

The Anti-inflammatory Property of *Mitracarpus villosus* (Sw.) DC. Extract is Responsible for its use in the Treatment of Ring Worm Infection

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ABSTRACT

Background: Microbial pathogens continuously mutate in order to survive the activities of new antimicrobials. This is the reason new antimicrobials are easily rendered useless after introduction. Continuous search for new antimicrobial agents is therefore an imperative until a permanent solution is found. Natural products have contributed immensely to the development of new drugs. The extract of *Mitracarpus villosus* (Sw.) DC., family: Rubiaceae, which is used traditionally for topical treatment of eczema and ring worm infections is a potential source of new antimicrobial compounds. **Method:** The methanol-methyl chloride extracts of the whole plant and leaves were partitioned into n-hexane, ethyl acetate, n-butanol and aqueous fractions. Both the crude and fractions were tested for antifungal and anti-inflammatory properties. The cytotoxicity property of the leaf extract was also investigated. **Results:** Results showed that the whole plant extract had no bioactivity. The aqueous fraction also showed no activity while the n-butanol fraction showed considerable anti-inflammatory activity. There was no antifungal activity in both crude and fractions. The leaf extract and fractions also showed no antifungal properties but exhibited strong anti-inflammatory properties. *M. villosus* is used mainly for the treatment of ring worm which is a fungal infection. **Conclusion:** Based on the absence of *in vitro* antifungal properties in the entire specimen tested, we propose that the effectiveness of the herb as an anti-fungal agent is derived from its anti-inflammatory property.

Key words: *Mitracarpus villosus*, Antifungal, Anti-inflammatory, COX, PGE₂, Prostaglandins.

INTRODUCTION

The continuous emergence of resistant genes in pathogenic organism has posed the greatest challenge to the efficacy of the current battery of antimicrobial agents and this has made the continuous search for new antibiotics with different mechanisms of action an imperative.¹ Plant extracts and secondary metabolites from plants have been used as antimicrobial remedies and a good number of medicines used in orthodox therapy are derived from them. *M. villosus* (Sw.) DC., (English name: girdle pod) family: Rubiaceae is one of such plants. It is popularly known as “ogwu-ngwo” or “obinezi” by the Igbos of Nigeria due to its ethnomedicinal use as an antifungal agent used for the treatment of ringworm and eczema.² It is also used ethnomedicinally for the treatment of sore throat, headaches, toothaches, amenorrhoea, dyspepsia, hepatic disease, venereal diseases and leprosy.³ Phytochemical screening has shown that the leaves of *M. villosus* contain alkaloids; glycosides, flavonoids, tannins, saponins, phenols, essential oils, cardiac glycosides, steroid, triterpenoids⁴ most of which have intrinsic bioactivity.

Previous reports from antimicrobial sensitivity testing of the leaf extract suggested that it possesses antibacterial⁵ and antifungal properties.⁶ However, the high minimum inhibitory concentrations reported in those reports do not adequately justify the efficacy of this herb as an antifungal and antibacterial agent. In this work, methanol: methyl chloride extract of the whole herb and its fractions as well as the crude extracts and fractions of the leaves were evaluated for their anti-inflammatory and antifungal properties. We propose for the first time the possible anti-inflammatory mechanism of antifungal action of *M. villosus*.

MATERIALS AND METHODS

Sample collection, identification and preparation

M. villosus (whole plant) was collected in December 2014 from Agulu in Anambra State, Nigeria. The geographical location of Agulu is 6°07'N 7°04'E in Nigeria. The sample was identified by Mrs Antho-

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nia Emezie of the Department of Pharmacognosy, UNIZIK, Awka and was given voucher number PCG00556. It was deposited at the Faculty of Pharmaceutical Sciences' Herbarium. The specimen was air dried at room temperature and pulverized to fine-sized particles using a locally fabricated laboratory mill.

Chemicals and reagents

Analytical grades of methanol, n-hexane, ethyl acetate, methyl chloride, chloroform (JHD, China) were used for extraction and fractionation. Other chemicals used were xylene and Tween 80 (BDH, England). All laboratory reagents were freshly prepared and freshly distilled water was used when required.

