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Research Article

Chemical Composition, Cytotoxic and Antimicrobial Activity of Essential Oil from *Tetracarpidium Conophorum* Leaves

Seun F Akomolafe*

Department of Biochemistry, Ekiti State University, Ado-Ekiti, Nigeria

Abstract

Essential oil extracted using solvent free microwave extraction method from the leaves of *T. conophorum* was evaluated for its chemical composition, cytotoxic and antibacterial activities for the first time. The Gas Chromatography/Mass Spectrometry (GC/MS) analysis revealed the main constituents of the essential oil of *T. conophorum* to be 2-Furancarboxaldehyde-5-methyl-hydroquinone (4.12%), Benzeneacetaldehyde (5.57%), 2-Methoxy-4-vinylphenol (12.23%), 6,10-Dimethyl-5,9-undecadien-2-one (4.40%), Bicyclo [3.1.1] heptane,2,6,6-trimethyl (14.20%), 3,7,11,15-Tetramethyl-2-hexadecen-1-ol (4.66%) and Hexadenoic acid, methyl ester (6.48%), all other compounds were either present in trace quantity or in amounts less than 3%. The oil of *T. conophorum* leaves was effective against 5 of the 9 fertility infecting pathogens tested within the limit of concentration considered, with MIC values ranging from 0.312 to 5.0mg/ml. The oil had high (87.02µg/ml) cytotoxicity levels against brine shrimp. At 50µg/ml the oil was not toxic. While maximum mortalities happened at 200µg/ml, the least mortalities were observed at 100µg/ml. It is apparent that the bioactivity of the essential oil of *T. conophorum* leaves contributes to the use of this plant in folk medicine. Therefore, part of the mechanisms through which this plant is used as fertility enhancing plant may be through their positive effect on fertility infecting pathogens.

Keywords: Antibacterial; Cytotoxicity; Essential oil; Solvent free microwave extraction; *T. conophorum*

Introduction

Infertility is a major clinical problem, affecting people medically and psychosocially. Published literature data indicates that 15% of all couples in the United States are infertile, and the male factor is responsible for 25% of these cases [1]. Infections of the male genitourinary tract account for up to 15% of cases of male infertility [2]. Acute and chronic infections and consequent inflammation in the male reproductive system may compromise the sperm cell function and the whole spermatogenetic process [3,4], causing qualitative and quantitative sperm alterations. Recent studies have shown that the simple presence of bacteria in semen samples may compromise the sperm quality. The microorganisms responsible for semen contaminations generally originate from the urinary tract of patients or can be transmitted by the partner via sexual intercourse [5].

The problems of multidrug resistances exhibited by human pathogenic microorganisms and the side effects of antibiotics have led scientists to search for alternatives such as medicinal plants. The phenolic compounds present in most plants have a broad spectrum of biological activities whereby their antimicrobial actions stand out [6,7].

The genus Euphorbiaceae is a pantropical genus comprises about nineteen known species belonging to tribe Plukenetieae of subfamily Acalypphoideae. The genus is unusual in the Ephorbiaceae for its four carpellate ovary, vine habit and widely distributed in the new world. They are referred to as English walnut (Juglandaceae) and African walnut (Euphorbiaceae). Each family has its own peculiar characteristics but they have some things in common such as the nuts. Juglandaceae is mostly found in the Southeast Europe, to Japan. Among these species, *Tetracarpidium conophorum* ((Mull. Arg.) Hutch. & Dalz) is a perennial climbing shrub found in the forest regions of India, Nigeria, Congo, Gabon and Liberia.

In Nigeria, it is found in Uyo, Etinam, Akpabuyo, Lagos and Ibadan. The plant is cultivated principally for the nuts which are cooked and consumed as snacks. The leaves are used as male fertility agent and in the treatment of dysentery in southern Nigeria. Some studies have been reported in the literature that present the proximate, ascorbic acid, heavy metals, amino acids and fatty acid components of the nut [8]. The use of its leaf juice for the treatment of prolonged and constant hiccups has been reported [9]. The oil from the nut could serve as a source of energy for growing seedlings and has been reported to be used in the formulation of wood varnish, stand oil and vulcanized oil. In the present study the chemical composition and antimicrobial activity of essential oil from *T. conophorum* leaves against fertility infecting pathogens (Gram-positive and Gram-negative bacteria) as well as cytotoxic effect were reported for the first time.

