

**EFFECTS OF METHYL ACETATE, BUTANOL AND N-HEXANE FRACTIONS OF
TERMINALIA CATAPPA (RED LEAF) EXTRACT ON *ONCHOCERCA VOLVULUS*
BY MODIFIED IN-VITRO BIOASSAY**

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ABSTRACT

Purpose: The study was to determine the effects of various concentrations of Methyl acetate, butanol and N-hexane fractions of *Terminalia catappa* (Tropical Almond) red leaf extract on adult *Onchocerca volvulus*, by modified in-vitro bioassay using the parameters of paralysis and death. This is an observation with fractions of ethyl acetate, n-hexane, and butanol, to determine the polar constituents the more potent fraction.

Method: A total of 252 adult worms (*Onchocerca volvulus*) sourced via nodulectomy were used for the study. The crushed red leaves of *Terminalia catappa* was extracted using 95% ethanol, filtered, and evaporated (Tabassam method). The dried extracts were dissolved in sterile dilutions and prepared in best fraction concentration doses of 20mg/ml and 40mg/ml methanolic *T. catappa* extract. Six labeled beakers were used for the study; six worms assigned per beaker. A positive control beaker contained Albendazole and a negative control beaker, contained saline water. The worms were observed for paralysis and death time as the procedure was repeated three times.

Results: Observation with the fraction doses showed that ethyl acetate is more potent on *O. volvulus* at mean average paralysis time of 122.5mins and death at 181.5mins. Positive control (Albendazole) showed mean average *O. volvulus* paralysis time of 58 mins \pm 8.96mins and death at 93.7 \pm 9.59mins. The average negative control (saline water) showed a mean average paralysis time of *O. volvulus* at 154 \pm 5.97 and death at 235 \pm 4.21mins.

Conclusion is that ethyl acetate fraction is of concentration dependent anthelmintic effects on *Onchocerca volvulus*

Keywords

Terminalia catappa, *Onchocerca volvulus*, Methyl acetate, Butanol, N-hexane,

1. INTRODUCTION

Onchocerciasis is recognized as a major deleterious disease of massive public health and socioeconomic concern^[5]. It is classified as a Neglected Tropical Disease by the World Health Organisation (WHO), with hundreds of thousands of people blind and an increasing number of individuals at risk of more infection. Empirical findings revealed that it is the second leading cause of blindness caused by infection with the *Onchocerca volvulus* nematode^[6]. This disease has ravaged the *Simulium damnosum* (black fly) endemic local communities of South Eastern Nigeria ^[1]. About 99% of cases are found in Africa where 85million people live in endemic areas ^[2]. Nigeria has the highest number of persons with onchocerciasis, accounting for over one-third of the global prevalence ^[3]. According to the records of the African Program on Onchocerciasis Control (APOC), 508 of 779 Local Government Areas in Nigeria have indicators of serious onchocerciasis infection that about 35,210 communities are endemic with onchocerciasis and on the whole, more than 25 million Nigerians are at risk of infection. The Nigerian Federal Ministry of Health surveyed in 1993 - 1994 to determine the level of endemicity nationwide; Imo State in the southeastern region of Nigerian was found to be highly endemic with over 1.1 million persons at risk of getting infected with onchocerciasis^[4].

Herbs have medicinal property due to the presence of different active constituents like alkaloids, volatile essential oils, glycosides, resins, oleoresins, steroids, tannins, terpenes and phenols. Medicinal plants are continuously revolutionizing the face of the earth through all the distinctive benefits they render ^[7]. The World Health Organization (WHO) observed that about 74% of 119 plant-derived pharmaceutical medicines are used in modern medicine. It is also estimated by the ^[5] that 4 billion people (80% of the world population) presently use herbal medicine for health-care purposes. Most of these plants contain potent active compounds that inhibit and cure several ailments ^[7].

