

Antifungal studies of methanol leaf extract and fractions of *Tapinanthus globiferus***(A. Rich) Tiegh****Abstract**

Fungal infections are the major cause of many skin diseases especially in developing countries. Medicinal plants represents a rich source of antimicrobial agents. *Tapinanthus globiferus* (Loranthaceae) has been extensively used in ethnomedicine to treat hypertension, ulcer, diabetes, cancer and fungal infections without scientific basis. This work was aimed at screening the phytochemical constituents and evaluating the antifungal activity of methanol leaf extract and its fractions (ethylacetate and *n*-butanol) of *T. globiferus* against some clinical fungal isolates including *Candida albicans*, *Trychophyton mentagrophytes*, *Trychophyton rubrum* and *Aspergillus niger* using agar well diffusion and broth micro-dilution methods. Preliminary phytochemical screening of the extract and fractions revealed the presence of carbohydrates, alkaloids, flavonoids, tannins, saponins, glycosides, steroids and triterpenes. The extract and its fractions exhibited good antifungal activity against all the test organisms with mean zone of inhibition range of $27.83 \pm 0.16 - 14.46 \pm 0.29$ mm which was higher compared to that of the standard drug, Fluconazole ($26.1 \pm 0.44 - 18.49 \pm 0.16$ mm). The minimum inhibitory concentration (MIC) and minimum fungicidal concentration (MFC) of the methanol extract ranged between 6.25 – 25.0 mg/mL; ethylacetate fraction had 3.13 – 25.0 mg/mL while *n*-butanol fraction had the least MIC ranged between 0.39 – 12.5 mg/mL against the test organisms. *T. globiferus* have demonstrated good antifungal activity validating the ethnomedicinal claim of the use of the plant in the treatment of fungal infections.

Keywords: Antifungal; Phytochemical screening; *Tapinanthus globiferus*

Introduction

Fungal infection and their complications continue to remain a burden on the health system of many nations and have claimed the lives of many people especially in Africa with the most frequent fungal pathogens being *Candida*, *Aspergillus*, *Pneumocystis* and *Cryptococcus* spp. It is estimated that these fungal species cause at least 1.4 million deaths worldwide per year (Brown *et al.*, 2012). Skin diseases are associated with mortality rates of 20,000 in Sub-Saharan Africa in 2001 (WHO, 2005). Malnutrition, poor environmental sanitation, lack of portable water supply are the contributing factor to the burden of skin disease in Africa (Mahé *et al.*, 1998; Doe *et al.*, 2001; Adebola, 2004) and Nigeria inclusive. Medicinal plants have been considered as the primary source of healing and are widely used for the treatment of various ailments; this has been attributed to their affordability, accessibility and lesser side effects (Farnsworth *et al.*, 1991; Haile and Delenasaw, 2007). About 80 percent of world population still rely on plant-based traditional medicines for some aspect of their primary health care. The discovery of many potent drugs being used in modern clinical practices have been achieved through research and development from traditional medicinal preparations (Burkill, 2000). *Tapinanthus globiferus* (Loranthaceae) is a semi-parasite that grows mostly on the branches of a large number of tree species including *Vitellaria paradoxa*, *Kola*, *Citrus*, *Combretum*, *Acacia*, *Aloe* and *Terminalia* as host trees (Waterberg *et al.*, 1989; Polhill and Wiens, 1998). It is widely distributed throughout tropical and subtropical region of Western and Eastern African.

The plant is used in ethnomedicine to treat itching (Burkill, 2000), tumour (Haile and Delensaw, 2007), hypertension, ulcers, epilepsy, diabetes, weakness of vision and promoting muscular relaxation before delivery (Bassay, 2012) and it is also used to remove placenta after parturition (Sher and Alyemini, 2011). In spite of its wide spread usage, literature search revealed the paucity of research conducted on the plant, hence the need to evaluate the antifungal effect of the plant in order to validate the ethnomedicinal claim of its use in treatment of fungal infections.

Materials and Methods

The solvents/reagents used were of analytical grade and were distilled prior to use, they include methanol, n-butanol, ethylacetate, chloroform, n-hexane and dimethyl sulfoxide (DMSO; Lobal Chemie Pvt Ltd, India). Sabouraud dextrose agar and broth (Himedia Laboratoies Pvt Ltd, India). UV spectrophotometer (Abrera BARCELONA Spain). Ohaus digital weighing balance (Champ 11 CH15R, Ohaus Corporation, Pinebrook NJ, USA), Metler balance (Model P162 supplied by Gallenamp). 96 well Micro-titre plate, single and multi-channels micro pipette (HUAWAI LAB), Vertical automatic electro thermal pressure steam sterilizer (LX-C35L. HEFEI HUATAI Medical equipment Co. LTD). Microplate Reader (2100-C, Optic Ivyman System) and fluconazole powder (Cat No. F8929, Sigma Aldrich, U.S.A.)