Materials and Methods

Collection and Identification of Plant Sample

Fresh samples of *T. conophorum* leaf were obtained from a farm land near Akure metropolis, Nigeria during wet season around July and August. Authentication of the sample was carried out at the Department of Plant Science, Ekiti State University by Mr Ajayi where voucher specimen (number UHAE 335) was deposited in the herbarium of the same Department.

***Corresponding author:** Seun F Akomolafe, Department of Biochemistry, Ekiti State University, Ado-Ekiti, Nigeria, Tel No: +234 8030460149; E-mail: purposefulseun@yahoo.co.uk / seun.akomolafe@eksu.edu.ng

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Solvent-Free Microwave Extraction

The essential oil was extracted using solvent-free microwave extraction. Briefly, Solvent-Free Microwave Extraction (SFME) was carried out in a milestone Dry DIST (2008) microwave apparatus. The multimode microwave reactor has a twin magnetron (2,800 W, 2,450 MHz) with a maximum delivered power of 1000W in 10W increments. A rotating microwave diffuser ensures homogenous microwave distribution throughout the plasma-coated PTFE cavity (35 cm x 35 cm x 35 cm). The temperature is monitored by a shielded thermocouple (ATC-300) inserted directly into the corresponding container. Temperature is being controlled by feedback to the microwave power regulator. In a typical SFME procedure, a known amount (50g) of the dried powdered sample is placed in the reactor. The essential oil is collected after the extraction time which lasted for about 30 minutes.

Gas Chromatography-Mass Spectrometry (GC-MS)

The GC-MS of essential oil was performed using an Agilent 7890 GC coupled to an Agilent 5977 MSD with a Zebron-5MS column (ZB-5MS 30m x 0.25mm x 0.25um) (5%-Phenyl)-methylpolysiloxane. GC grade Helium was used as a carrier gas at a flow rate of 2mL/min; splitless 1 ul injections were used. Injector temp, 280˚C; source temp, 280˚C. Oven temp was 70˚C oven, ramp 15˚C/minute to 120˚C, ramp at 10˚C/minute to 180˚C then ramp at 20˚C/minute to 270˚C and hold for 3 minutes. The chemical compositions of the essential oil of the leaves of *T. conophorum* was determined according to their retention time, and data was gathered with Chem station.

Bacteria Samples and Culture Preparation

Nine reference strains of bacteria used in this study were chosen based on their pathological effects on excessive contamination of human semen. Gram positive bacteria, *Streptococcus faecalis* (Laboratory strain), *Bacillus cereus* (Laboratory strain), *Staphylococcus aureus* (ATCC 2593), *Streptococcus pyogene* (Laboratory strain) and Gram negative bacteria *Pseudomonas aeruginosa* (ATCC 27853), *Escherichia coli* (Laboratory strain), *Shigella flexneri* (Laboratory strain), *Proteus vulgaris* (KZN) and *Proteus mirabilis* (ATCC 7002). The microorganisms were retrieved from the Microbiology unit of MPED Research Centre, Botany Department, University of Fort Hare. The bacteria isolates were sub-cultured on nutrient agar (SAARCHEM, Gauteng SA) plates and incubated at 37°C for 24 hours. A loopful of bacterial cells from the nutrient agar plates was inoculated into 50mL of a nutrient broth (DIFCO, California, USA) in a 250mL sidearm Erlenmeyer flask and incubated at 37°C for 16 hours with vigorous shaking (Orbital incubator, S150, UK). After incubation, the culture was diluted with fresh media to give a D_{600nm} of 0.1. One hundred microliters of the culture cells was added onto the plate and spread into a bacterial lawn using a sterile glass spreader.

