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# Antifungal Activity and Acute Toxicity of the Methanolic Crude Extract and Fractions of *Croton zambesicus* Muell. Arg. (Euphorbiaceae)

**Vincent Ngouana • Thierry Ngouana Kammalac • Patrick Valère Tsouh Fokou • Valerie Flore Donkeng Donfack • Fabrice Fekam Boyom* • Paul Henri Amvam Zollo**

Laboratory of Phytobiochemistry and Medicinal Plants Study, Faculty of Science, University of Yaoundé 1, P.O. Box 812, Yaoundé, Cameroon

*Corresponding author*: * **ffefe@yahoo.com**

## ABSTRACT

Fungal infections are increasingly a public health concern in the developing World. Due to drug resistance, high cost and side effects of available drugs, the development of new antifungals is an urgent issue. The aim of this study was to evaluate the antifungal activity and the acute toxicity of the stem bark of *Croton zambesicus*. The methanolic crude extract was fractionated by flash chromatography. After a phytochemical screening of the crude extract and fractions, their antifungal activity was assessed on three yeasts (*Candida albicans*, *Candida krusei*, *Candida glabrata*) and three dermatophytes (*Microsporum langeronii*, *Microsporum gypseum*, *Trichophyton mentagrophytes*). The acute toxicity was evaluated on Wistar male and female rats, aged about 8 weeks. The phytochemical screening revealed the presence of alkaloids, phenols, flavonoids, saponins, tanins and anthraquinones. The crude extract and fractions were found to be active on all fungal strains, with MIC values ranging from 0.048 to 0.195 mg/ml for yeasts and 3.125 to 6.250 mg/ml for dermatophytes. Almost all the fractions showed fungicidal action against the dermatophytes. No death was recorded up to a dose of 12 g/kg, showing that the crude extract is less toxic according to the WHO standards. The results achieved confirm the traditional use of *C. zambesicus* against fungal infections.

**Keywords:** biological activity, *Croton zambesicus*, fungi, toxicity
**Abbreviations: DMSO**, dimethyl sulfoxide; **MIC**, minimal inhibitory concentration; **MFC**, minimal fungicidal concentration

## INTRODUCTION

Dermatophytes include the *Microsporum*, *Trichophyton* and *Epidermophyton* genera that cause cutaneous mycoses. Yeasts like *Candida* species and other filamentous fungi (*Aspergillus* spp.) are responsible for subcutaneous and invasive fungal infections. It has been reported that superficial and invasive mycoses are increasing since the last two decades, and constitute a major type of infections encountered in immunocompromised and diabetic persons and are classified at the fourth range of nosocomial infections (Sylvie 2003; Bouguerra *et al.* 2004; Chabasse *et al.* 2004; Barchiesi *et al*. 2006). The development of resistance to drug by pathogens and toxic side effects of available antifungal therapies (Ghannoum and Rice 1999; Sanglard and Odds 2002; Bouguerra *et al.* 2004; Chabasse *et al.* 2004; Barchiesi *et al*. 2006; Thiel 2007; Azor 2007; Martinez-Rossi *et al*. 2008; Perlin 2009) have emphasized the search for new efficient and non-toxic antifungal drugs. Plants are a good source of antimicrobial agents (Adjanohoun *et al.* 1996; Facheux *et al.* 2003). Among them, *Croton zambesicus*, a Guineo-Congolese species of the large family of Euphorbiaceae, is used by traditional healers in the treatment of many infections. Its antidiabetic, vasorelaxant, antimalarial, antiulcer and anti-convulsive activities have been demonstrated (Ngadjui *et al.* 2002; Okokon *et al*. 2005, 2006; Baccelli *et al.* 2007; Okokon and Nwafor 2009). The petroleum ether extract of leaves has an antifungal activity (Abo *et al.* 1999) and the alkaloidal fraction of leaves' ethanolic extract inhibits *Aspergillus* and *Microsporum* species (Block *et al.* 2004). Essential oils of the bark, leaves and roots were analyzed and contain terpenoids (Boyom *et al.* 2002). We report in this paper, the antifungal activity and acute toxicity of the stem bark of *C. zambesicus*.