Culture media and other reagents used in the microbiological analyses

Sabouraud dextrose agar (Oxoid, UK), and dimethyl sulfoxide (DMSO) (Uvasol Merck) were used for the antifungal assay. McFarland turbidity standards were prepared from barium chloride, sulfuric acid and distilled water.

Test Animals

Adult albino mice of both sexes (8 weeks old) were obtained from the laboratory animal facility of the Department of Pharmacology and Toxicology, Nnamdi Azikiwe University, Awka, Nigeria. They were fed standard mice pellet diet (Ladokun Farms, Nigeria) and water *ad libitum*; and were observed under 12 h light/dark cycles in metabolic cages in a well-ventilated rodent cubicle. The animals were handled according to the guidelines approved by the Ethical Committee on animal research, Nnamdi Azikiwe University, Awka, Nigeria with protocol number 112/2014. The animals were randomly placed into various experimental groups.

Test Microorganisms

The microorganisms used in this study were *Aspergillus niger*, *Candida albicans*, and two *Dermatophytes* (*Tinea spp.*). These were clinical isolates obtained from the Pharmaceutical microbiology laboratory of Nnamdi Azikiwe University.

Extraction

Whole Plant: Exactly 1.5 kg of the pulverized whole plant specimen was extracted over 48 h with 5 L of methanol: methyl chloride (2:1) combination. The solution was filtered using Buckner funnel and concentrated to dryness under vacuum at 50°C using rotary evaporator. The crude extracts were stored at 4°C until needed.

Leaf extract: Exactly 900 g of the pulverized leaf specimen was extracted over 48 h with 3 L of methanol: methyl chloride (2:1) combination. The solution was filtered using Buckner funnel and concentrated to dryness under vacuum at 50°C using rotary evaporator. The crude extract was stored at 4°C until needed.

Liquid-Liquid Fractionation

Whole Plant: The crude methanol/methyl chloride (170 g) was dispersed in 250 mL of distilled water and subjected to liquid-liquid fractionation using 250 mL each of n-hexane, ethyl acetate and n-butanol in increasing order of polarity. The dispersion was extracted three times with each of the organic solvents. All of the fractions so obtained were filtered twice using Whatman No.1 filter paper in a Buckner funnel. A rotary evaporator was used to concentrate the fractions at 45°C. The fractions obtained were stored at 4°C until used.

Leaf: The crude methanol/methyl chloride (52 g) was dispersed in 250 mL of distilled water and subjected to liquid-liquid fractionation using

250 mL each of n-hexane, ethyl acetate and n-butanol in increasing order of polarities. The dispersion was extracted three times with each of the organic solvents. All of the fractions so obtained were filtered twice using Whatman No.1 filter paper in a Buckner funnel. A rotary evaporator was used to concentrate the fractions at 45°C. The fractions obtained were stored at 4°C until used.

High Pressure Liquid Chromatography Experiment

The liquid-liquid fractions were further subjected to HPLC analysis. Exactly 1 mg/mL of the fractions was dissolved in HPLC grade methanol, sonicated for 10 min and centrifuged at 3000 rpm for 5 minutes. A 1:5 serial dilution was carried to get 0.2 mg/mL solution and 20 µL of this solution analysed in Dionex HPLC system equipped with photodiode array detector (UVD340s, Dionex Softron GmbH, Germany) using 125 mm Eurospher-10 C18 prefilled column (Knauer, Germany) with 4 mm internal diameter and 5 µm particle size. The mobile phase comprised of nano-pure water adjusted to pH 2 with formic acid and methanol. Separation was monitored at 254 nm and peaks were identified by dereplication.