Minimum Inhibitory Concentration (MIC) Determination

The Minimum Inhibitory Concentration (MIC) of the extracts was determined using agar dilution method as described by Eloff [10], with a slight modification. The bacterial strains were grown at 37°C overnight and maintained on nutrient agar. Inoculums of the test organisms were prepared in normal saline (9g/L) compared with 0.5 McFarland standard to achieve 5 x 10^5 (CFU/mL). A stock concentration of 100µl from essential oils (20mg/mL) was prepared in 10% DMSO (Sigma) and further diluted in molten MHB agar at 50°C to give a final concentration ranged from 0.3125-5mg/mL [11,12], after pouring into the plates and allowed the agar to set, the plates were inoculated with standardized inocula of the test bacteria. The plates were further incubated at 37°C for 24 hours under aseptic conditions. The MIC was recorded as the lowest concentration at which no visible microbial growth was observed. Bacteria treated with Amoxicilin and Ciprofloxacin, were used as positive controls.

Brine Shrimp Lethality Test

The brine shrimp lethality test was used to predict the toxicity of the oils and was conducted according to the methods of Oloyede et al. [13], with a slight modification using brine shrimp eggs obtained from Ocean Star International, Inc. Company USA. The shrimp eggs were hatched and matured to naupili in a hatching tank filled with sea water in 48 hours at room temperature. The naupili (harvested shrimps) were attracted to one side of the vials from which they could be collected for the assay with a light source. Solutions of the oils were made in Dimethyl Sulfoxide (DMSO), at varying concentrations (50, 100, 150 and 200µg/ml) and incubated in triplicate vials with the brine shrimp larvae. Twenty brine shrimp larvae placed in a mixture of sea water and 1% DMSO without the oil served as control. After 48 hours, the vials were examined against a lighted background and the average number of larvae that survived in each vial was counted and IC_{50} values were obtained using non-linear regression curve.

Results and Discussion

The percentage yield of the essential oil (which was yellowish in colour with strong burnt odour and refractive index of 1.664) was 1.43% (weight/dry weight). The percentage oil yield of *T. conophorum* leaves observed in this study was lower than that reported for the nuts of this same plant [14]. The chemical composition and constituent percentage of the essential oil of the leaves is presented in table 1. A total of 24 constituents (representing 97.74%) were identified in the oil of *T. conophorum* leaves. The ubiquitous terpenoid compounds were mostly (36.58%) represented among the volatile, followed by fatty acids (31.92%) and aromatic compounds (21.92%).

The major components were 2-Furancarboxaldehyde-5-methyl-hydroquinone (4.12%), Benzeneacetaldehyde (5.57%), 2-Methoxy-4-vinylphenol (12.23%), 6,10-Dimethyl-5,9-undecadien-2-one (4.40%), Bicyclo [3.1.1]heptane,2,6,6-trimethyl (14.20%), 3,7,11,15-Tetramethyl-2-hexadecen-1-ol (4.66%) and Hexadenoic acid, methyl ester (6.48%). On the contrary, Buttery et al. [15], identified a total of 45 volatile compounds from whole green mature walnut husks of which the major constituents were (E)-4, 8-dimethyl-1,3,7-nonatriene, pinocarvone, pinocarveol, myrtenal, myrtenol, (E,E)-4,8,12-trimethyl-1,3,7,11-tridecatetraene, caryophyllene epoxide, verbenol, verbenone, and terpinolene.

It is apparent from our study that Bicyclo [3.1.1] heptane,2,6,6-trimethyl (14.20%) and 2-Methoxy-4-vinylphenol (12.23%) occurred in higher amounts in *T. conophorum* leaf oil. Bicyclo [3.1.1] heptane,2,6,6-trimethyl which also known as Pinene is one of the most common bicyclic monoterpenes in nature, they played significant roles in the biology of plants and animals in which they exhibit bactericidal activity and act as natural insect pheromones [16]. 2-Methoxy-4-vinylphenol is a naturally occurring phenolic compound used as a flavoring agent and has been found in buckwheat, apple, peanut, clove and curry [17]. It has been reported that this compound possess antioxidant, anti-inflammatory effects [18], and can induce the arrest of abnormal cell cycle progression [19].

