



## Phytochemical analysis and antifungal property of *Mallotus oppositifolius* (Geiseler) Müll.Arg. (Euphorbiaceae)

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### ABSTRACT

The emergence of resistant fungi to available drugs highlights the need for new antifungal drugs. The present study aimed to evaluate the antifungal activity of the isolated compounds, fractions and crude extract from the leaf of *Mallotus oppositifolius* (Geiseler) Müll. Arg. Three pure compounds labelled **1-3** were isolated from the methylene chloride / methanol (1/1) extract of the leaf of this plant using chromatography techniques. These compounds were identified using analytical spectroscopic methods as betulinic acid (**1**), quercetine (**2**) and quercitin (**3**). The crude extract, fractions and compounds were tested against pathogenic yeasts (*Candida albicans*, *Candida glabrata*, *Candida krusei*) and dermatophytes (*Trichophyton rubrum*, *Trichophyton soudanense*, *Microsporum audouinii*, *Microsporum langeronii*) using agar well diffusion and dilution methods. The safety of the crude extract was studied on Wistar rats according to the WHO guidelines. The minimal inhibitory concentration (MIC) values ranged from 48 to 781 µg/ml against yeasts for crude extract and fractions, and 1.86 to 25000 µg/ml against dermatophytes for pure compounds, fractions and crude extract. The antifungal activity of pure compounds was not determined against yeasts. The crude extract of leaf was found to be safe in rat at up to 12 g/kg. The results achieved supported the traditional use of *Mallotus oppositifolius* leaf for the treatment of fungal infections.

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**Keywords:** *Mallotus oppositifolius*, antifungal activity, safety, phytochemical screening.

### INTRODUCTION

Fungal infections account amongst neglected tropical diseases (NTDs) of growing

public health importance worldwide. They affect more than a billion people, resulting in approximately 11.5 million life-threatening

infections and more than 1.5 million deaths yearly (Tudela and Denning, 2017). Among fungal diseases, candidiasis and dermatophytosis are the most frequently and widely diagnosed (Gnat et al., 2019).

Dermatophytosis is a major public health problem, that represents one of the common infectious diseases worldwide, especially in developing countries. An increase in the incidence of dermatophytosis is noted worldwide, with particular emphasis in low-income countries. Children between 4 and 16 years old are more at risk due to more frequent contact with different sources and inadequate amount of fungi-inhibiting fatty acids synthesized in prepubescent children, predisposing them to dermatophytic infections (Araya et al., 2020). In recent years, the epidemiology of yeast infections has steadily changed worldwide. This notably includes emergence of several novel pathogenic *Candida* species. Invasive candidiasis for instance is estimated to affect more than 250 million people causing more than 50,000 deaths worldwide every year. Epidemiological data unveils an incidence of 2 to 14 cases of candidemia per 100,000 individuals with a mortality range of 10 to 20% (Ghazi et al., 2019). Such infections occur as opportunistic consequences of other health problems including surgical operations, asthma, malnutrition, AIDS, cancer, alcoholism, organ transplantation, diabetes, corticosteroid therapies and the widespread drug resistance (Nowak and Szyfter, 2008; Tudela and Denning, 2017; Bongomin et al., 2017; Ghaddar et al., 2020).

Of note, the growing resistance of fungi to therapeutics is a serious health threat in both developed and developing worlds. Introduction of alternative antifungal drugs is desperately needed to mitigate the tendency of drugs to quickly develop drug-resistant pathogens (Institute of Medicine (US) Forum on Emerging Infections, 2003). Therefore, drug discovery advocates the identification of novel antifungal compounds that can facilitate the development of better dosing regimens and novel strategies to mitigate

drug resistance and quench the dissemination of resistant fungi.

Indeed, natural products remain credible sources for drug discovery against fungal infections (Da Silva Filho et al., 2008; Pacciaroni Adel et al., 2008; Goly et al., 2015; Afolabi and Kareem, 2019; Mbunde et al., 2021). A recent ethnomedical survey identified the medicinal plant, *Mallotus oppositifolius* (Euphorbiaceae) as the most recommended species for the treatment of skin diseases including fungal skin diseases (Bernadine et al., 2015; Kouadio et al., 2015). Though the antifungal potential of *Mallotus oppositifolius* extracts is reported (Bernadine et al., 2015; Kouadio et al., 2015), their active principles are still to be elucidated as hit purification is crucial within the framework of potential drug discovery prerequisite identification. The present study was carried out to prepare natural products from *Mallotus oppositifolius* leaf and evaluate their antifungal effect against selected pathogenic dermatophytes and yeasts.

## MATERIALS AND METHODS

### General

Isolates were weighed using an electronic balance (MELTER TELEDON PC 2000, Switzerland). Melting points were determined on a Büchi-540 melting point apparatus. The mass spectra were generated with electronic impact (EI) at higher resolution on a Mass Spectrometer (Flinnigan MAT SSQ-7000, Germany) under 70eV. <sup>1</sup>H- and <sup>13</sup>C-NMR spectra were run on a NMR Bruker spectrometer (Germany) equipped with 5 mm <sup>1</sup>H and <sup>13</sup>C probes operating at 300 MHz and 75 MHz respectively. The solvent used was acetone-d<sub>6</sub>. Silica gel 60 (Merck, particle size 0.040-0.060 mm) and LH-20 Sephadex gels (Merck, Switzerland) were used for flash and column chromatography, while pre-coated aluminium silica gel 60 F<sub>254</sub> sheets were used for TLC with different mixtures of ether, hexane, ethyl acetate, methanol and acetone as eluents; spots were visualised under UV lamps (254 and 365 nm) or by heating after spraying with 50% H<sub>2</sub>SO<sub>4</sub> reagent.