Antifungal Assay

The antifungal assay of each of the crude extracts and their respective fractions was carried out on Sabouraud dextrose agar using the agar well diffusion assay as described by Portillo and co-workers.⁷ The antifungal sensitivity testing was carried out against four fungi isolates as listed above. A 0.5 McFarl and standard suspension of each of the test isolates was prepared as described by Jain *et al.*⁸ and these formed the fungi stock solutions used in the agar well diffusion assays. The test doses were obtained by 2-fold serial dilution. For the whole plant assay, the doses were 50, 25, 12.5, 6.25, and 3.125 mg/mL while the doses for the leaf assay were 1, 0.5, 0.25, 0.125, 0.0625 and 0.03125 mg/mL. The cultures were incubated at 27°C for 48hrs for the yeasts and 7 days for the dermatophytes.

Evaluation of the Anti-inflammatory Activity of the Extracts and Fractions

Whole Plant: The effect of methanol/methyl chloride extract of *M. villosus* on topical inflammation was evaluated using the method of Atta and Alkahafi.⁹ Adult Albino mice of either sex were divided into seven groups with ten animals in five groups each and five animals each in the remaining two groups. Group 1 received crude extract, group 2 received butanol fractions, group 3 received n-hexane fractions, group 4 received aqueous fractions, group 5 received ethyl acetate fractions (EAC), group 6 received diclofenac (the positive control) while group 7 received the vehicle (negative control).

The ten animals in groups 1-5 were divided into subgroups of 5 animals each. Subgroups 1 and 2 of each group respectively received 500 µg and 250 µg/ear of the appropriate extract or fraction. The treatments were applied to the anterior surface of the right ear while positive control was applied at the dose of 250 µg/ear. The negative control group received 0.05 mL of the vehicle. While positive control group received 250 µg/ear of diclofenac. Topical inflammation was instantly induced on the posterior surface of the same ear by application of xylene (0.01 mL).

Two hours after induction of inflammation, the mice were sacrificed by overdose of ether anaesthesia and both ears removed. Circular sections (7 mm diameter) of both the right (treated) and left (untreated) ears were punched out using a cock borer and weighed using a sensitive weighing balance. Oedema was quantified as the weight difference between the two earplugs.

The anti-inflammatory activity was evaluated as percentage oedema reduction/inhibition in the treated animals relative to control animals¹⁰ using the relationship:

$$\text{Oedema inhibition/reduction (\%)} = 1 - \frac{R_t - L_t}{R_c - L_c} \times \frac{100}{1}$$

Where R_t = mean weight of right ear plug of treated animals; L_t = mean weight of left ear plug of treated animals; R_c = mean weight of right ear plug of control animals; L_c = mean weight of left ear plug of control animals.

Microculture Tetrazolium (MTT) Assay

Cytotoxicity tests of the leaf extract was carried out at the laboratory of Prof. Dr. W. E. G. Müller, Institut für Physiologische Chemie und Pathobiochemie, University of Mainz, Mainz, Germany. The cytotoxicity was tested against L5178Y mouse lymphoma cells (ATCC CRL 1722) using the microculture tetrazolium (MTT) assay, and compared to that of untreated controls.¹¹

Cell Cultures

L5178Y mouse lymphoma cells (ATCC CRL 1722) were grown in Eagle's minimal essential medium supplement with 10% horse serum in roller tube culture. The medium contained 100 units/mL penicillin and 100 µg/mL streptomycin. The cells were maintained in a humid atmosphere at 37°C with 5% CO₂.

MTT Colorimetric Assay

Stock solutions in ethanol 96% (v/v) of *M. villosus* leaf extract and fractions were prepared. Exponentially growing cells were harvested, counted and diluted appropriately. Of the cell suspension, 50 µL containing 3750 cells were pipetted into 96-well microtiter plates. Subsequently, 50 µL of a solution of the test samples (10 µg/mL) containing the appropriate concentration was added to each well. The small amount of ethanol present in the wells did not affect the experiments. The test plates were incubated at 37°C with 5% CO₂ for 72 h. A solution of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was prepared at 5 mg/mL in phosphate buffered saline (PBS; 1.5 mM KH₂PO₄, 6.5 mM Na₂HPO₄, 137 mM NaCl, 2.7 mM KCl; pH 7.4) and from this solution, 20 µL was pipetted into each well. The yellow MTT penetrates the healthy living cells and in the presence of mitochondrial dehydrogenases, MTT is transformed to its blue formazan complex. After an incubation period of 3h 45 min at 37°C in a humidified incubator with 5% CO₂, the medium was centrifuged (15 min, 20°C, 210 x g) with 200 µL DMSO, the cells were lysed to liberate the formed formazan product. After thorough mixing, the absorbance was measured at 520 nm using a scanning microtiter-well spectrophotometer. The colour intensity is correlated with the number of healthy living cells.

