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Extraction and Nanoencapsulation of Ocimum Gratissimum Leaf Extract and Its Anti-Mycobacterial Activities

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Abstract
Isolation of saponin from the methanol extract of *Ocimum gratissimum* leaves, its identification with High-Performance Liquid Chromatography (HPLC), application of the saponin in the synthesis of nanoemulsion and their antimicrobial activities were carried out. 0.7mg was isolated from 40g of powdered leaves mixed with 70% of methanol. The methanol and saponin extracts were subjected to HPLC analysis. The methanol extracts revealed 15 peaks and the saponin revealed 8 peaks with a total elution time of 30 minutes each. The saponin was used to synthesize nanoemulsion (emulsifier). The nanoemulsion and saponin were subjected to antituberculosis activity. The nanoemulsion has better anti-tuberculosis activity than the saponin due to its Minimum Inhibitory Concentration (MIC). This goes to confirm the importance of nanomedicine in the drug delivery system and its application on diverse areas such as food, cosmetics, pharmaceuticals, and material synthesis. The biological synthesis is an eco-friendly alternative to the chemical and physical methods.
Keywords: Extraction, Natural Product, Nanoencapsulation, *Ocimum gratissimum*, Anti-Mycobacterial, Tuberculosis.
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1. Introduction

Medicinal plants also known as medicinal herbs have been found and applied in traditional medicine practices since prehistoric time. The World Health Organization (WHO) has been coordinating a network called the International Regulatory Cooperation for Herbal Medicines to try and improve the quality of medical products made from medicinal plants and the claims made for them (W.H.O, 2017). The plant and the essential oil of *Ocimum gratissimum* are used in traditional medicine especially in Africa and India (Nakamura, et. al, 1999; Holets, et. al, 2003). The extracted essential oil of *O.gratissimum* has been reported to possess a spectrum of antifungal properties (Dubey, et. al, 2000). The blood cholesterol-lowering properties of dietary saponins are of particular interest in human nutrition (Windaus, 1909). Triterpene saponins have a list of pharmacological activities, antiulcer, analgesic, anti-allergic, sedative, anti-viral, spermicidal, and piscicidal (Hostettmann and Marston, 1995).

Development of nanoemulsions and polymer micelles-based delivery systems to achieve enhanced water solubility/dispersibility, oral bioavailability and biological benefits for phytochemicals was reviewed (Donsì, et. al, 2010). Additionally, nanoemulsion has been formulated using green synthesized silver nanoparticle from *Azadirachta indica* leaf extract (Ashish, et. al, 2014).

Hence, this study was conducted to investigate the anti-mycobacterium activities of saponin extracted from *Ocimum gratissimum* leaves and its nanoemulsion against two strains of Mycobacterium tuberculosis

2. Methodology

2.1 Collection of the Sample

The plant material *O.gratissimum* was purchased from Dei-Dei International Market Abuja of the Federal Capital Territory, Nigeria in May 2018.

2.2 Sample Preparation

The plant material was originally identified and authenticated by a taxonomy botanist at the National Institute of Pharmaceutical Research and Development Abuja. The plant materials (leaves) were air dried at room temperature for two weeks and grounded with a blender. The powder was packaged into a container covered with lid and store in a dry and well-ventilated room until used.

2.3 Extraction

The 40g of grounded dry leaves were weighed in a 250ml conical flask. 100ml of 70% methanol was heated for 4 hours at 55^{o}C. The residue was re-extracted with 200ml of 70% methanol. The extract was concentrated on water bath till the volume reduced to 40ml. the extract was transferred into a separating funnel and mixed with 20ml of hexane in a separating funnel. The separating funnel was allowed to stand still. There is a development of an aqueous and hexane layer. Aqueous layer portion was collected, and the hexane layer was discarded. 60ml of n-butanol was added and properly mixed by vigorous shaking. The n-butanol extract was treated with 10ml of 5%

sodium chloride solution. It formed a precipitate. The precipitate solution was concentrated on a water bath. The concentrated precipitate saponin was dried in an oven. The saponin precipitate was weighed (Ajuru, et. al, 2017).

