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GC-MS Analysis of Raw and Roasted Seeds of Chrysophyllum Albidum, a Medicinal Plant Used for the Treatment of Tuberculosis

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Abstract
Tuberculosis is a multi-systematic disease with myriad presentations and manifestations and is the most common cause of infectious disease-related mortality worldwide. The disease is treated with the roasted cotyledonous part of *Chrysophyllum albidum* seeds by traditional practitioners in Ogun state, Nigeria. The phytoconstituents of the hexane fraction and the hexane extracts of the roasted seeds at 50^o C, 100^o C and 120^o C were investigated using GC-MS analysis. The results showed the hexane fraction of the raw seeds contained 92.71 % by proportion of esters of long chain fatty acids. The hexane extract of roasted seeds (50^o C) contained 68.69 % of long chain fatty acids, hexane extract of roasted seeds (100^o C) contained74.54 % while the hexane extract of roasted seeds (120^oC) contained 91.65 %.The traditional method of application of roasted seeds instead of raw seeds in treating tuberculosis enhances the availability of free fatty acids. The main fatty acids content at 120^o C are Hexadecanoic acid and 9, 12-Octadecadienoic acid. Fatty acids had been reported to be toxic to Mycobacterium tuberculosis. Thus the efficacy of the treating of tuberculosis with the roasted seeds of *Chrysophyllum albidum* is due to the presence of hexadecanoic acid and 9,12-Octadecadienoic acid which could work together or work synergistically with other antimicrobial effectors to bring about macrophage-mediated killing of the pathogen.

Keywords: Tuberculosis, antimicrobial, phytoconstituents.

Introduction

Tuberculosis is an airborne infectious disease that primarily affects the lungs. It can affect other parts of the body. The causative agent of tuberculosis is Mycobacterium tuberculosis. The bacterium infects macrophages and it adapts to the hostile intracellular milieu of this cell by exploiting the plasticity of its central carbon metabolism machinery (Mehrotra et al, 2014). Infection with Mycobacterium tuberculosis (MTB) is accompanied by an intense local inflammatory response which may be critical to the pathogenesis of tuberculosis (Toossi, 2000). The *Mycobacterium tuberculosis* possesses a distinct cell wall which is necessary for the bacteria's survival because it contains a fatty acid called mycolic acid which provides a strong lipid barrier (Cantrell, 2013). The ability of the organism to remain dormant or persistent within host cells for many years with the potential to be activated allows the bacterium to escape the immune system of the host (Meena and Rajini, 2010). Survival mechanisms of the bacterium include prevention of phago-lysosome fusion (Pieters, 2008), prevention of cell acidification (Queval et al, 2017) and protection against reactive nitrogen intermediates (RNI) (Rousseau et al, 2004). Although drug-sensitive TB is a treatable disease, the current standard treatment requires 6–8 months of a multi-drug regimen to achieve relapse-free cure. This long course of

treatment is often associated with toxicity, poor adherence and development of drug resistance (Lee, 2017). The emergence of extensive drug-resistant tuberculosis (XDR-TB) has significantly threatened to jeopardize global efforts to control TB, especially in HIV endemic regions (Prasad et al, 2017). There is need for anti-tuberculosis agents that could help in adjunct therapy to bring about shortening the treatment regimen, minimizing the side effects of the current anti-tubercular drugs and effective against antibiotic resistant strains. In laboratory settings, plant extracts have been shown to have a variety of pharmacological effects, including anti-inflammatory, vasodilatory, antimicrobial, anticonvulsant, sedative and antipyretic effects (WHO, 2002).

