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Acute Toxicity Studies, Physicochemical and GC/MS Analyses of *Monodora myristica* (Gaertn.) Dunal Oil

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A – research concept and design; B – collection and/or assembly of data; C – data analysis and interpretation; D – writing the article; E – critical revision of the article; F – final approval of article.

Abstract

Background: The seeds of *Monodora myristica*, a tropical plant belonging to the family Annonaceae, are widely used as condiments in the preparation of a number of African delicacies, to impart or enhance flavour. Their use is largely due to the volatile and fixed oils contained in the nuts.

Objective: To screen for the acute toxicity (LD_{50}) profile, determine the physicochemical properties, as well as carry out GC-MS analysis of the seed oil of *Monodora myristica.*

Method: The acute toxicity screening was done using Lorke's method. Physicochemical profile of the oil was determined using standard methods. GC-MS analysis of the oil sample was also carried out, using Shimadzu GCMS-QP2010SE.

Results: The acute toxicity study showed that the oil sample had an LD_{50} of 316 mg/kg. The acid value was determined to be 9.27 mg KOH/g oil, while saponification value was 194.95 mg KOH/g oil. GC-MS analysis of the oil revealed that it contained fatty acids such as n-hexadecanoic acid, cis-vaccenic acid and 9,12-octadecadienoic acid; as well as terpinoids such as alpha-terpineol and alpha-cadinol.

Conclusion: Results of the toxicological screening indicates that *Monodora myristica* oil is moderately toxic as the LD_{50} was within 50 – 500 mg/kg range. The oil has a relatively high tendency to go rancid due to the high acid value of 9.27 mg KOH/g oil.

Keywords: Physicochemical; *Monodora myristica;* GC-MS analysis; LD_{50}; Condiments

INTRODUCTION

Medicinal plants have always, and will for a very long time, continue to play very significant roles as sources of drugs. The reliance on traditional medicine, particularly medicinal plants, as a trusted means for maintaining good health in most developing countries, is well known. A rise in dependence on the use of medicinal plants, even in the developed nations, can be traceable to the successful extraction and development of numerous anti-infective and medicinal agents from them (plants), as well as from traditional herbal remedies (Hoareau and DaSilva, 1999).

Monodora myristica, also known as the calabash nutmeg, or African calabash, is a tropical perennial tree belonging to the family Annonaceae. Local Nigerian names for the calabash nutmeg include ehuru, ariwo, awerewa, ehiri and airama (Onyenibo *et al.*, 2015). The calabash nutmeg is native to East and West Africa, and is found abundantly in the African evergreen forests of Angola, Cameroon, Liberia, Nigeria and Western Kenya (Okafor, 1987).

The seeds are similar to that of true nutmeg (*Myristica fragrans*) in odour and the flavour they impart (Weiss, 2002). It is one of the most commonly utilized spices

in the eastern and southern parts of Nigeria. The seeds are widely used as condiments for preparing pepper soup, stew and a number of other African delicacies, mainly for their stimulating flavour. The use of this plant possibly revolves around the volatile and fixed oils found in the nuts.

The low moisture content of the seeds ensure that they can withstand long-term storage and not easily deteriorate. The properly dried seeds can be safely stored for years and still retain their quality. This is also suggestive of minimal susceptibility to microbial degradation. Hence, rural dwellers without electricity or modern storage facilities, are able to store and use the seeds all year round (Agiriga and Siwela, 2017).

Trado-medical uses associated with *M. myristica include* toothache, cough, headache, fever, constipation, dysentery, chest pain and skin diseases. These uses and several other medicinal applications of this plant, are primarily associated with Indigenous Knowledge Systems (IKS) (Agiriga and Siwela, 2017).

The seeds of *M. myristica* have been reported to contain aluminum, calcium, copper, iron, magnesium, manganese, phosphorus, potassium, sodium and zinc (Ekeanyanwu *et al.*, 2010).

Several phytochemical classes, e.g saponins, flavonoids, steroids, tannins, alkaloids, etc., have been reported to be present in different parts of this plant (Nwaoguikpe *et al.*, 2014).