Cell survival was calculated using the formula:

$$\text{Survival \%} = 100 \times \frac{\text{Absorbance of Treated Cells} - \text{Absorbance of Culture Medium}}{\text{Absorbance of untreated Cells} - \text{Absorbance of Culture Medium}}$$

All experiments were carried out in triplicates and repeated three times. As controls, media with 0.1% EGMME/DMSO were included in the experiments.

Statistical Analysis

Results were analysed by SPSS Version 16.0 using Student's *t*-tests and expressed as mean ± standard error of mean (SEM). Differences between means were considered significant at $P < 0.05$.

RESULTS

The extract and fractions of *M. villosus* whole plant were subjected to antifungal and anti-inflammatory assays. Also, the leaf extract and fractions were investigated for anti-inflammatory, cytotoxic and antifungal properties.

Whole Plant: Results (crude whole plant extract) showed no antifungal and anti-inflammatory properties (Table 1 and Figure 1). Similarly, no antifungal activity was detected in any of fractions of the whole plant (Table 1). Conversely, Figure 1 shows the percentage inhibition of oedema formation exhibited by the n-butanol fraction which was almost twice the value exhibited by the positive control at 250 µg/mL (diclofenac). This indicates a potent anti-inflammatory activity from the butanol fraction.

Leaf Extract: The results of the antifungal and anti-inflammatory assays of the leaf extract and fractions are shown in Table 2 and Figure 2 respectively.

Again, the crude extract as well as the fractions showed no antifungal activity corroborating the results obtained earlier with the whole plant extract. This further confirms that the extract has no direct fungistatic or fungicidal properties.

The anti-inflammatory activity of the crude leaf extract was profound (Figure 2) in contrast to the total absence of anti-inflammatory activity observed in the crude whole plant extract (Figure 1).

Furthermore, the butanol and ethyl acetate fractions had anti-inflammatory activities that were better than those observed for diclofenac and indomethacin used as the positive controls in the studies.

The result of the cytotoxicity assay of the leaf extract and fractions showed inhibition of mouse lymphoma cells not more than 25% at the tested concentration (10 µg/mL).

The HPLC analysis of the extract showed three major peaks and numerous other smaller peaks (Figure 3).

DISCUSSION

There was no antifungal activity in any of the samples tested. The absence of antifungal activity in both the crude extracts and fractions suggests that although the plant was effective in the treatment of fungal infections, the extracts has no *in vitro* antifungal activity which contradicts previous reports of antifungal property from *M. villosus* extract.⁶ The absence of *in vitro* antifungal property of the plant was however corroborated by Nwosu and Okafor¹² who reported no activity even at high concentration. On the other hand, a strong anti-inflammatory activity

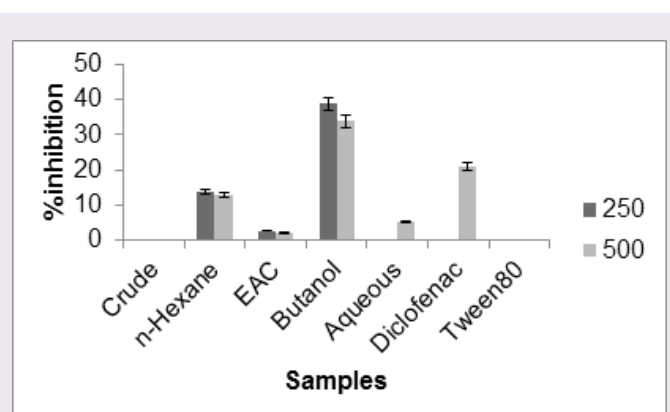


Figure 1. Percentage oedema inhibition of whole plant extract and fractions of *Mitracarpus villosus*.

