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GC-MS Analysis and Antifungal Activity of *Morinda Lucida* Extract

Isaac Aiyonoguan.[a], OsaroIyekowa[a*], Ruth O. Oghomwen[b], Oghomwen T. Michael[b], Lilian E. Amoren[c], Isimemen F. Akhidenor[a], Lucky O. Eduwuirofo[a], Agholor M. Oligie[a], Ferdinard O. Amayo[a], Justina O. Akinbolade[a] , Ndubuisi P. Egboluche[a] and Ogbodhu C. Uzoma-Odoji-Kpasa[d]

[a]Department of Chemistry, University of Benin, Benin City, Nigeria
[b]Department of Science Laboratory Technology, University of Benin, Benin City, Nigeria.
[c]Department of Chemical Engineering, Edo State Polytechnic, Usen, Edo, Nigeria.
[d]Department of Chemical Engineering, Delta State University of Science and Technology, Ozoro, Nigeria
*Corresponding Author: osaro.iyekowa@uniben.edu

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Abstract

The increasing climatic and environmental change and injudicious use of antibiotics has led recently to increase fungal infections resistance to available pharmaceutical antifungal drugs. Consequently, research for alternative local medicinal source of plant origin is on the increase, thus, the aim of this research work is to determine the chemical constituents and antifungal activity of Morindalucidaextracts. The Morindalucidaleaves were collected, dried under shade, pulverized and extracted with hexane, ethylacetate and methanol solvents using soxhlet extractor in order of increasing polarity. The three crude extracts were concentrated using rotary evaporator and the phytochemical screening was conducted according to standard methods while the antifungal analysis was conducted using the agar well diffusion method. Vacuum liquid chromatography was used for isolation of the oil from the hexane extract, which was characterized by gas chromatography-mass spectrometry (GC-MS). The result shows the presence of glycoside, saponins, phenolics, alkaloids, terpenoids, steroids, eugenols and flavonoids with the exception of tanins in all the extracts. The hexane, ethylacetate and methanol extracts of Morindalucidaexhibit moderate, strong, and very strong antifungal inhibitory activities respectively against the tested fungi strains of Candida auris, Candida albicans, Aspergillusfumigatus, Trichophytonmentagrophytes and Epidermophytonfloccosum. The GC-MS analysis of the hexane extractshows 48 compounds including hexadecanoic acid methyl ester, and squalene among others, which have reported antifungal activity. This study corroborates the use of the plants as antifungal agents by traditional people.

1. Introduction

Since the beginning of time, man has been afflicted by numerous infectious diseases, which have historically affected civilization's growth and development [1]. Chicken pox, measles, malaria, tetanus, influenza, TB, HIV and Aids, coronavirus, meningitis, and other microbiological illnesses are examples of such infectious disorders. Fungi are prevalent in the environment and infection caused by fungus-related pathogens has increased [2]. Fungi infections are common microbial infections that are getting more resistant, challenging, and hard to treat [3] According toWHO [3],

the majority of these infectious diseases have a tendency to become resistant to the synthetic medications used to treat them over time. Finding native medicinal plants with strong bioactive phytochemicals is therefore crucial for treating many ailments, particularly fungal infections caused by microbes. The indiscriminate antibiotic usage and rising fungal infection cases has recently doubled in the twenty-first century [4]. These have led to an upsurge in fungal infections that are now harder to treat due to resistance to prescription antifungal medicines. Local medicinal plants and fauna species with powerful bioactive compounds are increasingly in demand as treatments for resistant fungal diseases [5]. Various local medicinal plants that can treat fungal infections are available; one of such plant is *Morindalucida,* which is found in local habitats such as bushes and forest in Nigeria and West Africa [6]

Plate 1: *Morindalucida*leaves

*Morindalucida*also referred to as brimstone tree belongs to the family of Rubiaceae. It is clear from the foregoing that the well-known *Morindalucida* is a medicinal plant, which has been used as traditional medicine to mitigate varieties of health risks particularly in the African continent. According to Adeleye [7], the entire *Morindalucida*has a special medicinal function. Numerous studies have been done on *Morindalucida*antibacterial properties. A number of anthraquinones and terpenes, particularly those that can treat fungal infections, have been identified from the *Morindalucida* [7].*Morindalucida*root DCM extract has antifungal properties because it contains separated antrhaquinones. Sleeping sickness and congestive heart failure can both be treated with the *Morindalucida*plant (Dahunsi *et al*., 2020). The present study is aimed at analysing the oil constituents by GC-MS Analysis and determining the antifungal activity of *Morinda Lucida* leavesextracts.

