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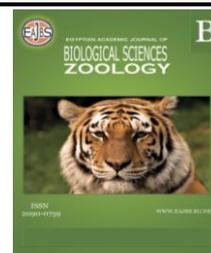


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Extraction and Identification of Bioactive Compounds from *Archachatina marginata* (Giant African Land Snail).

Ovioma, G. O. ^{*1}; Ojokuku, S. A. ², Kuteyi T. R. ³, Saanu A. I. ⁴, Olusegun-Joseph, T. S. ⁵ and Apena, L. O. ¹

1- Department of Biological Sciences, School of Science, Yaba College of Technology, Yaba Lagos.

2- Department Chemical Science, College of Technology, Lagos.

3- Department of Chemical Science, Yaba College of Technology, Yaba Lagos.

4- Analytical Chemist, Yaba College of Technology Central Laboratory.

5- Department of Science Laboratory Technology, Yaba College of Technology, Lagos.

E-mail*: godwin.oviola@yabatech.edu.ng

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ABSTRACT

Molluscs are important natural sources of many biologically active compounds. Giant snails (*Achatina marginata*) have been used in folklore medicine to treat liver diseases, anaemia, and constipation among other medicinal uses. The aim of this research is to extract and identify bioactive compounds present in *Archachatina marginata*. Ten giant African land snails were collected from a snail farm during the dry season at Badore, Ajah, Lagos. 20g of fresh tissue of the snails was used for extraction with methanol. Methanolic extraction using Chellaram, Gas Chromatography and Mass Spectrometer (GC-MS), and Fourier Transform Infra-Red Spectroscopy (FT-IR) was conducted in the identification of the bioactive compounds. The FT-IR analysis provided seven peaks for the two samples, and the spectra of both samples were discovered to be in wavenumber range between 4000-600 cm⁻¹ with a resolution of 4cm⁻¹. The GC-MS in comparison of the mass spectra of the constituents, with the National Institute of Standard and Techniques (NIST14), identified twenty chemical constituents including carotenoids, esters, fatty acid, phenols, and Oleanolic Acid-amino Acids Derivatives and phthalates were characterized. The methanol-based extracts exhibited significant bioactive constituent levels, and their anti-oxidant characteristics find great application in traditional medicine and pharmaceutical industries. They act as antioxidants which play important roles in inhibiting and scavenging free radicals, thus providing protection to humans against infections and degenerative diseases. Conclusively, twenty bioactive compounds were extracted and identified in *Achantina marginata*. The novel compounds obtained from *A. marginata* will be of high potential for antioxidant activity.

INTRODUCTION

The use of crude or more sophisticated products from nature in order to acquire health benefits is folkloric; nevertheless, the expenditure of many of these products has been scientifically proven to offer protection against several human diseases (Gayathri *et*

al., 2017). Molluscs are an abundant and significant group in the trophic chain of the animal kingdom. Among molluscs, gastropods including snails and slugs, represent the most abundant class. Snails have adapted extreme environmental conditions for more than 600 million years, due to their capacity to adapt to different environments and to reach dry land (González *et al.*, 2007). For years, snails have been used as food, and as for a variety of medicinal conditions (Ulagesan and Kim, 2018). Molluscs are very good sources of biomedically important products and have developed very effective mechanisms that are part of their innate immunity (Tincu and Taylor, 2004). Bioactive compounds discovered in molluscs include peptides, sterols, terpenes, polypropionate, nitrogenous compounds, fatty acid derivatives, miscellaneous compounds and alkaloids (Blunt *et al.*, 2009). Molluscs are predominantly investigated for their antimicrobial, cytotoxic, anti-leukemic, anti-neoplastic and antiviral properties, they are also reported as one of the important sources to derive bioactive compounds that exhibit, anti-inflammatory and antioxidant properties (Nagash *et al.*, 2010). Hence the present study was designed to identify the bioactive compounds present in the fleshy tissue extract of terrestrial gastropod, *A. marginata*.