## MATERIALS AND METHODS

### Plant material

The stem bark of *C. zambesicus* was collected at Mount Eloumdem around Yaoundé Cameroon on January 04, 2008 and authenticated at the National Herbarium of Cameroon (Yaoundé) were a voucher specimen was deposited under the reference number 8204/SFR/CAM.

### Extraction and fractionation

500 g of dried-powdered plant material were macerated with 98% methanol at room temperature for 48 h. After filtration and concentration under reduced pressure, using a rotary evaporator HEIDOPH WB 2000, the obtained crude extract was further fractionated by flash chromatography over silica gel, using hexane (Hex), ethyl acetate (EtOAc) and methanol (MeOH) solvent systems. The crude extract and subsequent fractions were used for antifungal and phytochemical screening.

### Phytochemical screening

The crude extract and fractions were subjected to qualitative phytochemical screening for the presence of alkaloids, phenols, flavonoids, triterpenoids, saponines, anthraquinones, tannins, anthocyanins, coumarins, essential oils, steroids and lipids according to Harborne (1976) and Odeyibi and Sofowora (1978).

### Antifungal tests

The fungal strains used in this study were obtained from the "Centre Pasteur du Cameroun", Yaoundé.

Inhibition of yeasts by the crude extract and fractions was as-

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Research Note

sessed by the agar well diffusion method (Ngono *et al*. 2000) and MIC values were determined by the broth dilution methods (Berghe and Vlietnick 1991). Percentages of inhibition and MIC values were determined on dermatophytes by the food poisoning technique. MFC values were determined after sub-culturing the fungi.

## Agar well diffusion method

Sterilized culture medium was poured on 90 cm Petri dishes. After solidification, an inoculum of yeasts strains standardized at 2.5 × $10^5$ CFU/ml on Malassiez cell was spread on the solid medium. After a pre-incubation time of 15 min, wells were hollowed and 100 µl of the crude extract (50 mg/ml), fractions (25 mg/ml), positive control Amphotericine B (Sigma-Aldrich) (100 µg/ml) were individually introduced in separate wells and in triplicate. Inhibition zone diameters were measured after 48 h of incubation at 37°C.

## Microdilution assay

The MIC values were determined using a microdilution method in 96 multi-wells microtiter plates, as previously described (Sarker *et al.* 2007), with slight modifications. The stock solutions of extracts were first diluted to the highest concentration to be tested, and thereafter diluted following a two-fold factor, using the nutrient broth, and 2.5% phenol red indicator The final concentrations were 25 to 0.024 mg/ml for the crude extract and 12.5 to 0.012 mg/ml for fractions. Finally, 10 µl of standardized fungal suspension was added to each well to obtain inoculums of 2.0 × $10^4$ CFU/ml. Amphotericine B was used as positive control and 10% DMSO as negative control. Plates were incubated in triplicate at 37°C for 48 h. MIC values were evaluated as the lowest concentration at which color change from red to yellow occurred.

## Food poisoning assay

The antidermatophytic activity was assessed according to the agar dilution method (Favel *et al.* 1994) on SDA. Stock solutions in 10% DMSO (50 and 25 mg/ml for crude extract and fractions respectively) were incorporated into the growth medium and serially two-fold diluted and allowed to solidify. The resulting concentrations ranged from 50 to 3.125 mg/ml for the crude extract and 25 to 1.562 mg/ml for fractions. The so-prepared dishes were inoculated in triplicate with 7 days-old dermatophyte explants of 6 mm in diameter and incubated for 10 days at 30°C. Percentages of inhibition were determined as previously described (Ajaiyeoba *et al.* 1998). The MIC values defined as the lowest concentrations that show no visible fungal growth after the incubation time was recorded.