Fournier *et al.*, (1999) in their review of existing literature, reported the presence of terpenes, including *β*-phellandrene, 1,8-cineole, *α*-phellandrene. *α*-pinene, limonene, myrcene, *α*-thujene, p-cymene, linalool, germacrene D-4-ol, spathulenol, δ-cadinene, *β*-caryophyllene. *α*-humulene, *α*-copaene and *α*-cubebene in the fruit, leaf and seed extracts of *M. myristica.*

More chemical constituents of *M. myristica* were highlighted in the report of koudou *et al.,* (2007) on the chemical constitution and hypotensive effects of the essential oil of this plant, some of which include sabinene, (E)-*β*-ocimene, piperitol, thymoquinone, carvacrol and *α*-santalene.

In a comparative study of the essential oils of *M. myristica* from Nigeria, Owokotomo and Ekundayo (2012) also reported identifying several compounds in the essential oils from the seed and stem bark, including germacrene-D-4-ol, copaene, tricyclo [5.2.1(1,5)] dec-2-ene, δ-cadinene, γ-cadinene, α-terpineol, α-cubebene, caryophyllene and γ-muurolene. They also observed that caryophyllene and γ-muurolene were the only two compounds present in both the seed and stem-bark oils.

Feyisayo and Oluokun (2014), in their work that compared the phenolic profile of *M. myristica* and *Monodora* tenuifolia, confirmed the presence of several compounds in both species, such as myristicin, safrole, elemicin, caffeic acid, catechin, kaempherol, quercetin and eugenol. Their report also highlighted very high myristicin composition (over 40%) amongst phenolic compounds identified in both species.

Most of the compounds identified to be present in *M.* myristica are known bioactive compounds, and are believed to be responsible for the many medicinal and pharmacological properties associated with this plant (Agiriga and Siwela, 2017).

Several authors have reported safe or low toxicity levels for extracts from different parts of *M. myristica.* Isiogugu *et al.*, (2018), who worked on the root bark, reported LD_{50} value above 5000 mg/kg. Ajayi *et al.*, (2013), investigated the short-term toxicological effects of the seed oil of *M. myristica*, and concluded that there were no toxic effects on albino rats. Feyisayo *et al.*, (2014) who evaluated the acute and sub-lethal toxicological profile of the ethyl acetate fraction of the seed extract, also reported LD_{50} greater than 5000 mg/kg. However, the high content of myristicin which has been reported to possess cytotoxic properties, calls for concern (Lee *et al.*, 2005).

Evidently, significant variations have been reported in the chemical composition of this plant. This study is being undertaken with a view to further confirm, or augment available information concerning the chemical constituents, as well as the toxicity profile of *M. myristica*.

METHODOLOGY

Collection of Plant Material

The dried seeds of *Monodora myristica* (Gaertn.) Dunal, were purchased from Opolo market, Yenegoa, Bayelsa state, Nigeria.

Prepararion of Plant Material

The seeds were sun dried, sorted, peeled and pulverized. It was weighed using a kitchen weighing scale and was air dried to a constant weight.

Extraction of Plant Material

The seeds were extracted with dichloromethane. 250ml portions of the solvent were used to macerate the plant material for 72 hours with intermittent gentle shaking. It was then filtered using cotton wool and filter paper. The filtrate was then concentrated by evaporating to constant weight on the laboratory bench at room temperature.

Acute toxicity study

The study was done in two phases, using a modified Lorke's method. Twelve white albino mice were purchased from the Department of Pharmacology, Faculty of Basic Medical Sciences, Niger Delta University, Wilberforce Island, Bayelsa State.

About 1g of the already concentrated dichloromethane fraction of *Monodora myristica* was dissolved in 2 ml of dimethyl sulfoxide (DMSO) and diluted with distilled water to make up 10 ml. Hence, a stock solution of 100 mg/ml was prepared, from which suitable dilutions were made for the lower doses of 10 mg/kg and 100 mg/kg.

