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Radical Scavenging Capacity and Efficacy of *Myristica fragrans* (Nutmeg) Metabolites on *Cladosporum herbarum* of Food Origin

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Authors' contributions

This work was carried out in collaboration between all authors. Author SOJ designed the study, performed the statistical analysis, wrote the protocol and wrote the first draft of the manuscript. Authors OHLP and KAA managed the analyses of the study. Authors SOJ and OHLP managed the literature searches. All authors read and approved the final manuscript.

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ABSTRACT

Aim: To determine radical scavenging capacity and efficacy of *Myristica fragrans* metabolites on fungi of food origin.
Study of Design: Pretreatment and processing of *Myristica fragrans,* solvent extraction technique, phytochemical screening, radical scavenging activity, total phenolic concentration assay, essential oil extraction and evaluation of antifungal activity.
Place and Duration of Study: Microbiology Unit, Department of Biological Sciences, College of Natural and Applied Sciences, Fountain University Osogbo, Osun State, Nigeria between October, 2015 and July, 2016.

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Methodology: Crude extract of *Myristica fragrans* seed was obtained using organic solvent (distilled water, ethyl acetate and ethanol) with solvent extraction technique and preliminary phytoconstituents was determined. The essential oil was extracted by hydrodistillation technique and isolated with petroleum ether. Metabolites present in the essential oil were quantified using Gas Chromatography Flame Ionization Detection (GCFID). Antifungal activity of *Myristica fragrans* oil and crude extract were investigated using agar well diffusion method. Folin-Ciocalteu and 2,2, diphenyl picryl hydrazyl (DPPH) radical scavenging assays were employed to determine total phenolic content and antioxidant activity respectively.

Results: Preliminary phytochemical screening of *Myristica fragrans* seed revealed the existence of alkaloid, phenols, flavonoids, terpenes, saponins, glycosides, tannins, steroids and phenolic compounds. The percentage yield of *Myristica fragrans* oil extracted was 3.25%. Thirty-six metabolites were quantified in *Myristica fragrans* essential oil using GCFID among which are sabinene (26.58%), myristicin (13.55%), alpha-pinene (11.84%), terpinene-4-ol (9.35%), limonene (5.74%), safrole (5.40%), alpha-terpineol (4.51%), alpha-myrcena (3.82%), gama- terpinene (3.71%), alpha-terpinolene (3.19%), pinene-2-ol (1.84%), elimicin (1.27%) and isoeugenol (1.13%) respectively. Highest scavenging and antifungal activities were observed in ethyl acetate extract of *Myristica fragrans* compared to Beta-carotene and antifungal drug (Fluconazole) used as control at varying concentrations.

Conclusion: Presence of thirty-six different phytoconstituents (metabolites) in *Myristica fragrans* essential oil poses the potential of providing useful drugs for treating food-borne infection and reduction of oxidative stress in the body other than its general uses as spices and flavoring agent.

Keywords: Myristica fragrans; sabinene; beta-carotene; flavonoid; antioxidant; fluconazole.

1. INTRODUCTION

Spices are defined as vegetable substances of indigenous origin which are aromatic or have a hot piquant taste, used to enhance the flavor of foods or adding stimulating ingredients to the foods [1]. Herbs and spices are considered safe and proven to be effective against certain health conditions. They are extensively used particularly in many Asian, African and other developing countries and in recent years, the use of herbs and spices have extended to the developed countries because of their beneficial aspects [2].

Myristica fragrans (Nutmeg) is stimulant, carminative; it is used in tonic and electuaries and form a constituent of the preparation prescribed for dysentery, nausea, rheumatism and early stage of leprosy [3]. It also has anti-depressant activity on mice, anti-diabetic, anti-oxidant activity and memory- enhancing activities [4]. Nutritionally nutmeg is rich in carbohydrates, proteins, dietary fibre, vitamins A, C and E electrolytes (sodium and potassium), minerals (calcium, copper, iron, magnesium, manganese, zinc and phosphorus) and phytonutrients (carotene-B and crypto-xanthin B) [5]. Due to the fact that nutmeg is used worldwide as a flavoring agent, its antioxidant property and ability to inhibit the growth of fungi in ready-to-eat foods were analyzed in this research.

2. MATERIALS AND METHODS

2.1 Pretreatment and Processing of *Myristica fragrans*

Damaged and spoiled seeds were removed from dried *Myristica fragrans* seed purchased at Orisumbare market in Osogbo, Osun State while the wholesome seeds were rinsed, air-dried, blended into smooth powder and stored in an air-tight container at 28℃ for further analysis.