Chrysophyllum albidum, African star apple, belongs to the plant family Sapotaceae. The plant is a lowland rain forest tree species that grows up to 25 to 37 m in height at maturity with a girth varying from 1.5 to 2 m (Oboh et al, 2009). The plant extracts possesses hepatoprotective activity (Adebayo et al, 2011).The ethanol root bark extract showed anti-fertility activity (Onyeka et al, 2012). The seeds of the plant could remove metal ions from aqueous solution (Oboh et al, 2009). The roots, barks and leaves of C. albidum is/are widely used as an application to sprains, bruises and wounds in southern Nigeria (Olorunnisola et al, 2008; Mac Donald et al, 2014). The bark is used for the treatment of yellow fever and malaria. The root and stem barks are used in urinary related infections (Florence and Adiaha, 2015). The leaf is used as an emollient and for the treatment of skin eruption, stomacheache and diarrhea (Adisa, 2000) and for cancer remedy in Cuba (Mac Donald et al, 2014). Treatment of diabetic rats with ethanolic extract of the C. albidum seed cotyledon lowered blood glucose level (Olorunnisola et al, 2008). The methanolic extract and fractions of C. albidum possessed antiplasmodial effect (Adewoye et al, 2010).The cotyledons from the seeds of Chrysophyllum albidum are used as ointments in the treatment of vaginal and dermatological infections in Western Nigeria. The cotyledonous part of the seeds of Chrysophyllum albidum are roasted and put in honey by traditional practitioners in Ogun state part of Nigeria for the treatment of tuberculosis.

The aim of this study is to scientifically validate the therapeutic claim of the seeds by conducting antimycobacterium tuberculosis test and using GC-MS to investigate possible antitubercular agents present in the seeds.

Materials and Methods

Extraction and Fractionation of *Chrysophyllum Albidum* Seeds

The air-dried cotyledonous part of the seeds of C. albidum were grounded into powder. 60 g of the powder were put in 200 mls of 80 % ethanol solution and shaken intermittently at room temperature for 72 hours. Then, the mixture was filtered and the filterate was evaporated using an oven evaporator set at 42.5 ^{o} C. The 2.9 g yield of the ethanolic extract was subjected to sequential liquid partitioning to give 1.94 g of butanol fraction, 0.48 g of ethylacetate fraction and 0.01 g of hexane fraction. The fractions were tested for anti-mycobacterium tuberculosis.

Preparation of Samples of Roasted seeds of *Chrysophyllum albidum*

In line with the folklore usage of the seeds of Chrysophyllum albidum in treating tuberculosis infection, 100 seeds of the plant were put in a closed crucible and heated for 30 minutes at 50^{o} C, 100^{o} C and 120^{o} C separately. The cotyledonous part of the seeds were removed from their shells after heating and were grounded to powder for hexane extraction at room temperature. The yields were 0.13g, 0.15g and 0.16g of hexane extracts respectively for the temperatures. Various concentrations of the 50^{o}C and the 120^{o} C were subjected to anti-mycobacterium tuberculosis test.

The Test Organisms

The reference Mycobacterium tuberculosis strain $H_{37}R_V$ labeled PT_{12} and the local isolates labeled PT_{10} were used. The local isolates were isolated from TB patients using standard methods (Salami and Oluboye, 2002). The organisms were sub-

cultured in Middle Brook 7H9 broth supplemented with OADC at 37^{o} C for 21-28 days and were confirmed acid fast gram positive bacillus using Ziehl Nelson stain.

Anti-mycobacterium Tuberculosis Test

The anti-mycobacterium tuberculosis test was done using proportion method. 5mls of the filtered extract solutions (DMSO as solvent) were added to 15mls of the homogenized egg LJ media to arrive at various concentrations ranging from 50 mg/ml to 0.5 mg/ml. Each 20 mls medium was divided into 10 mls in universal containers. Standard drugs, isoniazid and rifampicin, at 0.2µg/ml and 0.4µg/ml respectively, were added to LJ media accordingly. The media were slanted to form slopes. The LJ slopes without extracts and drugs were used as control. The slopes were insipissated at 85^{o} C for 45 minutes, cooled and stored in a refrigerator at 4^{O} C. Sterility and viability check were carried out before inoculation.

Inoculation of slopes with the bacteria

Bacterial dilutions 10^{-5} mg/ml and 10^{-3} mg/ml were prepared for inoculation. 0.1 ml of the chosen bacterial dilutions was inoculated into all the labelled LJ slopes (Adeleye et al, 2008). The universal containers were loosely closed with caps to allow evaporation and were incubated at 37^{o} C. The specimens were checked on the 7th, 14th, and 21st days to ensure no contaminations. Readings were done on the 28th day.

Nitrate Reduction Test

Nitrate reduction test was performed on all the slopes after 28 days. This involved addition of 2mls Nitrate Substrate Broth, incubation at 37^{o} C for 2 hours, addition of 1 drop of 50 % hydrochloric acid, 2 drops of AFB Nitrate Reagent A (sulfanilamide, 0.2 %) , 2 drops of AFB Nitrate Reagent B (Naphthylethylenediamine Dihydrochloride, 0.1 %) and a pinch of Nitrate Reagent C (Zinc dust). Colour change was examined for resistance while no colour change for sensitive.