## Subculture

Subculture was performed on non supplemented medium for 10 days using dishes where no visible growth was observed. The lowest concentration at which no growth was observed was defined as MFC. The MFC/MIC ratio was calculated to determine the type of activity exhibited by the considered extract.

## Acute toxicity

Acute toxicity was assessed according to the WHO (2000) guidelines.

Male and female albino Wistar rats weighing about 140 g and aged around 8 weeks from the animal house of the Laboratory of Toxicology and Pharmacology, Faculty of Science, University of Yaoundé 1 were used for the study. They were grouped into 5 animals per cage for each sex with free access to food and water and acclimatized for 7 days prior to the experiment. Doses of 4, 8, and 12 g/kg body weight of crude extract were orally administered. Control group was given distilled water. The animals were observed after the 2nd, the 24th and the 48th hour for any toxic symptoms or death.

**Table 1** Results of the phytochemical screening of the crude extract and fractions.

| | $^{g}C_b$ | $^{h}C_1$ | $^{i}C_2$ | $^{j}C_3$ | $^{k}C_4$ | $^{l}C_5$ | $^{m}C_6$ | $^{n}C_7$ | $^{o}C_8$ | $^{p}C_9$ |
|---|---|---|---|---|---|---|---|---|---|---|
| Alkaloids | + | 0 | - | - | + | + | + | + | - | + |
| Phenol | + | 0 | + | + | + | + | + | + | + | + |
| Flavonoides | + | 0 | + | + | + | - | + | - | + | + |
| Triterpenoids | - | 0 | - | - | - | - | - | - | - | - |
| Saponines | + | 0 | - | - | - | - | - | + | + | + |
| Anthraquinones | + | 0 | + | + | + | + | + | + | + | + |
| Tanins | + | 0 | - | - | + | + | - | + | - | - |
| Anthocyanes | + | 0 | - | - | + | + | - | + | - | - |
| Coumarins | - | 0 | - | - | - | - | - | - | - | - |
| Essential oil | + | 0 | + | + | - | - | - | - | - | - |
| Steroids | + | 0 | + | + | - | - | - | - | - | - |
| Lipids | + | 0 | + | + | - | - | - | - | - | - |

(+): present, (-): absent, 0: not evaluated. g: crude extract, h: 100% Hex fraction, i: 25% Hex-EtOAc fraction, j: 50% Hex-EtOAc fraction, k: 75% Hex-EtOAc fraction, l: 100% EtOAc fraction, m: 5% EtOAc-MeOH fraction, n: 10% EtOAc-MeOH fraction, o: 15% EtOAc-MeOH fraction, p: 100% MeOH fraction

## Statistical analyses

The results are presented as means ± SD. Data were analyzed using the SPSS 10.1 software for Windows. The mean values were compared using student's *t*-test at $P < 0.05$.

# RESULTS

## Phytochemical screening

The phytochemical analysis showed the presence of alkaloids, phenols, flavonoids, saponines, anthraquinones, tannins, anthocyanins, essential oils, steroids and lipids, and the absence of triterpenoids and coumarins. Phenols and anthraquinones were concurrently found in the crude extract and fractions (**Table 1**).

## Antifungal activity

The antifungal activity parameters of *C. zambesicus* extracts are summarized in **Tables 2**, **3**, **4** and **5**, in which the inhibition zone diameter, MIC, and MFC are presented. The crude extract and almost all the fractions were found to exhibit antifungal activity on dermatophytes and yeasts. They showed a broad range of inhibition zone diameters ranging from 0 to 24 mm (**Table 2**), the crude extract possessing the highest inhibition zone diameter on *C. albicans* (24.0 ± 0.6 mm), followed by fractions $C_3$ (18.0 ± 0.3 mm on *C. albicans*), $C_4$ (18.0 ± 0.6 mm on *C. krusei*) and $C_8$ (19.0 ± 1.1 mm on *C. glabrata*). Amphotericin B showed inhibition zone diameters ranging from 21 to 23 mm on all the yeasts. On the other hand, *T. mentagrophytes*, *M. gypseum* and *M. langeronii* were susceptible to the crude extract (50 mg/ml) and fractions (25 mg/ml) with 100% inhibition. MIC values for the crude extract and fractions were found to range from 0.048 to 1.562 mg/ml on yeasts. The crude extract was the most active on *C. albicans* (MIC = 0.048 mg/ml). It showed less potency on dermatophytes with MIC value of 12.5 mg/ml, compared to the fractions that showed MIC values ranging from 3.125 to 6.25 mg/ml (**Table 3**).