No	Name of Compounds	Molecular Formula	Molecular Weight	RT	% Peak Area	KI
1	Furfural	$C_5H_2O_2$	96	3.20	3.36	830
2	2- Hexanal	$C_6H_{10}O$	98	3.33	1.93	856
3	2- Furancarboxaldehyde- 5- methyl- Hydroquinone	$C_6H_6O_2$	110	4.20	4.12	962
4	Benzeneacetaldehyde	C_8H_8O	120	4.89	5.57	1049
5	Mequinol	$C_7H_8O_2$	124	5.24	1.50	1183
6	Ethanone-1,2- methylphenyl	$C_9H_{10}O$	134	6.03	0.99	1188
7	Indole	C_8H_7N	117	6.84	1.38	1288
8	2-Methoxy-4-vinylphenol	$C_9H_{10}O_2$	150	6.96	12.23	1313
9	Naphthalene-1,2,3,4- tetrahydro-1,1,6- trimethyl-	$C_{13}H_{18}$	174	7.48	2.14	1340
10	5,9-Undecadien-2-one- 6,10- dimethyl- (E)-	$C_{13}O_{22}O$	194	7.84	4.40	1436
11	2H-1-Benzopyran- 3,5,6,8a- tetrahydro-2,5,5,8a- tetramethyl- trans-	$C_{13}H_{20}O$	192	8.14	1.33	1620
12	2- Naphthalenemethanol-1,2,3,4,4a,5,6,8a-octahydro-.alpha.,alpha.,4a,8-tetramethyl-, [2R- (2.alpha.,4a.alpha.,8a.beta.)]-	$C_{15}H_{26}O$	222	9.24	0.87	1652
13	Bicyclo [3.1.1]heptane,2,6,6-trimethyl-	$C_{10}H_{18}$	138	10.05	14.2	1656
14	2-Pentadecanone- 6,10,14-trimethyl	$C_{18}H_{36}O$	268	10.09	2.89	1660
15	1-Hexadecyne	$C_{16}H_{30}$	222	10.18	2.33	1664
16	3,7,11,15-Tetramethyl-2-hexadecen-	$C_{20}H_{40}O$	297	10.28	4.66	2114
17	Hexadecanoic acid, methyl ester	$C_{17}H_{34}O_2$	270	10.49	6.48	2127
18	Dibutyl phthalate	$C_{16}H_{22}O_4$	278	10.75	1.01	2132
19	9,12-Octadecadienoic acid (Z,Z)-methyl ester	$C_{19}H_{34}O_2$	294	11.33	1.57	2092
20	9,12,15-Octadecatrienoic acid, methyl ester	$C_{19}H_{32}O_2$	292	11.37	3.54	2098
21	Phytol	$C_{20}H_{40}O$	297	11.42	3.51	2099
22	Heneicosane	$C_{21}H_{44}$	297	13.81	2.10	2100
23	Pyridine-3-carboxamide, oxime, N- (2-trifluoromethylphenyl)-	$C_{14}H_{11}F_3N_2O_2$	296	14.44	1.96	2107
24	Eicosane	$C_{20}H_{42}$	283	14.69	2.56	2109
	Total	97.74%				
	Monoterpene Hydrocarbon	16.59				
	Sesquiterpene Hydrocarbon	17.59				
	Diterpenoid	2.42				
	Aromatic Compounds	21.92				
	Fatty Acids	31.92				
	Others	7.32				

Table 1: Volatile constituents of the essential oil of *T. conophorum* leaves.

Note: KI=Kovat retention index; RT= Retention time.

The identified compounds possess many biological properties. For instance, Hexadecanoic acid, methyl ester can be an antioxidant, antiandrogenic, hypocholesterolemic, nematicide, pesticide, lubricant activities and hemolytic 5-alpha reductase inhibitors. 3,7,11,15-Tetramethyl-2-hexadecen-1-ol which also known as Phytolditerpene is an antimicrobial, anticancer, anti-inflammatory and diuretic agent [20].