If a pure compound shows really interesting activity, further pharmacological assays (*in vitro*, *in vivo*, tolerated dose, and so on) and chemical work (structure elucidation, structure modification, *e.t.c.*) should be carried out in order to enter the development step (Riguera, 1997). It has been reported that for bio-prospecting, freeze-dried samples of marine organisms are solvent extracted, and the extract partitioned by various chromatographic techniques including thin layer chromatography, vacuum liquid chromatography, column chromatography and high-performance reversed-phase liquid chromatography (Ebada *et al.*, 2008). Gas Chromatography and Mass Spectrometer (GC-MS): which combines two different analytical techniques, Gas Chromatography (GC) and Mass Spectrometry (MS), is used to analyse complex organic and biochemical mixtures. FT-IR analysis is used for the identification of organic, inorganic, and polymeric materials utilizing infra-red spectra for scanning the samples. The most popular application is to investigate the functional groups present in the given compound (Kong and Yu, 2007).

Justification of the study, the fact that cancers and tumours have become the order of the day can only be an understatement, and the need for research into various possible remedies, and the identification of the bioactive compounds responsible for the remedies cannot be overemphasized. Such identification and structural elucidation can thus lead to the synthesis of effective drugs to treat the numerous ailments afflicting the human race.

MATERIALS AND METHODS

Sample Collection:

Ten giant African land snails- *Achatina marginata* species were collected from a snail farm during the dry season at Recreational Avenue, Lamgbasa 106104, Badore, Ajah, Lagos; Latitude: 6.4988, Longitude: 3.6155. The snails were transported to the laboratory in large sturdy basket they were identified according to the key provided by Brian Eversham(2018) and acclimatized for 2 days at room temperature before use. The weight of each snail was measured using a weighing balance, before and after the acclimatization period.

Extract Preparation:

The methanolic extract of fleshy tissue was prepared by the method of Chellaram (Chellaram and Edward, 2004). The specimens were taken out of the

container, and their soft bodies were carefully removed by breaking the shell, using a wooden hammer. The sample was rinsed with distilled water to wash off dirt and soil and was dried at 60°C, till the sample was dried enough to be ground into powder using a blender.

Twenty gram each, of the sample, was measured into the conical flasks labelled A and B respectively, and methanol was added to each sample to the point where the samples were submerged, the samples were stirred using a glass rod until a uniform mixture was obtained.

The samples were soaked in methanol and maintained for 3 days. The extracts were filtered through Whatman No.1 filter paper. The resultant extracts obtained were concentrated using a rotary vacuum evaporator with reduced pressure. The samples were accurately labelled, kept in airtight containers and stored at 4°C for further analysis.

Fourier Transform Infra-Red (FT-IR) Spectroscopic Analysis:

The functional group present in the extract isolated from *A. marginata* was determined using ATR-FT-IR spectroscopy (Aligent FT-IR-7600), equipped with a single bounce diamond crystal and a deuterated triglycine sulfate detector.

The FT-IR spectra of samples were determined to be in the infrared (IR) range of 4000-600 cm⁻¹ with a resolution of 4cm⁻¹. Each spectrum was collected from 32 scans in the transmittance mode. Triplicate measurements were made and the mean values were used. In order to obtain the FT-IR spectra, the transmittance values were plotted (y-axis), as a function of wave number (x-axis).

Identification of Bioactive Compounds by GC-MS:

Samples (A and B) originally extracted with Methanol were reconstituted the solvent to about 2mLs, and filtered with Whatman 125mm filter paper to remove undissolved particles. One microliter (1μL) of the filtered sample was then injected into the GC-MS for chromatographic analysis, using Agilent Technologies GC systems with GC-7820A/ MSD-5977E model (Agilent Technologies, Santa Clara, CA, USA), equipped with HP-5MS Ultra Inert capillary column (30 m in length × 0.250mm in diameter × 0.25 μm in thickness of film).

Pure helium gas (99.9%) was used as carrier gas at a flow rate of 1.0 mL/min, a pressure of 7psi and 40°C. Spectroscopic detection by GC-MS involved an electron ionization system that utilized high-energy electrons (70 eV), and a temperature program at an initial temperature of 40°C ramped to 250°C at a rate of 5°C/min for 10 minutes, and a transfer line temperature of 300°C. One microliter of the reconstituted sample extract was injected, in a split mode with a Ratio of 20:1, and a Split Flow of 20mL/min. The quantity of the compounds present was expressed as a percentage based on the peak area produced in the chromatogram. Chemical compounds extracted from the samples were identified based on GC retention time on the HP-5 MS column and matching of the spectra with computer software data of NIST 14 standard Library.

