Journal of Animal &Plant Sciences, 2017. Vol.34, Issue 2: 5413-5424
Publication date 30/10/2017, http://www.m.elewa.org/JAPS; *ISSN 2071-7024*

Profiling active phytochemical compounds of *Ziziphus abyssinica* herb responsible for antioxidant and antimicrobial activity

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Key words: Bioautography, antimicrobial, antioxidant,

1 ABSTRACT

This paper seeks to determine the various active compounds that are present in the *Ziziphus abyssinica* A. Rich that would elicit antioxidant and antimicrobial activity on foodstuff. The cold methanolic extract was separated using TLC method, the activity of compounds was done using autobiographic TLC identification and the active areas were reconstituted and identified using GC-MS. There were 21 compounds which exhibited antioxidant activity with the most prevalent being Benzenamine, 3,4-dimethyl-, Phenol, 2,5-bis(1,1-dimethylethyl)-, Butylated hydroxytoluene, Methyl decanoate, Hexadecanoic acid / Palmitic acid and Phenol, 2,2'-methylenebis[6-(1,1-dimethylethyl)-4-ethyl-. Compounds with antimicrobial activity were 24 out of which some had antioxidant and antifungal. The most active compounds isolated were 2-Furancarboxaldehyde $C_5H_4O_2$, 4-Methyl-2-hexanol, Sulphurous acid, cyclohexylmethyl undecyl ester, Decanoic acid (capric acid), Hydrazinecarboxamide, and N, N-diphenyl Aldehyde.
It is therefore worth to note that AZA has antioxidant and antimicrobial properties that can qualify it to be a natural preservative.

2 INTRODUCTION

The herbal plant *Ziziphus abyssinica* (AZA) locally known as Angau of class Rhamnaceae is mainly found in West Pokot County of Kenya. This herb has been used by the pastoralist of West Pokot for many decades to preserve their meat and meat products for a long time, a practice known to be in many traditional communities to date. It is the crushed, dried portions of the fruits that are added to meat and left to dry. Research has it that preservative properties of herbs are derived mainly from various phytochemical and physicochemical compounds that naturally occur within the herbs. These compounds are responsible for colour, flavour, texture and protection against microbial infestation, antioxidation and predation. (Doughari *et al.*, 2009; Polya 2003; Dahikar *et al.*, 2009). Therefore, antimicrobial and antioxidant activity exhibited by the herbs are presumably the main properties utilized for the purposes of preservation. Previous studies on phytochemical analysis of AZA indicated the presence condensed tannins, reducing compounds, sterols and steroids, alkaloids, saponins, flavonoids and polyphenolics (Nyaberi *et al.*, 2010). Saponins have detergent properties and serves as lytic agent that are well

known to exhibit anti-inflammatory properties, while alkaloids flavonoids and polyphenolics aid in defense mechanism of the plant and thus act as phytoprotective agent against invading microorganism (Doughari, 2009). Phytochemicals give plants their antioxidant activities (Eminagaoglu *et al*., 2007; Guleria *et al*., 2012 and Anwar *et al*., 2009). Research in plant oils of clove, oregano, rosemary, sage and lavender has reported the presence of antioxidant and anti-rancidity properties (Adorjan *et al*., 2010). Out of the many phytochemicals present in plants, only a few of them have been reported to be responsible for the antimicrobial and antioxidant activities. To identify these compounds many methods have been employed, with the most notable one being the TLC method where bioautography method is employed, due to its flexibility, simplicity and accuracy (Olech *et al*., 2012; Cimpoiu *et al*., 2006; Badarinath *et al*., 2010). It also allows localization of activity even in complex matrix and therefore facilitating target-directed isolation of active constituents (Rahalison *et al*., 1991). These TLC methods have been developed and applied successfully for qualitative and quantitative analysis of both antioxidants and antimicrobial compounds (Zhao *et al*., 2010 Jasprica *et al*., 2007). To identify antioxidant compounds the stable free radical 2, 2-diphenyl -1-picrylhydrazyl (DPPH) is often used as a derivatization reagent (Kusznierewicz *et al*., 2012). To be able to promote the use of AZA as a potential preservative then an investigation of its composition and activity is very important to validate its use (Nair *et al*,. 2006). This study will therefore, seek to determine the various active compounds that are present in the herb.