2.2 Preparation of *Myristica fragrans* Extract

Thirty grams of *Myristica fragrans* powder was mixed separately with 250 mL of distilled water, ethylacetate and ethanol separately using solvent extraction technique. The mixtures were subjected to intermittent shaking for 2 hours on an orbital shaker at 250 rpm which was terminated after 72 hours. Each mixture was filtered with vacuum pump after 72 hours and the filtrate was concentrated under reduced pressure in a rotary vacuum evaporator (NYC R-205D) at 40℃ until dried crude extract was obtained. The crude extract was reconstituted in Dimethyl Sulfoxide (DMSO) and stored in the refrigerator at 4℃ for phytochemical, antioxidant and antifungal analyses [6].

2.3 Phytochemical Screening of *Myristica fragrans* Extract

Phytoconstituents of *Myristica fragrans* crude extracts such as alkaloids, flavonoids, steroids, terpenoids, saponins, tannins, glycosides and phenol were determined according to the methodology of Sofowora [7].

2.4 DPPH Radical Scavenging Activity

One millimeter of 0.004% methanol solution of DPPH was added to 1 mL of various concentrations (0.2-1.0 mg/mL) of each crude extract in its extraction solvent (ethanol, ethyl acetate and distilled water) separately and incubated in the dark at 28℃ for 30 minutes. The absorbance was read against Beta-carotene (control) at 517 nm thus; Inhibition of free radical DPPH in percentage (I%) was calculated.

2.5 Total Phenolic Concentration

An aliquot (1 mL of each extract) and standard solution of Gallic acid was mixed with 9 mL of distilled water and reagent blank using distilled water was prepared. Folin-Ciocalteu phenol reagent (1 mL) was added and shaken thus 10 mL of 7% sodium carbonate solution was added to the mixture after 5 minutes and the volume was made up to the mark and incubated at 28℃ for 90 minutes. Absorbance against the reagent blank was determined at 550 nm thus, total phenolic content was expressed as milligram Gallic acid Equivalents (GAE) [8].

2.6 Extraction of *Myristica fragrans* Essential Oil

Myristica fragrans oil was extracted from 10% $^{w}/_{v}$ *Myristica fragrans* powder using hydro-distillation technique at 100℃ and continuously agitation with magnetic stirrer. The essential oil was separated from the distillate by mixing with petroleum ether in a separatory funnel; the lower layer (organic layer) contains *Myristica fragrans* oil and petroleum ether while the upper layer is the aqueous layer. Furthermore, organic layer was dried by mixing with 2 g of anhydrous Sodium sulfate and allowed to stand overnight [9]. The residue was removed by decanting while the volatile petroleum ether was separated from the essential oil by exposure to air, thus percentage yield of essential oil was determined and phytoconstituent with corresponding concentration was analyzed using Gas Chromatography-Flame Ionization Detector HP 6890 Powered with HP ChemStation Rev. A 09.01 [1206] Software. The chromatography was done in HP 5 MS column (0.25 µm interior diameter x 30 m long) with a particle size of 0.25 µm, at a flow rate of 1.0 mL/min using Flame Ionization Detector (FID) signal and hydrogen as the mobile phase at injection temperature of 150℃ and 300℃ detector temperature respectively [10].

2.7 Antifungal Activity of *Myristica fragrans* Crude Extracts and Oil

One hundred microliters (100 µL) of each fungal isolate was inoculated on Potato Dextrose Agar using spread plate method. The plates were allowed to dry and a sterile cork borer was used to bore wells in the agar at different points [11]. Twenty microliters (20 µL) of extracts of various concentrations (5, 10, 15 and 20 µg/mL) were introduced into the wells. Sterile dimethyl sulfoxide (20 µL) and Fluconazole (20 µL) served as negative and positive control respectively and agar plates were incubated at 28℃. Daily evaluations were carried out by measurement of colony diameter 24 hours after incubation until two-third of the plate surface of the control was covered by fungus [12]. The zones of inhibition were recorded to the nearest diameter according to Moreira et al. [13]. The isolates that showed higher zones of inhibition were subjected to essential oil effectiveness using agar well diffusion respectively.