2. 0. Materials and Method

2.1 Sample Collection and Treatment

*Morindalucida*fresh leaves were gathered from their native habitat in Sabon-gidaOra in Owan West Local Government Area of Edo State, Nigeria. The leaves were identified by a Taxonomist in the Department of Plant Biology and Bioteechnology, University of Benin. The leaves were washed with distilled water, air-dried for twenty-eight (28) days in the laboratory and pulverized

to a powdered form. Three hundred and thirty grams (330g) of the powdered sample were packed in thimbles and thoroughly extracted in a soxhlet extractor for eight (8) hours using 600 ml of n-hexane. The defatted leaves were re-extracted with increasing polarity using the ethylacetate and methanol solvents respectively in a soxhlet extractor. The extracts were concentrated separately using a rotary evaporator (Model, RE 200).

i. Phytochemical screening of hexane, ethylacetate and methanol extracts

The phytochemical screening was performed using standard methods and procedures prescribed by Sofowora (1993) and Trease and Evans (1989) to identify the presence of the bioactive chemical constituents such as flavonoids, alkaloids, glycosides, saponins, phenolics, eugenols, steroids and terpenoids.

ii. Isolation of oil from hexane extract

Vacuum liquid chromatography (VLC) was used to separate 41.5g of the hexane extract using silica gel (particle size: 200-450 mesh) as the solid phase and methanol and ethylacetate mixture (1:1) as the mobile phase. To recover the pure oil, the brown oily phase that was obtained was dried over sodium carbonate (Na_2CO_3) and concentrated.

iii. Gas Chromatography – Mass Spectrometry (GC-MS) Analysis

The GC–MS of the brown oil from hexane extract of *Morindalucida*was obtained on a shimadzu, GC-MS–QP2010.

iv. Microorganisms

The Microorganisms that was utilized in this study were obtained from the University of Benin Teaching Hospital, Benin City, Nigeria. These microorganisms includes clinical isolates of *Aspergillusfumigatus, Candida auris, Trichophytonmentagrophytes, Epidermophytonfloccosum and Candida albicans*

v. Media

Nutrient broth and nutrient agar, all products of himedia laboratories Mumbai (India) were used in this study. The composition of the medium was beef extract 3.0g, peptone 50g, sodium chloride 8.0g and agar 15.0g.

vi. Agar Well Diffusion

0.025g, 0.05g, 0.075g and 0.01g each of hexane, ethyl acetate and methanol extracts were weighed using a standard weighing balance. One ml (1ml) of sterile water was added to mix each extract. 24-hour culture of each fungi suspension was made using potatoes dextrose broth.

vii. Determination of Minimum Inhibitory Concentration (MIC)

Minimum inhibitory concentration is the lowest concentration of an antimicrobial agent that will inhibit the visible in-vitro growth of microorganism after overnight incubation. Minimum inhibitory concentrations are important in diagnostic of microorganism to an antimicrobial agent and also to monitor the activity of a new antimicrobial agent.

The MIC values of each plant extracts were determined using two fold micro-dilution to prepare concentrations of 25mg/ml, 50mg/ml, 75mg/ml and 100mg/ml of each extracts and a drop of the bacterial suspension that had been previously diluted to about 10^6 cfu/ml were aseptically incorporated into molten nutrient agar and allowed to set. The plates were incubated at 37℃ for 24 hours. The lowest concentration preventing visible growth for each of the test organisms was recorded as the MIC. The experiments were carried out in triplicate for each extracts concentration and fluconazole was used as a positive control.