### Plant material

The leaves of *Mallotus oppositifolius* were harvested on the 28<sup>th</sup> of January 2016 from Mount Eloumden, Centre Region of Cameroon. The plant was identified by Mr. Nana Victor at the National Herbarium of Cameroon (Yaounde), where a voucher specimen (1739/SRFK) is deposited.

### Extraction and fractionation

The leaves were chopped, air-dried and ground to fine powder using locally made grinder. Powdered plant material (2.5 kg) was successively extracted by cold maceration with a methylene chloride: methanol mixture (1:1) (CH<sub>2</sub>Cl<sub>2</sub>/MeOH) for 48 h and then with methanol for 8 h. The filtrates were combined and concentrated using a rotary evaporator (Rotavapor® R-100, Buchi, Germany) under reduced pressure to afford 160 g of extract.

The crude extract was subjected to silica gel column flash chromatography eluting successively with solvents of increasing polarity with pure hexane and then with hexane-ethyl acetate mixtures 25; 50 and 75% then with pure ethyl acetate and finally with 5% ethyl acetate-methanol mixture to afford 6 fractions indexed A, B, C, D, E and F that were evaporated under reduced pressure. Fractions B, C and D, were pooled based on their thin layer chromatography (TLC) profiles to form fraction G. Based on its promising biological activities, fraction G (23 g) was further chromatographed on a type 60 silica gel column and on LH-20 Sephadex with hexane, *n*-hexane-ethyl acetate, and ethyl acetate as eluent of increasing polarity to furnish **1**, Hex/EA (4:1); **2**, Hex/EA (3:2); and **3**, Hex/EA (3:7). The structures of these compounds were determined from their spectral data (<sup>1</sup>H NMR, <sup>13</sup>C NMR, mass spectroscopy) and by the use of two-dimensional multi-pulse NMR techniques (COSY <sup>1</sup>H -<sup>1</sup>H, HMBC, HMQC).

The phytochemical screening of the crude extract and fractions for flavonoids and triterpenes was carried out as previously described (Herborne, 1976; Odebiyi and Sofowora, 1979).

### Preliminary antifungal screening

The fungal clinical isolates were provided by the Centre Pasteur of Cameroon, Yaounde, and maintained in continuous culture in the Laboratory for Phytobiochemistry and Medicinal Plants Studies, University of Yaounde 1. The antifungal susceptibility testing of crude extract, fractions and pure compounds was performed against fungi including three yeasts (*Candida albicans*, *Candida glabrata*, *Candida krusei*), and four dermatophytes (*Trichophyton rubrum*, *Trichophyton soudanense*, *Microsporum audouinii*, *Microsporum langeronii*).

The agar dilution method was used for the initial screening of the antifungal activity against dermatophytes as previously described (Ngono Ngane et al., 2003), with some modifications. Plant extract and fractions and Amphotericin B used as positive control were individually mixed in the Sabouraud dextrose agar (SDA) culture medium to achieve final concentrations of 50 mg/ml for crude extract, 25 mg/ml for fractions, and 25 µg/ml for Amphotericin B and isolated compounds respectively. The medium was thereafter inoculated with 6 mm explants of 7-days old dermatophyte cultures and incubated in triplicate for 10 days at 25 °C and the mycelium growth diameter measured using a calliper. The antifungal activity of the inhibitors expressed as percentage of inhibition was calculated relative to the untreated cultures (negative control) (Singh et al., 1993).

The agar well diffusion method was used to evaluate the susceptibility of yeasts. Suspensions of yeasts were collected from 48 hours fresh cultures on SDA medium and enumerated using the Malassez cell counting chamber. The inoculum adjusted to 2.10<sup>4</sup> CFU/ml was poured on the surface of a solidified SDA medium in a Petri dish. After 15 minutes pre-incubation at room temperature, wells of 6 mm diameter were bored and 100 µl of 50 mg/ml crude extract, 25 mg/ml of fractions, and 25 µg/ml of Amphotericin B (positive control) and isolated compounds were respectively introduced. Petri dishes were incubated in triplicate for 48 hours

at 35 °C and the growth inhibition diameters thereafter measured using a calliper.

#### **Determination of the Minimal Inhibitory Concentration (MIC) and Minimal Fungicidal Concentration (MFC)**

The MIC values of inhibitors were evaluated by the agar dilution method and the broth micro-dilution method for dermatophytes and yeasts respectively, according to Singh et al. (1993) and Torres et al. (1993).