3. RESULTS

3.1 Phytochemical Screening of *Myristica fragrans* Extracts

The ethanolic extract of *Myristica fragrans* contained all phytoconstituents except glycosides and terpenoids. The ethylacetate extract also revealed the presence of alkaloids, flavonoids, tannins, saponins and phenols while glycosides, terpenoids and steroids are absent. In the aqueous extract of *Myristica fragrans,* alkaloids, glycoside and flavonoid were absent while others were present (Table 1).

3.2 Radical Scavenging Activity of *Myristica fragrans* Extracts

The DPPH scavenging activity obtained in the ethylacetate extract of *Myristica fragrans* was higher than the standard reference used (Beta-carotene). The standard reference had the lowest scavenging activity compared to other organic solvent extracts. At 1.0 mg/mL concentration, ethanol extract and distilled water had related inhibition percentage (Fig. 1). This assay is based on the reduction of DPPH solution in methanol in the presence of a hydrogen-donating antioxidant due to the formation of the non-radical from DPPH resulting to loss of purple color.

3.3 Total Phenolic Concentration

Total phenolic concentrations of 0.90±0.08, 0.40±0.16 and 0.37±0.02 mg GAE/gm were obtained from ethanolic, ethyl acetate and aqueous extracts of *Myristica fragrans* respectively. Results are means of triplicate analysis.

3.4 Quantification of *Myristica fragrans* Oil Using GC-FID

The percentage yield of *Myristica fragrans* essential oil using hydrodistillation technique was 3.25%. A total of thirty six compounds representing 100% of the essential oil were analyzed. The compounds with significant concentrations include sabinene which had the highest percentage (26.58%), myristicin (13.55%), alpha-pinene (11.84%), terpinene-4-ol (9.35%), limonene (5.74%), safrole (5.40%), alpha-terpineol (4.51%), alpha-myrcena (3.82%), gama- terpinene (3.71%), alpha-terpinolene (3.19%), pinene-2-ol (1.84%), elimicin (1.27%) and isoeugenol (1.13%) (Table 2).

3.5 Antifungal Effectiveness of *Myristica fragrans* Extracts and Essential Oil

Ethylacetate extract of *Myristica fragrans* inhibited all the test organisms (*Cladosporum herbarum, Penicillium chrysogenum* and *Penicillium digitatum* in increasing order of concentrations (5<10<15<20 µg/mL) while *Myristica fragrans* essential oil is effective towards *Cladosporum herbarum* only. Fluconazole and DMSO did not show any zone of inhibition (Table 3).

4. DISCUSSION

Preliminary phytochemical analysis helped to identify therapeutic compounds in plants. In this study, secondary metabolites such as saponin, alkaloids, tannins, phenol, flavonoids and steroids were present in different extracts of *Myristica fragrans* which is in agreement with the studies carried out by Assa et al. [14] and Rancy and Krishnakumari [5]. The presence of these phytoconstituents is linked to the antioxidant and antifungal activities of the *Myristica fragrans* extracts. Ethanol is an excellent organic solvent for extraction of metabolites from *Myristica fragrans* seed due to the fact that ethanolic extract contained more phytoconstituents than ethylacetate and aqueous extracts respectively (Table 1).

The total phenolics content and high DPPH radical scavenging activities of the ethylacetate and ethanol extracts of *Myristica fragrans* is due to the presence of tannins and flavonoid which serve as electron donor to scavenging of free reactive oxygen species according to the report of Gayathri and Anuradha, [15].

Table 1. Preliminary phytochemical screening of *Myristica fragrans* seed

Phytoconstituent	Ethanol extract	Ethylacetate extract	Aqueous extract
Alkaloids (Wagner's reagent)	+	+	-
Phenol	+	+	+
Flavonoids	+	+	-
Saponin	+	+	+
Tannins	+	+	+
Glycosides	-	-	-
Steroids	+	-	+
Terpenoids	-	-	+

Key: - = Absent; + = Present

The antifungal effectiveness of ethanol extract, ethylacetate extract and *Myristica fragrans* essential oil is due to presences of active constituent such as sabinene, myristicin, alpha-pinene, alpha-terpeneol, terpenene-4-ol, alpha-myrcena, limonene, safrole, gama-terpinolene, pinene-2-ol, Isoeugenol, elimicin and alpha-terpinolene in *Myristica fragrans* which act by inactivating microbial adhesion, enzymes and cell wall protein synthesis [16,4].

The results of this research justify the importance of *Myristica fragrans* as an antioxidant, antimicrobial and other ethno-medicinal uses. Presence of thirty-six different phytoconstituents (metabolites) in *Myristica fragrans* essential oil poses the potential of providing useful drugs for treating food-borne infection and reduction of oxidative stress in the body other than its general uses as spices and flavoring agent.