2.4 High-performance liquid chromatography analysis

The methanol and saponin extract of *O.gratissimum* were analyzed by High-Performance Liquid Chromatography (HPLC) with UV Diode Array Detector (UV-DAD). The HPLC consisted of Ultra-Fast LC-20AB equipped with SIL-20AC auto sampler; DGU-20A3 degasser; SPD-M20A UV-Diode Array Detector; column oven CTO-20AC, system controller CBM-20Alite and Windows LC solution software (Shimadzu Corporation, Kyoto Japan); column, 5µm VP-ODS C18, and dimensions (4.6 x 150 mm). The chromatographic conditions included mobile phase: 0.2% v/v formic acid and acetonitrile (20:80); mode: isocratic; flow rate 0.6 ml/min; injection volume 10 µl of 100 mg/ml solution of extract in water; detection UV 254 nm. The HPLC operating conditions were programmed to give solvent B: 20%. Column oven temperature was 40^0C. No standard reference was employed for identification purpose (Okhale, et. Al, 2017).

2.5 Preparation of O.gratissimum nanoemulsion

Tween 80, sodium dodecyl sulfate and sodium carboxymethyl cellulose were used as excipients. A 10 % concentration of cocktail-excipients in water was prepared. A 2 % solution of *O.gratissimum* in dichloromethane was prepared and slowly dropped to the aqueous solution of the excipients that were stirred (600 rpm). Then the system was stirred (600 rpm) for 10 min at 35°C, after which the mixtures were transferred to a shaking bath where they were mixed again for 40 min, and the simultaneously organic solvent was evaporated and air-dried.

2.6 Anti-tuberculosis activity of saponin and synthesized nanoemulsion (emulsifier) of Ocimum gratissimum

The anti-tuberculosis activity of saponin and synthesized nanoemulsion (emulsifier) of saponin from the leaves of *O.gratissimum* was carried out on *Mycobacterium bovis* BCG strain (ATCC 35737) and *Mycobacterium smegmatis* (650) using broth dilution method. These two strains of tuberculosis are acceptable as surrogates in drug research development against tuberculosis due to lack of containment of pathogenic tuberculosis. A 5mg of saponin and nano emulsified saponin from *O.gratissimum* were dissolved in 1 ml of sterilized Middle 7H9 broth and 50% aqueous Tween 80 respectively and centrifuged for 20 minutes at 13,000 rpm. 100 µl of each of the solutions was introduced into 1st well of a 96-well micro-titer plate, from where a two-fold dilution was performed by transferring 50 µl from 1st well into well 2 containing 50 µl 7H9 broth, with thorough mixing and repeated through to well 11 where 50 µl was discarded, leaving column 12 for the negative control. A 50 µl of inoculum prepared by diluting a 5 -7-day old culture of *M.bovis* BCG and *M. smegmatis* (OD 0.2-0.3) 1:1000 (by adding 50 µl of cell culture into 50 ml 7H9/ADC medium) was added to all the wells and incubated for 14 days at 37^0C. The MIC determinations were done in duplicate. Post incubation period the plates were stained by 25 µl of 3-(4, 5-dimethylthiazol- 2-yl)-2, 5 diphenyltetrazolium for color change in wells where there is no inhibition of M. tuberculosis cells. The last well where there is no color change is regarded as the MIC of the test agents against M. tuberculosis.

3. Result

Table 1. High-Performance Liquid Chromatography of methanol extracts

Retention time	**Peak Area**	**Area %**
3.153	5573039	5.49
3.913	8276049	8.15
4.734	11695133	11.52
6.265	9277444	9.14
7.501	10621211	10.46
8.480	5154479	5.08
9.207	3063118	3.02
10.469	2861382	2.82
11.530	33840691	33.32
14.413	261709	0.26
17.179	6715101	6.61
19.794	2208725	2.17
22.802	691808	0.68
24.702	423058	0.42
27.858	890978	0.88

Table 2. High-Performance Liquid Chromatography of Saponin extract.