GC-MS Analysis

Constituents in the hexane extracts and hexane fractions of the plant were elucidated using GC-MS performed on Agilent Technologies 7890 A GC coupled with Agilent Technologies 5975 C MS. Helium was used as carrier gas and sample was injected in split less mode at 70 ev in a column HP 5 MS, length 30 meters, internal diameters 0.320 mm, column thickness 0.25µm. The initial temperature was 50^{o} C, held for 2 minutes, flow rate 10^{o}/min, final temperature 240^{o} C, held for 6 minutes. The resulting GC-MS was analyzed using commercially available standards.

Results and Discussion

Chrysophyllum albidum fruits are sold in local markets in South western Nigeria during December to April. The cotyledonous part of the seeds is roasted, powdered and put in honey for treatment of tuberculosis by traditional practitioners in Abeokuta, Ogun state part of Nigeria. Fractions from the 80 % ethanolic extract were tested for anti-mycobacterium tuberculosis activity. Table 1 below shows that only the hexane fraction was sensitive to the drug susceptible Mycobacterium tuberculosis strains used while the butanol and ethyl acetate fractions did not inhibit the growth of the bacterium.

Table 1: Result of anti-mycobacterium tuberculosis activity of fractions from C. albidum

Fractions	**Weight**	**Mycobacterium tuberculosis**	
		PT_{12}	PT_{10}
Butanol	50 mg/ml	R	R
Ethylacetate	10 mg/ml	R	R
Hexane	0.5 mg/ml	S	S
Isoniazid	0.2 µg/ml	S	S
Rifampicin	0.4 µg/ml	S	S

Key: R means Resistant (Bacteria growth was not inhibited).

S means Sensitive (Bacteria growth was inhibited).

PT_{12} Mycobacterium tuberculosis $H_{37}Rv$ strain

PT_{10} Local isolate of Mycobacterium tuberculosis from TB patient

The seeds were roasted in a closed crucible at three different temperatures, 50° C, 100° C and 120° C. The hexane extracts of the roasted cotyledonous part for 50° C and 120° C were tested for anti-mycobacterium tuberculosis and the results obtained are shown in Table 2. All the concentrations tested for anti-mycobacterium tuberculosis activity gave positive result, inhibited the growth of the Mycobacterium tuberculosis.

Table 2: Result of anti-mycobacterium tuberculosis activity of Hexane extracts (Roasted seeds)

S/Ns	Samples tested	Concentrations	Mycobacterium tuberculosis	
			PT_{12}	PT_{10}
1.	Hexane extract (50° C)	2 mg/ml	S	S
		1.5 mg/l	S	S
		0.4 mg/ml	S	S
2.	Hexane extract (120° C)	2 mg/ml	S	S
		1.5 mg/ml	S	S
		0.4 mg/ml	S	S
3.	Rifampicin	0.4 µg/ml	S	S
4.	Positive control	Agar inoculated only	R	R
5.	Negative Control	Agar not inoculated	-	-

GC-MS analysis was carried out for the antimycobacterium tuberculosis fractions and extracts. Figure 1 shows the chromatogram of the active hexane fraction while figures 2, 3 and 4 are the chromatograms of the hexane extracts for the seeds roasted at 50° C, 100° C and 120° C respectively. Figure 5 showed structures of some compounds identified by the GC-MS analysis.