Identification of Components:

Interpretation of mass spectrum GC-MS was conducted using the database of the National Institute of Standard and Technology (NIST14) Library. The spectrum of the unknown component was compared with the spectrum of the known components stored in the NIST14. L. The relative percentage amount of each bio-component was calculated by comparing its average peak area to the total area. The name, molecular weight, molecular formula and structure of the components of each chemical constituent were ascertained.

RESULTS

The results obtained in this research work include of data, tables, and graphs Findings were interpreted as shown. Seven (7) peaks of functional groups present in the methanolic extract were identified by the FT-IR spectra images are represented by the wave number and intensity respectively. The GC-MS result identified twenty (20) constituents including phenols, benzol and esters, and this was further used in characterizing the activity of chemical constituents separately from another.

FT-IR- spectra of methanolic fleshy tissue extract of *A. marginata*:

The Fourier transform infrared spectroscopic analysis represented by the graph (Fig. 1.) shows the peak of each functional group, with the wave intensity (transmittance) on the y-axis and the wave number (cm^{-1}) on the x-axis for sample A, of the methanolic fleshy tissue extract.

FT-IR- spectra of methanolic fleshy tissue extract of *A. marginata*.

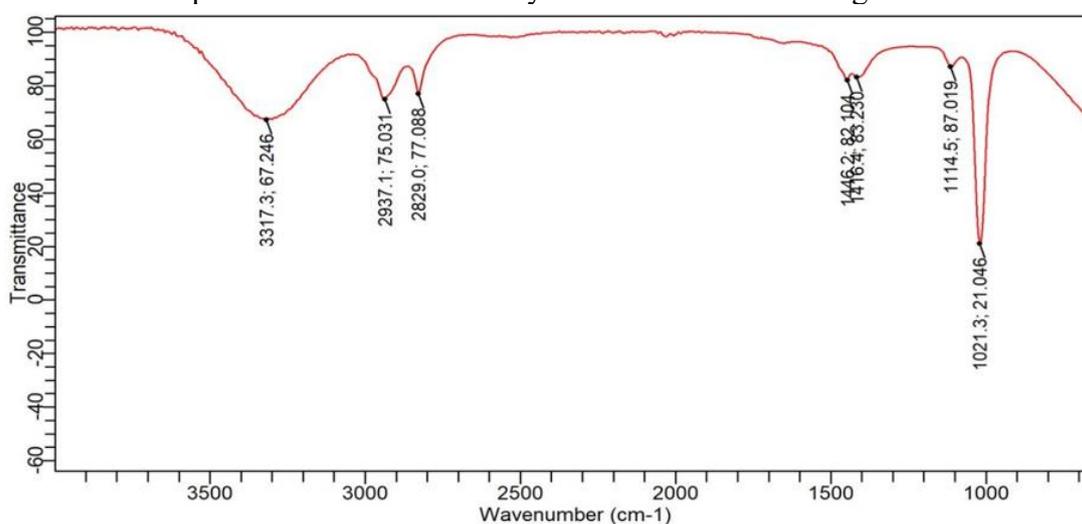


Fig. 1. FT-IR spectroscopic analysis of methanolic fleshy tissue extract- sample A.

The Table 1 illustrates the peak number, wave number (cm^{-1}), and wave intensity of each functional group as identified in the FT-IR spectrum.

Table 1. FT-IR result for sample A

Peak Number	Wave number (cm^{-1})	Intensity
1	1021.29057	21.04630
2	1114.47401	87.01875
3	1416.38838	83.23009
4	1446.20708	82.10429
5	2829.04942	77.08750
6	2937.14222	75.03073
7	3317.33068	67.24559

Figure (2) The Fourier transform infrared spectroscopic analysis represented by the graph shows the peak of each functional group, with the wave intensity (transmittance) on the y-axis and the wave number (cm^{-1}) on the x-axis for sample B, of the methanolic fleshy tissue extract.

