ejpmr, 2016,3(2), 95-99

SJIF Impact Factor 2.026

EUROPEAN JOURNAL OF PHARMACEUTICAL AND MEDICAL RESEARCH

www.ejpmr.com

Research Article

ISSN 3294-3211

EJPMR

PHYTOCHEMICAL ANALYSIS OF THE ETHYLACETATE-SOLUBLE CONSTITUENTS OF MONODORA MYRISTICA SEED ISOLATED BY PREPARATIVE THIN LAYER CHROMATOGRAPHY (PTLC)

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Article Received on 10/12/2015 **Article Revised on 01/01/2016** **Article Accepted on 22/01/2016**

ABSTRACT

Monodora myristica (fam. Annonaceae) is one of the antimalarial plants in Ibo-Nigeria folkloric medicine with claims of antimicrobial and antifungal properties which have been confirmed by the authors in preceding preliminary studies. The Monodora plant is an ornamental tree with a height of up to 30m high, dense foliage and spreading crown. It flowers from September to April at which time the new leaves appear. The fruits are produced between April and September. They are about 15cm in diameter, green, round, woody and are suspended in a long stalk. The pulp is white and contains numerous seeds of about 2.5cm long. Previous preliminary studies on the plant's seed by the authors showed that the ethylacetate-soluble fraction of its methanolic extract possess significant and higher antimicrobial properties than the ethylacetate-insoluble fraction. The study was therefore designed to evaluate the antimicrobial properties and potential therapeutic applications of the the ethylacetate-soluble constituents of the plant's seed. Monodora myristica seeds were sun-dried, milled and extracted by cold maceration with 95% methanol. This methanolic extract was further fractionated with ethylacetate to obtain an ethylacetate-soluble fraction and an ethylacetate-insoluble fraction. The constituents of the ethylacetate-soluble fraction were then isolated by PTLC using pre-coated alumina and silica gel GF_{254} plates.

KEYWORDS: Monodora myristica, Phytochemistry, Ethylacetate fractions, Preparative thin layer chromatography, Ibo-Nigeria folkloric medicine.

1. INTRODUCTION

Monodora myristica seed has been identified as a seed that possess antimicrobial properties.[1] The seed of the plant yields a colorless volatile oil which has a pleasant taste and aroma. As such it is used as a condiment for soup. It is added into snuff as a flavoring agent. The seeds are also used to treat migraine by external application to the fore-head. It is used as a stomachic and by mixing it with palm-oil, it is equally used as a stimulant. Pomade made from the pulverized seeds fried in oil as well as the powder is used to treat Guinea worm and other sores. A related specie M. tenuifolia is used as an anthelmintic and in Yoruba-Nigeria traditional medicine for the treatment of toothache. Some other family members are also used in the treatment of malaria. The pulverized seeds have also been employed as pesticides in the preservation of some agricultural products. It exhibits significant antimicrobial properties especially against the fungi and thus can be employed for the treatment of fungi infections. It is also used as a flavouring agent (local spice) in the preparation of the pepper source (ose orji) used for eating Garden egg by Ibo-Nigerians. It is also of value in the prevention of biodegradation of plant agricultural products e.g Okra (Abelmoschus spp) where it has shown significant ability to inhibit the growth of fungi isolated from a deteriorating Okra sample. It has also been associated with anti-protozoal and anti-parasitic activities.

The seed oil gave a high yield of saturated and unsaturated fatty acids on saponification, gas-liquid chromatography revealed large amounts of C-16 and C-18 fatty acids with traces of other fatty acids while the iodine values indicated that both ethanol and hexane extraction produced excellent yields of fatty acids with hexane showing more efficiency as a crystallization solvent at a solvent-oil ratio of 3:1 at 50^{o}C. The essential oil of the seed has also been found to be mainly monoterpenic, the major component being alpha-phellandrene (Sabinene) and Myrcene while the oils from the leaves was mainly sesquiterpenic with the major constituent being beta-caryophylene.

Chromatography refers to a number of highly efficient techniques for the separation of a wide variety of substances ranging from inorganic ions to complex biopolymers. For all types of chromatography, the distribution of solute between the phases results from the

balance of forces (polar, dispersive or ionic forces) between the molecules of the solute and the molecules of each phase.