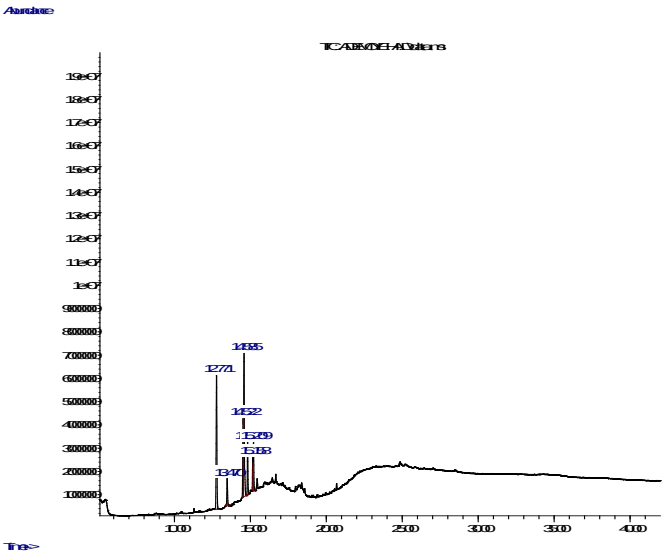


Fig 1: Hexane fraction of C. albidum

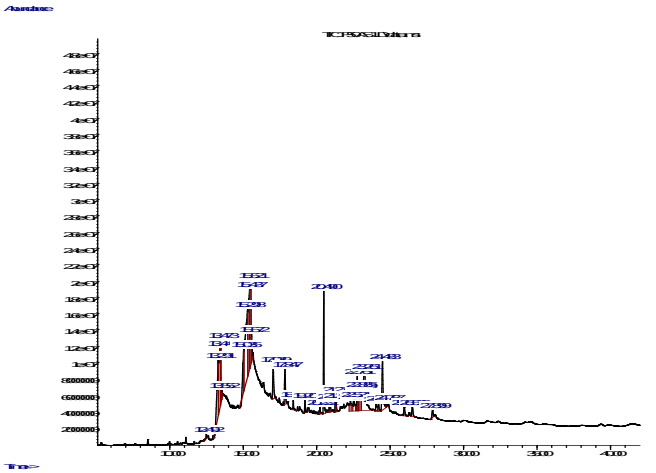


Fig 2: Hexane extract C. albidum roasted seeds 50 deg

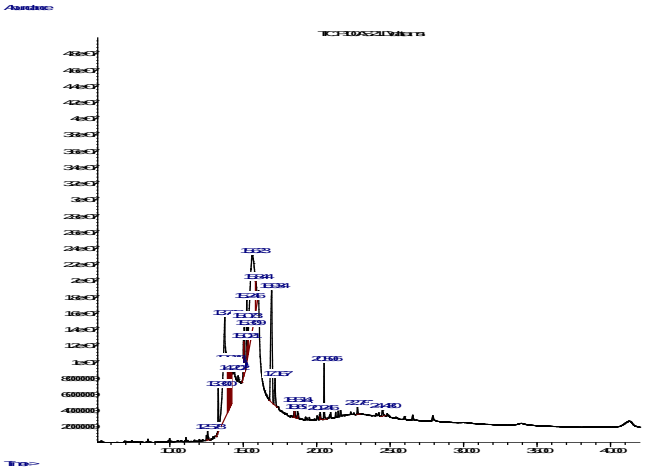


Fig 3: Hexane extract C. albidum roasted seeds 100 deg

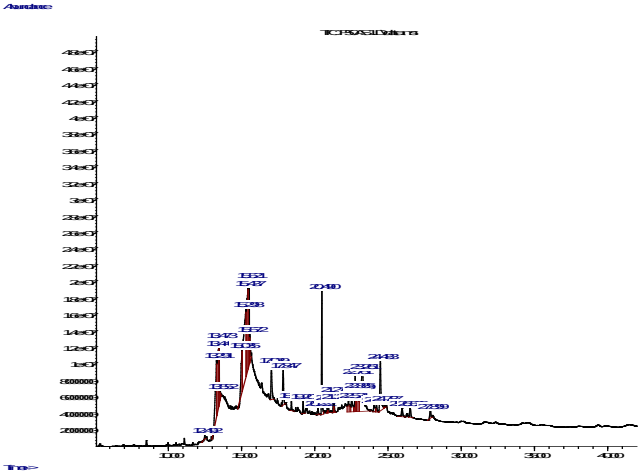


Fig: 4 Hexane extract C. albidum roasted seeds 120 deg

Esters of long chain saturated and unsaturated fatty acids constituted 92.76 % of the total constituents of the hexane fraction. The methyl ester of unsaturated fatty acids constituted 43.86 % (11-Octadecenoic acid methyl ester 28.47 % and 9,12-Octadecadienoic acid methyl ester 14.39 %). The ethyl ester of unsaturated fatty acid is 4.99 % (Linoleic acid ethyl ester). The methyl ester of saturated fatty acids constituted 39.31 % (Hexadecanoic acid methyl ester 30.01 % and Octadecanoic acid methyl ester 9.30 %). The ethyl ester of satuirated fatty acid is 5.60 % (Hexadecanoic acid ethyl ester). This analysis shows that the methyl esters of unsaturated fatty acids are more than the methyl esters of the saturated fatty acids. Again, the methyl esters of both saturated and unsaturated fatty acids are more than the ethyl esters of the fatty acids.

