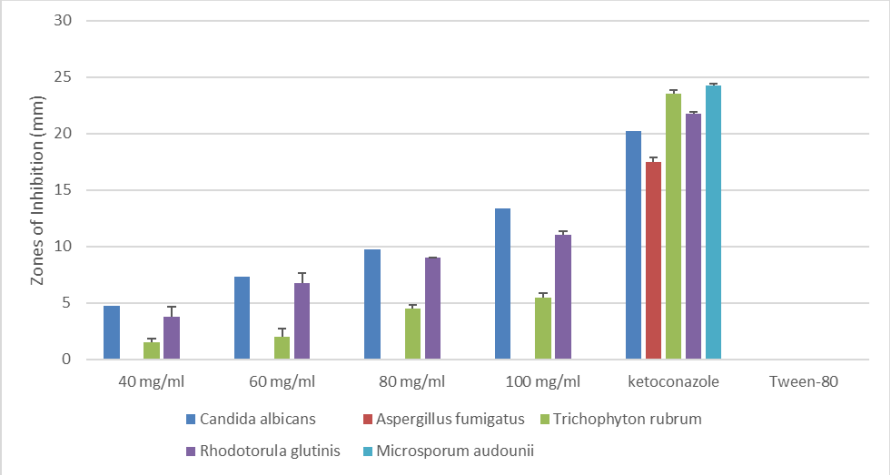

Figure 4. Zones of inhibition in mm of different concentrations of the ethylacetate fraction of EC.

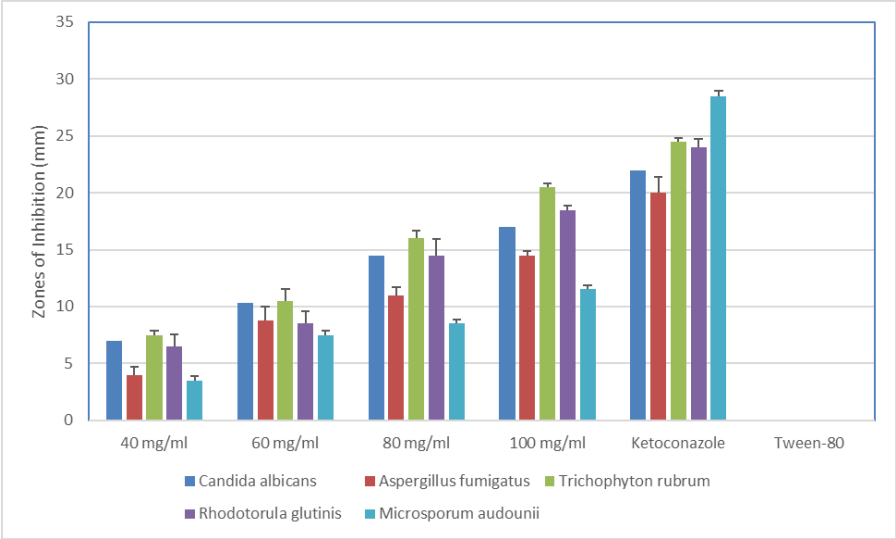

Figure 5. Zones of inhibition in mm of different concentrations of the aqueous extract of EC.

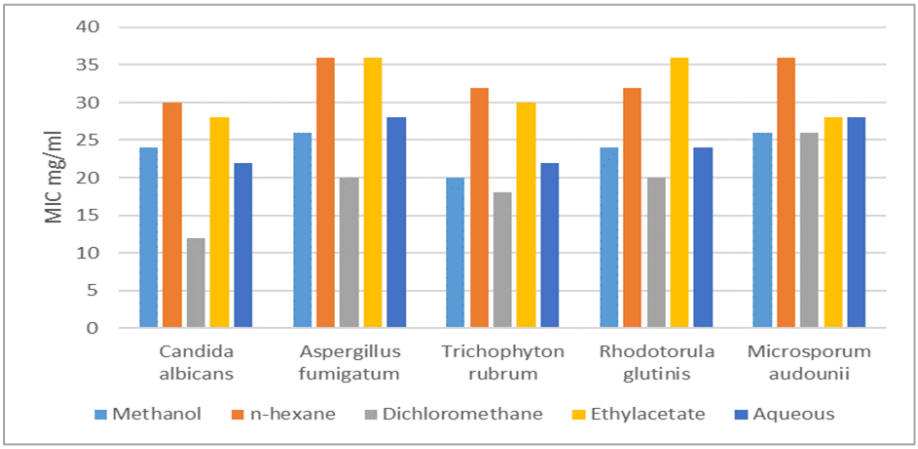

Figure 6. Minimum inhibitory concentrations (MIC) (mgml^{-1}) of methanol extract and fractions of EC against *Aspergillus fumigatus, Trichophyton rubrum, Candida albicans, Microsporum audounii* and *Rhodotorula glutinis.*