Phase I: Nine (9) albino mice were used in this phase. The nine white albino mice were divided into three (3) groups of three (3) mice per group. Each group was administered different doses (10, 100 and 1000 mg/kg) of the test substance intraperitoneally (IP). The mice were kept under observation for 24 hours to monitor their behavior for toxicity signs and for mortality.

Phase II: Three (3) albino mice were used in this phase. They were distributed into three groups of one mouse in each group. They were then administered higher doses (1600 mg/kg, 2900 mg/kg and 5000 mg/kg) of the test substance intraperitoneally (IP) and then observed for 24 hours to monitor their behavior for toxicity signs as well as mortality.

The specific dose for each animal was calculated following the example below, and documented as shown in Table 1.

PHASE 1 Group 1; 10 mg/kg

Weight of first mouse = 25 g approximately 0.025 kg

1 kg = 10 mg

Therefore 0.025 kg = 0.25 mg

1 ml contains 100 mg of extract

Therefore 0.0025 ml = 0.25 mg.

Table 1: Volumes and doses of the extract administered to each animal

Phase	Group	Weight (kg)	Dosage (mg/kg)	Individual dose (mg)	Dilution	Volume administered (ml)
1	1	0.025	10	0.25	100	0.25
	1	0.029	10	0,29	100	0.29
	1	0.033	10	0.33	100	0.33
	2	0.030	100	3.00	10	0.30
	2	0.029	100	2.90	10	0.29
	2	0.031	100	3.10	10	0.31
	3	0.025	1000	25.00	nil	0.25
	3	0.027	1000	27.00	nil	0.27
	3	0.030	1000	30.00	nil	0.30
2	1	0.030	1600	48.00	nil	0.48
	2	0.028	2900	81.20	nil	0.812
	3	0.025	5000	125.00	nil	1.25

The LD_{50} was then calculated using the formula:

$LD_{50} = \sqrt{(D0 x D100)}$

Where D0 = Highest dose that gave no mortality,

D100 = Lowest dose that produced mortality.

Quantitative analysis

Determination of Acid Value

Acid value was determined by dissolving 2 g of the oil sample in a mixture of 25 ml of ethanol (95%) and 25 ml of ether. 1 ml of phenolphthalein solution was added, and the mixture titrated with 0.5 M ethanolic KOH. The 0.5 M ethanolic KOH was added in a drop wise pattern until a pale pink colour (which persisted for 15 seconds) was obtained. The procedure was repeated twice and the average titre value determined. The acid value was obtained from the formula:

Acid value = 5.61 x a/ Weight (in 2 g) of sample

Where a = volume of 0.5 M KOH required (titre value) (Olaniyi and Ogungbamila, 1998).

Determination of Saponification Value

About 2 g of the oil was weighed and transferred into a 250 ml Quick fit flask containing 25 ml of freshly prepared 0.5 M ethanolic KOH. A reflux condenser was attached and the mixture refluxed for 1hr on a water bath, while swirling the contents frequently. The flask was removed from the water bath, cooled and 1 ml of phenolphthalein indicator was added. The content of the flask was then titrated with 0.5 M HCl. A blank titration without the oil was also performed under the same conditions. The same procedure was repeated twice and the average titre value obtained was used to calculate the saponification value thus;

Saponification value = 28.05 x (b–a)/ Weight (in g) of the substance.

Where a = titre value for the test sample; b = titre value for blank titration (Olaniyi and Ogungbamila, 1998).

Determination of Ester Value

The ester value of the oil sample was obtained by subtracting the acid value from the saponification value.

Ester value = saponification value - acid value (Olaniyi and Ogungbamila, 1998).

GC-MS Analysis

GC/MS analysis of the oil sample was done using Shimadzu GCMS-QP2010SE. 1 µL of the dilute sample was withdrawn from the sample vial using a clean GC syringe and injected into the injection port of the machine by splitless injection mode with a sampling time of 2.00 min. and a purge flow of 3.0 ml/min. The operating conditions were as provided below: column oven temperature - 100℃, injection temperature - 250℃, carrier gas was helium (He) at a flow of 3.10 ml/min. The mass-to-charge ratio ranged from 45-700 m/z.