3.0. Results and Discussion

The phytochemical constituents in the extracts of *Morindalucida* are shown in Table 1.

Table 1: Phytochemical constituents in extract of *Morindalucida*

S/N	Phytochemical const8ituents	Hexane extract	Ethyl acetate extract	Methanol extract
1	**Glycosides**	-	+	+
2	**Saponins**	+	-	+
3	**Phenolics**	+	+	+
4	**Alkaloids**	-	+	+
5	**Terpenoids**	+	+	+
6	**Steroids**	+	-	-
7	**Eugenols**	+	+	+
8	**Flavonoids**	+	+	+
9	**Tannins**	-	-	-

Key: + = present - = absent

The results in Table 1, shows the presence of phenolics, terpenoidss, eugenols and flavonoids in hexane, ethylacetate and methanol extracts. Alkaloids and glycosides were present in ethylacetate and methanol extracts but absent in hexane extract. Saponin was present in hexane and methanol extracts but absent in ethylacetate extract. Steroid was present in hexane extract but absent in ethylacetate and methanol extracts. From the qualitative phytochemical analysis of the threeextracts (hexane, ethyl acetate and methanol), it shows that tannins was conspicuously absent which correlate with the report of [6] that stated the absence of tannins in *Morindalucida*leaves.

3.1. GC-MS Analysis

The GC-MS chromatogram of the isolated brown oil given in figure 1 showed fifty six (56) peaks indicating from the search list of the chemical abstract service fifty six compounds. The chemical compounds identified (according to NIST Library) in the oil fraction are presented in Table 2.