Similar types of compounds among the twenty four compounds of this present study were identified in *Caesalpinia sappan* ethanol extract and *Cassia italic* leaf methanol extract by Sarumathy et al. [21], and Sermakkani and Thangapandian [22].

Similarly, Maria Jancy Rani et al. [23], observed the presence of phytol in the leaves of *Lantana camara* and Sridharan et al. [24], observed its presence in *Mimosa pudica* leaves. Similar result was also observed in the leaves of Lantana camara [25]. Phytol was observed to have antibacterial activities against Staphylococcous aureus by causing damage to cell membranes as a result there is a leakage of potassium ions from bacterial cells [26]. Phytol is a key acyclic diterpene alcohol that is a precursor for vitamins E and K1. It is used along with simple sugar or corn syrup as a hardener in candies.

In-vitro Anti-Microbial Activity

Table 2 shows the antibacterial activities of the essential oil of T. *conophorum* leaves. The essential oil exhibited appreciable antibacterial activity against *S. faecalis, B. cereus, S. aureus, S. pyogene, P. aeruginosa, E. coli, S. flexneri, P. vulgaris* and *P. mirabilis*.

The most frequently isolated microorganism in male patients with genital tract infections or semen contamination are *E. coli*, *S. faecalis, S. aureus* and *P. aeruginosa*. The negative influence of these species on sperm quality has been investigated and found to be partially due to its effect on motility [27], and to the impaired acrosomal function, as demonstrated at the ultrastructural level by Diemer et al. [28].

Bacterial Species	TC Oil (mg/ml)	Amoxicilin (mg/L)	Ciprofloxacin (mg/L)
Streptococcus faecalis (Laboratory strain)	0.625	1.25	0.312
Bacillus cereus (Laboratory strain)	5.000	0.312	0.312
Staphylococcus aureus (ATCC 2593)	0.625	1.25	0.312
Streptococcus pyogene (Laboratory strain)	>5.000	1.25	0.312
Pseudomonas aeruginosa (ATCC 27853)	0.312	1.25	0.312
Escherichia coli (Laboratory strain)	0.625	0.625	0.312
Shigella flexneri (Laboratory strain)	>5.000	1.25	0.312
Proteus vulgaris (KZN)	>5.000	1.25	0.312
Proteus mirabilis (ATCC 7002)	>5.000	1.25	0.312

Table 2: Minimum Inhibitory Concentration (MIC) of the essential oil of *T. conophorum* leaves against fertility infecting pathogens

It was observed that the essential oil showed strong antibacterial activity against *S. faecalis, S. aureus, P. aeruginosa* and *E. coli* at a minimum inhibitory concentration of 0.625, 0.625, 0.312 and 0.625 mg/ml respectively. The oil showed a greater activity against *S. faecalis, S. aureus* and *P. aeruginosa* when compared to Amoxicillin whereas Ciprofloxacin effect was more pronounced than the oil. Also, the oil showed equal activity on *P. aeruginosa* as the standard drug, Ciprofloxacin but higher than that of Amoxicillin while the oil showed equal activity on *E. coli* as the standard drug, Amoxicillin but lower than that of Ciprofloxacin.

The oil caused inhibition of *B. cereus* growth only at higher concentration (5mg/ml) while the oil was found to be inactive against other organisms (*S. pyogene, S. flexneri, P. vulgaris and P. mirabilis*) within the limit of concentration considered. However, it could be active at concentration greater than 5mg/mL. Orhan et al. [14], reported that the oil extracted from walnut seed displayed inhibitory potential towards the isolated strains of *Escherichia coli, Pseudomonas aeruginosa, Proteus mirabilis, Klebsiella pneumoniae, Acinetobacter baumannii, Staphylococcus aureus,* and *Enterococcus faecalis* as well as the fungi *Candida albicans* and *C. parapsilosis.*

It is therefore apparent that this oil under in vitro study has broad spectrum antibacterial activity and it contains components that could be exploited to eliminate invading microorganisms in male genitals which could contribute to the deterioration of the sperm quality of infertile men.