2. EXPERIMENTAL

2.1. Materials

2.1.1. Plant Materials: This consisted of the seeds of Monodora myristica. They were collected in September at Nsukka in Enugu state of Nigeria by Mr Paulinus Ugwu and Mr J.E Ekekwe both of Botany department of University of Nigeria, Nsukka. They were then prepared by cutting, sun - drying and milling. The powdered forms were then used in the experiments.

2.1.2. Reagents: Sulphoric acid, Chloroform, Ammonia solution, Ferric chloride, Fehling's solution 1 and 11, Ethylacetate, Hydrochloric acid, Glacial acetic acid, Aluminium chloride, Ethanol, Bromine water, Mayer's reagent, Distilled water, Sodium hydroxide, Tollen's reagent, 2,4 – dinitrophenylhydrazine, Acetic anhydride, acetic acid, silica gel GF_{254}.

2.1.3. Solvents

Methanol, Ethylacetate, Methylethylketone (MEK), MEK / Hexane, Dimethyl sulphoxide (DMSO), Chloroform and Ethanol.

2.1.4. Instrumentation: Uniplan TLC spreader, Chromatographic tank, Aluminium plates, Silica plates, Seperating funnel, Evaporating dish, Rotary evaporator, Water bath, Capillary tubes, Test tubes, Conical flasks, Measuring cylinders, Beakers, Pipettes, Funnels, Filter papers, Weighing balances, Glass chromatoplates, UV lamp, Bunsen burner and Spatula.

3. METHODS

3.1. EXTRACTION AND FRACTIONATION OF MONODORA MYRISTICA

The extraction was carried out by cold maceration using 95% methanol. The powered drug was cold macerated with the solvent for 24 hours. The extract was filtered off. The process repeated several times until the constitutions were completely extracted, indicated by the colorlessness of the extraction solvent.

The extract was then evaporated to dryness under reduced pressure, using the rotary evaporator. The extract was then washed with several portions of ethylacetate to get the ethylacetate-soluble and ethylacetate-insoluble fractions. The solution of the ethylacetate-soluble fraction was evaporated to dryness with the rotary evaporator.

3.2. PREPARATION OF PTLC PLATES

Five glass plates were washed using detergent solution and allowed to dry. The plates were arranged in a row in the spreading rail and cleaned with acetone-soaked cotton wool. 40g of the silica was mixed with distilled water in the ratio of (1:2) and vigorously shacked in a conical flask to form slurry. This was poured into the spreading rail and the spreader moved to coat the plates to 0.7mm thickness. The coated plates were allowed to air-dry at room temperature and then activated at 110^{o}C for 1hr before being used.

3.3. ANALYTICAL TLC OF THE FRACTIONS

Analytical TLC of the ethylacetate-soluble and insoluble fraction was carried out using pre-coated alumina and silica gel GF_{254} plates. The solutions of the ethylacetate-soluble and ethylacetate-insoluble fractions were prepared in dimethylsulphoxide. About 3 drops of each solution was placed about 8mm from one end of the plate. On drying the plate was gently dipped into a small chromatographic tank previously equilibrated with the developing solvent system for development. After the solvent had moved a reasonable distance, the plate was removed, allowed to dry, viewed and marked under both the UV (254 and 365nm) light.

3.4. ISOLATION OF ETHYLACETATE-SOLUBLE FRACTION CONSTITUENTS BY PTLC

The ethylacetate-soluble fraction was then separated by applying the solution as a band about 4cm from the edge of the glass plate using a capillary tube. The spotted band was allowed to dry and the plate gently placed into the developing solvent system, Hexane-MEK (3:1). The tank was securely closed and the plate developed by allowing the solvent to run up the spotted plate. After the solvent front had moved to the edge of the plate, it was removed from the tank and marked under UV-light. The different bands were crapped out into different bottles and labeled. The entire isolation process is represented in figure 3.4 below.

3.5. PHYTOCHEMISTRY OF THE PTLC ISOLATES

The various phytochemical tests that gave positive results with the crude monodora extract in the preliminary study were carried out on all the isolated bands of the ethlacetate-soluble fraction. Desorption/extraction from the adsorbent was done using the appropriate solvents for each phytochemical test. The tests carried out were tests for glycosides, sterols and triterpenoids, aldehydes, and unsaturated compounds.