Fatty acids and their derivatives had been reported as antimicrobial agents (Kabara, 1972).

Table 3: Hexane fraction from C. albidum

S/N	Compound	RT	%
1.	Hexadecanoic acid methyl ester	12.771	30.01
2.	Hexadecanoic acid ethyl ester	13.470	5.60
3.	9,12-octadecadienoic acid methyl ester	14.522	14.39
4.	11-Octadecenoic acid, methyl ester	14.585	28.47
5.	Octadecanoic acid, methyl ester	14.814	9.30
6.	Linoleic acid ethyl ester	15.158	4.99
7.	1-Nonadecene	15.209	7,29

The GC-MS analysis of the hexane extract of the roasted seeds at 50 ^{o} C showed the presence of long chain fatty acids in high concentration compared to the hexane fraction that showed only the esters of the fatty acids. Again, more compounds were identified in the hexane extract, eighteen compounds compared to only seven compounds identified in the hexane fraction. The dienoic acids, 9,12-Octadecadienoic acid, constituted the highest percentage, 47.85 %. This is followed by a saturated fatty acid, n-Hexadecanoic acid 17.46 %. The third fatty acid identified is the monoenoic acid, oleic acid 3.38 %. Polyunsaturated fatty acids had been reported to be bactericidal (Knapp and Melly, 1986).

Table 4: Hexane extract of roasted seeds (50^{o} C)

S/N	Compound	RT	%
1.	Tetracontane	12491	0.18
2.	n-Hexadecanoic acid	13.292-13.550	17.46
3.	9,12-Octadecadienoic acid (Z,Z)	15.037-15.570,23.250	47.85
4.	Oleic acid	17.040, 18.408	3.38
5.	9,17-Octadecadienal (Z)	17.847	2.73
6.	2-methyl-Z,Z-3,13-Octadecadienol	19.209, 24.785	0.97
7.	Z,E-2,13-Octadecadien-1-ol	20.222, 24.050	1.27
8.	2,6,10,14,18,22-Tetracoshexaene-2,6,10,15,19,23-	20.491	6.51

	hexamethyl- (all E)		
9.	11,13-Dimethyl-12-tetradecen-1-ol acetate	20.857, 22.522	2.03
10.	Cis-Vaccinic acid	20.925	0.56
11.	Farnesol isomer a	21.269	0.84
12.	5,9,13-Pentadecatrien-2-one,6,1014-trimethyl (E,E)	21.326	0.56
13.	Z,E-3,13-Octadecadien-1-ol	22.299, 24.210	1.73
14.	Vitamin E	22.762	2.296
15.	9-Octadecyne	22.859	4.04
16.	Stigmasta-7,16-dien-3-ol(3. Beta.,5.alpha)	24.484	5.03
17.	2(1H)-Naphthalenone,octahydro-4a-methyl-7-(1-methylethyl)-,(4.alpha.,7.beta.,8a.beta)-	25.96, 26.521	1.93
18.	5. alpha-androstan-3-beta.,17.beta.dioldiacetate	27.900	0.65

Other compounds of biological importance identified in the hexane extract of the seeds roasted at 50^{o} C which are individually more than 1 % include a stigmasterol (Stigmasta-7,16-dien-3-ol(3.Beta.,5.alpha), Squalene (2,6,10,14,18,22-Tetracosahexaene-2,6,10,15,19,23 hexamethyl-), vitamin E, 9-Octadecyne and unsaturated long chain alcohol (Z,E-3,13-Octadecadien-1-ol). A stigmasterol isolated from Knowltonia vesicatoria had been reported to be active against drug sensitive Mycobacterium tuberculosis (Labuschagné et al, 2012), 9-Octadecyne (4.04 %) had been reported to possess good solubility, good inhibition ability, high penetration potential and low binding energy to target protein and thus had been recommended as a drug molecule (Upgade et al, 2014). Squalene, a triterpene and a precursor for synthesis of sterols, has antioxidant, chemopreventive and antitumor activities (Spanova and Drum, 2011; Das et al, 2008). Vitamin E is antiaging, analgesic, antidiabetic, anti-inflammatory, antioxidant, anticancer (Narayanamoorthi et al, 2015).The use of the cotyledonous in treating vagina infections could be due to the presence vitamin E. The 2,13-Octadecadien-1-ol and the 3,13-Octadecadien-1-ol are pheromones (Naka et al, 2006).