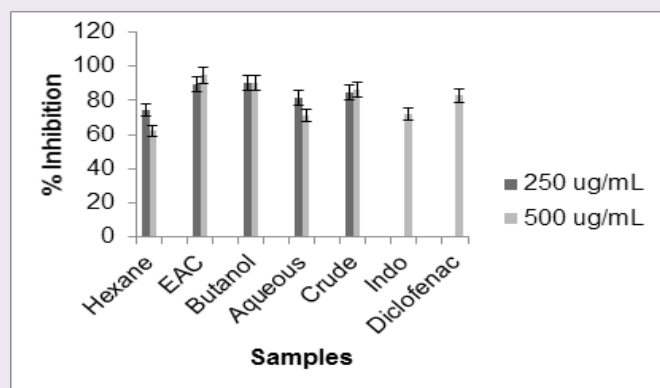


Figure 2. Showing the result of anti-inflammatory properties crude and fractions of *M. villosus* leaf extract.

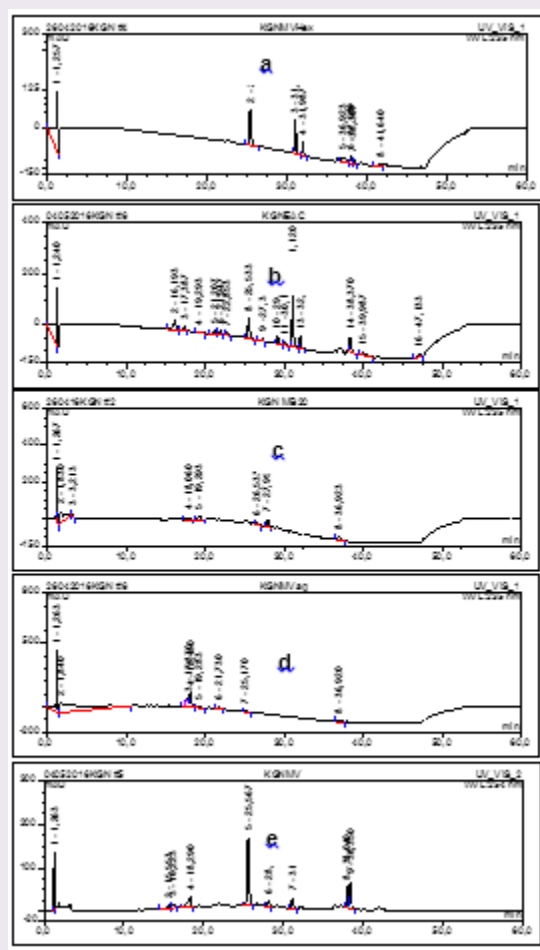


Figure 3. Showing the chromatograms of the (a) n-hexane, (b) ethylacetate, (c) n-butanol, (d) aqueous fractions respectively and (e) crude extract of *Mitracarpus villosus*.

was observed in the whole plant fractions. In traditional practice, only the leaves of *M. villosus* are majorly used for the treatment of fungal infections suggesting that the absence of anti-inflammatory activity in the whole plant extract could be due to dilution from artefacts from other parts of the plant. Further studies were carried out with the leaves of the plant alone.

Table 1: Antifungal activity of crude extract and fractions of *Mitracarpus villosus* whole plant showing the mean Inhibition Zone Diameters (IZDs)(mm) produced against test organisms.

Test Organisms	Fluconazole (50 µg/mL)	Miconazol (50 µg/mL)	DMSO
<i>C. albicans</i> 1	0	20	0
<i>C. albicans</i> 2	0	18	0
<i>A. niger</i> 1	8	10	0
<i>A. niger</i> 2	9	11	0
<i>T. mentagrophytes</i>	5	8	0
<i>M. audonii</i>	6	8	0

The crude extract and all the fractions recorded no activity; DMSO = Dimethyl sulphoxide.

Table 2: Antifungal activity of the crude extract and fractions of *Mitracarpus villosus* leaf against some selected clinical isolates.