Brine Shrimp Cytotoxicity Assay

Brine shrimp lethality assay is frequently used as a model system to measure cytotoxic effects of a variety of toxic substances and plant extracts against brine shrimps nauplii [29]. In the present study, the result for the IC_{50} of the oil extracted from T. *conophorum* leaves (87.02µg/ml) suggested that the plant oil may not be highly toxic. However, at 50µg/ml, which was the least concentration used, the oils were not toxic, as all the brine shrimps survived (Figures 1-3). The toxicity of this oil may probably be due to the dominance of hydrocarbons present in this oil, this observation is similar to the one made by Fashola et al. [30], and Okoh and Afolayan [31]. It has been reported that secondary metabolites from plants which are active medicinally are most times toxic to brine shrimp larvae [13,32]. This significant lethality of the plant oil (IC_{50} value less than 100ppm or µg/ml) to brine shrimp is indicative of the presence of potent cytotoxic and probably insecticidal compounds [29], which may serve as natural therapy for the treatment of infectious diseases. Therefore, further isolation of the highly active compounds from T. *conophorum* leaves may lead to the discovery of new cytotoxic compounds [33,34].

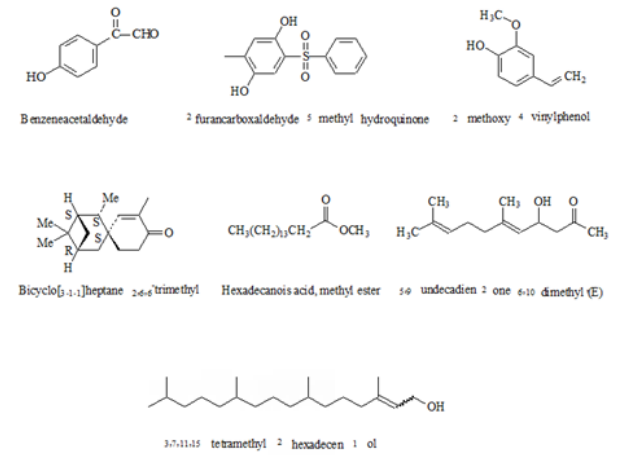

Figure 1: Structure of major compounds of the essential oils of *T. conophorum* leaves.

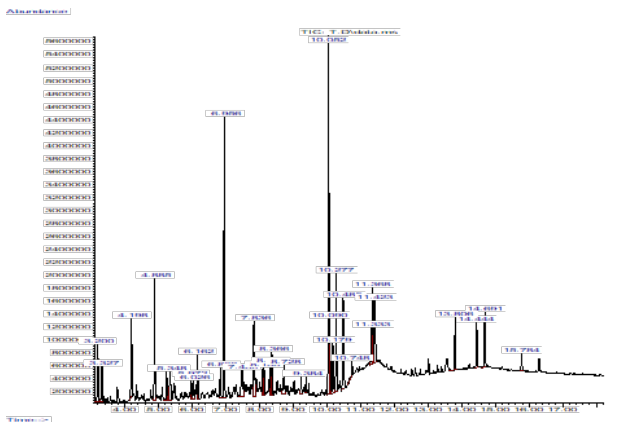

Figure 2: Representative Gas Chromatography-Mass Spectrometry (GC-MS) profile of *Tetracarpidium conophorum* leaf oil.

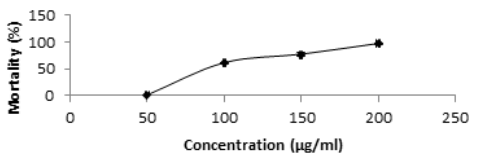

Figure 3: Effect of the essential oil of *T. conophorum* leaves on brine shrimps.

Conclusion

This study suggests that the essential oil of leaf of *T. conophorum* may be a potent source of bioactive and antibacterial compounds which may be of good advantageous in pharmaceutical industries.

The lethality of the plant oil to brine shrimp may be an indicative of the presence of potent cytotoxic and probably insecticidal compounds which may serve as natural therapy for the treatment of infectious diseases. Therefore, there is need for further studies to ascertain the in vivo biological activities of this essential oil and the compounds responsible for such activities.

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