Plant sample

Plant sample of *T. globiferus* growing on *Vitex doniana* was collected from Dange Shuni Local Government Area of Sokoto State, Nigeria in December 2016. It was identified and authenticated by Namadi Sanusi of the Herbarium Section, Department of Botany, Ahmadu Bello University Zaria, with a voucher (No.900107). The plant material was air dried, pulverized, labelled and stored in a polythene bag for further use.

Preparation of plant material

The powdered leaf of *T. globiferus* (2.0 kg) was exhaustively extracted with 3 L of 90 % methanol for 6 days. The extract was filtered using Whatman No. 1 filter paper and the filtrate was evaporated to dryness using rotary evaporator at 40 °C to afford crude methanol leaf extract (140 g). Some part of the extract (120 g) was partitioned into n-hexane, chloroform, ethylacetate and n-butanol fractions.

Preliminary Phytochemical Screening

Preliminary phytochemical screening was carried out on the methanol leaf extract *T. globiferus* and its ethylacetate and n-butanol fractions in accordance with the procedures described by Trease and Evans (1996) and Edeoga *et al.* (2005) to identify the presence of some secondary metabolites.

Antifungal studies

Test organisms

Four (4) clinical fungal isolates of *Candida albicans*, *Aspergillus niger*, *Trychophyton rubrum* and *Trychophyton mentagrophyte* were obtained from the Department of Clinical Microbiology, Usmanu Danfodiyo University Teaching Hospital, Sokoto.

Preparation of test organisms

Test organisms were sub-cultured and grown on 10 mL Sabouraud dextrose agar slants and was eventually kept in the refrigerator at 2 – 8°C.

Preparation of reference antifungal agent

Stock solutions (5 mg/mL) of fluconazole was prepared by dissolving 50 mg of its powder in 10 mL dimethyl sulfoxide (DMSO) from which 0.05 mg/mL (50 µg/mL) working concentration was prepared.

Preparation of plant extract/fractions

Stock concentrations of 100 mg/mL was prepared with 10% DMSO by dissolving 0.5 g each of the methanol extract and its fractions (ethylacetate and *n*-butanol) in 5 mL 10% DMSO and two-fold serial dilution was carried out to obtain three more concentrations of 50, 25 and 12.5 mg/mL.

Preparation of culture media

The Sabouraud dextrose agar (SDA) and broth as growth media were weighed and prepared with distilled water according to the manufacturer's specifications. SDA was gently heated to aid its dissolution, it was dispensed into sterile petri dishes and allowed to cool and solidify. These were kept aseptically until ready for use.

Determination of antifungal activity of *T. globiferus*

Cultivation and standardization of the test organisms

Eighteen-hour solid culture of *Candida albicans* was suspended in sterile Sabouraud dextrose liquid medium. It was standardized in accordance with Clinical Laboratory Standards Institute (CLSI, 2002) by inoculating in normal saline to compare its turbidity to 0.5 McFarland standard which is approximately 1.0×10^6 CFU/mL. *Trichophyton* spp. and *Aspergillus niger* were harvested from 6 day old Sabouraud dextrose agar (SDA) slant cultures, the spore suspension was standardized to 1.0×10^6 spores / mL using spectrophotometer at 530 nm.

Antifungal screening of *T. globiferus*

The antifungal activity of the crude methanol extract, ethyl acetate and *n*-butanol fractions were determined according to the method described by Olowosulu *et al.* (2005). Sabouraud dextrose agar (SDA) as the growth medium was prepared according to Manufacturer's instructions and autoclaved at 121°C for 15 min, the media was transferred into sterile petri dishes and allowed to cool and solidify. Wells were punched on the plates using a sterile cork borer of 8 mm diameter. 0.1 mL of the inoculum was seeded and spread evenly over the surface of the sterilized media using a sterile cotton swab, the wells were filled separately with 200 µL solution of the graded concentration of extract/fractions and 0.05 mg/mL Fluconazole which served as positive control, 10% DMSO was used as negative control, plate were incubated at 27 °C for 48-72 h, zone of inhibition was measured using transparent ruler. The experiment was carried out in triplicates.