Subculture permitted the evaluation of the MFC values (**Table 4**) for active extracts. Moreover, the crude extract and fractions were fungicidal on almost all tested dermatophytes (**Table 5**).

## Acute toxicity

The oral administration of a single dose varying from 4-12 g/kg in acute toxicity study showed no toxicity signs or death of animals after 48 h. The oral $LD_{50}$ value was considered to be above 12 g/kg in rats.

**Table 2** Inhibition zone diameters (mm) of crude extract and fractions on yeasts strains.

| | $^{g}C_b$ | $^{h}C_1$ | $^{j}C_3$ | $^{k}C_4$ | $^{l}C_5$ | $^{m}C_6$ | $^{n}C_7$ | $^{o}C_8$ | $^{p}C_9$ | $^{q}A$ |
|---|---|---|---|---|---|---|---|---|---|---|
| *C. albicans* | $24.0 \pm 0.6^{a}$ | $0 \pm 0^{f}$ | $18.0 \pm 0.3^{b}$ | $10.0 \pm 0.8^{d}$ | $10.0 \pm 1.2^{d}$ | $12.0 \pm 0.6^{d}$ | $0 \pm 0^{f}$ | $11.0 \pm 1.1^{d}$ | $0 \pm 0^{f}$ | $23.0 \pm 1.0^{a}$ |
| *C. krusei* | $15.0 \pm 0.5^{c}$ | $9.0 \pm 0.6^{e}$ | $12.0 \pm 1.2^{d}$ | $18.0 \pm 0.6^{b}$ | $15.0 \pm 0.5^{c}$ | $12.0 \pm 0^{d}$ | $9.0 \pm 0.3^{e}$ | $16.0 \pm 0.6^{c}$ | $14.0 \pm 0.8^{c}$ | $22.0 \pm 1.0^{a}$ |
| *C. glabrata* | $0 \pm 0^{f}$ | $15.0 \pm 1.0^{c}$ | $16.0 \pm 0.8^{c}$ | $18.0 \pm 1.1^{b}$ | $10.0 \pm 0.6^{d}$ | $0 \pm 0^{f}$ | $12.0 \pm 1.2^{d}$ | $19.0 \pm 1.1^{b}$ | $11.0 \pm 1.0^{d}$ | $21.0 \pm 1.0^{a}$ |

Value express in mean ± SD in mm**;** g: crude extract, h: 100% Hex fraction, j: 50% Hex- EtOAc fraction, k: 75% Hex- EtOAc fraction, l: 100% EtOAc fraction, m: 5% EtOAc -MeOH fraction, n: 10% EtOAc -MeOH fraction, o: 15% EtOAc -MeOH fraction, p: 100% MeOH fraction, q: Amphothericine B. a, b, c, d, e and f connect values that are not significantly different according to the Student's *t*-test ($P < 0,05$)