Retention time	Peak Area	Area %
3.057	18909496	41.03
3.834	8051052	17.47
4.594	11113079	24.11
6.283	2819077	6.12
7.544	3527815	7.66
11.687	1429325	3.10
14.363	98912	0.21
17.284	136168	0.30

Table 3. Anti-tuberculosis activity of saponin and nanoemulsion (emulsifier) against *M.bovis* and *M. smegmatis*

Samples	Starting conc. µg/ml	Micro well plate											
		1	2	3	4	5	6	7	8	9	10	11	MIC µg/ml
Saponin Against *M. bovis* BCG	5000	-	-	-	+	+	+	+	+	+	+	+	1250
Saponin against *M. smegmatis*	5000	-	-	-	-	+	+	+	+	+	+	+	625
Saponin (emulsifier) against *M. bovis* BCG	5000	-	-	-	-	-	+	+	+	+	+	+	312.5
Saponin (emulsifier) against *M. smegmatis*	5000	-	-	-	-	-	-	-	-	+	+	+	39

KEY: - = Activity, no growth, + = Growth of organisms.

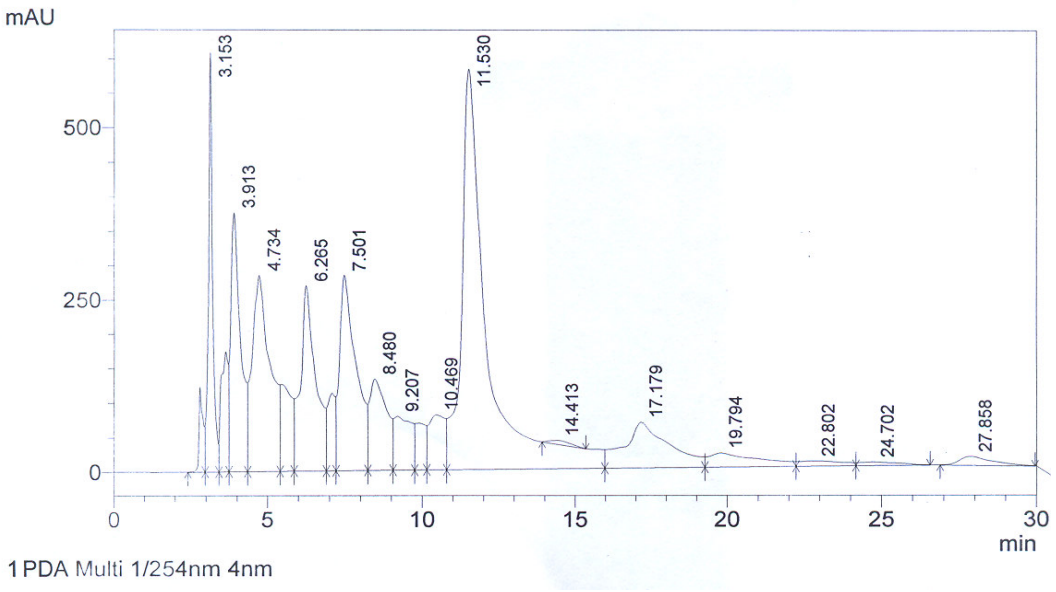

Figure 1. HPLC spectrum of methanol extract of *O.gratissimum*

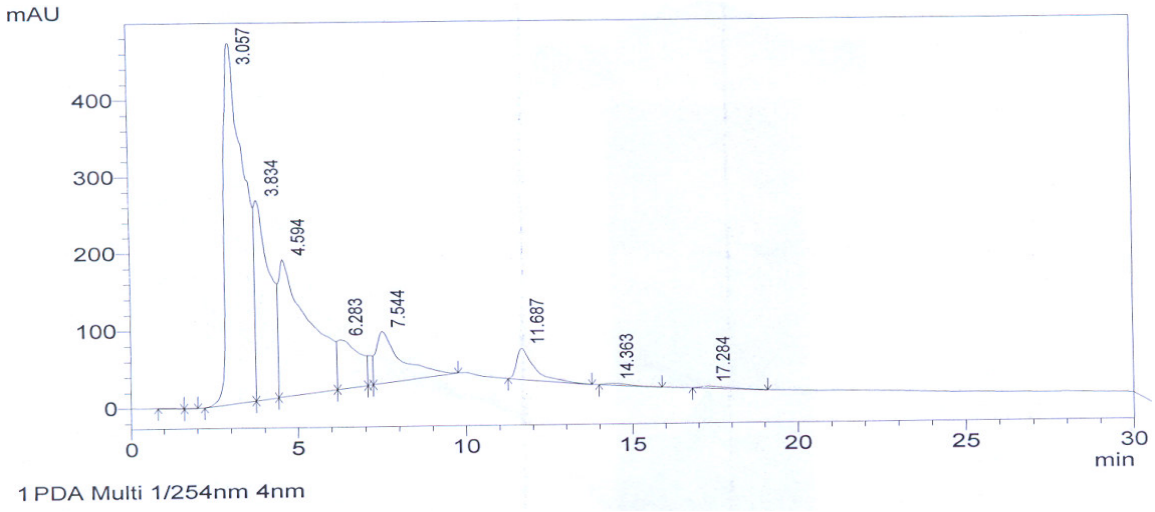

Figure 2. HPLC spectrum of saponin from *O.gratissimum*

4. Discussion

The result of the work indicates that 0.7g of saponin mixed with 70% methanol was recovered from 40g of *O.gratissimum* leaves. HPLC analysis for saponin extract revealed eight peaks, each with a total elution time of 30mins. Each peak also eluted at different retention time. The number of peaks present in the methanol extract is more than the number of peaks present in the saponin extract. This shows that saponin was extracted from the methanol mixture which contains other constituents outside saponin; more peaks are contained in the methanol extract. After 7.544 mins, there was a pause in the spectrum of the saponin which has a small peak area. In the methanol extract, small peaks were observed before a sudden rise of 11.530 peak. This shows that there is a mass transfer of saponin compounds from the methanol mixture during the extraction of saponins. Moreover, the number of peaks decreased to eight peaks from fifteen peaks observed in the methanol extracts chromatogram. This justified the concentration of the saponin increased in the saponin fraction. Standard references of saponin were not used. It is expected that we run the saponin standard reference to confirm the compounds and type of saponin present in the sample.