##### **Microdilution method**

The MIC measurement was achieved using the microdilution method in 96 multi-well microtiter plates, as described previously by Torres et al. (1993) with slight modifications. A standardized yeast suspension at  $2.0 \times 10^4$  CFU/ml was treated with serially diluted concentrations of samples in culture medium supplemented with phenol red. The final concentrations of the crude extract ranged from 25000 µg/ml to 12.20 µg/ml while fractions and Amphotericin B were tested at 12500 µg/ml to 0.61 µg/ml. Negative control consisted in medium with yeast but without any treatment. Plates were prepared in triplicate, and incubated at 35 °C for 48 h. The lowest concentration at which the medium color change from red to yellow occurred was considered as the MIC. Of note, phenol red is a pH indicator that changes color from red to yellow when pH decreases, indicating microbial growth. Wells without visible growth (no color change) were sub-cultured in inhibitor-free medium and incubated for additional 48 h. The lowest concentration at which no visible growth was observed was defined as MFC. Then, the MFC/MIC ratio was calculated to determine the fungicidal or fungistatic feature of the inhibitor (Carbannelle et al., 1987).

##### **Agar dilution method**

The antidermatophytic activity was assessed according to the agar dilution method on SDA. Tested samples were two-fold serially diluted into the culture medium to achieved concentrations starting from 25 mg/ml for crude extract, 12.5 mg/ml for fractions, and 6.25 µg/ml for Amphotericin B and isolated compounds. SDA plates were individually

inoculated with 6 mm in diameter of dermatophytes obtained from 7-days cultures and incubated for 10 days at 30 °C. All individual tests were performed in triplicate.

The diameter of the dermatophyte mycelial growth zone was measured in two perpendicular directions through the centre of the explant to determine the percent inhibition (PI) (Reyes, 1997).

$$PI = \frac{dt - dx}{dt} \times 100$$

PI = percent inhibition expressed (%)

dt = mean colony diameter in the negative control plate (without antifungal agent)

dx = average colony diameter in the assay plate containing the extract

The minimal inhibitory concentration (MIC) was determined as the lowest concentration that produced no visible fungal growth. The MFC values were thereafter evaluated through sub-culture from plates without noticeable growth, and determined as the smallest concentration of inhibitor that kills the fungi.

#### **Determination of the acute toxicity of plant samples**

Wistar albino male rats aged 6-8 weeks and weighing 160–180 g were used for the experiments. Animals were bred in animal house of the Laboratory of Animal Physiology of the University of Yaoundé 1. They were maintained at room temperature on a 12 h light-dark natural cycle. Food and water were given *ad libitum* during experimentation. All experiments were carried out with approval of Institutional Ethical Committee, which approved all procedures recommended by the European Union on the protection of animals used for scientific proposes (CEE Council 86/609; Ref N° FWA-IRD 0001954).

The acute toxicity of the crude extract of leaves of *M. oppositifolius* was studied on male and female Wistar rats according to the guidelines of the WHO (W.H.O., 1992). Animals were divided into 4 groups of 5 per sex, and received orally extract doses of 0, 4, 8,

and 12 g/kg body weight in corn oil. They were monitored after drug administration for 48 hours and toxicological parameters recorded. The median lethal dose (LD<sub>50</sub>) was determined as the highest dose of extract at which no death occurred.

### Statistical analysis

Data from antifungal assay were presented as mean ± SEM and statistical significance was achieved by one-way analysis of variance (ANOVA method) using GraphPad statistical analysis software InStat. The 5% probability threshold was considered significant. The Duncan's test was used to compare values in the toxicity study.

## RESULTS

### Characterization of purified compounds

The structures of the three known compounds were established as betulenic acid (a pentacyclic triterpene) (**1**), quercetin (a flavonoid) (**2**), (Kapche, 2000), and quercitrin (a symmetric macrocyclic flavonoid glycoside) (**3**) (Figure 2) (Agrawal, 1992).

*Betulenic acid (compound 1)*: White sequins, mp 301-304 °C. Liebermann-Burchard- Positive (Triterpene). EI-MS *m/z* : 456 [M<sup>+</sup>]; 438 [M - HO<sub>2</sub>]<sup>+</sup> ; 423 [M - H<sub>2</sub>O - CH<sub>3</sub>]<sup>+</sup> ; 410; 395; 248; 220; 207; 189; 187; 175; 173. <sup>1</sup>H NMR spectrum (200 MHz, CDCl<sub>3</sub>): δ<sub>H</sub> (ppm); 0.85 (3H, s), 0.91 (3H, s); 0.96 (3H, s); 0.98 (3H, s); 0.99 (3H, s); 1.68 (3H, s, H-30); 3.20 (1H, dd, H-3); 4.76 (1H, m, H-29); 4.63 (1H, m, H-29). <sup>13</sup>C NMR spectrum (50 MHz, CDCl<sub>3</sub>): δ<sub>C</sub> 37.0(C-1), 27.0 (C-2), 78.7 (C-3), 38.5 (C-4), 55.1 (C-5), 18.1 (C-6), 32.0(C-7), 40.5(C-8), 50.3(C-9), 36.9 (C-10), 20.7(C-11), 25.3 (C-12), 38.1 (C-13), 42.2 (C-14), 29.5 (C-15), 34.1 (C-16), 56.0(C-17), 46.7 (C-18), 49.0(C-19), 150.4(C-20), 30.4(C-21), 38.0(C-22), 27.7 (C-23), 15.1 (C-24), 15.9(C-25), 15.7 (C-26), 14.4 (C-27), 179.1 (C-28), 109.3(C-29), 19.1(C-30).