Determination of minimum inhibitory concentration (MIC)

The minimum inhibitory concentration (MIC) was determined using a 96 wells microtitre plate as previously described by Khatoon *et al.* (2014). 100 µL of Sabouraud Dextrose broth was added into each micro well of the microtitre plate. 100 µL of the extract/fractions was added into well-1 to make 200 µL total volume. 100 µL of the mixture (extract/fractions) and media was taken from well-1 to well-2 and serially diluted (2-fold) up to well-10 where 100 µL finally discarded from the last well, well 11 (extract blank) served as negative control and well-12 (media + inoculum) which served as positive control. 100 µL of the fungal spore suspension approximately (1×10^6 spores mL⁻¹) was added to each well except for well-11 of the microplate. The microplates was covered with aluminum foil and incubated at 27 °C for 48 hr. The experiment was performed in triplicate. The MIC of the extract/fraction is the lowest concentration that caused growth inhibition of more than 90% after 48 h of incubation (Paola *et al.*, 2010).

Determination of Minimum fungicidal concentration (MFC)

Twenty (20 µL) of each well that showed no visible growth after MIC determination was sub-cultured onto the solid media (SDA) and incubated at 27 °C for 48 h. The lowest concentration

of the extract/fraction that does not yield any fungal growth on the solid medium used was taken as the MFC (Espinel-Ingroff *et al.*, 2002).

Statistical Analysis

The results obtained were expressed as mean \pm standard error of mean and it was analyzed for significant using analysis of variance (ANOVA); values were considered significant at $P < 0.05$.

Results and Discussion

Preliminary phytochemical screening of the methanol leaf extract and fractions of *T. globiferus* growing on *Vitex doniana* revealed the presence of saponins, tannins, alkaloids, cardiac glycosides, carbohydrates, steroids/triterpenes and flavonoids which varies in the fractions (Table 1). This is in agreement with what was reported by Abubakar *et al.* (2016) on *T. globiferus* growing on other host plants. These phytochemical constituents were reported to be responsible for different pharmacological and physiological activities of plants (Cragg and Newman, 2005).

The results of antifungal screening indicated that the fungal isolates were significantly inhibited by the methanol extract and its fractions (ethylacetate and *n*-butanol). The activity increases with the increase in the concentration of the extract and fractions, ethylacetate fraction exhibited the highest mean zone of inhibition range of 27.83 ± 0.16 – 27.00 ± 0.57 mm against all the test organisms except *A. niger* (17.33 ± 0.88 mm); this activity was higher than that of the fluconazole drug (26.1 ± 0.44 – 18.49 ± 0.16 mm) against the same organism, while methanol leaf extract recorded the least mean zone of inhibition (Table 2). The MIC and MFC of the extract and fractions ranged between 0.39 – 25 mg/mL (Table 3); *n*-butanol fraction had the lowest value of 0.39 mg/mL against *C. albicans*, hence the effect was fungistatic while the ethylacetate fraction had an MIC and MFC value of 3.13 mg/mL against *T. rubrum*. The lower MIC and MFC values suggests that the fractions have good antifungal activity. The highest activity observed by the ethylacetate fraction might be due to the concentration of moderately polar compounds such as flavonoids and their derivatives that have been reported to possess antifungal activity (Harborne *et al.*, 1993). Of all the fungal isolates used *C. albicans*, *T. mentagrophyte* and *T. rubrum* were the most susceptible to ethylacetate fraction. *C. albicans*, *T. mentagrophytes* and *T. rubrum* are implicated in diseases such as candidiasis, *Tinea capitis*, *Tinea pedis*, *Tinea corporis*, *Tinea barbae* and *Tinea cruris* (Macura, 1993; Moran *et al.*, 1997). Interestingly, *n*-butanol fraction with zone of inhibition (24.50 mm) when compared to ethyl acetate fraction (27.16 mm) recorded the lowest MIC against *C. albicans* suggesting that the *n*-butanol fraction might have better antifungal activity at lower concentration. Fungal species involving *C. albicans* and *A. niger* are the major causative agents of infections such as oral candidiasis, oesophageal candidiasis, vaginal thrush, lung diseases and otomycosis (Abarca *et al.*, 1994; Schuster *et al.*, 2002 and Oladele and Denning, 2014). Pandima *et al.* (2010) reported that the fungicidal effect of extract may be due to the inhibition of fungal growth by interfering with the production of fungal protein, DNA replication and through various cellular metabolisms of the organisms. Fungicidal effect of the plant extract could be as a result of the damage caused to the cell membrane (Himratul-Aznita *et al.*, 2011).