RESULTS

Acute Toxicity Study

Potential toxic effects of the dichloromethane fraction of *Monodora myristica* in albino mice are presented in Table 2.

Table 2: Acute lethal effects of intraperitoneally administered dichloromethane fraction of *Monodora myristica* on albino mice.

Experimental Phase	Dose (mg/kg)	Mortality after 24 hrs.
Phase I	10	0/3
	100	0/3
	1000	1/3
Phase II	1600	1/1
	2900	1/1
	5000	1/1

Some behavioral signs of toxicity observed after administration of different doses of the test sample, are presented in Tables 3.

Table 3: Behavioral toxicity signs observed in Phases I and II post-administration of *M. myristica* extract.

Behavioral toxicity signs	Group 1 (mg/kg)			Group 2 (mg/kg)			Group 3 (mg/kg)			Group 1 (mg/kg)	Group 2 (mg/kg)	Group 3 (mg/kg)
	10	10	10	100	100	100	1000	1000	1000	1600	2900	5000
Seizure	-	-	-	-	-	-	++	+++	++	+++	+++	+++
Paralysis	-	-	-	-	-	-	+++	+++	+++	+++	+++	+++
Hyperventilation	-	-	-	-	-	-	+++	++	+++	+++	+++	+++
Loss of motor coordination	-	-	-	-	-	-	+++	+++	+++	+++	+++	+++
Depression	-	-	-	-	-	-	++	++	++	+++	+++	+++
Immobility	+	+	+	+	+	+	+++	+++	+++	+++	+++	+++
Staggered movement	-	-	-	-	-	-	+++	+++	+++	+++	+++	+++

KEY: + = slightly present; ++ = moderately present; +++ = abundantly present.

Calculation of LD_{50}

D_0 = 100mg/kg

D_{100} = 1000mg/kg

$LD_{50} = \sqrt{(100x1000}$

= 316 mg/kg

Quantitative Analyses

Results of the quantitative analyses of *M. myristica oil* are represented in Table 4.

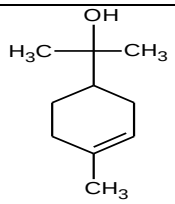
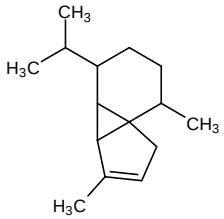
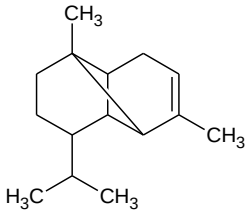
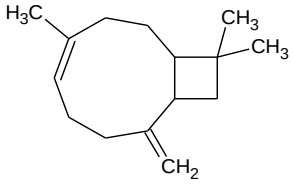
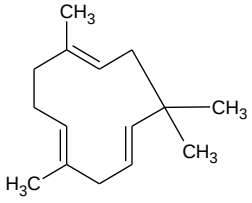
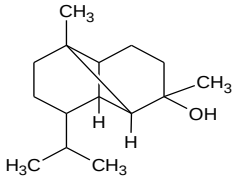
Table 4: Results of the quantitative analyses of *M. myristica* oil.

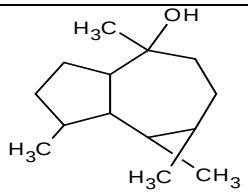
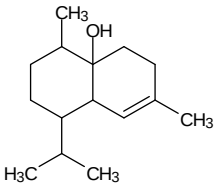
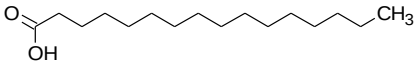
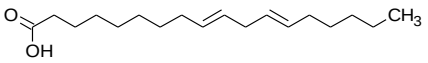
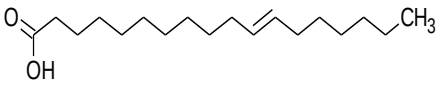
S/N	Parameter	Result (mg KOH/g)
1	Acid value	9.27
2	Saponification value	194.95
3	Ester value	181.18

GC-MS Analysis

GC/MS analysis shows that *M. myristica* potentially contains compounds such as terpineol, humulene, hexadecenoic acid and vaccenic acid. Some of the library suggested compounds are presented in Table 5.