3.5.1. TEST FOR GLYCODSIDES (HYDROLYSIS TEST)

0.1g of 2ml of aqueous solution methanolic extract of the powder was boiled with 3ml of dilute sulphuric acid for 15 minutes. The filtrate was neutralized with 20% a sodium hydroxide and about 5ml of equal volumes of fehling's solution A and B mixture was added to the mixture and boiled. This was observed when cold for some brick red precipitate.

3.5.2. TEST FOR STEROLS AND TRITERPENOIDS

0.1g of the extract was dissolved in 5ml chloroform.

(i) 2ml chloroform solution was evaporated to dryness, and re-dissolved with a 5 parts conc H_2SO_4 – 1 part

water solution and then observed for a dark green colour. (molechott test).

(ii) 2ml chloroform solution was concentrated and about 1ml of conc H_2SO_4 was added and the mixture observed for a brown colour at the interface. (Salkowski's test).

3.5.3. TEST FOR ALDEHYDES (TOLLEN'S REAGENT TEST)
To 2ml methanol extract was added about 1ml of tollen's reagent (prepared by precipitating the Ag from $AgNo_3$ with NaoH and re-dissolution with ammonium solution) and heated for about 20 minutes. This was then observed for the precipitation of silver ions (sliver mirror).

3.5.4. TEST FOR UNSATURATION (BROMINE WATER TEST)
To about 2ml. aqueous ethanol solution of the sample was added about 2ml of bromine water. This was then observed for bromine water decolourization.