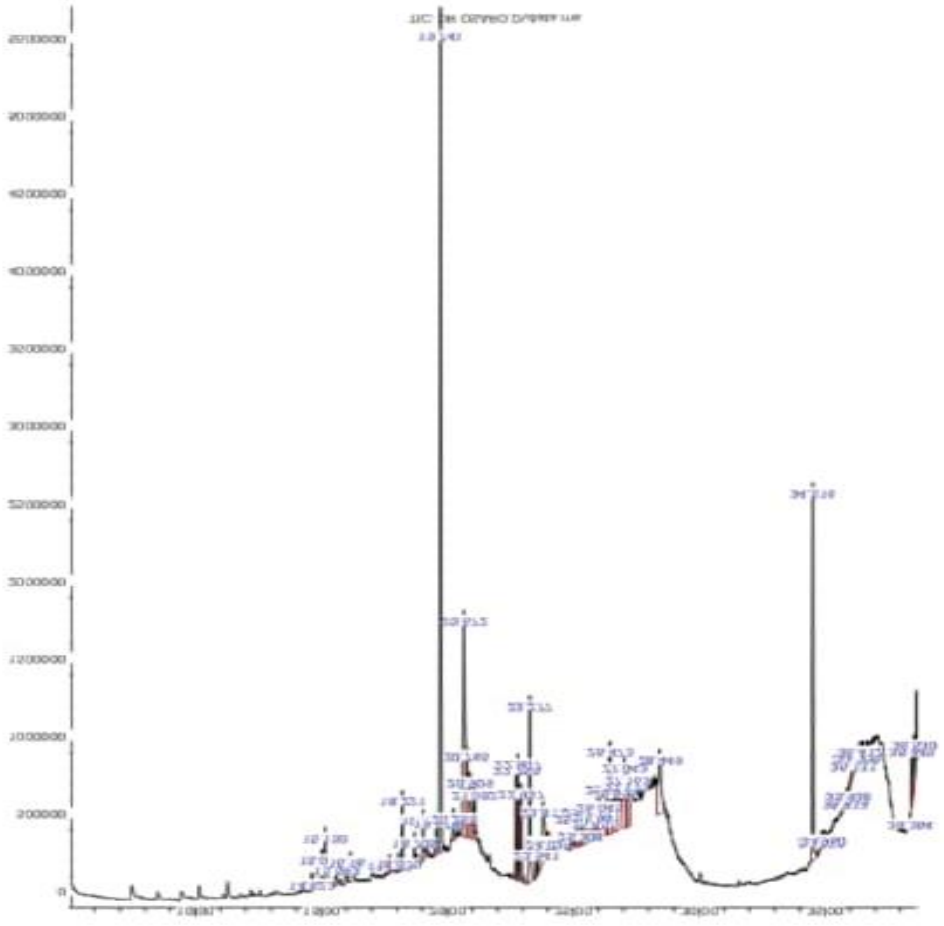

Figure 1: GC-MS Analysis of isolated brown oil of *Morindalucida*leav

Table 2: Compounds In oil fraction of hexane extract of *Morindalucida*

Peak No.	Retention Time (Rt)	Name of compounds	Area Percent (%)
1	14.6233	Cyclopentaneacetaldehyde	0.4075
2	15.0241	7–methylene bicycle (4.2.0) octane	1.0861
3	15.1498	3–cyclcopropenoic acid	2.0289
4	15.5926	16–methyl heptadecanoic acid	0.6561
5	16.163	Guaifenesin	1.2841
6	17.7047	9–methyl tetradecanoate	0.4317
7	18.0697	6,10,14–trimethyl trans-2,7–dimethyl–3,6–octadien–2-ol.	0.4071
8	18.2207	Neophytadiene	1.7784
9	18.7048	5–methyl–trans–cyclohexanol	0.5733
10	19.0473	1,8-Nonadiene	1.1762
11	19.5779	9–Hexadecanoic acid methyl ester (z)-	0.9996
12	19.7412	Hexadecanoic acid, methyl ester	17.4561
13	20.2551	Phytol	0.7969
14	20.6715	n-Hexadecanoic acid	8.3785
15	20.7655	n-Hexadecanoic acid	2.8614
16	20.9088	n-Hexadecanoic acid	2.5738
17	21.0824	(2R,4S,6R)-(-)- Hexadecanoic acid, ethyl ester	1.538
18	22.7495	9,12-Octadecadienoic acid, methyl ester	2.4587
19	22.8075	7,10,13-Hexadecatrienoic acid, methyl ester	2.4704
20	22.9373	9-Octadecenoic acid, methyl ester	2.0445
21	23.2767	Phytol	4.5348
22	23.5411	Methyl stearate	0.6087
23	23.8106	9-Octadecenoic acid	3.5972

24	23.982	9-Eicosenoic acid,	0.5132
25	24.007	cis-Vaccenic acid	0.2099
26	24.0388	Oleic Acid	0.3063
27	25.1634	Erucic acid	0.9371
28	25.308	(S)(+)-Z-13-Methyl-11-pentadecen-1-ol acetate	0.7299
29	25.4958	9,12,15-Octadecatrienoic acid, methyl ester (z, z, z)	1.997
30	25.8508	trans-13-Octadecenoic acid	1.1107
31	25.9661	Methyl -9,12-heptadecadienoate	0.6052
32	25.9914	Z,Z-4,15-Octadecadien-1-ol acetate	0.4142
33	26.0451	6,9-Octadecadienoic acid, methyl ester	1.2343
34	26.3833	2-Acetyl-2-allylpent-4-enoic acid ethyl ester	1.4427
35	26.4734	2-Methyl-Z,Z-3,13-octadecadienol	4.2011
36	26.6957	(z) - trans-13-Octadecenoic acid	2.0212
37	27.0435	Methyl 11-oxo-9-undecenoate	3.0579
38	27.1275	(z) -9-Methyl-10,12-hexadecadien-1-ol acetate	0.5022
39	27.1628	9-Eicosenoic acid	2.4251
40	28.4493	p-Menth-8(10)-en-9-ol	3.7797
41	34.5164	Squalene	8.6833
42	34.8588	1,3,5-Benzenetriol Phosphonic acid	0.6472
43	34.9213	2,2-dimethyl-maltol	0.2922
44	34.9597	2-Hexenal	0.109
45	35.813	3-ethyl-2-hydroxy, 2-Cyclopenten-1-one	0.0964
46	35.8416	Oleic Acid	0.1463
47	35.938	1-(ethenyloxy)- Z-2-Octadecen-1-ol	0.1249
48	36.1312	(E) – Oleic acid	0.8141
49	36.1996	Erucic acid	0.5155