Table 5: Summary of results of GC-MS analysis of *M. myristica oil.*

S/N	Retention time	Base peak	Library recommended compound	Structure
1.	6.554	59	L-alpha-terpineol	
2.	8.916	105	alpha-cubebene	
3.	9.281	105	copaene	
4.	9.825	93	caryophyllene	
5.	10.226	93	humulene	
6.	10.677	161	cubedol	

7.	11.535	43	1H-cycloprop[e]azulene-4-ol, decahydro-1,1	
8.	12.122	119	cubenol	
9.	15.297	73	n-hexadecanoic acid	
10.	16.841	55	9,12-octadecadienoic acid (Z,Z)-	
11.	16.892	55	cis-vaccenic acid	

DISCUSSION

Acute Toxicity Study

The acute toxicological screening showed that the oil sample had an LD_{50} of 316 mg/kg (intraperitoneal). This value indicates that the dichloromethane extract of *Monodora myristica* is moderately toxic as the LD_{50} was within the range 50 – 500 mg/kg. This may explain why multiple behavioral signs of toxicity, including loss of motor coordination, depression, immobility, staggered movements, hyperventilation, seizures and paralysis, were observed within 24 hours after administration of the extract. Instructively, these signs were absent in group 1 and group 2 of Phase I (Hodge and Sterner, 1943; Loomis and Hayes, 1996; Letsara *et al.*, 2020). In contrast to this study, however, several authors have reported different parts of *M. myristica*, extracted with different solvents, to be very safe (Feyisayo *et al.*, 2014; Isiogugu *et al.*, 2018; Ajayi *et al.*, 2013).

Quantitative Analysis

The acid value was determined to be 9.27 mg KOH/g oil. This is a bit higher than the value reported by Akinyede *et al.* (2016), as well as that recommended for cold pressed and virgin oils by the FAO-WHO Codex Alimentarius Commission (CXS 210, 1999). A high acid value may indicate an appreciable level of hydrolysis of the triglycerides which leads to increased amounts of free fatty acids (Moore *et al.*, 2020). Since hydrolysis is implicated as a pathway in rancidification, the oil may be prone to going rancid on prolonged storage. (Imoisi and Michael, 2020). The acid value is however lower than some reported values in literature (Ajayi *et al.*, 2004). The saponification value obtained was 194.95 mg KOH/g oil. The value is higher than that reported by Adolf *et al.*, (2018), for *M. myristica* (150.07 mg KOH/g). Several studies have also reported higher saponification values (Ajayi *et al.*, 2004; Bello *et al.*, 2014; Akinyede *et al.*, 2016). The saponification value shows that *M. myristica* oil can potentially find usefulness in the cosmetic industry.

GC-MS Analysis

The results of the GCMS analysis shows that the oil contains fatty acids such as n-hexadecanoic acid, cis-vaccenic acid and 9,12-octadecadienoic acid; as well as terpinoids such as alpha-terpineol and alpha-cadinol. The values of medicinal plants can be assessed or inferred from the phytochemical components found in them. Hence, as more bioactive compounds are confirmed to be present in *M. myristica*, more medicinal potentials of the plant would become evident (Okwu, 2001; Enabulele and Ehiagbonare, 2011)

CONCLUSION

The LD_{50} (intraperitoneal) of the dichloromethane extract of *Monodora myristica* seed was determined to be 316 mg/kg, indicating that the extract had moderate acute toxicity on the test animals. Hence, the test subjects exhibited toxicity-related behaviours such as seizures, paralysis, hyperventilation and immobility. The results of the GCMS analysis shows that the extract contains several bioactive fatty acids and terpenoids. From the results of the quantitative analysis, there are variations from recommended standards for commonly used vegetable oils. Therefore, the oil sample is not recommended for use in cooking food.

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