4. RESULTS

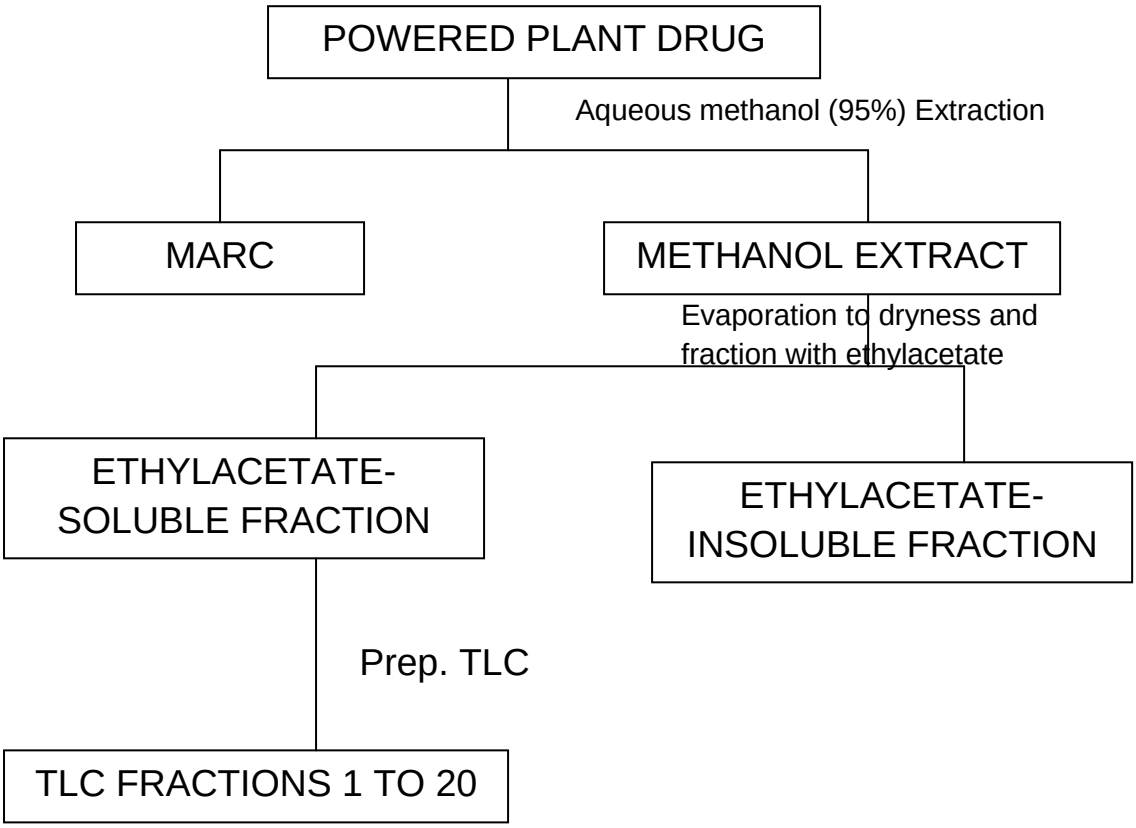

Figure 1. Flow chart for isolation

4.1. RESULTS OF PTLC OF ETHLACETTE SOLUBLE FRACTION.
The solvent system – MEK (3:1) separated the ETOAC – soluble fraction into 20 bands with some bands poorly separated and hence combined for the anti-microbial and phytochemical tests.

The results of the separation are shown in figure 2 below. Table 2 shows the band colors, sizes and Rf values.

Table 4.1

BANDS	COLOURS (UV)		SIZES (MM)	DISTANCES (MM)	RF VALUES
	254nm	365nm			
1	Yellow	Reddish brown	1.85	0.85	0.0531
2/3	Yellowish gray	Purple	2.20	2.70	0.1688
3x	Grey	Purple brown	0.20	2.72	0.1700
4/5	""	Light Fl blue	0.60	4.90	0.3063
6	Light yellow	Grey	1.80	5.50	0.3438
7/8	Yellowish grey	Purple	0.55	7.30	0.4563
9	Light yellow	Grey	1.05	7.85	0.4906
10	Grey	Violet	0.60	8.90	0.5563
11	White	Fl blue	1.30	9.50	0.5938

12/13	Light yellow	Purple grey	130	10.80	0.750
14	Light yellow	Sky blue	0.85	12.10	0.7563
15	Yellow	Yellow	0.333	13.28	0.8094
16	White	White	0.42	13.70	0.8300
17	Grey	Violet	1.60	14.30	0.8563
18	White	Sky blue	0.45	14.30	0.8938
19	White	Purple	0.40	14.75	0.9219
20	White	Sky blue	0.85	15.15	0.9469

NB: SOLVENT FRONT = 16.00mm

Rf = Distance / Solvent front

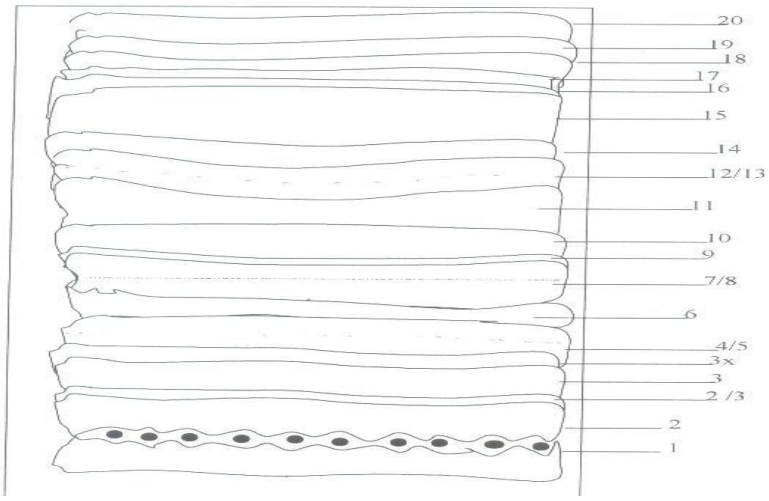

Figure 2. PTL Chromatogram of ETAC-Soluble fraction of M. myristica seed on Silica gel (GF_{254}) using Hexane-Mek (3:1) as solvent system

4.2. RESULTS OF PHYTOCHEMICAL TESTS ON PTLC FRACTIONS

The results of the phytochemical tests on the various PTLC bands are given in table 4.2 below.