50	36.2586	Octadecane	0.2559
51	36.3316	2-(octadecyloxy)ethyl ester	0.2765
52	36.3695	Heptadecanoic acid, heptadecyl ester	0.0977
53	36.4124	4-Methyldocosane	0.0197
54	38.3838	2-Heptadecanol	0.0632
55	38.4492	Ergost-22-en-3-ol	0.9605
56	38.5301	Dodecanoic acid, 1,2,3-propanetriyl ester	1.2616

The main constituents detected from the isolated brown oil of *Morindalucida*obtained from the GC-MS analysis of hexane fraction revealed the key compounds that might be responsible for the observed pharmacological activity, some of these constituents identified have been reported with significant antifungal activity. Detected compounds includes hexadecanoic acid methyl ester (Retention time (Rt): 19.74, 17.46%), n-hexadecanoic acid (13.81%) squalene (Rt: 34.52, 8.68%), phytol (Rt: 23.28, 4.53%) and 2-methyl–Z,Z–3,13–octadacadienol (Rt: 26.47, 4.20%).

Hexadecanoic acid methyl ester has been reported in the leaf of *M. lucida* [11]. It has been reported to show antibacterial and antifungal activity [12]. N–henxadecanoic acid a fatty acid and has been reported to be present in the root and stem of *M. lucida* (Oluwole *et al*., 2022) and has been reported to exhibit antimicrobial and antifungal biological activities [11]

Squalene is a triterpene that is an intermediate in the cholesterol biosynthesis pathway. Triterpenes (squalene) have been isolated from the different parts of the genus *Morinda* [12]. Squalene has been shown to have excellent antioxidant, anticancer antibacterial and antifungal biological activities [13].

Phytol is diterpeneterpenoids. It has been isolated from leaf, stem-bark and fruit of *M. lucida* [14]. Explained that phytol from plant extract reduce the mitochondrial content of fungus, which could alter the ATP generation and level of reactive oxygen species. Consequently, the mitochondria of the fungus became dysfunctional. It is reported that phytol could disrupt the cell membranes of the fungus, resulting in K^+ ions leaking from the cells, and causing fungus hyphae to wither [10]

2-methyl-Z,Z-3,13–octadecadienol is a fatty alcohol, a terpenoid. Its presence in *M. lucida* might probably not have been reported in literature. It antimicrobial and antifungal activity might probably not have been reported in literature but it is known to be used as pesticide, herbicides, insecticide and pheromone. These properties might make it to be toxic and lethal to fungi.

Moreover, some of the chemical constituents that appear in lower amounts in this hexane extract such as fatty acids tend to possess antifungal properties against diverse mycological pathogens. Since the fungal tissue is lipophilic, in nature, the fatty acid will attract the absorption of the fungus more easily. Other minor phytochemical components other than fatty acids such as terpenoid, steroid etc possibly contributed to antifungal effect by working synergistically with the major compounds.

The result in Table 3, shows that the hexane extract at 100mg/ml demonstrate very strong inhibitory activity against *Candida auris*with zone of inhibition of 18mm. the hexane extract showed strong inhibitory activity against *Aspergillusfumigatus, and Trichophytonmentagrophytes*with zone of inhibition of 14mm, and 15mm. It also demonstrates moderate inhibitory activity against *Epidermophytonfloccosum* with zone of inhibition of 11mm. The hexane extract showed very weak inhibitory activity at 100mg/ml against *Candida albicans* with zone of inhibitory of 5.5mm. This results support the work of [6] who reported low activity for non-polar extract of *Morindalucida*as shown in Figure 2. In Table 4 and Figure 3, the ethyl acetate extract at 100mg/ml demonstrate a very strong inhibitory activity against *Candida albicans* with zone of inhibition of 19.5mm which is very close to that of the fluconazole control drug. The ethyl acetate extract showed moderate inhibitory activity against *Candida auris*and *Trichophytonmentagrophytes*at 100mg/ml with zone of inhibition of 12.5mm and 10.7mm. The ethyl acetate extract exhibit weak inhibitory activity against *Aspergillusfumigatus*and *Epidemophytonfloccosum*at 100mg/ml with zone of inhibition of 7.3mm and 7.5mm respectively. The minimum inhibitory concentration (MIC) of the ethyl acetate extract was 25mg/ml for all the fungal isolates.