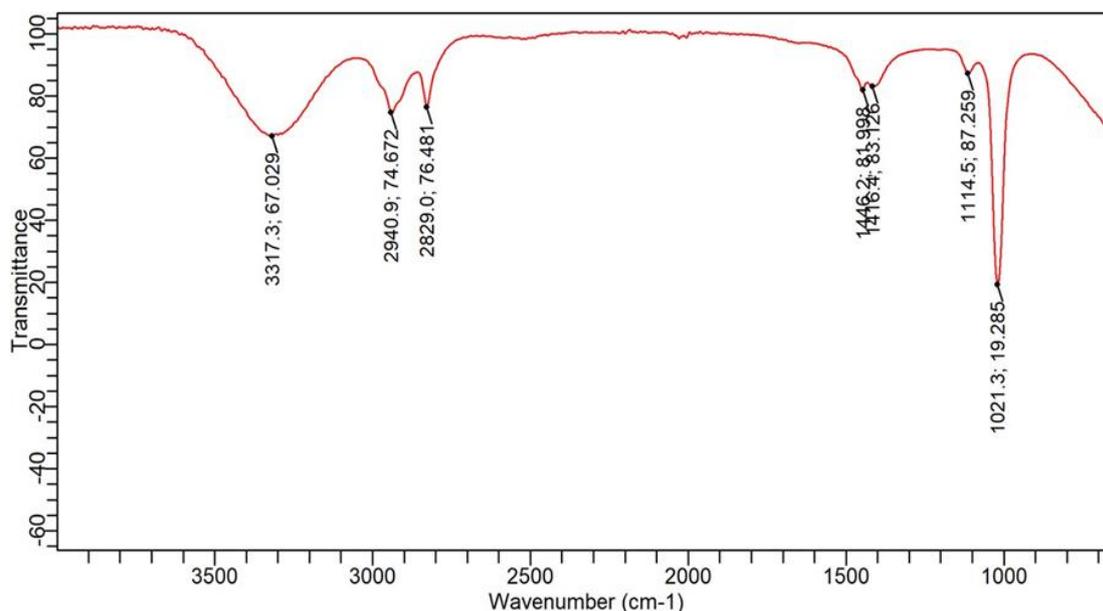


Fig. 2. FT-IR spectroscopic analysis of methanolic fleshy extract- sample B.

The Table 2 illustrates the peak number, wave number (cm^{-1}), and wave intensity of each functional group as identified in the FT-IR spectrum.

Table 2. FT-IR result for sample B

Peak Number	Wave number (cm^{-1})	Intensity
1	1021.29057	19.28501
2	1114.47401	87.25889
3	1416.38838	83.12621
4	1446.20708	81.99816
5	2829.04942	76.48138
6	2940.86955	74.67203
7	3317.33068	67.02876

Gas Chromatography-Mass Spectra of Methanolic Fleshy Tissue Extracts of *Achatina marginata*

The studies on the active principles in *A. marginata* whole animal methanol extract carried out by GC-MS analysis detected the presence of some compounds which are clearly illustrated. (Fig. 3), (Table 3) for sample A, and (Fig. 4), (Table 4) for sample B. The active principles with their peak number, retention time (RT), and peak area (%). The molecular formula (MF), molecular weight (MW), and compound structure, and name, and activities were also identified.

Figure 3 illustrates the Gas Chromatography and Mass Spectrum (GC-MS) of the methanolic fleshy tissue extract of sample A, with the abundance (area) of the chemical constituent identified on the y-axis, while the retention time (minutes) is on the x-axis.

Figure 4 graph illustrates the Gas Chromatography-Mass spectrum (GC-MS) of the methanolic fleshy tissue extract of sample B, with the abundance (area) of the chemical constituent identified on the y-axis, while the retention time (minutes) is on the x-axis.