		PTLC BANDS																
Phytochemical classes		1	2	2/3	$^{4}/_{5}$	3	3x	$^{7}/_{8}$	9	10	11	$^{12}/_{13}$	14	15	26	17	18	19	20
1	Alkaloids	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
2	Glycosides	+	-	+	-	-	-	-	-	-	+	+	-	-	-	-	-	-	-
3	Tannins	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
4	Saponins																		
5	Flavonoids	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
6	Sterold and triterpenoids	+	+	-	-	-	-	-	-	-	+	+	+	+	+	+	+	+	-
7	Aldehydes	-	+	++	*	+	+	+	+	++	-	++	-	+	-	-	-	-	-
8	Steroidal aglycores	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
9	Carbonyl Compound	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
10	Unsaturated Compounds	+	+	+	+	+	+	+	+	+	-	+	+	+	+	+	-	-	-

* could not be determined because it was in trace amounts.

5. DISCUSSION

The phytochemistry of the isolates, like in the preliminary study, showed the presence of glycosides, sterols, triterpenoids, aldehydes and unsaturated compounds. Also the test for alkaloids, tannins, saponins, flavonoids, steroidal aglycones and carbonyl compounds gave negative result.

Bands 1 and 2/3 tested positive for glycosides. Glycosides are a large and varied group of polar compounds occurring mainly in higher plants and also to a small extent in lower forms of plant life. They are characterized by the possession of a glycosidic linkage between one or more sugar moieties and anon-sugar moiety (the aglycone or genin) in the molecule. Though the possession of a sugar unit gives them a nature group identity, they however vary much in their physical, chemical and pharmacological properties due to the varied nature and complexity of their aglycones. Their pharmacological actions include purgation, cardioactivity and antimicrobial activities.[2]

Bands 1 and 2 tested positive for sterols/triterpenoids. Sterols are modified steroids in which the side chain is an aliphatic one containing one or more hydroxyl groups attached in alycyclic linkage. Steroids form a group of structurally compounds widely distributed in animals and plants. They include sterols, vitamin D, the bile acids, a number of sex hormones, the adrenal cortex hormones, some carcinogenic hydrocarbons, certain sapogenins etc. Triterpenoids are terpenoids whose carbon skeleton are based on six isoprene units derived from the acyclic C_{30} hydrocarbon, squalene. They are colorless, crystalline, optically active often with high melting point and generally difficult to characterize due to their chemical unreactivity. They occur in waxy coatings of leaves and on fruits such as apple and pear. They serve a protective function in repelling insects and microbes. Large quantities of triterpenes are found in the latex and resins of many plants and they serve as a chemical defence against pathogens and herbivores. They are expected to act against certain human and animal pathogens but for their their hydrophobic nature which limits their therapeutic application. The recent advances in drug solubilization techniques is expected to overcome this problem. The biological activities of triterpenes include anti-tumour anti-inflammatory, antiviral and antibacterial activity.[3]

Bands 2, 2/3, 3, 3x, 7/8, 9, 10, 12/13, 15, and 17 all tested positive for aldehydes. Aldehydes are simple organic compounds which contain a carbonyl group – carbon-oxygen double bond. They are simple in the sense that they don't have other reactive groups like –OH or –Cl directly attached to the carbon atom in the carbonyl group. Eg methanal, ethanol, propanal, 2-methylbutanal.[4] They are formed by partial oxidation of primary alcohols and form carboxylic acids when further oxidized. They are used for the manufacture of synthetic resins like Bakelite, and for making dyestuffs, flavourings, perfumes and other chemicals. Some are used as preservatives and disinfectants.[5]

Bands 11, 17, 18, 19 and 20 tested positive for unsaturated compounds. Unsaturated compounds are chemical compounds that contain carbon-carbon double bonds or triple bonds.[6] They can also have functional groups.[7] Thus the constituents of M. myristica seed may be triterpenoids existing as steroidal aglycones or glycosides. The authors however are conducting further studies on this plant by separating its constituents and analyzing the antimicrobial and phytochemical characteristics of the isolates.

These results indicate that the constituents of these bands are triterpenoids existing as steroidal aglycones or glycosides. We however recommend further in-vitro and in-vivo studies on the extracts of this plant's seed to confirm these findings, particularly the ethylacetate-insoluble fraction which the authors did not follow up in details in this serial study.

6. CONCLUSION

The study concluded that the constituents of Monodora myristica may be triterpenoids existing as steroidal aglycones or glycosides.

7. ACKNOWLEDGEMENT

Mr Paulinus Ugwu and Mr J.E Ekekwe of Botany department, University of Nigeria, Nsukka for assisting in the identification and collection of the plant materials.

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