*Quercetin (compound 2)*: yellow powder, mp 320-321 °C. Shinoda test positive (Flavonoid). EI-MS *m/z* : 302 [M<sup>+</sup>]; 301; 286; 285; 153; 137; 109. <sup>1</sup>H NMR (300 MHz, acetone d<sub>6</sub>) δ<sub>H</sub> 12.19 (OH, s, H-5), 6.27 (d, *J* = 2.0 Hz, H-6), 6.53 (d, *J* = 2.0 Hz, H-8), 7.84 (d,

*J* = 2.2 Hz, H-2'), 7.0(dd, *J* = 8.5 & 2.2 Hz, H-5'), 7.71 (d, *J* = 8.5 Hz, H-6'). <sup>13</sup>C NMR (75 MHz, acetone d<sub>6</sub>) δ<sub>C</sub> 147.3 (C-2), 137.1 (C-3), 176.9 (C-4), 158.2 (C-5), 99.5 (C-6), 165.4 (C-7), 94.8 (C-8), 162.6(C-9), 104.5(C-10), 124.2 (C-1'), 116.1 (C-2'), 146.2 (C-3'), 148.7 (C-4'), 116.6 (C-5'), 121.8 (C-6').

*Quercitrin (compound 3)*: Beige colored crystals, mp 232 - 234 °C. Shinoda test positive (Flavonoid). <sup>1</sup>H NMR (300 MHz, acetone d<sub>6</sub>) δ<sub>H</sub> 12.19 (OH, s, H-5), 6.27 (d, *J* = 2.1 Hz, H-6), 6.48 (d, *J* = 2.1 Hz, H-8), 7.51 (d, *J* = 2.1 Hz, H-2'), 7.0(d, *J* = 8.3 Hz, H-5'), 7.41 (dd, *J* = 8.3 & 2.1 Hz, H-6') 5.53 (d, *J* = 1.1 Hz, H-1''), 4.23 (dd, *J* = 3.3 & 1.5 Hz, H-2''), 3.74 (dd, *J* = 8.8 & 3.3 Hz, H-3''), 3.37 (dd, *J* = 8.8 & 9.5 Hz, H-4''), 3.41 (dd, *J* = 9.3 & 5.7 Hz, H-5''), 0.92 (d, *J* = 5.7 Hz, H-6''). <sup>13</sup>C NMR (75 MHz, acetone d<sub>6</sub>) δ<sub>C</sub> 158.4(C-2), 136.2 (C-3), 179.7 (C-4), 163.6 (C-5), 99.9 (C-6), 165.4 (C-7), 94.9 (C-8), 158.8 (C-9), 106.2(C-10), 123.3 (C-1'), 116.2 (C-2'), 146.3 (C-3'), 149.4 (C-4'), 116.6 (C-5'), 122.1 (C-6'), 102.2(C-1''), 71.0 (C-2''), 71.6 (C-3''), 72.5 (C-4''), 70.8 (C-5''), 17.3 (C-6'').

### Antifungal activity of tested samples

The antifungal activities of *Mallotus oppositifolius* extracts are summarized in tables 1&2 below, where the percent inhibition, growth inhibition diameter, MIC, and MFC are presented. These results indicate that almost all extracts, fractions and isolated compounds were found to exhibit antifungal activity on dermatophytes and yeasts.

#### Activity against yeasts

Globally *M. oppositifolius* extracts and fractions showed inhibitory activity on yeasts with growth inhibition diameters ranging from 8±0 to 18±1 mm (Table 1). The highest inhibition diameters were obtained with the crude extracts of stem and leaf on *C. albicans* and *C. krusei* respectively. Fractions of the leaves extract of *M. oppositifolius* exerted variable inhibitory activity on yeasts (inhibition diameter of 0 to 18 mm) compared to the crude extract (10 to 18 mm). Fractions **C** (Ø15 mm) and **G** (Ø 18 mm) were the most active on *C. albicans* and *C. krusei* respectively and were considered for MIC and MFC

determination. The MIC and MFC of fraction **G** against *C. krusei* were 48 µg/ml (Table 1). The MFC/MIC ratio indicated that fraction **G** was fungicidal on *C. krusei* (Table 1).

**Antidermatophytic activity**

Overall, the crude extract and fractions from *M. oppositifolius* leaves showed high anti-dermatophytic activity with percentage radial growth inhibition of 36-100%. The fraction **C** and quercetin were the most potent on the tested dermatophytes with 100% inhibition at 25 mg/ml, and 25 µg/ml respectively (Table 2). *M. langeronii* showed susceptibility to the crude extract and all the fractions and compounds.

MIC values on dermatophytes ranged from 1.86 to 12500 µg/ml, with betuleninic acid exerting the strongest effect (1.86 µg/ml) on *M. langeronii*. Quercitrin and lupeol displayed MIC values of 3.125 µg / ml. The isolated compounds, **1-3** with MIC values ranging from MIC 1.86 µg/ml to 6.25 µg/ml exhibited improved potency compared to their parent fractions (MIC 6250-12500 g/ml), comparable to an extent to the potency of the standard drug,

amphotericin B (MIC of 1.22-4.8 µg/ml). Apart from fraction **C**, all the inhibitors showed fungicidal activity against dermatophytes with MFC/MIC ratio smallest than 4.