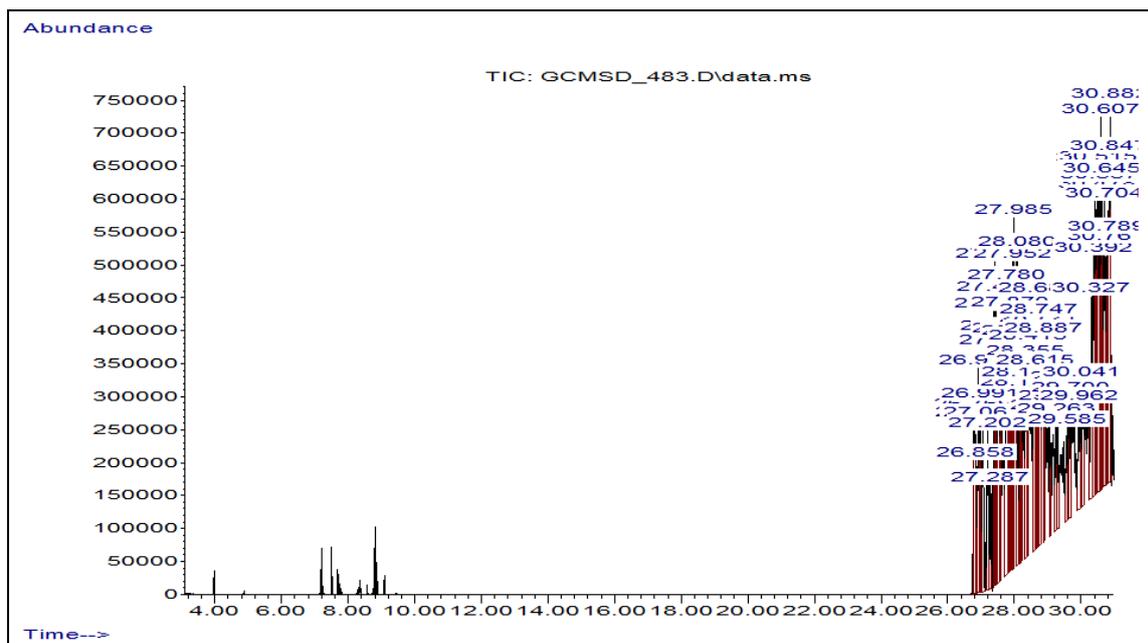


Fig. 3. GC-MS analysis of methanolic fleshy tissue extract- sample A.

The Table 3 represents the GC-MS result indicating the peak number, retention time (min.), peak area (%), molecular formula (MF), molecular weight (MW), name, and compound structure of each chemical constituent identified in this analysis.

Table 3. Show bioactive compounds identified in *A. marginata* methanol extract- (sample A).

S/N	Peak Number	Retention Time (min.)	Peak Area (%)	Molecular Formula (M F)	Molecular Weight (MW-g/mol)	Compound Name
1	2	26.820	1.94	C ₁₅ H ₁₂ Br ₄ O ₂	539.757076	Phenol, 4,4'-(1-methylethylidene) bis [2,6-dibromo-
2	6	26.991	1.10	C ₂₇ H ₂₀ Cl ₄ N ₄	540.04421	N,5-Bis[3,4-dichlorophenyl]-3,5-dihydro-3-[[1-methylethyl]imino]-2-phenazinamine
3	9	27.287	0.72	C ₂₇ H ₃₇ F ₆ NO ₃	537.267765	Isonipecotic acid, N-(2,5-di(trifluoromethyl)benzoyl)-, dodecyl ester
4	9	27.287	0.72	C ₄₀ H ₅₆	536.4382	.beta. Carotene
5	20	27.952	1.57	C ₁₀ Cl ₁₀ Fe	525.623463	Ferrocene, decachloro-
6	23	28.148	0.74	C ₃₃ H ₅₀ O ₆	542.36074	Oleanan-29-oic acid, 3-(acetyloxy)-12,13-epoxy-11-oxo-, methyl ester, (3.beta.,12.beta.,20.beta.)-
7	36	29.585	1.15	C ₃₇ H ₇₂ O ₂	548.55323	Cyclopropanepentadecanoic acid, 2-octadecyl-, methyl ester
8	37	29.618	0.57	C ₄₀ H ₆₄	544.5008	.psi.,.psi.-Carotene, 7,7',8,8',11,11',12,12'-octahydro-
11	51	30.704	2.51	C ₃₃ H ₄₆ Cl ₂ N ₂	540.303806	Cholest-3-eno[3,4-b]quinoxaline, 6',7'-dichloro-

The Table 4 represents the GC-MS result indicating the peak number, retention time (min.), peak area (%), molecular formula (MF), molecular weight (MW), name, and compound structure of each chemical constituent identified in this analysis.

Table 4. Show bioactive compounds identified in *A. marginata* methanolic fleshy tissue extract- (Sample B).