3 MATERIALS AND METHODS

3.1 Preparation of the samples: A 250 g dried portion of the fruit AZA was extracted using the cold methanolic method. From the extract obtained, 20 g was taken and dissolved in 50 ml absolute methanol and left to stand overnight. The mixture was taken and filtered using Whitman filter paper No. 41 along with 2 gm sodium sulphate, which had been wetted with absolute methanol to remove sediments and traces of water in the filtrate. A 50 ml portion of hexane was added to the filtrate to remove organic impurities. The mixture was shaken and the hexane portion discarded. The filtrate was then concentrated by bubbling with nitrogen to reduce the volume to 1ml, from the filtrate, obtained phytochemical analysis to identify active compounds was performed using bioautography TLC analysis.

3.2 Extraction and separation by the TLC system: Analyses of the chemical constituents in the extracts were performed by separation on glass -backed thin layer chromatography (TLC) plates (Merck, silica gel 60 F254). Three eluent systems were used to develop the TLC plates under saturated conditions i.e., ethyl acetate/methanol/water (40:5.4:5): [EMW] for polar and neutral compounds, chloroform/ethyl acetate/formic acid (5:4:1): [CEF] for intermediate polarity and acidic compounds, benzene/ethanol/ammonia hydroxide (18:2:0.2): [BEA] for non-polar and basic compounds (Kotze and Eloff, 2002). The chemical compounds separated were detected by spraying acidified vanillin (0.1 g vanillin: 28 ml methanol: 1ml sulphuric acid). Upon spraying, the chromatograms were heated at 110°C in an incubator to allow for optimal colour development (Kotze and Eloff, 2002).

3.3 Separation of Compounds using Bio-autography: Several TLC plates were loaded with methanol extract (1 mg/ml, 2 µl) in a narrow spot directly onto the plate and eluted using the three different mobile solvent systems CEF, BEA, EMW and a control for each solvent system.

3.4 Separation of Antimicrobial Compounds: The developed plates were dried under hot air of about 80°C in an oven with fast moving air for 5 days to remove traces of solvent on the plates. One-week-old cultures of

microorganisms grown on Synthetic Define agar (SD) were each transferred into 250 ml of freshly prepared broth using a sterile swab. Densities of the cultures used for *C. albicans*, were approximately 8×10^6 cells/ml respectively, In the case of bacteria, overnight cultures grown on Muller Hilton broth were used and the densities of bacterial organism used for *E. coli*, and *S. aureus* were approximately 3×10^{11}, and 3×10^{12} cfu/ml, respectively. The prepared chromatograms were sprayed with the fungal or bacterial suspension until wet. This process was carried out in a bio-safety Class II cabinet (Labotec, SA) for fungi, and Laminar flow cabinet (Labotec, SA) for bacteria. Thereafter, the plates were incubated overnight at 35°C and 100% relative humidity in the dark and then sprayed with a 2 mg/ml solution of p-iodonitrotetrazolium violet (Sigma®) (INT) (Begue and Klein, 1972). White bands, were an indication of areas where reduction of INT to the coloured formazan did not take place due to the presence of compounds that inhibited the growth of tested organisms. Portions that were identified to have antimicrobial effect were scrapped from the TLC plates and reconstituted in methanol. The reconstituted samples were left to stand for thirty minutes before the mixture was filtered using No. 1 Whitman filter paper. The samples were then passed through Millipore (Billerica, MA, USA) membranes (0.22 and 0.45 µm) before being injected into the HPLC for analysis and identification of compounds using MS (Suleiman *et al.*, 2010).

3.5 Separation of antioxidant Compounds using Bio-autography: Analysis of the chemical constituents in the extracts was performed by separation on glass -backed thin layer chromatography (TLC) plates (Merck, silica gel 60 F254). Three eluent systems were used to develop the TLC plates under saturated conditions. In situ 2.54-mM DPPH methanol was sprayed on the developed plates, the plates were observed with visible light under UV 254 nm, and UV 366 nm. Samples producing yellowish bands on the red/purple background were considered as antioxidant, usually, the purple background colour was visualized after spraying the plate with DPPH reagent (Ruiz-Terán *et al*, 2010; Lihua *et al.*, 2009 and Rumzhum *et al.*, 2012). The background colour of the plate changed from purple to red after 12 hrs in darkness. The red background makes the yellowish bands clearly visible. Portions that were identified to have antioxidant effect were scrapped from the TLC plates and reconstituted in methanol. The reconstituted samples were left to stand for thirty minutes before the mixture was filtered using No. 1 Whitman filter paper. The samples were then passed through Millipore (Billerica, MA, USA) membranes (0.22 and 0.45 µm) before being injected into the HPLC for analysis and identification of compounds using MS (Suleiman *et al.*, 2010). Consequently GC-MS analysis.