**Acute toxicity of *M. oppositifolius* leaf crude extract**

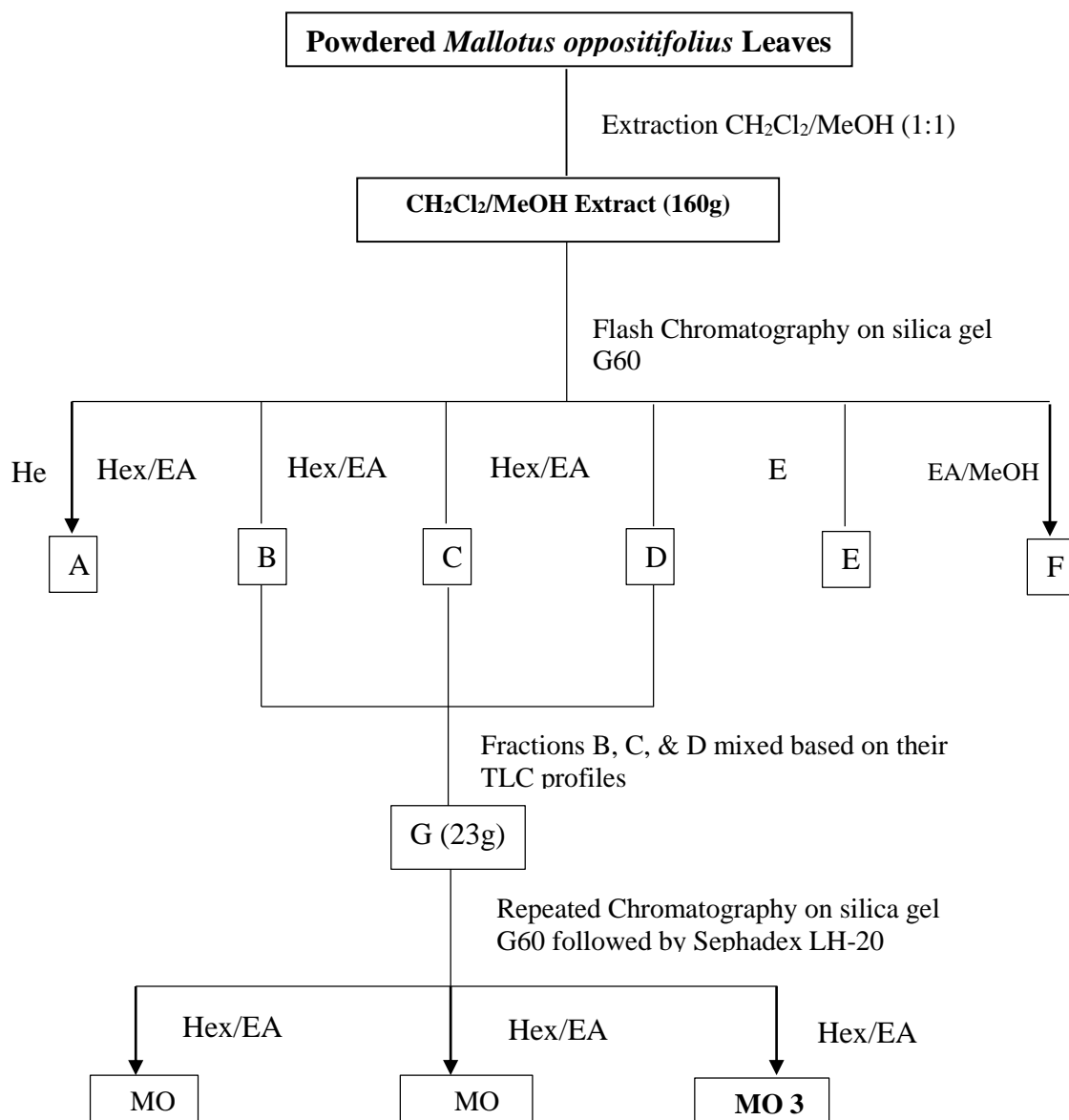
In a perspective to further develop the crude extract from *Mallotus oppositifolius* leaves into a standardized phytomedicine, it was evaluated for acute toxicity in rats. Oral administration of the crude extract at increasing doses showed no mortality nor behavioral changes and/or significant adverse effects in *Wistar* albinos rats, up to a dose of 12 g/kg body weight, suggesting its median lethal dose (LD<sub>50</sub>) to be greater than 12 g/kg. However, it is noteworthy that at the dose of 12 g / kg of body weight, there was a slight decrease in physical sensitivity, the degree of mobility and aggressiveness during the first hour post-administration. But these signs disappeared as from the second hour in treatment conditions.