Terminalia catappa (Linn) has been investigated in various pharmaceutical studies as it contains a variety of chemical components ^[9]. The plant extracts exhibit anthelmintic as well as biological activities, including antioxidant (punicalagin, punicalin, terfluvina A and B, chebulic acid,

benzoic acid, cumaric, and its derivatives) ^[10]. It also contains anti-inflammatory (triterpenic acids, especially ursolic acid and its derivatives) ^[11], antimicrobial (flavones and flavanols) ^[12] and hepato-protective activities (punicalagin, punicalin), ^[13]. In India, a plaster of *T. catappa* leaves is used to treat scabies, leprosy wounds and other skin diseases ^[12]. Its traditional uses especially in India, the Philippines and Malaysia include the treatment of diarrhoea and fever ^[13]. There have been studies done previously that suggested that the most polar fractions gotten from *T. catappa* leaves are effective against bacteria ^[8] and fungi ^[14].

11. MATERIALS AND METHODS

COLLECTION AND EXTRACTION OF LEAF

Terminalia catappa leaves were collected from the plants naturally grown in Umudurunna Abba, Nwangele LGA, Nigeria. The specimens were collected during the evening period, at the time when the leaves were freely falling off the tree. The leaves were packaged in a bag and taken to the Nnamdi Azikiwe University Agulu campus, Anambra state Nigeria, the next morning for identification at the department of Biological Sciences, of the same University. Afterwards, the *Terminalia catappa* leaves were washed thoroughly in distilled water and a known quantity (850gm) were dried at room temperature for 1 week. The red leaves were ground into fine powder form using clean laboratory motor and pestle. 850g of the pulverized plant sample was macerated in 2.5L of methanol for 48 hours with intermittent shaking. It was filtered using a muslin cloth and further filtered through cellulose filter paper. The filtrate was concentrated using rotary evaporator (Buchi Labortechnik) under reduced pressure at 40°C.

Purchase Albendazole / Reagents

The drug Albendazole 100mg (anthelmintic) was purchased from a registered pharmaceutical store, Hashem Pharmacy in Owerri, Imo state. While the other reagents used for the study as n-hexane, butanol, methanol, distilled water, were purchased from a local chemical store dealer.

PREPARATION OF FRACTIONS

Methanol extract was prepared following the methods of Tabassam. The samples were sequentially subjected to liquid-liquid partition method, with *n*-hexane, followed by ethyl acetate, then *n*-butanol, resulting in three fractions with different polarities: the hexane fraction (FHEX), which was the least polar fraction; the ethyl acetate fraction (FACOEt) the most polar fraction; and the *n*-butanol fraction (FBuOH), with intermediate polarity. 100mg/ml of the crude extract in methanol was mixed with 150ml of distilled water and was poured in a separate funnel.

500ml of *n*-hexane was poured into the funnel and shook vigorously, releasing pressure at intervals. It was allowed to stand for 30 minutes for proper separation. Then the fraction was collected in a clean beaker. 500ml of ethyl acetate was poured in the residue and was shaken vigorously. It was also allowed to stand proper separation upon collection in a clean beaker. Then the butanol was finally poured in the residue, shook and then allowed to stand for 30 minutes for separation and collection. Finally, the resulting fractions were collected using a water bath at 40⁰c for further use.

COLLECTION OF PARASITES

Adult *Onchocerca volvulus* nematodes were obtained from the contents of the skin nodules of one hundred volunteers / afflicted individuals from Umuokwara, Ugwuaku and Aku ihube villages in Okigwe LGA, Imo state Nigeria. A total of 252 worms were collected. The volunteer donors aged between 29 – 65 years old, have lived in the villages of Ezinnachi Autonomous Community in Okigwe LGA (Umuokwara, Aku ihube, and Ugwuaku villages) for ten years and above and have not received any medication for Onchocerciasis in the past 5 years and thereby qualified the study criteria.

The choosing of study sample was without bias for gender, sex, occupation and stratification. The donors provided written formal consent. Skin snip biopsy and microscopic investigation to confirm presence of microfilariae load were performed for diagnosis. The Skin biopsy test was after a simple palpation test conducted to ascertain that the nodular content was possibly the *Onchocerca volvulus* nematode. The content of the nodule was pressed with the index finger and observed for a movement. Movement indicated the presence of the *Onchocerca worm*. The

Onchocerca volvulus was sourced via nodulectomy procedure and for which head nodules were particularly targeted.

Skin snip biopsy was first performed on the identified individuals by elevating a small cone of skin (3 mm in diameter) with a needle and shaving it off with a scalpel. This resulted in the removal of around 2 mg of tissue. The tissue was then incubated in normal saline at room temperature for 24 hours to allow the microfilariae (larvae) to emerge. The microfilariae were then identified microscopically. The sites for the skin snip were over the scapula and the lower extremities. A day later, nodulectomy was performed on the patient that enabled the identification of macrofilariae (adult worms) in the tissue.