Test organisms	Gentamicin 10 µg/mL	Nystantin 50 µg/mL	Griseofulvin 10 µg/mL
<i>Aspergillus niger</i>	NA	13	NA
<i>Candida albicans</i>	NA	16	NA
<i>Dermatophyte spp</i>	NA	NA	NA
<i>Dermatophyte spp</i>	NA	NA	NA

The crude extract and all the fractions recorded no activity; NA = No activity.

As was reported in the result section above, both the crude leaf extracts and fractions had profound anti-inflammatory activities. In fact, the ethyl acetate and *n*-butanol fractions exhibited profound activities that were better than or similar to those observed for the positive control diclofenac. This is encouraging considering that the extract and fractions are still in the crude state compared to the pure compounds used as the positive control. This confirms that the anti-inflammatory constituent(s) are largely present in the leaf which is used traditionally. The absence of *in vitro* antifungal properties in the leaves suggests that the only possible mechanism of antifungal action is anti-inflammatory activity or derived from cytotoxicity of the extract. Cytotoxicity assay of the leaf extract and fractions showed not more than 25% inhibition of mouse lymphoma cells at 10 µg/mL suggesting that the leaf extract has inconsiderable cytotoxic property. The test carried out at only one dose of 10 µg/mL was a preliminary test that we frequently use to determine if further testing is necessary in our laboratory. A 25% inhibition is not considered encouraging enough for further elaborate investigation.

The anti-inflammatory property of *M. villosus* from other assay methods has been reported¹³ thus establishing the anti-inflammatory property of the leaf extract.

Based on these results, we are strongly convinced that the efficacy of *M. villosus* in the management of inflammatory mycosis is derived mainly from its anti-inflammatory property rather than any direct fungicidal or fungistatic property. Studies have shown that some antifungal agents have inherent anti-inflammatory properties. For instance, Rosen and co-workers reported that some allylamine antifungal preparations and ketoconazole have anti-inflammatory activities that were superior to that of 5% hydrocortisone¹⁴ suggesting that anti-inflammation could be a mechanism of antifungal activity. They however were not sure of the mechanism of anti-inflammatory action. It has been reported that *Candida albicans* use arachidonic acid released during infection as the sole source of carbon and utilize prostaglandin E2 derived from arachidonic acid for its germ tube development.^{15,16} The development of PGE2 involves COX

enzymes. Most anti-inflammatory agents especially the non-steroidal anti-inflammatory agents inhibit the activities of this enzyme. This could be the mechanism of anti-fungal activities of *M. villosus* extract.

Anti-inflammatory agents can only have antifungal effect due to their anti-inflammatory properties if inflammatory processes are involved in fungal colonization which in turn promotes pathogenesis. It has been reported that the extent of fungal colonization in the gut of healthy members of a family with history of Crohn's disease, an inflammatory bowel disease, was significantly higher than was observed in the gut of healthy control from Crohn's disease free families.¹⁷ Another report had it that fungal colonization delayed the healing process of inflammatory lesions.¹⁸ It therefore means that some fungal species depend on the inflammation of surrounding tissues to propagate. This means that any agent that can inhibit inflammation will reduce the rate of propagation and colonization of the invading cells enabling the body defence system to easily cordon off the infected area and kill off the invaders.

This is most likely the case with ring worm which starts with ring-like outline with a small diameter from where it spreads peripherally to surrounding tissues. The diameter and the area of the skin covered gradually increases in size as inflammation rendering the surrounding areas fertile for fungal propagation.

The HPLC-DAD screening could not sufficiently identify any components of the extracts. However, it has shown that the major components are concentrated on the ethyl acetate and butanol fractions which in turn showed best activities. Further work on this extract would be best carried out with ethyl acetate or a mixture of both to enable maximal extraction of the active component.

CONCLUSION

In conclusion, the studies carried out have shown that the extract and fractions of *M. villosus* leaves have strong topical anti-inflammatory activity better than that exhibited by diclofenac with the ethyl acetate and n-butanol fractions showing the best anti-inflammatory potencies.

CONFLICT OF INTEREST

There is no conflict of interest to disclose.

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