**Table 1:** Anti-yeast activity of *Mallotus oppositifolius* leaf crude extract, fractions and compounds (µg/ml).

|             | Strain                  | MOL  | A   | B    | C    | D    | E    | F    | G    | 1  | 2  | 3  | AmB   |
|-------------|-------------------------|------|-----|------|------|------|------|------|------|----|----|----|-------|
| ID (mm)     | <i>Candida albicans</i> | 8±1  | 0±0 | 8±1  | 15±0 | 0±0  | 0±0  | 10±0 | 10±1 | ND | ND | ND | 23±1  |
|             | <i>Candida krusei</i>   | 12±0 | 9±0 | 12±0 | 8±1  | 12±1 | 12±0 | 12±0 | 18±1 | ND | ND | ND | 22±1  |
|             | <i>Candida glabrata</i> | 0±0  | 0±0 | 8±0  | 8±0  | 11±1 | 12±0 | 12±1 | 12±1 | ND | ND | ND | 21±1  |
| MIC (µg/ml) | <i>Candida albicans</i> | ND   | ND  | ND   | ND   | ND   | ND   | ND   | ND   | ND | ND | ND | ND    |
|             | <i>Candida krusei</i>   | ND   | ND  | ND   | ND   | ND   | ND   | ND   | 48   | ND | ND | ND | 39.06 |
|             | <i>Candida glabrata</i> | ND   | ND  | ND   | ND   | ND   | ND   | ND   | ND   | ND | ND | ND | ND    |
| MFC (µg/ml) | <i>Candida albicans</i> | ND   | ND  | ND   | ND   | ND   | ND   | ND   | ND   | ND | ND | ND | ND    |
|             | <i>Candida krusei</i>   | ND   | ND  | ND   | ND   | ND   | ND   | ND   | 48   | ND | ND | ND | 39.06 |
|             | <i>Candida glabrata</i> | ND   | ND  | ND   | ND   | ND   | ND   | ND   | ND   | ND | ND | ND | ND    |

|                |                         |    |    |    |    |    |    |    |    |    |    |    |    |
|----------------|-------------------------|----|----|----|----|----|----|----|----|----|----|----|----|
| <b>MFC/MIC</b> | <i>Candida albicans</i> | ND | ND | ND | ND | ND | ND | ND | ND | ND | ND | ND | ND |
|                | <i>Candida krusei</i>   | ND | ND | ND | ND | ND | ND | ND | 1  | ND | ND | ND | 1  |
|                | <i>Candida glabrata</i> | ND | ND | ND | ND | ND | ND | ND | ND | ND | ND | ND | ND |

MOL: Crude leaf extract; A-G: fractions; 1: Betulinic acid; 2: Quercitrin; 3: Quercetin; *M.L.* : *Microsporium langeronii*, *M.A.* : *Microsporium audouini*, *T.R.* : *Trichophyton rubrum*, *T.S.* : *Trichophyton soudanense*, *C.A.* : *Candida albicans*, *C.G.* : *Candida glabrata*, *C.K.* : *Candida krusei*;; AmB : Amphotericin B; %I: Percent inhibition; ID: Inhibition Diameter; MIC: Minimal Inhibitory Concentration; MFC: Minimal Fungicidal Concentration; ND: Not determined.



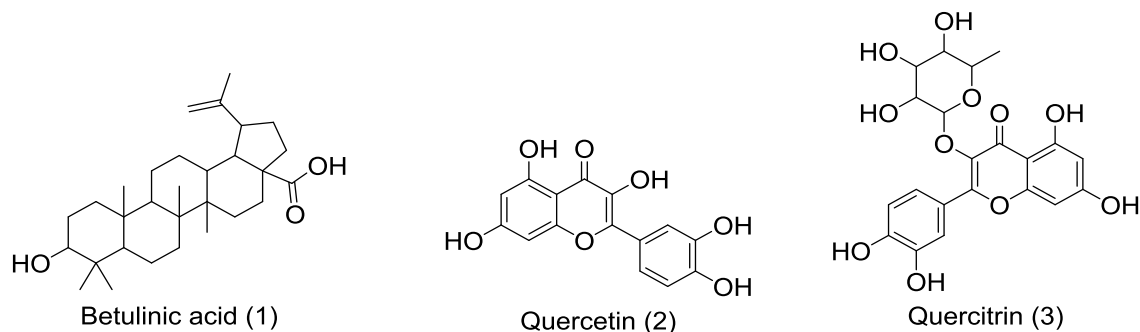
**Figure 1:** Flowchart summarizing extraction and fractionation procedure of *M. oppositifolius* leaves. MO: *M. oppositifolius*; A-G: Fractions; MO<sub>1,2,3</sub>: Pure compounds afforded from fractionation of MO leaf extract. Hex: Hexane; EA: Ethyl Acetate; MeOH: Methanol.

**Table 2:** Antidermatophytic activity of *Mallotus oppositifolius* leaf crude extract, fractions and compounds ( $\mu\text{g/ml}$ ).