The extracted bundle of worms still in its natural sac was further preserved in 0.5% carboxymethyl cellulose and taken to the department of pharmacy laboratory of Nnamdi Azikiwe University, Agulu campus, Anambra state Nigeria, where it was authenticated by a parasitologist. The sac was then incised to recover the nematodes, which were placed in a beaker containing 0.5% carboxymethyl cellulose.

Data Analysis

The time of paralysis and death of the *Onchocerca volvulus* of the various extract treatment are given in a table. Paralysis occurred when the worms do not revive when vigorously shaken. Death was recorded when the worms lost their motility followed with fading away of their body colour. The results are dose-dependent, on comparing the fractions of methanolic extracts of the plant leaf showing the highest anthelmintic activity with death time.

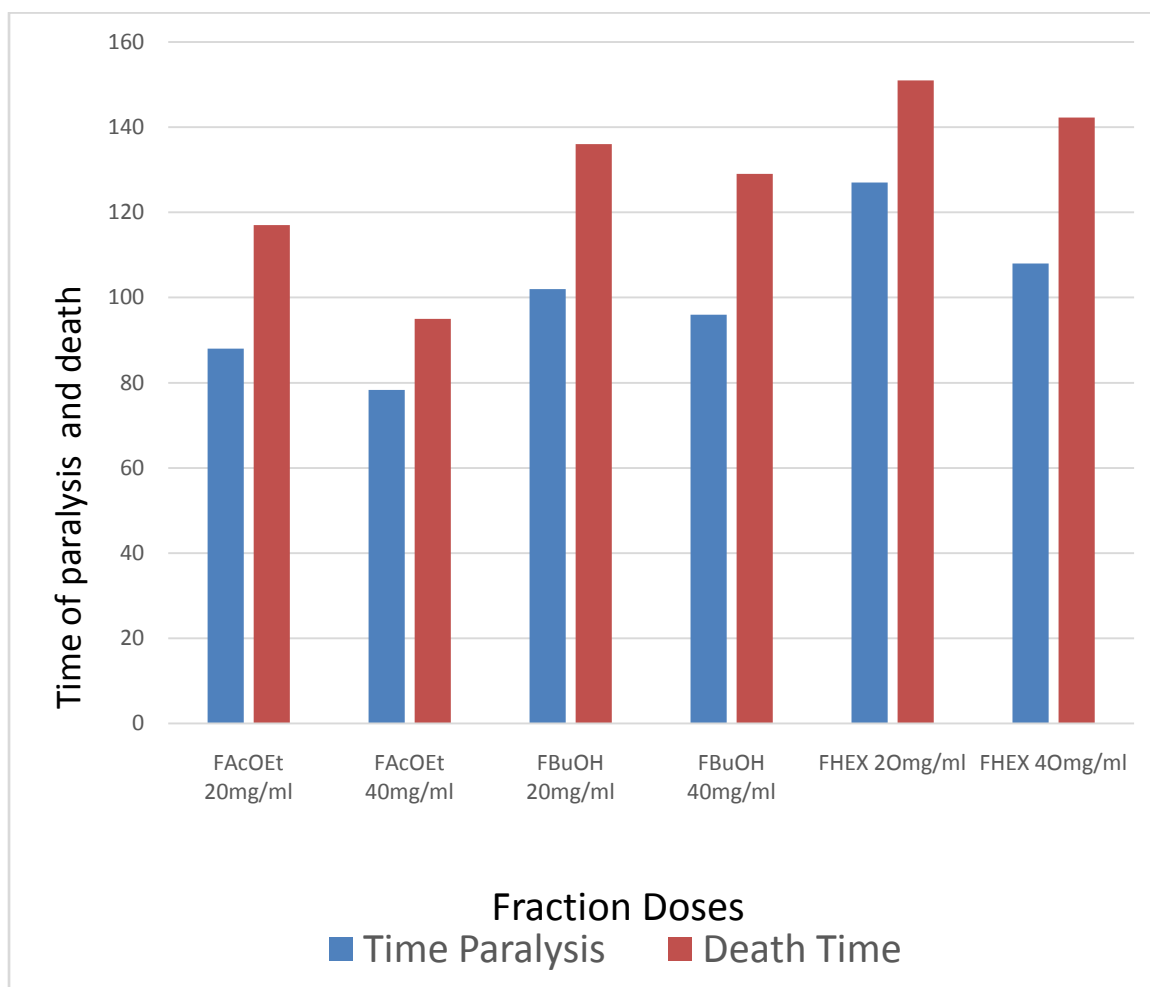
Each experiment was performed in triplicate and results were expressed as Mean \pm Standard Deviation (SD). And to evaluate the effects of the various fractions on *Onchocerca volvulus*, the Shapiro-Wilk test for normality was applied. Once the results were confirmed to be normally distributed, ANOVA followed by Dunnett's test analyzed data obtained. The significance level was set at 5% for all tests