While in Table 5, the methanol extract at 100mg/ml exhibit moderate inhibitory activity against *Aspergillusfumigatus with zone of inhibition of 10.8mm, while at 100mg/ml it exhibit strong inhibitory activity against Candida auris, Trichophytonmentagrophytes* and *Epidermophytonfloccosum* with zone of inhibition of 15.5mm, 14mm and 13mm respectively This high activity has also be reported for polar solvents has also be reported by [5]. The methanol extract exhibit very weak inhibitory activity against *Candida albicans*with zone of inhibition of 6.2mm.In Figure 4, the antifungal inhibitory activity of methanol extract was low compare to the fluconazole control drug on the fungal isolates. However strong inhibitory activity was observed against *C. auris* which corroborates the work of [6].

Antifungal activity of hexane extract of *M. lucida*

Table 3: Minimum inhibitory concentration (MIC) mg/ml

Fungal isolates	**25mg/ml**	**50mg/ml**	**75mg/ml**	**100mg/ml**	**Fluconazole (0.01mg/mL)**
Zone of inhibition (millimeters, mm)					
A. fumigates	0.0	0.0	12.0	14.0	28
C. auris	10.0	12.0	15.0	18.0	29
T. mentagrophytes	8.0	11.0	12.0	15.0	29
E. floccosum	7.0	9.0	9.5	11.0	27
C. albicans	0.0	0.0	0.0	5.5	22

Key:
< 9mm = weak inhibitory activity
9mm – 12mm = moderate inhibitory activity

13mm – 17mm = strong inhibitory activity
> 17mm = very strong inhibitory activity
Source: [16]

