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COMPARATIVE EVALUATION OF LARVICIDAL PROPERTIES OF METHANOL EXTRACTS AND FRACTIONS OF *MYRISTICA FRAGRANS* SEED HOUTT (MYRISTICACEAE) AND *THYMUS VULGARIS* LEAF LINN (LAMIACEAE) ON THE FOURTH INSTAR LARVAE OF *CULEX QUINQUEFASCIATUS* LINN AND THE CONTROL OF FILARIASIS

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ABSTRACT

Introduction: Larvicides are generally chemical substances used to kill mosquitoes when in their larval stage, this is done by exhibiting certain acute toxic effects thus preventing its further progression to pupa and adult stages, thereby preventing the spread of diseases caused by mosquitoes. **Aim**: The aim of this work is to compare the larvicidal properties of methanol extracts and solvent partitioned fractions of *Myristica fragrans* HOUTT. seeds and *Thymus Vulgaris* Linn. leaves on the fourth instar larvae of *Culex quinquefasciatus* vector of filariasis. **Materials & Methods**: The *Myristica fragrans* seeds were obtained, crushed into coarse powder and defatted in n-hexane for 24 hours and air-dried. The defatted seeds and leaves powders were separately marcerated in 100% redistilled methanol for 72 hours. The percentage yields were obtained and the toxicity evaluation was done by exposing the fourth instar larvae of *Culex quinquefasciatus* to varying concentrations of reconstituted methanol

crude extracts and solvent partitioned fractions of *M.fragrans* seeds and *T. Vulgaris* leaves. The larval mortalities were determined using the non-linear regression analysis of a statistical package graph pad prism®. **Results**: The results of the LC_{50} of the methanol crude extracts of *M. fragrans* was 0.23mg/mL while that of the *T. Vulgaris* was 0.64mg/mL. The LC_{50} of the fraction of *M. fragrans* for n-hexane was 0.01mg/mL and 0.37mg/mL for Chloroform fraction respectively, while *T.vulgaris* had LC_{50} of 0.18mg/mL of n-hexane fraction and 0.55mg/mL for chloroform fraction, compared to that of nicotine with LC_{50} of 0.01mg/mL. Moreover, the larvicidal effects of the ethyl acetate and that of aqueous fractions were not feasible. **Conclusion**: The methanol crude extracts, n-hexane and chloroform fractions of *M. fragrans* considering differences in their percentage mortalities and LC_{50} had the highest larvicidal activity than *T. vulgaris*, but n-hexane fractions of *M. fragrans* and *T. vulgaris* offer great potentials in the vector control of culex mosquitoes that cause filariasis, even though n-hexane fraction of *M. fragrans* has a higher activity than *T. vulgaris* of n-hexane fraction comparing their LC_{50} of 0 01 mg/mL and 0.18mg/mL respectively and 0.01 mg/mL of the positive control, the nicotine.

KEYWORD: *Myristica fragrans, Thymus vulgaris*, Filariasis, Larvicides, Toxicity, Mortality, *Culex quinquefasciatus* and Nicotine.

INTRODUCTION

Insect-transmitted diseases are a major source of illnesses and deaths worldwide. Mosquitoes alone transmit diseases to more than 700 million people annually.[1] Therefore the control of mosquitoes is an important health concern. Several compounds obtained from plants possess potential insecticidal growth deterrent or repellant characteristics.[2] Essential oils and secondary metabolites are the useful components of plants which are complex mixtures of monoterpenes, sesquiterpenes and biological related phenolic compounds which are toxic to the larvae. Hence larvicides are generally chemical substances used to kill mosquitoes when in their larval stage. Filariasis is a disease common in the tropics and subtropics caused by the presence in the lymphatic vessels of the parasitic nematode worms *Wuchereria banchrofti* and *Brugia malayi* commonly called filarial worms. The worms which are transmitted to humans by culex mosquitoes bring about inflammation and eventual blocking of lymphatic vessels, caused the surrounding tissues to swell resulting in elephantiasis.[3] The rupture of urinary lymphatics may lead to the presence of chyle in the urine resulting in development of series of serious medical conditions such as heart failure, portal hypertension, cirrhosis and