Zones of inhibition of the microbial isolates by ethylacetate fraction of EC is highlighted in Figure **4**. *Aspergillus fumigatus* and *Microsporum audounii* showed resistance, while *Candida albicans* was the most sensitive at the different test concentrations with zones of inhibition measuring 4.75±0.37 mm, 7.35±0.37 mm, 9.75±0.18 mm and 13.35±0.37 mm respectively. These zones of inhibition were significantly different (P<0.5) with an approximate measurement of 3.00 mm. *Trichophyton rubrum* was the least sensitive among the isolates that showed sensitivity, with the zones of inhibition measuring 1.50±0.35 mm, 2.00±0.71 mm, 4.50±0.35 mm and 5.50±0.35 mm respectively as the concentration increases from 40 mg/ml to 100 mg/ml.

Candida albicans, *Aspergillus fumigatus*, *Trichophyton rubrum*, *Rhodotorula glutinis* and *Microsporum audounii* showed sensitivity at different concentrations of the aqueous extract of EC (Figure **5**). However *Trichophyton rubrum* showed the highest susceptibility to this extract as the concentrations varies, while *Microsporum audounii* was least susceptibility to the extract at 40 mg/ml to 100 mg/ml.

Perusing through Figure **6**, it is observed that methanol extract and the different fractions display varying MIC values. However dichloromethane extract display the lowest MIC value against the different clinical isolates evaluated. Against *Candida albicans*, it show an MIC of 12 mg/ml, against *Trichophyton rubrum*-16 mg/ml, against *Aspergillus fumigatus*-20 mg/ml, against *Rhodotorula glutinis*-20 mg/ml in and against *Microsporum audounii*-26 mg/ml . These implies that 12 mg/ml of dichloromethane extract of EC prevents the growth of *Candida albicans*. Showing that dichloromethane extract is more effective against *Candida albican*s. By implication dichloromethane fraction of EC could be more effective in the treatment of *Candida albican* related infection.

Ethylacetate and n-hexane fractions show high MIC values above 35 mg/ml against *Aspergillus fumigatum, Rhodotorula glutinis and Microsporum audounii.* Implying that over 35 mg/ml of these extract and fractions are required to prevent the growth of *Aspergillus fumigatum, Rhodotorula glutinis and Microsporum audounii*. Implying that at this concentration (35 mg/ml), these clinical isolate will be resistant to the action of the methanol extract and fraction.

DISCUSSION

Methanol is a general-purpose solvent used in extraction with subsequent evaluation of its pharmacological action of the extract after its evaporation. The molecule has both the polar hydroxyl part and the non-polar methyl part. The combination of these two parts in the molecule enable it to extract both polar (hydrophilic) and non-polar (hydrophobic) components in a sample. Although the presence of the hydroxyl group in the molecule have made the molecule polar due to the ability of the hydroxyl group to form inter molecular hydrogen bonding with the molecules of the extracts.

Water and gin (ethanol) are mostly used by traditional medicine practitioners in the preparation of herbal decoction due to their extractive potential. Methanol which belongs to the same homologous series with ethanol is use in most extraction to simulate how the plant parts are been extracted in folkloric medicine. Decoction from EC obtained from water extraction or soaked in palm wine over time tend to simulate the scientific use of these solvents (methanol, ethanol) and pulverization of the plant parts enhances the proper coming together of the solvent and pulverised plant material. The extractive method used in this study was cold maceration, it involves soaking of the plant material overtime with initial agitation. This extractive method has the advantage of keeping intact the volatile components of the plant, since heat was not involved, though the process may not be exhaustive in extracting the compounds present in the plant powder.

The yield obtained from the methanol extract (28.45%) showed EC to contain high organic compounds. The natures of these organic compounds will determine what part of the solvent (n-hexane-non-polar, dichloromethane and ethylacetate -polar) it will dissolve in. The nature of the n-hexane fraction is mostly hydrophobic; in fact, some authors may refer the process of using n-hexane as defatting process. The yield obtained with n-hexane fraction (43.10%) was high when compared to other fractions, implying high level of fatty or straight chain compounds may be present in the fraction. Dichloromethane fraction (6.92%) was low in yield, signifying low semi-polar compounds (alkaloids). Ethylacetate and aqueous fractions gave average yield of about 20.00%, implying moderate yield of polar

compounds (phenols, flavonoids) in EC. Addition of these percentage yields could result in moderate yield similar to that obtained with n-hexane. Thus, if the yields were to be broadly grouped into non-polar to polar fractions, the ratio will approximately be 2 to 1.