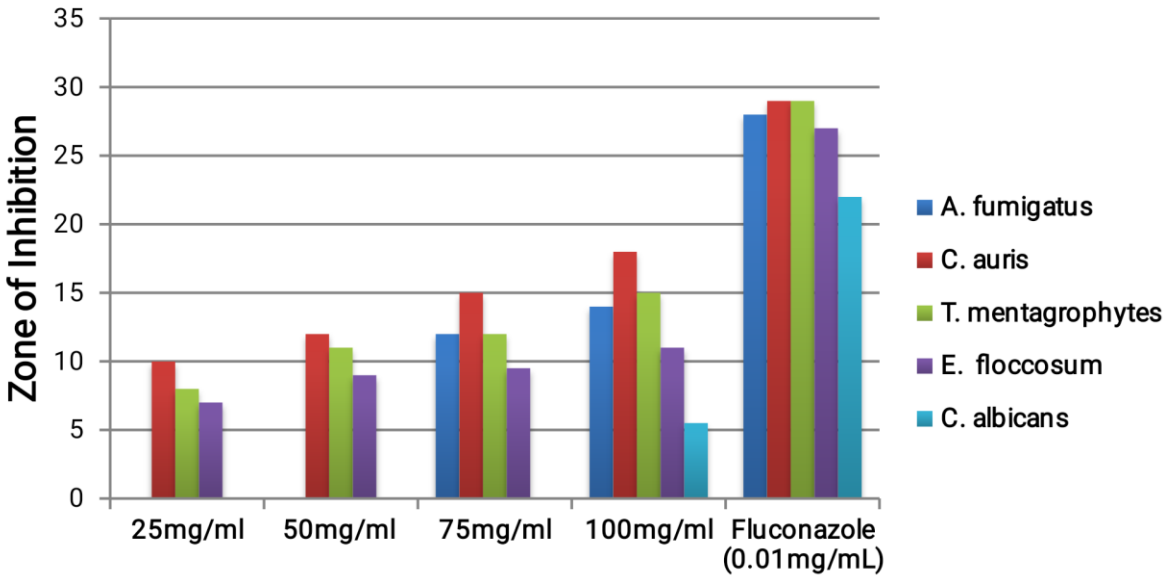

Figure 2: Antifungal activity of hexane extract of *M. lucida*

Table 4: Antifungal activity of ethyl acetate extract of *M. lucida*

Minimum inhibitory concentration (MIC) mg/ml

Fungal isolates	**25mg/ml**	**50mg/ml**	**75mg/ml**	**100mg/ml**	**Fluconazole (0.01mg/mL)**
Zones of inhibition (millimeters, mm)					
A. fumigatus	5.5	6.0	6.9	7.3	28
C. auris	5.0	6.0	7.5	12.5	29
T. mentagrophytes	6.8	7.5	10.0	10.7	29
E. floccosum	5.0	6.2	6.8	7.5	27
C. albicans	10	14	17	19.5	22

Key:

< 9mm = weak inhibitory activity

9mm – 12mm = moderate inhibitory activity

13mm – 17mm = strong inhibitory activity

> 17mm = very strong inhibitory activity

Source: [16]

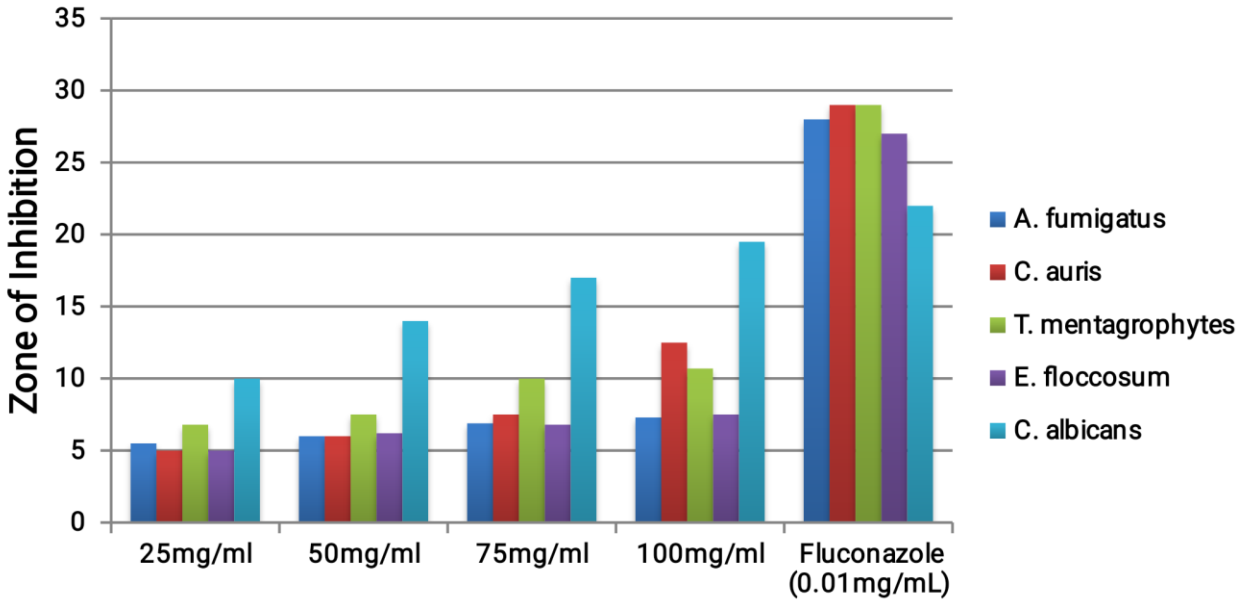

Figure 3: Antifungal activity of ethyl acetate extract of *M. lucida*

Table 5: Antifungal activity of methanol extract of *M. lucida*

Minimum inhibitory concentration (MIC) mg/ml

Fungal isolates	**25mg/ml**	**50mg/ml**	**75mg/ml**	**100mg/ml**	**Fluconazole (0.01mg/mL)**
Zones of inhibition (millimeters, mm)					
A. fumigatus	0.0	0.0	9.0	10.8	28
C. auris	11.0	12.5	13.0	15.5	29
T. mentagrophytes	8.0	11	12	14	29
E. floccosum	9.0	12.1	12.8	13.0	27
C. albicans	0.0	0.0	0.0	6.2	22