various cancers. A primary cause of this is the rapid and unplanned growth of cities which creates numerous breeding sites for the mosquitoes that transmit the disease.[4] When a mosquito with infected stage larvae takes a blood meal, the parasites are deposited in the human's body where they migrate to the lymphatic vessels and develop into adult worms over a period of 6-12 months, causing damage to and dilatation of the lymphatic vessels. The adult filariae live for several years in the human host, during this period they produce millions of immature microfilariae that circulate in the peripheral blood and are ingested by mosquitoes that bite the infected human. The larvae further develop inside the mosquito before becoming infectious to man.[5] *Myristica fragrans* HOUTT (nutmeg) are dried seeds of the plant, from the family Myristicaceae, an evergreen tree about 10 to 20 m high, indigenous to Molucca Islands. The plant is cultivated in Indonesia, Malaysia (Mollucca Islands, Sumatra, Java and Penang), Ceylon and West Indies(Grenada). It is used as a psychotropic agent. Pharmacological studies revealed that it has aphrodisiac, antioxidant, anti-inflammatory, anticancer, sedative, antilipemic, anticaries, antidiarrhoeal properties.[6] It has been reported to contain myristicin and elemicin components, the formal relationship of these compounds to Amphetamins, others include terpenes, alcohols and phenols.[7] *Thymus vulgaris* Linn. is an evergreen herbaceous shrub growing 15 to 30 cm high. It has woody, branched stems and very small, opposite leaves, hairy on the underside linear to oval in shape. The plant has an agreeable aromatic smell and a warm pungent taste. The fragrance of its leaves is due to an essential oil which gives its flavouring value for culinary purposes and is also the source of its medicinal properties. It is in flower from May to August.[8] Pharmacological studies revealed its antispasmodic, anti-inflammatory, antiamoebic and antibacterial properties. Its other important chemical constituents include thymol, carvacrol, camphene and limonene.[9]

A **B**

Figure 1. (A) *Myristica fragrans* seeds, (B) *Thymus vulgaris* leaves.

METHODS: *Myristica fragrans* seeds (BRAND-BENO NUTMEG) and Thymus Vulgaris leaves (BRAND-TIGER THYME) were bought from Itam market in Itu Local Government Area, Akwa Ibom state, Nigeria.

LARVAL COLLECTION: Larvae were collected from a breeding site in Ewet Housing Estate in Uyo Local Government Area, Akwa Ibom State, Nigeria and were reared in plastic buckets.

PREPARATION OF METHANOL CRUDE EXTRACTS AND PRELIMINARY LARVAL TOXICITY TESTS

The seeds of *Myristica fragrans* were crushed and the coarse powdered product of 1374g was defatted with n-hexane by cold marceration at room temperature for 24 hours, filtered and the filtrate evaporated to dryness, leaving behind the oil obtained from the seeds of *M.fragrans*. The powder was dried and re-marcerated with 100% redistilled methanol by cold marceration at room temperature, with regular stirring for 72 hours, it was filtered and concentrated to dryness. Similarly, the dried leaves of *Thymus Vulgaris* of 740g was extracted with 100% redistilled methanol by cold marceration at room temperature for 72 hours, filtered and the filtrate concentrated to thick extract. These extracts were used in preliminary larval toxicity tests.[10]

Bioassay-guided fractionation of methanol crude extracts: The active methanol crude extracts of *M. fragrans* seeds and *T. Vulgaris* leaves of 30g were separately dissolved in methanol - water in the ratio 3:1 and partitioned successively with n-Hexane, chloroform, ethyl acetate and the residues were considered as aqueous fractions. All the fractions were concentrated to solid residues and percentage yields obtained and recorded.[10]

PREPARATION OF STOCK SOLUTIONS

Stock solutions of crude extracts and fractions of n-Hexane, chloroform, ethyl acetate and aqueous were prepared as follows: The methanol extract and the partitioned fractions of 0.2g were dissolved in 1mL of ethanol and subsequently into 99mL of dechlorinated water to make up to 100mL and a stock solution of 2mg/mL.[10]

LARVAL TOXICITY TEST

The prepared stock solution of each extract and fraction of 50mL, 25mL, 12.5mL, 6.25mL and 3.125mL were dispensed into sterile cups and were serially diluted with declorinated

water to 100mL in each disposable cup to the test concentration of 0.0625 to 1.000mg/mL as activity-guided screening. 20 fourth instar larvae were released into each cup of 100mL solution and toxicity of the extracts and solvent partitioned fractions were obtained by percentage (%) mortality. After 24 hours contact, the number of dead larvae in cups were counted and recorded. Positive and negative experiments were also done with Nicotine and 1% of ethanol respectively. All the experiments were done in duplicates.[10]

Statistical Analysis: Results were expressed as means ± SEM of two independent experiments. Larval toxicities were reported as LC_{50} obtained from Graph Pad Prism® Statistical Software.