Table 5: Hexane extracts of roasted seeds (100^{o} C)

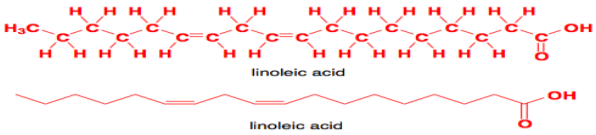
S/N	Compound	RT	%
1.	Hexadecanoic acid, methyl ester	12.577	0.54
2.	Hexadecanoic acid, ethyl ester	13.298	2.31
3.	n-Hexadecanoic acid	13.750-15.312	41.98
4.	9,12-OCtadecadienoic acid ethyl ester	15.020	1.62
5.	Ethyl oleate	15.072	2.82
6.	9,12-Octadecadienoic acid (Z,Z)	15.621, 15.844	29.91
7.	9-Octadecenal (Z)-	16.931	13.61
8.	Cis-13-Octadecenoic acid	17.166, 24.478	2.65
9.	9-Octadecenoic acid (E)	18.470	0.45
10.	cis-10-Heptadecenoic acid	18.516	0.73
11.	Z,E-2,13-Octadecadien-1-ol	18.699	0.17
12.	3-Eicosene	20.244	0.30
13.	2,6,10,14,18,22-Tetracosahexaene,2,6,10,15,19,23-hexamethyl-(all E)-	20.507	2.48
14.	Vitamin E	22.779	0.41

The GC-MS analysis of the hexane extract of the seeds roasted to 100^{o} C showed the fatty acids to be the main constituents as similar to that of 50^{o} C roasted seeds. However, there was an increase from 68.69 % of fatty acids in 50^{o} C to 71.89% in 100^{o}C. The GC-MS analysis of the hexane extract

of that of 120^{o} C showed the highest concentration of the fatty acids with 92.19 %. Other compounds with more than 1 % percentage abundance are 9,17-Octadecadiena 2.44 % and squalene 1.52 %. The two main fatty acids that had been consistent in all the roasted seeds extraction are Hexadecanoic acids and 9,12-Octadecadienoic acid. Unsaturated fatty acids inhibited growth of methicillin-resistant staphylococcus aureus (Ohta et al, 1995). Fatty acids had been characterized in the anti-tuberculosis activity of ink extracts of cuttlefish, sepiella inermis (Ravitchandirane et al, 2013). The antitubercular activity of the fixed oil of Moringa oleifera seed had been attributed to the oleic acid and palmitic acid (Egharevba et al, 2015). Unsaturated fatty acids had been reported to kill Mycobacterium in a short time (Kanetsuna, 1985).

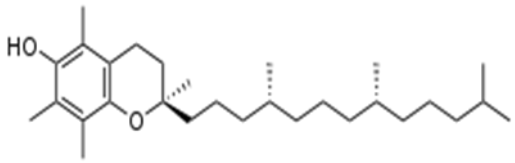
Table 6: Hexane extract of roasted seeds (120^{o} C)