3.6 Identification of the antimicrobial and antioxidant compounds using HPLC method : The HPLC system consisted of a solenoid pump with a photo diode array detector (PDA), C18 column was used as the stationary phase, The flow rate of the pump was 1 ml/min and a backpressure of < 100 KV with a sample size of 0.2 µl. The mobile phase consisted of 90% methanol, while the PDA detector was set at a wavelength range of 200 – 400 nm. The output signals were monitored and processed using Shimadzu CFR software. The run time of the method was about 7 minute and all analyses were separated within the run time.

3.7 Gas Chromatographic Analysis: Four reconstituted samples of AZA extract were taken, two that exhibited strong antioxidant activity and two that exhibited strong antimicrobial activity against *Staphylococcus aurous* with the bio autography tests were randomly chosen from the EMW (polar) samples. These samples were analysed by the GC-MS to identify the active compounds responsible for the antimicrobial and antioxidant effects of AZA. The sample extracts were subjected to Gas chromatographic analysis, carried out on Shimadzu GC-14 A chromatograph equipped with flame Ionization

detector fitted with 25 m× 0.22 mm (id.) SE-30 capillary column at carrier gas flow rate of 2 ml/min with split ratio was 1:100 and sample size 0.2l. The column temperature was programmed at 70˚C for 4 min. with4˚C/min rise to220˚C while detector and injector temperature were maintained at 300˚C and 220˚C respectively. Percentage composition of individual components was calculated, based on peak area using Shimadzu C-R4A chromate pac electronic integrator, all from Shimadzu Corporation, Japan.

4 RESULTS AND DISCUSSION

4.1 Thin layer chromatography: The TLC plates eluted with CEF and BEA gave results that could not be visible, on the other hand, the results of EMW were clearly visible as shown in plate 1. These results revealed several spots as shown, which indicated that the extract contained various compounds some of which may be responsible for the antimicrobial and antioxidant activity of the extract.