RESULTS

Table 1: Extraction yields of methanol crude extracts.

Plant	Morphological part used	Quantity Extracted(g)	Quantity Yield(g)	Percentage Yield (%)
Myristica Fragrans	Seeds	1374	Oil=>448.9 (520ml) Extract=>103.1	32.7 7.5
Thymus Vulgaris	Leaves	740	80.7	10.9

Table 2: Larvicidal effect of methanol extract of *Myristica fragrans*(seed) on the fourth instar larvae of *Culex quinquefasciatus* after 24 hours of exposure (0.2g).

Conc. mg/mL	Dead	Alive	Mean± SEM of Dead Larvae	%Mortality ± SEM
1.0000	20 17	0 3	18.5±1.50	92.5±1.50
0.5000	17 11	3 9	14.0±3.00	70.0±3.00
0.2500	11 10	9 10	10.5±0.50	52.5±0.50
0.1250	9 4	11 16	6.5±2.50	32.5±2.50
0.0625	8 4	12 16	6.0±2.00	30.0±2.00

Table 3: Larvicidal effect of methanol extract of *Thymus vulgaris* (leaf) on the fourth instar larvae of *Culex quinquefasciatus* after 24 hours of exposure (0.2g).

Conc. mg/mL	Dead	Alive	Mean± SEM of Dead Larvae	%Mortality ± SEM
1.0000	17 13	3 7	15.0±2.00	75.0±2.00
0.5000	8 8	12 12	8.0±0.00	40.0±0.00
0.2500	8 6	12 14	7.0±1.00	35.0±1.00
0.1250	6 4	14 16	5.0±1.00	25.0±1.00
0.0625	6 2	14 18	4.0±2.00	20.0±2.00

Table 4: Larvicidal effect of Nicotine (positive control) and 1% Ethanol (negative control) on the fourth instar larvae of *Culex quinquefasciatus* after 24 hours of exposure (0.2g).

Conc. mg/mL	Dead	Alive	Mean± SEM of Dead Larvae	%Mortality ± SEM
1.0000	20 20	0 0	20.0±0.00	100±0.00
0.5000	20 20	0 0	20.0±0.00	100±0.00
0.2500	20 20	0 0	20.0±0.00	100±0.00
0.1250	20 20	0 0	20.0±0.00	100±0.00
0.0625	20 20	0 0	20.0±0.00	100±0.00
0.0313	20 20	0 0	20.0±0.00	100±0.00
0.0156	13 11	7 9	12.0±1.00	60.0±1.00
0.0078	10 8	10 12	12.0±1.00	60.0±1.00

0.0039	2 4	18 16	3.0±1.00	15.0±1.00
0.0020	1 3	19 17	2.0±1.00	10.0±1.00
1% Ethanol	1 1	19 19	1.0±0.00	5.0±0.00

Table 5: Median lethal concentrations (LC_{50}) for the Methanol extracts of M. *fragrans*, T. v*ulgaris*, and Nicotine.

Plant	Median lethal concentrations(LC_{50}) mg/mL
M.*fragrans*	0.23
T.*vulgaris*	0.64
Nicotine	0.01

Table 6: Solvent partitioned fractions yield.

Plant	Quantity used(g)	Solvents used in partitioning	Yield (g)	Percentage Yield (%)
M.fragrans	30	N-Hexane	7.1	23.67
		Chloroform	5.6	18.67
		Ethylacetate	4.5	15.00
		Aqueous	11.0	36.67
T.vulgaris	30	N-Hexane	2.2	7.33
		Chloroform	7.2	24.00
		Ethylacetate	4.2	14.00
		Aqueous	9.4	31.33

Table 7: Median Lethal concentrations (LC_{50}) for solvent partitioned fractions.

Solvent partitioned fractions	M.*fragrans* LC_{50} (mg/mL)	T.*vulgaris* LC_{50} (mg/mL)
n-Hexane	0.01	0.18
Chloroform	0.37	0.55
Ethylacetate	N.F	N.F
Aqueous	N.F	N.F

N.F=Not Feasible

Table 8: Larvicidal effect of n-Hexane fraction of *Myristica fragrans* (seed) on the fourth instar larvae of *Culex quinquefasciatus* after 24 hours of exposure.