The sensitivity and resistance of clinical isolates against extract from plants are evaluated by measuring Zones of inhibition, after culturing the microbes in the presence of the extract on agar over 24 hours. Results (Figure **1-5**) showed activity for extract and fractions from EC against selected fungi isolates tested but to different extent. Dichloromethane fraction and methanol extracts showed high activities against clinical isolates. Though the activity observed by ketoconazole (positive control) were slightly higher than dichloromethane fraction but was not statistically significant. Previous studies have shown activity of methanol and aqueous leaf extract against *Candida albicans* and *Asperigillus niger.* In that work potato dextrose agar was used as the medium of culture[21]. In another study, *Candidia albicans* was observed to be susceptible to ethylacetate fraction of EC at a concentration of 125 mg/ml[22]. This research implying that EC may contain phytochemicals with activity against these fungi isolates, thus could be used to treat infections caused by these microbes.

The dichloromethane extract recorded antifungal activity that was higher than that of the aqueous extract at a concentration of 100 mg/mL ($p < 0.05$). This could be due to the presence of more active constituents in the dichloromethane extract. Results of the inhibition zone diameters were all concentration dependent as the inhibition zones produced varied with the extract concentration used. This study supports previous findings that an increase in Zones of inhibition can be linked to increased extract or fraction concentration[23]. The controls used in this work acted as guide to check the sensitivity or resistance of the isolates. Ketoconazole used as positive control in this study recorded showed Zones of inhibition to different extent for the extract or fraction, while tween-80 which was used as negative control, showed no inhibition. Implying that ketoconazole is a broad-spectrum antifungal agent since it is effective against these fungal isolates.

Antifungal susceptibility testing against pathogenic fungi is an important information for when determining the proper antifungal drug and measuring resistant strains. The MIC is the lowest concentration of drug showing no visible growth, while MFC is the lowest concentration of drug which reduced the colony forming units by a definite value[24]. Although both the MIC and MFC are well known parameters of antifungal susceptibility, the MIC is by far the more highly referred in clinical settings. In fact, while methods for determining the MIC have been standardized in developed countries to ensure accuracy and reproducibility of data, by contrast, scant attention has been paid to standardizing methods for determining the MFC[25].

The results of the MICs (Minimum Inhibitory Concentrations) of the extract and fractions correlate well with those shown by the inhibition zone diameters. The effectiveness of an agent with activity against microorganisms is quantitatively measured making use of MIC [26]. Potent antimicrobial agents are known to have lower MIC values and as shown in this study, the dichloromethane fraction recorded lower MIC values against the fungal isolates tested. The lowest MIC values was recorded against *Candida albicans* and *Microsporum audounii* implying that the isolates were the most susceptible of all the isolates tested.

The fungal in this study are known as natural human microbiome, that can live harmlessly with human for a lifetime[24]. However under certain conditions such as low immunity, they begin to infect of the skin, nails and hairs. However, improper and prompted treatment is not seek, they could cause life-threaten systemic conditions. Thus, the sensitivity of these fungi to EC extract and fractions, verify the ethno-medicinal claim of the use of EC.

CONCLUSION

This study was able to show that the methanol extract of the aerial parts EC possess antifungal potential against the studied microbes. The fractions from the methanol extract also showed varying antifungal potential, prominent among which are the activity exhibited by n-hexane fraction against *Rhodotorula glutinis*. Dichloromethane fraction against *Candida albicans* and *Microsporum audounii* at high concentration (100 mg/ml). Ethylacetate fraction against *Candida albicans* and aqueous fraction against *Trichophyton rubrum.* The minimum inhibitory concentration were lowest with Candida albican for both the extract and fractions. This study also have showed high

proportion of non-polar constituents in the n-hexane fraction which may be responsible for the activity investigated. The species of fungi used in this study are encountered on a regular basis and activity against them could immensely reduce incidence of fungal infection. There is need for the particular phytochemicals responsible for this activity to be identified through isolation and characterization in further studies.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

FUNDING SOURCE

None

ACKNOWLEDGEMENT

Special thanks to the laboratory staff of the Department of Pharmaceutical Microbiology for providing the needed assistance to enable this research to have taken place.

LIST OF ABBREVIATION

None

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