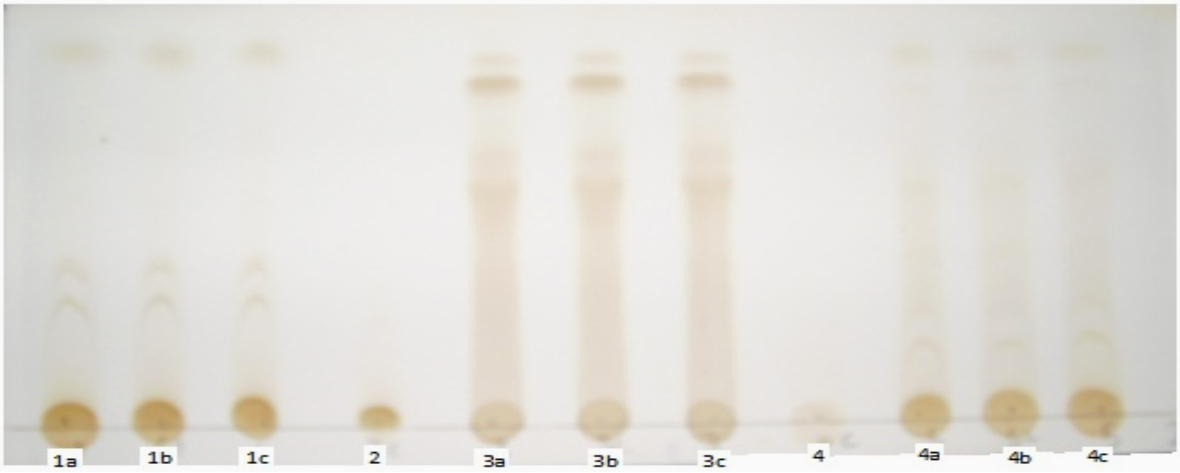


Plate 1: Indicates how each of the extracts separated upon elution with EMW

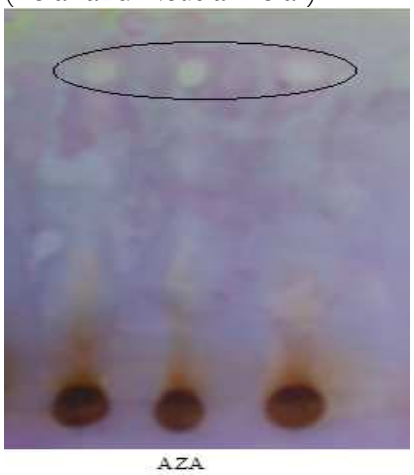
The first three samples 1a, 1b and 1c were in triplicate, three compounds separated as seen from the three spots. Sample 3a, 3b and 3c are AZA in triplicate, from this sample five compounds separated. When the same sample was filtered with active charcoal as seen in sample No. 4 Nothing separated. In order to identify the activity of the compounds visualized on the TLC plate, autographic and Bio-autographic TLC was undertaken.

3.8 Mass Spectrometry: A Jeol model JMS-AX505H mass spectrometer combined with Hewlett Packard 5890 gas chromatograph were used for GC-MS analysis. The filtered extract was injected on a 25 m × 0.22 mm BPS (5% phenyl-methyl silicone) capillary Column using helium as a carrier gas with split ratio1:100 and interface temperature 230°C. Data acquisition and processing were performed by Jeol JMADA 5000 system. Various components were identified by their retention time and peak enhancement with standard samples and MS library search.

4.2 Identification of antimicrobial Compounds using Bio-autography: The bio-autographic TLC analysis with EMW solvent system, targeted the polar and neutral polar compounds in the extracts of AZA. This analysis produced a distinct area of inhibition in triplicate when *S. aureus* and *Candida* were inoculated, indicating the presence of a compound or compounds that inhibited the growth of the two microorganism at Rf of 8.2 and 9.2 respectively. The growth of *E. coli* was not inhibited by the

compounds that separated in the TLC solvent system (Plate 2).

A. Antimicrobial *S. aureus*-EMW (Polar and Neutral Polar)



B. Antimicrobial *C. albicans*-EMW (Polar and Neutral Polar)

C. Antimicrobial *S. aureus*-BEA (Non Polar)

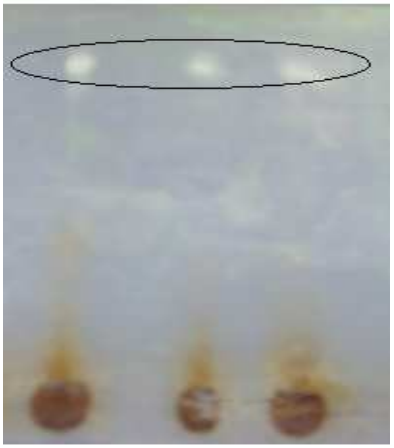
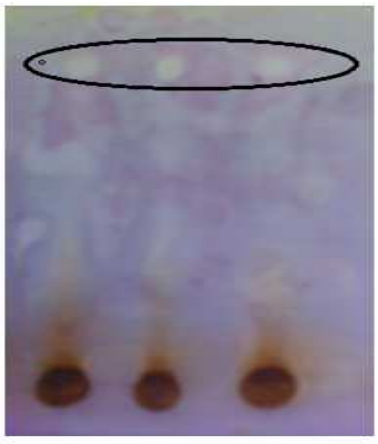


Plate 2: Autographic images of plates inoculated with *S. aureus*, and *C. albicans* that gave positive results showing the area of inhibition upon illusion with EMW and BEA

The autobiographic BEA solvent system targeting non-polar compounds in the solvents AZA produced a distinct area of inhibition with *E. coli*, *S. aureus* and *C. albicans* this indicated that there were compounds that inhibited the growth of the three microorganism *E. coli*, *S. aureus* and *Candida albicans* at an Rf of 7.9, 7.8 and 7.4 respectively.