Conc. mg/mL	Dead	Alive	Mean± SEM of Dead Larvae	%Mortality ± SEM
1.0000	20 20	0 0	20.0±0.00	100±0.00
0.5000	20 20	0 0	20.0±0.00	100±0.00
0.2500	20 20	0 0	20.0±0.00	100±0.00
0.1250	20 20	0 0	20.0±0.00	100±0.00
0.0625	19 19	1 1	19.0±0.00	95±0.00
0.03125	19 19	1 1	19.0±0.00	95±0.00
0.0156	19 19	1 1	19.0±0.00	95.0±0.00
0.0078	10 7	10 13	8.5.0±1.50	42.5±1.50

Table 9: Larvicidal effect of n-Hexane fraction of *Thymus vulgaris* (leaf) on the fourth instar larvae of *Culex quinquefasciatus* after 24 hours of exposure.

Conc. mg/mL	Dead	Alive	Mean± SEM of Dead Larvae	%Mortality ± SEM
1.0000	20 20	0 0	20.0±0.00	100±0.00
0.5000	20 20	0 0	20.0±0.00	100±0.00
0.2500	13 13	7 7	13±0.00	65.0±0.00
0.1250	8 7	12 13	7.5±0.50	37.5±0.50
0.0625	2 2	18 18	2±0.00	10.0±0.00

Table 10: Larvicidal effect of Chloroform fraction of *Myristica fragrans* (seed) on the fourth instar larvae of *Culex quinquefasciatus* after 24 hours of exposure.

Conc. mg/mL	Dead	Alive	Mean± SEM of Dead Larvae	%Mortality ± SEM
1.0000	20 20	0 0	20.0±0.00	100±0.00
0.5000	16 13	4 7	14.5±1.50	72.5±1.50
0.2500	5 7	15 13	6.0±1.00	30±1.00
0.1250	1 0	19 20	0.5±0.50	2.5±0.50
0.0625	1 0	19 20	0.5±0.50	2.5±0.50

Table 11: Larvicidal effect of Chloroform fraction of *Thymus vulgaris* (leaf) on the fourth instar larvae of *Culex quinquefasciatus* after 24 hours of exposure.

Conc. mg/mL	Dead	Alive	Mean± SEM of Dead Larvae	%Mortality ± SEM
1.0000	20 19	0 1	19.5±0.50	97.5±0.50
0.5000	9 9	11 11	9±0.00	45.0±0.00
0.2500	1 1	19 19	1±0.00	5±0.00
0.1250	1 1	19 19	1±0.00	5±0.00
0.0625	1 1	19 19	1±0.00	5±0.00

Table 12: Larvicidal effect of Ethyl acetate fraction of *Myristica fragrans* (seed) on the fourth instar larvae of *Culex quinquefasciatus* after 24 hours of exposure.

Conc. mg/mL	Dead	Alive	Mean± SEM of Dead Larvae	%Mortality ± SEM
1.0000	6 6	14 14	6±0.00	30±0.00
0.5000	4 2	16 18	3±1.00	15±0.00
0.2500	3 3	17 17	3±0.00	15±0.00
0.1250	2 1	18 19	1.5±0.50	7.5±0.50
0.0625	2 1	18 19	1.5±0.50	7.5±0.50

Table 13: Larvicidal effect of Ethyl acetate fraction of *Thymus vulgaris* (leaf) on the fourth instar larvae of *Culex quinquefasciatus* after 24 hours of exposure.

Conc. mg/mL	Dead	Alive	Mean± SEM of Dead Larvae	%Mortality ± SEM
1.0000	2 0	18 20	1.0±1.00	5±1.00
0.5000	1 0	19 20	0.5±0.50	2.5±0.50
0.2500	1 0	19 20	0.5±0.50	2.5±0.50
0.1250	1 0	19 20	0.5±0.50	2.5±0.50
0.0625	1 0	19 20	0.5±0.50	2.5±0.50

Table 14: Larvicidal effect of Aqueous fraction of *Myristica fragrans* (seed) on the fourth instar larvae of *Culex quinquefasciatus* after 24 hours of exposure.

Conc. mg/mL	Dead	Alive	Mean± SEM of Dead Larvae	%Mortality ± SEM
1.0000	2 4	18 16	3±1.00	15±1.00
0.5000	1 4	19 16	2.5±1.50	12.5±1.50
0.2500	3 1	17 19	2±1.00	10±1.00
0.1250	3 1	17 19	2±0.00	10±1.00
0.0625	1 1	19 19	1±0.00	5±0.00

Table 15: Larvicidal effect of aqueous fraction of *Thymus vulgaris* (leaf) on the fourth instar larvae of *Culex quinquefasciatus* after 24 hours of exposure.