Results

The effect of methyl acetate, butanol, and N – hexane fractions of methanolic *T. catappa* (red leaf) extract on *O. volvulus* revealed to be dose concentration-dependent. Methyl acetate, butanol, and N – hexane fractions of methanolic *T. catappa* leaf extract also showed maximum efficacy at a concentration of 40mg/ml for this study, which is comparable to the reference drug (Albendazole 100mg). Observation with the fraction doses showed that ethyl acetate is more potent on *O. volvulus* at mean average paralysis time of 122.5mins and death at 181.5mins.

Positive control (Albendazole) showed mean average *O. volvulus* paralysis time of 58 mins \pm 8.96mins and death at 93.7 \pm 9.59mins. The average negative control (saline water) showed a mean average paralysis time of *O. volvulus* at 154 \pm 5.97 and death at 235 \pm 4.21mins. Results further showed that methyl acetate fractions of methanolic *Terminalia catappa* red leaf extracts

are less potent than the standard drug Albendazole. The research work recorded 100% mortality in different concentrations, of the total 36 *Onchocerca volvulus* (nematodes) used for the research work.



Bar chart : Effects of fractions of Terminalia catappa red leaf extract on Onchocerca volvulus using parameters of paralysis and death

Key indications;

- FAc OEt – Fraction of Ethyl acetate
- FBuOH – Fraction of Butanol
- FHex – Fraction of Hexane

The table shows the determination of effect of methanolic fractions of Terminalia catappa red leaf extract on *O. volvulus*. Ethyl acetate fraction in best doses of 20mg/ml and 40mg/ml shows shortest paralysis and death time. The ethyl acetate (20mg/ml) crude fraction

paralyzed the worms at 88 ± 3.78 mins and killed the nematodes at 117 ± 3.78 mins. And the 40mg/ml dose of ethyl acetate fraction paralyzed the worms at 78.3 ± 31.85 mins and death at 95 ± 2.08 mins.

Butanol (20mg/ml) crude fraction paralyzed of worms recorded at mean average time of 102.3 ± 3.28 mins and death at 136.3 ± 3.28 mins. While the 40mg/ml dose of butanol paralyzed the worms at 96 ± 3.46 mins and death of worms was record at mean average time of 129 ± 0.57 mins

The n-hexane fraction dose of 20mg/ml paralyzed the *onchocerca* worms at 127.6 ± 2.81 mins and recorded the death at 151.6 ± 4.91 mins mean average time. And the 40mg/ml dose of n-hexane fraction paralyzed the worms at 108 ± 2.64 mins and killed the worms at mean average time of 142.3 ± 3.28 mins. All results are statistically significant at $P>0.05$

Conclusion

Observation with the fraction doses showed that ethyl acetate is more potent, over the butanol and n-hexane fractions on *O. volvulus* at mean average paralysis time of 122.5mins and death at 181.5mins. Bar chart; shows a result of the effects of fractions of methanolic *Terminalia catappa* red leaf extract on *O. volvulus*. It also shows a greater potency concentration dependent anthelmintic effects on *Onchocerca volvulus*.

Acknowledgement : None

Funding None

Availability of data and materials

The dataset(s) and other materials can be accessed upon request via the authors email handler (please put your email)

Ethics approval and consent to participate

An ethical clearance and approval was obtained from the the Ethics Committee,public health department Imo State Ministry of Health Owerri (Protocol number: 105/2014).

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