Table 1: Phytochemical screening of the methanol leaf extract, ethyl acetate and *n*-butanol fractions of *T. globiferus*

| Constituents | Test | M.E | EAF | BTF |
|---------------------|---------------------|-----|-----|-----|
| Carbohydrates | Molisch | + | + | + |
| Anthraquinones | Bontrager | - | - | - |
| Steroid/Triterpenes | Liebermann-Burchard | + | - | - |
| Glycoside | Keller- Killiani | + | + | + |
| Saponins | Frothing | + | + | + |
| Tannins | Ferric chloride | + | + | + |
| | Lead acetate | + | + | + |
| Flavonoids | Shinoda | + | + | + |
| | Ferric chloride | + | + | + |
| Alkaloids | Dragendoff | + | - | - |
| | Mayer | - | - | - |

Key: - = absent; + = present; M.E=methanol extract, EAF=ethylacetate fraction, BTF=*n*-butanol fraction.

Table 2: Susceptibility test of ME, EAF and BTF of *T. globiferus* against selected fungal species

| Fraction | Conc. (mg/mL) | Test organisms | | | |
|-------------|---------------|-------------------|-------------------------|------------------|-----------------|
| | | <i>C. candida</i> | <i>T. mentagrophyte</i> | <i>T. rubrum</i> | <i>A. niger</i> |
| ME | 100 | 18.83±0.44 | 15.83±0.16 | 16.50±0.28 | 14.46±0.29 |
| | 50 | 17.16±0.16 | 12.83±0.16 | 13.50±0.28 | 11.50±0.28 |
| | 25 | 14.16±0.44 | 11.50±0.28 | 12.50±0.28 | 10.33±0.16 |
| | 12.5 | 11.66±0.33 | 9.63±0.33 | 10.33±0.16 | 8.00±0.00 |
| Fluconazole | 0.05 | 25.16±0.44 | 21.00±0.57 | 20.16±0.72 | 18.83±0.44 |
| EAF | 100 | 27.83±0.16 | 27.16±0.44 | 27.00±0.57 | 17.33±0.88 |
| | 50 | 22.33±0.16 | 24.16±0.16 | 24.50±0.28 | 15.50±0.20 |
| | 25 | 18.83±0.44 | 17.83±0.16 | 22.00±0.57 | 13.16±0.44 |
| | 12.5 | 15.00±0.57 | 8.00±0.00 | 18.83±0.29 | 11.50±0.28 |
| Fluconazole | 0.05 | 26.16±0.44 | 20.00±0.57 | 22.16±0.72 | 18.93±0.24 |
| BTF | 100 | 24.50±0.28 | 15.50±0.28 | 15.85±0.44 | 18.83±0.29 |
| | 50 | 23.66±0.33 | 10.33±0.16 | 13.33±0.33 | 17.16±0.16 |
| | 25 | 21.00±0.57 | 9.38±0.33 | 11.50±0.28 | 13.16±0.28 |
| | 12.5 | 19.33±0.33 | 8.00±0.00 | 10.33±0.16 | 11.66±0.33 |
| Fluconazole | 0.05 | 24.16±0.16 | 22.00±0.53 | 21.26±0.72 | 18.49±0.16 |

Values are mean inhibition zone (mm) ± S.E of three replicates; Key; M.E=methanol extract, EAF=ethylacetate fraction, BTF=*n*-butanol fraction

Table 3: MIC and MFC of ME, EAF and BTF of *T. globiferus* against selected fungal species

| Organisms | ME | | EAF (mg/mL) | | BTF | |
|-------------------------|------|-------------------|----------------|-------------------|------|-------|
| | MIC | MFC | MIC | MFC | MIC | MFC |
| <i>C. albicans</i> | 6.25 | 12.5 ^η | 12.5 | 12.5* | 0.39 | 0.39* |
| <i>T. mentagrophyte</i> | 12.5 | 12.5* | 25.0 | 25.0* | 12.5 | 12.5* |
| <i>T. rubrum</i> | 6.25 | 6.25* | 3.13 | 3.13* | 6.25 | 6.25* |
| <i>A. niger</i> | 25.0 | 25.0 ^η | 12.5 | 12.5 ^η | 3.13 | 3.13* |

Key: * = fungicidal effect, η = fungistatic effect; ME=methanol extract, EAF=ethylacetate fraction, BTF=*n*-butanol fraction

Conclusion

The use of *Tapinanthus globiferus* as antifungal agent is promising as the methanol leaf extract and its fractions showed excellent antifungal activity against some selected fungal species with *n*-butanol fraction being the most active. This study indicated that *T. globiferus* has demonstrated good antifungal activity validating the ethno medicinal claim for the use of the plant in the treatment of fungal infections.

Conflict of interests

The authors declare that they have no financial or personal relationships which may have inappropriately influenced them in writing this article.

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