Conc. mg/mL	Dead	Alive	Mean± SEM of Dead Larvae	%Mortality ± SEM
1.0000	4 3	16 17	3.5±0.50	17.5±0.50
0.5000	1 1	19 19	1±0.00	5.0±0.00
0.2500	1 1	19 19	1±0.00	5.0±0.00
0.1250	0 0	20 20	0±0.00	0.0±0.00
0.0625	0 0	20 20	0±0.00	0.0±0.00

DISCUSSION

This research work has shown that plants do not serve only as a source of food, drug and shelter, but also as an active botanical larvicides/insecticides. Vector control is facing a threat due to emergence of resistance of vector (Mosquitoes) to conventional synthetic insecticides, warranting either counter measures or development of newer insecticides.[11] With the current development, botanical insecticides may serve as suitable substitutes for synthetic insecticides because of the bioactive chemicals they contain. They are biodegradable, readily

available in many parts of the world, and relatively safe. In the toxicity test of the methanol crude extracts of these two plants on larvae of *Culex quinquefasciatus* at the concentration range of 0.0625 to 1.0000mg/mL, the percentage mortality of *M. fragrans* ranged from 30.0± 2.00% to 92.5±1.50% (Table 2) with LC_{50} of 0.23mg/mL as shown in Table 4, while that of *T. vulgaris* ranged from 20.0± 2.00% to 75.0 ±2.00% (Table 3) with LC_{50} of 0.64mg/mL as shown in Table 4. It was also observed that every concentration of *M. fragrans* produced higher percentage mortality than that of *T. vulgaris* as in table 2 compared to table 3. This indicated that *M.fragrans* had a higher larvicidal activity than *T.vulgaris* when comparing their median lethal concentrations (LC_{50}). Hence, *M. fragrans* methanol crude extract was over 2 times higher than that of *T.vulgaris.* Larvicidal activities of the different solvent partitioned fractions of the two samples were also carried out with a concentration range of 0.0625 to 1.0000mg/mL. The n-Hexane fraction of *M.fragrans* which had a concentration range of 0.0078 to 1.0000mg/m because at the least concentration of 0.0625mg/mL, the LC_{50} was not attainable thus it was further diluted to 0.0078mg/mL. The results showed that even though the n-Hexane fractions of the two plants had 100% mortality at 1.0000mg/mL concentration, n-Hexane fraction of *M. fragrans* recorded higher percentage mortalities of 100± 0.00%, 100± 0.00%, 100± 0.00%, 100±0.00%, 95±0.00%,95±0.00%, 95±0.00%, and 42.5±0.50% with their LC_{50} of 0.01mg/mL compared to 100±0.00%, 100±0.00%, 37.5±0.50% and 10.0±0.00% with LC_{50} of 0.18mg/mL of *T.vulgaris* (Table 7). The Chloroform fraction of *M. fragrans* with LC_{50} of 0.37mg/mL was more active than the chloroform fraction of *T. vulgaris* with LC_{50} of 0.55mg/mL (table 7). Also, comparing the larvicidal activities of the other solvent partitioned fractions (which had no LC_{50}) were ethyl acetate fraction of *M. fragrans* which had a higher larvicidal activity than *T.vulgaris* based on their percentage mortality, and the activity of aqueous fraction of *M.fragrans* had a higher larvicidal activity than *T.vulgaris* based on their percentage mortality as shown in tables 12, 13, 14 and 15 respectively. Moreover, Nicotine was used as positive control which had LC_{50} of 0.01mg/mL and 1% ethanol used as negative control. Hence, it may be wise to isolate the active component(s) that gave the n-hexane fractions such great activities when compared to their percentage mortalities and LC_{50} of nicotine, the reference drug used.

CONCLUSION

From the results obtained, even though the methanol extracts and fractions *M. fragrans* were more active than those of *T.vulgaris*, but it can be conclude that n-Hexane fractions of *M.fragrans* and *T. vulgaris* offer great potentials as new vector control agents against *Culex*

mosquitoes based on their percentage mortalities and LC_{50} (median lethal concentrations). Now, based on the mechanism of action of larvicides, this mosquito-borne disease, filariasis can be successfully controlled since it acts or targets the transmission cycle of the vector involved.

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