|                          | Strains                        | MOL            | A              | B            | C           | D            | E              | F            | G              | 1            | 2           | 3              | AmB         |
|--------------------------|--------------------------------|----------------|----------------|--------------|-------------|--------------|----------------|--------------|----------------|--------------|-------------|----------------|-------------|
| % I                      | <i>Microsporum langeronii</i>  | 100 $\pm$ 0    | 93.5 $\pm$ 1.4 | 100 $\pm$ 0  | 100 $\pm$ 0 | 100 $\pm$ 0  | 100 $\pm$ 0    | 100 $\pm$ 0  | 100 $\pm$ 0    | 100 $\pm$ 0  | 100 $\pm$ 0 | 92.8 $\pm$ 0.5 | 100 $\pm$ 0 |
|                          | <i>Microsporum audouini</i>    | 83 $\pm$ 2.1   | 100 $\pm$ 0    | 80 $\pm$ 1.4 | 100 $\pm$ 0 | 92 $\pm$ 1.8 | 100 $\pm$ 0    | 71 $\pm$ 1.9 | 59 $\pm$ 3.4   | ND           | ND          | ND             | ND          |
|                          | <i>Trichophyton rubrum</i>     | 91.3 $\pm$ 0.7 | 100 $\pm$ 0    | 100 $\pm$ 0  | 100 $\pm$ 0 | 100 $\pm$ 0  | 96.2 $\pm$ 0.8 | 75 $\pm$ 0.6 | 87.1 $\pm$ 1.3 | 100 $\pm$ 0  | 100 $\pm$ 0 | 98 $\pm$ 0.1   | 95 $\pm$ 0  |
|                          | <i>Trichophyton soudanense</i> | 100 $\pm$ 0    | 98 $\pm$ 2     | 100 $\pm$ 0  | 100 $\pm$ 0 | 96 $\pm$ 1   | 94 $\pm$ 2.5   | 100 $\pm$ 0  | 36.3 $\pm$ 1.1 | 97 $\pm$ 0.5 | 100 $\pm$ 0 | 100 $\pm$ 0    | 82 $\pm$ 0  |
| MIC ( $\mu\text{g/ml}$ ) | <i>Microsporum langeronii</i>  | 6250           | 6250           | 6250         | 6250        | 6250         | 6250           | 6250         | 6250           | 1.86         | 3.125       | 3.125          | 1.22        |
|                          | <i>Microsporum audouini</i>    | 12500          | 6250           | 12500        | 6250        | 6250         | 12500          | 6250         | 6250           | ND           | ND          | ND             | 1.22        |
|                          | <i>Trichophyton rubrum</i>     | 12500          | 6250           | 6250         | 6250        | 12500        | 12500          | 6250         | 12500          | 6.25         | 3.125       | 3.125          | 4.8         |
|                          | <i>Trichophyton soudanense</i> | 12500          | 6250           | 6250         | 6250        | 6250         | 12500          | 6250         | 12500          | 3.125        | 6.25        | 3.125          | 1.22        |
| MFC ( $\mu\text{g/ml}$ ) | <i>Microsporum langeronii</i>  | 6250           | 12500          | 6250         | ND          | 6250         | 12500          | ND           | ND             | 3.125        | 6.25        | 3.125          | 1.22        |
|                          | <i>Microsporum audouini</i>    | ND             | 12500          | 25000        | 25000       | 12500        | 12500          | ND           | 6250           | ND           | ND          | ND             | 1.22        |
|                          | <i>Trichophyton rubrum</i>     | 12500          | 6250           | 12500        | 12500       | ND           | 25000          | 6250         | 25000          | 6.25         | 6.25        | 3.125          | 4.8         |
|                          | <i>Trichophyton soudanense</i> | ND             | 12500          | 12500        | 12500       | ND           | 25000          | 12500        | 25000          | ND           | ND          | 3.125          | 1.22        |
| MFC/MIC                  | <i>Microsporum langeronii</i>  | 1              | 2              | 1            | ND          | 1            | 2              | ND           | ND             | 2            | <u>2</u>    | 1              | 1           |
|                          | <i>Microsporum audouini</i>    | ND             | 2              | 2            | 4           | 2            | 1              | ND           | 1              | ND           | ND          | ND             | 1           |
|                          | <i>Trichophyton rubrum</i>     | 1              | 1              | 2            | 2           | ND           | 2              | 1            | 2              | 2            | <u>2</u>    | 1              | 1           |
|                          | <i>Trichophyton soudanense</i> | ND             | 2              | 2            | 2           | ND           | 2              | 2            | 2              | ND           | ND          | 1              | 1           |

MOL: Crude leaf extract; A-G : fractions; 1: Betulinic acid; 2: Quercitrin; 3: Quercetin; M.L. : *Microsporum langeronii*, M.A. : *Microsporum audouini*, T.R. : *Trichophyton rubrum*, T.S. : *Trichophyton soudanense*, C.A. : *Candida albicans*, C.G. : *Candida glabrata*, C.K. : *Candida krusei*; AmB : Amphotericin B; %I: Percent inhibition; ID: Inhibition Diameter; MIC: Minimal Inhibitory Concentration; MFC: Minimal Fungicidal Concentration; ND: Not determined





**Figure 2:** Structures of isolated compounds.

## DISCUSSION

A weak activity of *M. oppositifolius* isolates was recorded on yeasts with inhibition zone diameters between 10 and 18 mm compared to the reference drug (amphotericin B, 21-23 mm). Nevertheless, the elicited activity could be inherently attributed to the presence of antimicrobial secondary metabolites such as phenols, tannins, flavonoids, alkaloids, glycosides, anthraquinones, terpenoids and essential oils previously reported in *M. oppositifolius* (Gbedema et al., 2010; Herborne, 1976; Okwu and Okwu, 2004) either on an individual scale, or to their concerted contributions (Cowan, 1999; Latte and Kolodziej, 2000; Modafar et al., 2000; Charrouf and Guillaume, 2002). *C. albicans* was less susceptible than *C. krusei* to fraction G. Indeed, *M. oppositifolius* extracts previously showed varying degrees of *in vitro* antifungal activity against yeasts (Adekunle and Ikumapayi, 2006; Mosango, 2007). Though quercitrin (3) and quercetin (2) isolated from various sources, possess antifungal activity against fungi such as *C. albicans*, *C. glabrata*, *C. krusei*, *C. parapsilosis* and *C. tropicalis* with MIC values ranging from 7.8–256  $\mu\text{g/ml}$  (Báidez et al., 2006; Tempesti et al., 2011; Aboody and Mickymaray, 2020), they did not show anti-yeast activity in the current study up to 25  $\mu\text{g/ml}$ .