Key:

< 9mm = weak inhibitory activity
9mm – 12mm = moderate inhibitory activity
13mm – 17mm = strong inhibitory activity
> 17mm = very strong inhibitory activity Source: [16]

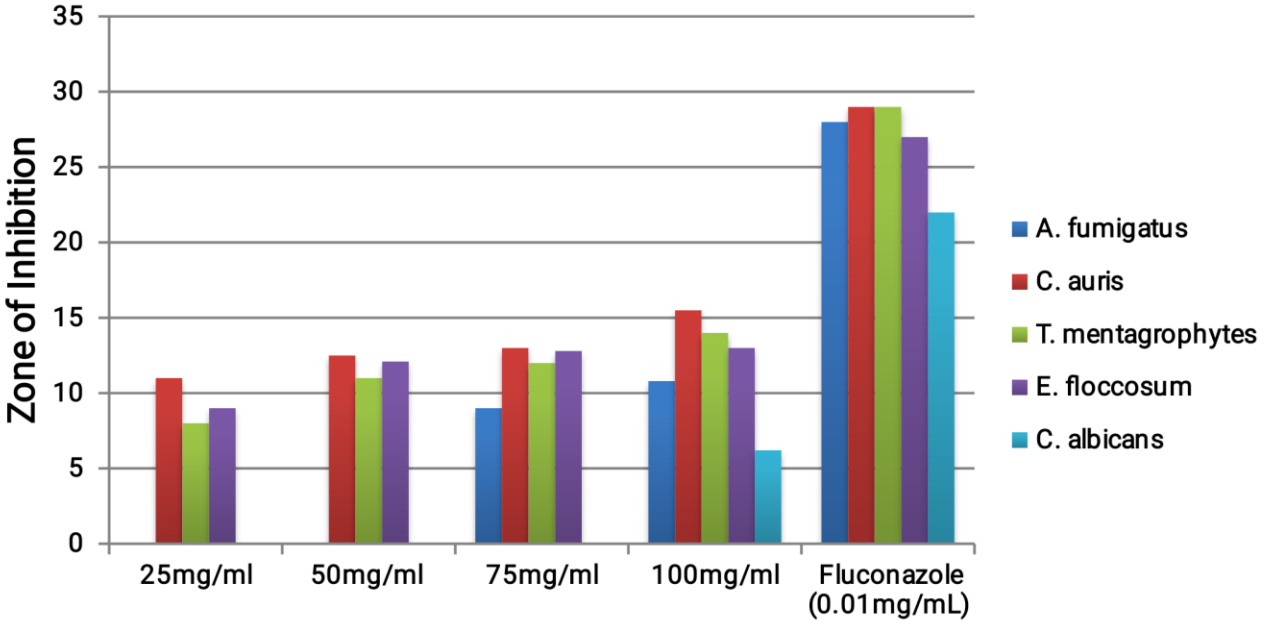

Figure 4: Antifungal activity of methanol extract of *M. lucida*

4.0. Conclusion

The phytochemical screening, antifungal activities and instrumental analysis (GC-MS) of the different fractionated extracts of *M. lucida*shows the presence of secondary metabolites (phytochemical) such as Glycoside, saponins, phenolics, alkaloids, terpenoids, steroids, eugenols and flavonoids and the presence of antifungal chemical agents (or chemical compounds) such as Hexadecanoic acid methyl ester, n-hexadecanioc acid, squalene, phytoletc have also been indicated from the results of GC-MS.. This study laid credence to the fact that *Morindalucida*plant is a potent antifungal plant as used by the local medicinal herbal practitioners.

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