Table 2. *Myristica fragrans* oil composition

Peak	Metabolites	RetTime [Min]	Area [pA∗s]	Amt/Area	Norm %
1.	Limonene	7.172	12.72329	1.927876e-4	5.740082
2.	Sabinene	7.899	474.37518	2.39441e-5	26.580480
3.	Alpha- Pinene	8.502	68.78395	7.35419e-5	11.837633
4.	Alpha- Myrcena	11.297	34.85354	4.68798e-5	3.823627
5.	Benzyl Alcohol	11.661	19.67177	1.26893e-5	0.584149
6.	Myrcene	12.820	18.96276	4.66350e-6	0.206946
7.	Cis Ocimene	12.933	36.32078	8.10124e-7	0.068857
8.	Allo Ocimene	13.286	36.03664	2.87497e-6	0.242449
9.	Pinene-2-ol	13.879	20.16058	3.90659e-5	1.843078
10.	Alpha- Thujene	14.241	11.21061	1.03251e-5	0.270874
11.	Gama- Terpinene	14.921	29.42255	5.38761e-5	3.709535
12.	Neral	15.309	23.07683	9.47327e-6	0.511587
13.	Geranial	15.396	28.01805	6.08708e-6	0.399107
14.	Isoartemisia	16.369	27.80702	3.70964e-6	0.241395
15.	1,8- Cineole	16.780	88.44959	2.81442e-6	0.582542
16.	Borneol	17.863	42.46359	4.94878e-6	0.491766
17.	Alpha- Terpinolene	18.133	90.11253	1.51237e-5	3.189242
18.	Linalool	18.352	116.46375	7.48476e-7	0.203991
19.	Alpha- Terpineol	19.102	112.65903	1.71031e-5	4.509033
20.	Terpinen- 4-ol	19.519	163.18135	2.44951e-5	9.353910
21.	Thymyl Methyl Ether	19.789	49.09733	2.78476e-6	0.319955
22.	Linalyl Acetate	20.788	106.63542	1.77339e-6	0.442537
23.	Ethyl Cinnamate	21.263	58.96811	4.15833e-6	0.573824
24.	Borneol Acetate	21.438	55.73929	5.53434e-6	0.721889
25.	Phenanthrene	21.821	137.35600	1.22924e-6	0.395119
26.	Linalyl Acetate	21.912	68.97568	0.00000	0.000000
27.	Safrole	22.231	90.23232	2.55830e-5	5.402016
28.	Isoeugenol	22.518	82.28958	5.85881e-6	1.128229
29.	Myristicin	22.602	144.04491	4.02119e-5	13.554887
30.	Elimicin	23.356	30.82641	1.76007e-5	1.269684
31.	Bi-Cyclogermacrene	24.614	53.00820	3.75255e-6	0.465493
32.	Alpha- Copane	24.728	26.35893	3.36140e-6	0.207344
33.	Alpha- Bergamotene	26.222	9.87211	1.78575e-5	0.412547
34.	Acetyleugenol	26.839	11.92313	7.35778e-6	0.205296
35.	Elemicin	27.063	6.84355	7.56197e-6	0.121104
36.	Benzyl Benzoate	27.756	3.84461	4.33249e-5	0.389752
	Total				100.0000

Table 3. Antifungal activity of *Myristica fragrans* extracts

Isolate	Concentration (µg/ml)	Mean diameter of zones of inhibition (mm)					
		Ethanol	Ethylacetate	Aqueous	Essential oil	Dimethylsulfoxide	Fluconazole
Penicillium chrysogenum	5	-	14	-	-	-	-
	10	-	19	-	-	-	-
	15	-	24	-	-	-	-
	20	-	29	-	-	-	-
Penicillium digitatum	5	22	-	-	-	-	-
	10	21	22	-	-	-	-
	15	24	30	-	-	-	-
	20	25	25	-	-	-	-
Cladosporum herbarium	5	22	-	-	-	-	-
	10	24	31	-	-	-	-
	15	27	35	-	-	-	-
	20	28	38	-	46.5	-	-

Values represent mean (n=2)
- = No zone of inhibition.
Non sensitive =total diameter less than 8 mm
Sensitive = total diameter between 9 – 14 mm
Very sensitive= total diameter between 15 – 19 mm
Extremely sensitive= total diameter higher than 20 mm