**Table 3** MIC values of the crude extract and fractions of *C. zambesicus* on the tested fungi (mg/ml).

| | $^{g}C_b$ | $^{h}C_1$ | $^{i}C_2$ | $^{j}C_3$ | $^{k}C_4$ | $^{l}C_5$ | $^{m}C_6$ | $^{n}C_7$ | $^{o}C_8$ | $^{p}C_9$ | $^{q}A$ |
|---|---|---|---|---|---|---|---|---|---|---|---|
| *C. albicans* | 0.048 | ND | ND | 0,390 | 0.781 | 1.562 | 0,781 | ND | 0.781 | ND | 0.002 |
| *C. krusei* | 0.195 | ND | ND | 0.781 | 0.195 | 0.390 | 0.781 | ND | 0.097 | 0.195 | 0.002 |
| *C. glabrata* | ND | ND | 1.562 | 0.390 | 0.390 | 1.562 | ND | 0.781 | 0.195 | 0.781 | 0.002 |
| *M. langeroinii* | 12.500 | ND | 6.250 | 6.250 | 6.250 | 6.250 | 6.250 | 6.250 | 6.250 | 6.250 | 0.003 |
| *M. gypseum* | 12.500 | ND | 6.250 | 3.125 | 6.250 | 3.125 | 3.125 | 3.125 | 3.125 | ND | 0.003 |
| *T. mentagrophytes* | 12.500 | ND | 3.125 | 6.250 | 6.250 | 3.125 | 3.125 | 3.125 | 6.250 | 3.125 | 0.003 |

ND: not determined. g: crude extract, h: 100% Hex fraction, i: 25% Hex- EtOAc fraction, j: 50% Hex- EtOAc fraction, k: 75% Hex- EtOAc fraction, l: 100% EtOAc fraction, m: 5% EtOAc -MeOH fraction, n: 10% EtOAc -MeOH fraction, o: 15% EtOAc -MeOH fraction, p: 100% MeOH fraction, q: Amphothericine B

**Table 4** MFC values of the crude extract and fractions of *C. zambesicus* on the tested fungi (mg/ml).

| | $^{g}C_b$ | $^{h}C_1$ | $^{i}C_2$ | $^{j}C_3$ | $^{k}C_4$ | $^{l}C_5$ | $^{m}C_6$ | $^{n}C_7$ | $^{o}C_8$ | $^{p}C_9$ | $^{q}A$ |
|---|---|---|---|---|---|---|---|---|---|---|---|
| *C. albicans* | 0.195 | ND | ND | 0.781 | 3.125 | >12.500 | 6.250 | ND | 6.250 | ND | 0.002 |
| *C. krusei* | 1.562 | ND | ND | 6.250 | 1.562 | 0.781 | 6.250 | ND | 0.781 | 0.781 | 0.002 |
| *C. glabrata* | N D | ND | 6.250 | 1.562 | 0.781 | 12.500 | ND | 3.125 | 1.562 | 6.250 | 0.002 |
| *M. langeroinii* | 50 | ND | 50 | 25 | 25 | 6.250 | 12.500 | 12.500 | 25 | 25 | 0.003 |
| *M. gypseum* | 25 | ND | 6.250 | 25 | 6.250 | 12.500 | 12.500 | 12.500 | 12.500 | ND | 0.003 |
| *T. mentagrophytes* | 12.500 | ND | 3.125 | 6.250 | 6.250 | 3.125 | 3.125 | 3.125 | 6.250 | 3.125 | 0.003 |

ND: not determined. g: crude extract, h: 100% Hex fraction, i: 25% Hex- EtOAc fraction, j: 50% Hex- EtOAc fraction, k: 75% Hex- EtOAc fraction, l: 100% EtOAc fraction, m: 5% EtOAc -MeOH fraction, n: 10% EtOAc -MeOH fraction, o: 15% EtOAc -MeOH fraction, p: 100% MeOH fraction, q: Amphothericine B