S/N	Compounds	RT	%
1.	Octacosane	12.949	0.10
2.	Hexadecanoic acid ethyl ester	13.298	0.65
3.	Hexadecanoic acid	13.859-15.341	42.24
4.	9,12-Octadecadienoic acid (E,E)-	15.644-16.090	49.41
5.	9,17-Octadecadienal	15.924-16.067	2.44
6.	trans-13-Octadecenoic acid	17.183	0.54
7.	Cyclohexane,1,3-dimethoxy-,cis	17.767	0.17
8.	9-Octadecenoic acid (E)-	18.471	0.18
9.	4-(3,4,,5,6-Tetrahydroxy-2-oxo-hexylamino-)-benzonitrile	19.255	0.26
10.	2,6,10,14,18,22-Tetracosahexaene,2,6,10,15,9,23-hexamethyl-(all E)-	20.519	1.52
11.	11,13-Dimethyl-12-tetradecen-1-ol acetate	20.891	0.10
12.	Hexadecane,1-iodo	20.954	0.12
13.	Cyclopropane carboxamide, 2-cyclopropyl-2-methyl-N-(1-cyclopropylethyl)-	21.297	0.17
14.	Vitamin E	22.779	0.40
15.	Z,E-2,13-Octadecadien-1-ol	24.067	0.21
16.	1-Cyclohexylnonene	24.233	0.29
17.	Ergosta-14,22-dien-3-ol,(3.beta.,5.alpha.,22E)-	24.496	0.59
18.	Cis-11,12-Epoxytetradecen-1-ol	24.811	0.16
19.	7-Pentadecyne	25.544	0.21
20.	Pregnan-20-one-3,11-dihydroxy-3.beta.,5.alpha.,11.alpha-	27.918	0.26

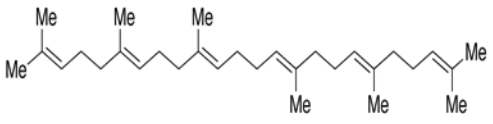


Linoleic acid- Antidiabetic-Hepatoprotective

n-Hexadecanoic acid-Anti-inflammatory



Vaginal lubrication, Anti-aging



Squalene- Antitumor

Z,E-3,13-Octadecadien-1-ol – a pheromone

9-Octadecyne

Figure 5: Structures of some compounds identified

It is possible that the saturated fatty acid, Hexadecanoic acid, work together with the unsaturated fatty acid to inhibit the growth of the Mycobacterium tuberculosis. The Hexadecanoic acid is anti-inflammatory, inhibiting the activity of phospholipase A2 (Aparna et al, 2002) and thereby possibly enhancing the activity of 9,12-Octadecadienoic acid in binding to Fatty acid synthase. Non-steroidal anti-inflammatory drugs had been reported to sensitize Mycobacterium tuberculosis to endogenous and exogenous antimicrobials (Gold et al, 2012). Also in line with this is a report that Aspirin and Ibuprofin enhance pyrazinamide treatment of murine tuberculosis (Byrne et al, 2007). The probable mechanism of mycobactericidal effect of the 9,12-Octadecadienoic acid in binding to the Fatty acid synthase had been reported (Weaks and Wakil, 1970). Pyrazinoic acid inhibits fatty acid synthase type 1 in replicating tubercle bacilli (Zimhony et al, 2007). A collaboration action among fatty acids and other antimicrobial effectors to bring about macrophage-mediated killing of mycobacterium had been suggested (Akaki et al, 1997). It could also be suggested that the fatty acids get involved in lipid peroxidation which in turn increases the toxicity of hydrogen peroxide (Sheridan et al, 1996). The hydrogen peroxide toxicity contributes to tuberculocidal effect especially if the whole process is accompanied with decline pH (Jackett et al, 1978). Thus, the anti-mycobacterium tuberculosis activity of the cotyledonous part of the seeds of Chrysophyllum albidum is due to its saturated and unsaturated fatty acids which seem to work synergistically to bring about the anti-tubercular activity. The method of roasting of the seeds by traditional practitioners also ensure the availability of the active ingredients, the saturated and the unsaturated fatty acids, in high concentration as implied in the GC-MS analysis of the 120o C roasted seeds.

Conclusion

The anti-mycobacterium tuberculosis property of the white cotyledonous part of Chrysophyllum albidum had been scientifically validated. The anti-tubercular activity of the seeds could be attributed to the presence of Hexadecanoic acid and 9,12-Octadecadienoic acid.

The GC-MS analysis of the raw seeds compared with that of the roasted seeds showed that the raw seeds contained 92.71 % of fatty acid ester while the roasted seeds contained the free fatty acids in increasing concentration as the temperature increases (Free fatty acids: 68.69 % at 50 deg, 74.54 % at 100 deg and 91.65 % at 120 deg). Thus, the traditional method of using the roasted seeds of Chrysophyllum albidum in treating tuberculosis enhances the availability of the free fatty acids in high concentration for the anti-mycobacterium tuberculosis effect.

Conflict of Interest

The authors declare no conflict of interest.

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