The anti-tuberculosis activity of saponin and synthesized nanoemulsion (emulsifier) of saponin from the leaves of *O.gratissimum* was carried out on *M. bovis* and *M. smegmatis*. The result shows the improved MIC from 1250µg/ml to 312.5µg/ml and 625µg/ml to 39µg/ml respectively. The saponin sensitivity of *M. bovis* has MIC at 1250µg/ml while *M.smegmatis* is 625µg/ml. Furthermore, the synthesized saponin nanoemulsion sensitivity of *M.bovis* has MIC at 312.5µg/ml while *M. smegmatis* is 39µg/ml. This shows that *M. bovis* is more resistant to saponin extract than *M. smegmatis*. We can observe that *M. bovis* is also more resistant to emulsifier than that of *M. smegmatis*. In the above result, we discovered that emulsifier has a better activity against the test organisms than saponin extract. The results of the Anti-tuberculosis activity of the saponin and nanoemulsified saponin of *O.gratissimum* shows that the extracts have anti-tuberculosis effects on the 2 strains of tuberculosis (*M. bovis* BCG and *M. smegmatis*) although at varying concentrations. The saponin had a MIC of 1250 µg/ml against *M. bovis* and MIC of 625 µg/ml against *M. smegmatis*. The activity of the nano emulsified saponin sample increased by 2 and 4 folds with MIC of 312.5 µg/ml against M. bovis BCG and MIC of 39 µg/ml against M. smegmatis

5. Conclusion

The methanol extract which was analyzed with HPLC shows resolution which gave 15 peaks while the saponin extract gave rise to 8 peaks. The nanoemulsion was synthesized with 250mg saponin. The study indicates that the synthesized emulsifier produced from saponin is more effective against tuberculosis test organisms (*M. bovis* and *M. smegmatis*) than saponin extract. Nanomedicine has a remarkable prospect in the improvement of diagnosis and treatment of human disease such as tuberculosis. Uses of microbes, plants, etc, in the biosynthesis of nanoproducts are an environmental acceptable procedure. Nanotechnology has turned a wide array of tools in biotechnology so that they are more personalizing, portable, cheaper, safer and easier to administer. Biological method of nanoemulsion and nanoparticles synthesis using microorganism (Klaus, et. al, 1999; Konishi, and Uruga, 2007), enzyme, fungus and plants or plants extracts is an eco-friendly alternative to the chemical and physical method.

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