**Table 5** MFC/MIC ratio of the crude extract and fractions of *C. zambesicus* on the tested fungi.

| | $^{g}C_b$ | $^{h}C_1$ | $^{i}C_2$ | $^{j}C_3$ | $^{k}C_4$ | $^{l}C_5$ | $^{m}C_6$ | $^{n}C_7$ | $^{o}C_8$ | $^{p}C_9$ | $^{q}A$ |
|---|---|---|---|---|---|---|---|---|---|---|---|
| *C. albicans* | 4 | ND | ND | 2 | 4 | ND | 8 | ND | 8 | ND | 1 |
| *C. krusei* | 8 | ND | ND | 8 | 8 | 2 | 8 | ND | 8 | 4 | 1 |
| *C. glabrata* | ND | ND | 4 | 4 | 2 | 8 | ND | 4 | 8 | 8 | 1 |
| *M. langeroinii* | 4 | ND | 8 | 4 | 4 | 1 | 2 | 2 | 4 | 4 | 1 |
| *M. gypseum* | 2 | ND | 1 | 8 | 1 | 4 | 4 | 4 | 4 | ND | 1 |
| *T. mentagrophytes* | 1 | ND | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 |

ND: not determined. g: crude extract, h: 100% Hex fraction, i: 25% Hex- EtOAc fraction, j: 50% Hex- EtOAc fraction, k: 75% Hex- EtOAc fraction, l: 100% EtOAc fraction, m: 5% EtOAc -MeOH fraction, n: 10% EtOAc -MeOH fraction, o: 15% EtOAc -MeOH fraction, p: 100% MeOH fraction, q: Amphothericine B

## DISCUSSION

### Phytochemical screening and antifungal activity

The phytochemical screening of the methanolic stem bark extracts of *C. zambesicus* revealed the presence of metabolites such as Phenols, flavonoids, anthraquinones, tannins, saponines, steroids and alkaloids. In a previous study, Datsu *et al*. (2009) have identified cardiac glycosides, flavonoids, terpenes and steroids in the ethyl acetate extract of the stem bark of the same plant. More recently, Okokon and Nwafor (2010) have analyzed the ethanolic extract of the roots and identified saponines, alkaloids, terpenes, cardiac glycosides, anthraquinones and noted a differential distribution of these metabolites in the fractions.

The antifungal activity exerted by the crude stem bark extract of *C. zambesicus* and fractions highlights its potential as a source of antifungal compounds. Previous findings have highlighted varying effects of extracts from *C. zambesicus* and elsewhere as antimicrobials (Ajaiyeoba *et al.* 1998; Abo *et al.* 1999; Adekunle and Ikunimapayi 2006; Ajayi and Akintola 2007; Reuben *et al.* 2008; Mohamed *et al.* 2009; Okokon and Nwafor 2010). In this study, fractions $C_3$, $C_4$ and $C_8$ showed to contain alkaloids, steroids, tannins, flavonoids, anthraquinones, essential oils, saponines or phenols at varying extents, but exhibited potencies against yeasts and dermatophytes.

Given that some of the above mentioned metabolites possess antimicrobial activities (Abo *et al*. 1999; Nwaogu *et al*. 2007; Datsu *et al*. 2009), their presence in *C. zambesicus* extracts may also elicit the observed antifungal activity. This activity may also be the result of synergistic interactions amongst the components. Of note, alkaloids, phenols, tannins and flavonoids have been shown to inhibit cell wall formation in fungi leading to the death of the organism. In addition, tannins can inhibit the growth of microorganisms by coagulating the protoplasm (Onodapo and Owonubi 1993; Barapedjo and Bunchoo 1995; Zacchino *et al.* 1998; Abo *et al.* 1999; Adekunle and Ikunimapayi 2006; Tapa *et al*. 2006; Oh *et al*. 2008; Effiong and Sanni 2009).

### Acute toxicity

The $LD_{50}$ of *C. zambesicus* crude extract was found to be above the dose of 12 g/kg, indicating it as less toxic orally ($LD_{50} > 5$ g/kg; Hodgson 2004), compared to the finding of Okokon and Nwafor (2008) who found a $LD_{50}$ of 273.86 mg/kg for the root extract of *C. zambesicus*.

## CONCLUSION

The results achieved from the current investigation clearly indicate that the antifungal activity of *C. zambesicus* vary with the fungi species and support a good correlation with the reported traditional medical uses of this plant as treatment for fungal infections. However, further investigation is required to purify the active principles and determine their role in the antifungal activity.

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