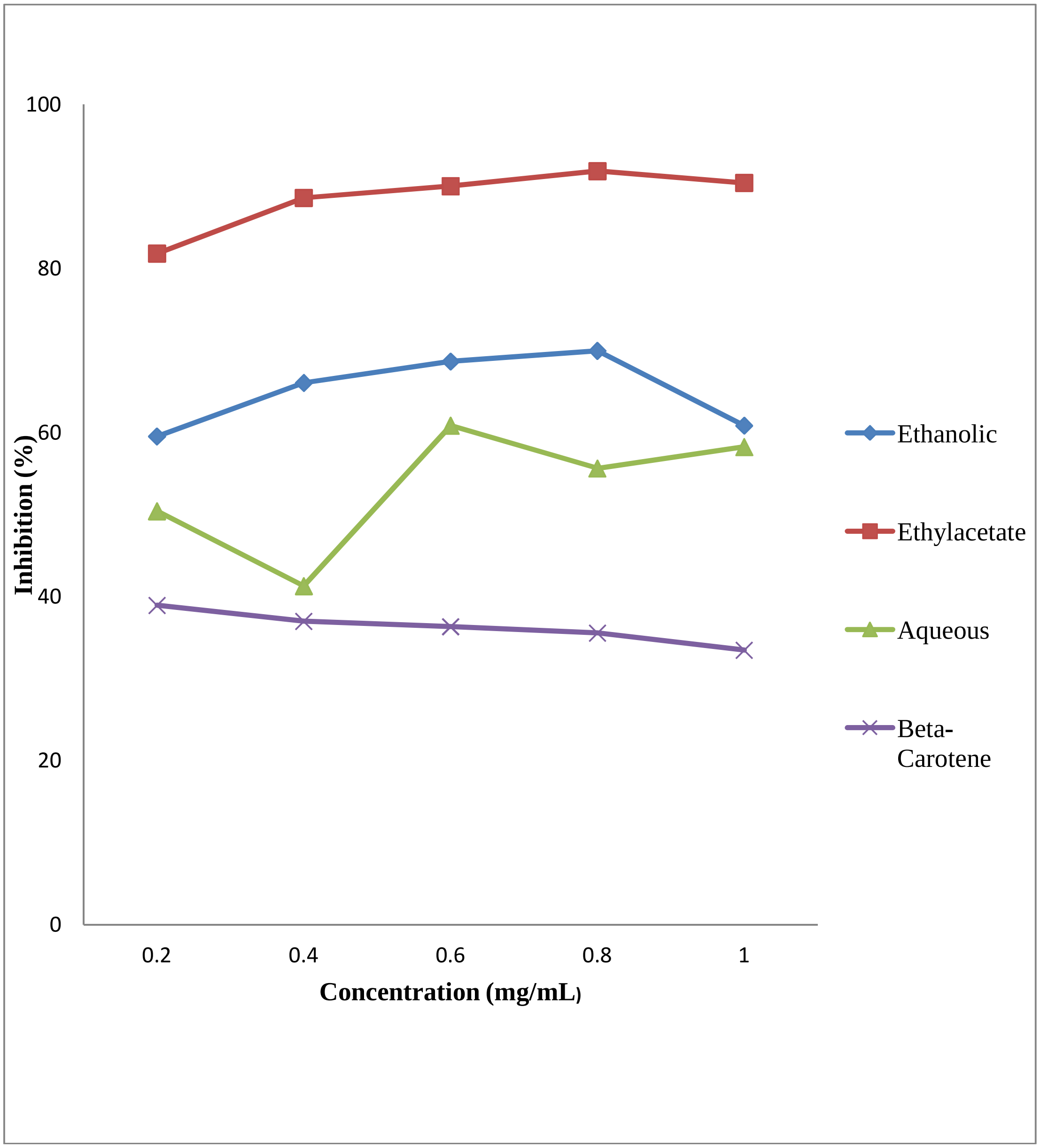

Fig. 1. Radical scavenging activity of *Myristica fragrans* extracts

5. CONCLUSION

The results of this research justify the importance of *Myristica fragrans* as an antioxidant, antimicrobial and other ethno-medicinal uses. Presence of thirty-six different phytoconstituents (metabolites) in *Myristica fragrans* essential oil poses the potential of providing useful drugs for treating food-borne infection and reduction of oxidative stress in the body other than its general uses as spices and flavoring agent.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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