S/N	Peak Number	Retention Time (Min.)	Peak Area (%)	Molecular Formula (MF)	Molecular Weight (MW-g/mol)	Compound Name
1	1	3.232	0.15	N D	N D	H,8H-Benzo[1,2-b:5,4-b'] dipyran-10-propanoic acid, 3,7-dibromo-5-methoxy-2,2,8,8-tetramethyl-, methyl ester
2	8	5.370	0.60	C ₁₅ H ₁₂ Br ₄ O ₂	539.757076	Phenol, 4,4'-(1-methylethylidene) bis [2,6-dibromo-
3	12	5.709	0.46	C ₃₁ H ₄₀ N ₄ O ₅	548.29987	7-Oxa-15,20,24,27-tetraazatetracyclo [13.9.6.2(8,11).1(2,8-6)] tritriaconta-2,4,6(33),8,10,12,31-heptaene-14, 9-26-dione, 20-acetyl-5-methoxy-, [s-(Z)]
5	25	6.830	0.99	C ₃₀ H ₁₈ O ₁₀	538.089996	Skyrin
6	64	11.081	1.38	C ₃₅ H ₄₆ N ₂ O ₃	542.35084	2-propenoic acid, 2-methyl-, 2-[4-[bis[4-(diethylamino)-2 3--methylphenyl] methyl] phenoxy]ethyl ester
7	101	15.216	0.11	C ₂₅ H ₄₈ N ₂ O ₂ Si ₄	520.279285	Aminoglutethimide, N, N, N, O-tetrakis(trimethylsilyl)deri
8	116	16.672	0.26	C ₂₉ H ₄₃ F ₄ N ₃ O ₃	529.317905	Isonipectic acid, N-(2-fluoro-3-trifluoromethylbenzoyl)-, pentadecyl ester
9	121	17.326	0.24	C ₃₄ H ₅₉ N ₃ O ₃	529.449493	Phthalic acid, monoamide, N, N -diundecyl, isobutyl ester
10	129	17.876	0.24	C ₃₄ H ₅₂ O ₅	540.381474	Olean-9(11)-en-12-one, 3. beta.,28-dihydroxy-, diacetate
11	130	17.995	0.07	C ₁₅ H ₆ Cl ₆ F ₄ O ₂	503.843508	phenol, 4,4'-[2-chloro-1-(chlorodifluoromethyl)-2,2-difluoroethylidene] bis [2,6-dichloro-
12	149	22.035	1.22	C ₃₆ H ₇₄ S	538.551125	Distearyl sulfide

Table 5. Activities of some identified bioactive compounds in the methanolic fleshy tissue extract of *Achatina marginata*

Compound name	Activities
Phenol, 4, 4'-(1-methylethylidene) bis [2,6-dibromo also known as TBBPA	Basic chemical manufacturing, flame retardant in electrical equipment, appliance and component manufacturing; plastic product manufacturing, aerospace product and parts manufacturing, artificial and synthetic fibers and filaments manufacturing, coating and adhesive manufacturing, textile and fabric finishing, converted paper product manufacturing, waste treatment and disposal, motor vehicle parts manufacturing and other miscellaneous manufacturing.
Ferrocene, decachloro-	Anti-cancer (breast and human lung cancer cell lines), fuel additive, as a ligand scaffold, and solid rocket propellant.
Beta. Carotene	Eye health, improved cognitive function, skin protection, anti-cancer (breast, lung and pancreatic)
Olean-9(11)-en-12-one, 3. beta.,28-dihydroxy-, diacetate	Anti-inflammatory, anti-ulcer, anti-allergic, antidote, anti-tumor, immunotropic activity, inhibits HIV-1, influenza A virus, herpes, hepatitis B and C, SARS-associated coronavirus and anti-viral activity
Distearyl sulphide	Secondary stabilizer and antioxidant in combination with phenolic antioxidant for polymers
Cyclopropanepentadecanoic acid, 2-octadecyl-, methyl ester	Anti-inflammatory, antibiotic and red blood cell stabilization
. psi., psi. -Carotene, 7,7',8,8',11,11',12,12'-octahydro-	Antioxidant, atherosclerosis prevention, exercise induced asthma, anti-cancer (pancreatic, prostate, bladder, colon, cervical and cancer cell growth in vitro)
Phthalic acid, monoamide, N, N -diundecyl, isobutyl ester	Plasticizer, additive, allelopathic, antimicrobial and insecticide activity
Isonipectic acid, N-(2,5-di(trifluoromethyl)benzoyl)-, dodecyl ester	Industrial and commercial use
Oleanan-29-oic acid, 3-(acetyloxy)-12,13-epoxy-11-oxo-, methyl ester, (3. beta.,12. beta.,20. beta.)-	Treatment of digestive tract ulcer, antibiotic to treat helicobacter infections, histamine H2 antagonist to reduce gastric acid secretion, antacid for symptomatic relief