Besides, *M. oppositifolius* leaf isolates exhibited more promising antifungal activity against the tested filamentous fungi compared

to the figures exhibited on yeasts with respect to the diameters of inhibition zone and the percentage of mycelial inhibition. At the concentration of 50 mg/ml, the crude extract inhibited the filamentous fungi (dermatophytes) with an average mycelial growth inhibition percentage of 93.57%. The MIC of the crude extract ranged 6250-12500  $\mu\text{g/ml}$ , and was 6250  $\mu\text{g/ml}$  for most fractions and 3.125  $\mu\text{g/ml}$  for quercetin and quercitrin on the tested dermatophytes. Besides, their MFC/MIC ratio were smaller than 4 suggesting their fungicidal activity (Carbonnelle et al., 1987). This is an indication that bio-guided fractionation has improved the antifungal potency.

The compounds betulinic acid (1), quercetin (2) and quercitrin (3) exerted very promising anti-dermatophytic activity. Of note, previous studies reported betulinic acid from *Eugenia umbelliflora* Berg. (Myrtaceae) to exert selective antifungal activity against dermatophytes (*E. floccosum*, *M. canis*, *M. gypseum*, *T. rubrum*, *T. mentagrophytes*), with MIC values between 200 and 1000  $\mu\text{g/ml}$ , and interestingly, to inhibit 4/5 species with MIC values below or equal to 500  $\mu\text{g/ml}$  (Machado et al., 2009). The piperazinyl derivative of betulinic acid was shown to exhibit antifungal profile similar to that of terbinafine against dermatophytes (Innocente et al., 2014). Also, it was alluded that betulinic acid exerts inhibitory effect by preventing topoisomerase I-DNA interaction resulting in non-formation of the 'cleavable complex' (Oliveira et al., 2016).

Quercitrin (**3**) and quercetin (**2**) isolated from various sources also showed antifungal activity against fungi such as *T. rubrum* and *T. beigelii* with MIC values ranging from 7.8–256 µg/ml (Aboody and Mickymaray, 2020).

It is noteworthy that fatty acid synthase is an enzyme essential for endogenous fatty acid synthesis in the membrane of fungi (Li et al., 2002) and is considered as a potential target for novel antifungal drugs due to its marked structural differences between fungal and mammalian cells (Bitencourt et al., 2013). Hence, quercetin was reported to have individual or synergistic antifungal properties with fluconazole, which is recognized as an inhibitor of fatty acid synthase. Additionally, quercetin was shown to inhibit *T. rubrum* growth with MICs of 125 µg/ml for the wild-type strain (MYA3108) and 63 µg/ml for the ABC transporter mutant strain ( $\Delta$ TruMDR2) respectively, suggesting a down-regulation of fatty acid synthase gene expression and a reduction of ergosterol content in *T. rubrum* by modulating the expression of FAS1 and ERG6 (Bitencourt et al., 2013). As well, quercetin was reported to modulate mitochondrial functions by inhibiting organelle enzymes or metabolic pathways (such as oxidative phosphorylation), by altering the production of mitochondrial ROS and by modulating the activity of transcription factors which regulate the expression of mitochondrial proteins. Quercetin also displayed both pro- and antioxidant activities and pro-apoptotic activity, mediated by the capability to directly cause the release of cytochrome c from mitochondria or indirectly by upregulating the expression of pro-apoptotic proteins of Bcl-2 family and down regulating antiapoptotic proteins. Interestingly, these effects are particularly evident on proliferating cancer cells and can have important therapeutic implications (Gibellini et al., 2015). The crystallographic data accompanied by kinetic analysis of the inhibition mechanisms for quercetin showed that this compound could inhibit nucleic acid synthesis in filamentous fungus *Cochliobolus lunatus* through the inhibition of a distinct binding mode defining the interaction between 17 $\beta$ -HSDcI and

quercetin (Cassetta et al., 2017; Aboody and Mickymaray, 2020). The acute toxicity study of the crude leaf extract of *M. oppositifolius* showed no noticeable variation in behavioral parameters. Besides, the monitoring of the animals over 7 days showed no recorded deaths.

## Conclusion

Overall, the antifungal activity of *Mallotus oppositifolius*, extract and derived compounds and toxicological profile eventually support its traditional use to treat skin fungal infections in Cameroon. The recorded data further support detailed pharmacological and toxicological investigations of the tested extracts as potential alternatives in the therapy of pathogenic fungi infections.

## COMPETING INTERESTS

The authors declare that they have no competing interest.

## AUTHORS' CONTRIBUTIONS

VG and FFB designed the study, VG, VFDD, EZM, and PVTf carried out the experiments and wrote the manuscript; GWF and BTN performed spectroscopic analyses and elucidate the structure of isolated compounds.

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