DISCUSSION

In this study, the FT-IR spectra of the *Achatina marginata* samples were determined to be in the infra-red (IR) range of 4000-600 cm^{-1} with a resolution of 4 cm^{-1} , and seven (7) peaks were recorded for samples A and B respectively and the functional groups identified were in the single bond region (wave number and transmittance 2829.0; 77.088, 2937.1;75.031 and 3317.3; 67.246), (wave number 2829.0; 76.481, 2940.9; 74.672 and 3317.3; 67.026) and the fingerprint region (wave number 1021.3; 21.046, 1114.5; 87.019, 1416.4; 83.230 and 1446.2; 82.104), (wave number 1021.3; 19.285, 1114.5; 87.259, 1416.4; 83.126 and 1446.2; 81.998). Each spectrum was collected from 32 scans in the transmittance mode. Triplicate measurements were made and the mean values were used. In order to obtain the FT-IR spectra, the transmittance values were plotted (y-axis), as a function of wave number (x-axis). Twenty (20) chemical constituents including fatty acid, phenols, esters, carotenoids, Amino acid derivatives, phthalic acid and Oleanolic acids were identified in the methanolic fleshy tissue extract of *A. marginata* by Gas Chromatography and Mass Spectrometer (GC-MS) analysis in samples A and B. The presence of various bioactive compounds justifies the use of snails, for various ailments by traditional practitioners. More than 2600 scientific studies have been carried out over the last 20 years testify to the important contribution of compounds extracted from gastropod snails to medicine and cellular biology (Pickrell, 2003). Chemical drugs may lead to adverse effects and recent researchers have focused on pharmacologically active compounds from plants and animals. GC-MS is used to identify the constituents of volatile matter, hydrocarbons, alcohols, acids and esters. The extraction and identification of the compounds shown in the result of this study confirm that snails are of significant health benefits because it contains biological constituents with anti-oxidant, anti-microbial, anti-cancer, anti-viral, and anti-inflammatory activities. (Akinnusi, 2002,) Studies indicate that high consumption of foods rich in bioactive compounds with antioxidant activity, including vitamins, phytochemicals, omega-3 fatty acids, and mainly phenolic compounds, such as flavonoids, and carotenoids, have positive impacts on human health and can reduce the risk of several acute to chronic diseases, (Akinnusi, 2002, Hassimotto *et al.*, 2009, Siriwardhana *et al.*, 2013). The tissue extracts from molluscs are very well known as immune-booster and possess anti-microbial, anti-fungal, anti-viral and anti-tumor activities (Bashir *et al.*, 2015, Boyanova *et al.*, 2012, Coates *et al.*, 2014, Dwek *et al.*, 2001, Rong *et al.*, 2013, Zhuang *et al.*, 2015).

Each chemical constituent identified in the result obtained has antioxidant properties such as carotenoid which improves eye health, improves cognitive function, skin protection, anti-cancer, Oleanolic acid has been reported to have anti-inflammatory, anti-ulcer, anti-allergic, and anti-tumor. (Hamzalioglu and Gokmen, 2016).

CONCLUSION

The result obtained from the FT-IR analysis showed mid-IR spectra with seven (7) peaks in the range of 600-4000 cm^{-1} with a resolution of 4 cm^{-1} , and the functional groups identified of which three (3) were in the single bond region and four (4) in the fingerprint region respectively, containing carbon constituents (C-O stretch) such as primary alcohol, phenol (C-H) compounds, methyl (C-H) and asymmetry, vinyl (C-H). Each chemical constituent identified by the GC-MS consisted of twenty (20) chemicals including amine (1), amino derivative (1), carotenoids (2), fatty acid (1), and acetate salt of acetic acid. The peak no., time (min.), area (%), compound name, molecular formula (MF), molecular weight (g/mol-1), structure and the activities of each compound were

determined. In conclusion, the chemical constituents extracted from *A. marginata* were identified to be of medical significance in the treatment of various ailments, and also of pharmaceutical importance in drug production based on report from previous study (Hamzalioglu and Gokmen, 2016).

Recommendation:

From this research findings, it is recommended that snail should be consumed regularly as source of protein, and essential bioactive substances. Further researches should be carried out to extract the active sub the bioactive compounds present in land snails (*Achatina marginata*), which can be regarded as high potential chemical constituents that may find application in the pharmaceutical industries in drug production, in modern medical practices and traditional medicine in the treatment of ailments and diseases.

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