Antimicrobial E coli-BEA



Antimicrobial Staphylococcus-BEA

Candida BEA

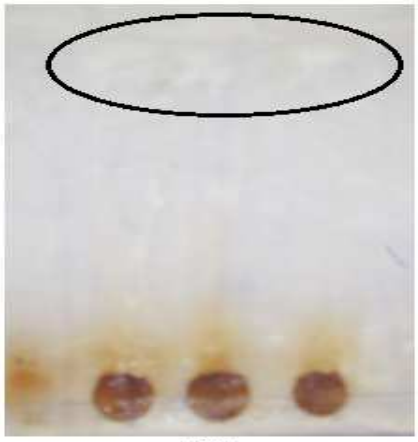


Plate 3: Indicating the area of inhibition caused by *E. coli, S. aureus* and *Candida* eluted by BEA

The autobiographic CEF solvent system targeting Intermediate polarity and acidic compounds in AZA extract indicated no activity on the three microorganisms *E. coli, S. aureus* and *Candida.* Most of the antimicrobial agents detected in this study were present in extracts of relatively non-polar solvents. These findings agreed with previously published results (Masoko and Eloff, 2005, 2006) that the substances responsible for the antimicrobial activity were mainly non-polar in nature (Suleiman *et al*., 2010).

4.2 Identification of antioxidant Compounds using autography: Various assays have been used to test for antioxidant activity but the most widely used methods are those that involve generation of free radicals, which are then neutralized by antioxidant compounds (Arnao *et al*., 2001). In qualitative analysis of antioxidant activity, the 2, 2, diphenyl-1-picrylhydrazyl (DPPH) assay on TLC plates is used as a screening test for the radical scavenging ability of the compounds present in different extracts.In the TLC autographic system, the solvent EMW was used to separate polar and Neutral polar compounds. The extract of AZA reported most activity at Rf value of between 4.8 and 7.4. This indicated that the compounds that separated were of varying volatility. The solvent system BEA had compounds separating at very low Rf value of between 1.2 and 2.9 respectively (Plate 4). The solvent system of CEF of intermediate polarity and acidic compounds had no antioxidant activity reported

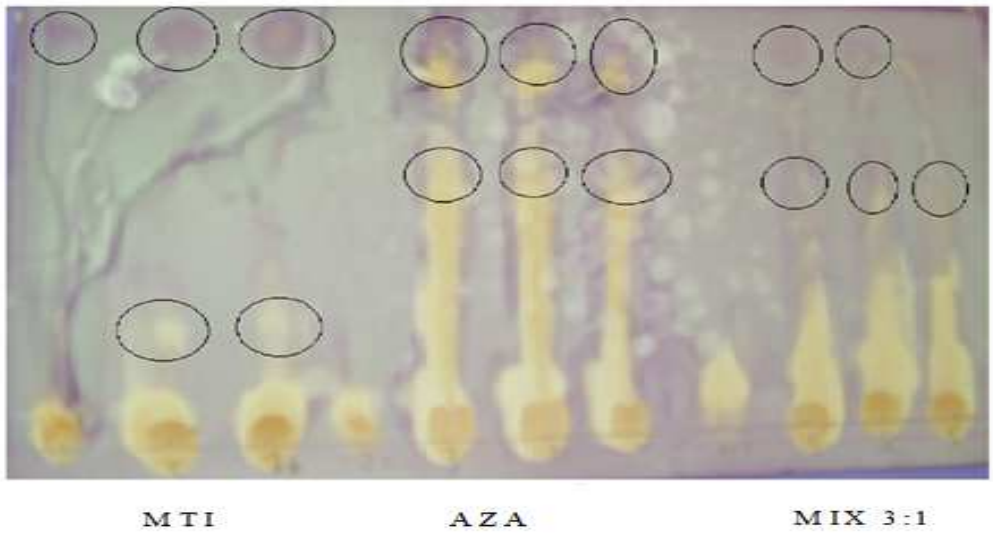


Plate 4: Antioxidation activity with the solvent system EMW (Polar and Neutral compounds)

4.3 Gas Chromatographic Analysis

4.3.1 Antioxidant activity: The sample that exhibited antioxidant effect after analysis produced twenty-one compounds. From the graph it can be noted that between the RT of 20 and 32 there lay most of the compounds. Some compounds appeared in very low pick area while others had high pick areas. All the compounds were individually investigated for their activity during previous encounters. It was noted that the compound with the highest antioxidant properties according to literature included *Benzenamine*, 3,4-*dimethyl*- MW 121.17, Butylated hydroxytoluene MW 220.35, Phenol, 2,5-bis(1,1-dimethylethyl)- MW 206.36, Phenol, 2,2'-methylenebis[6-(1,1-dimethylethyl)-4-ethyl- MW 368.55

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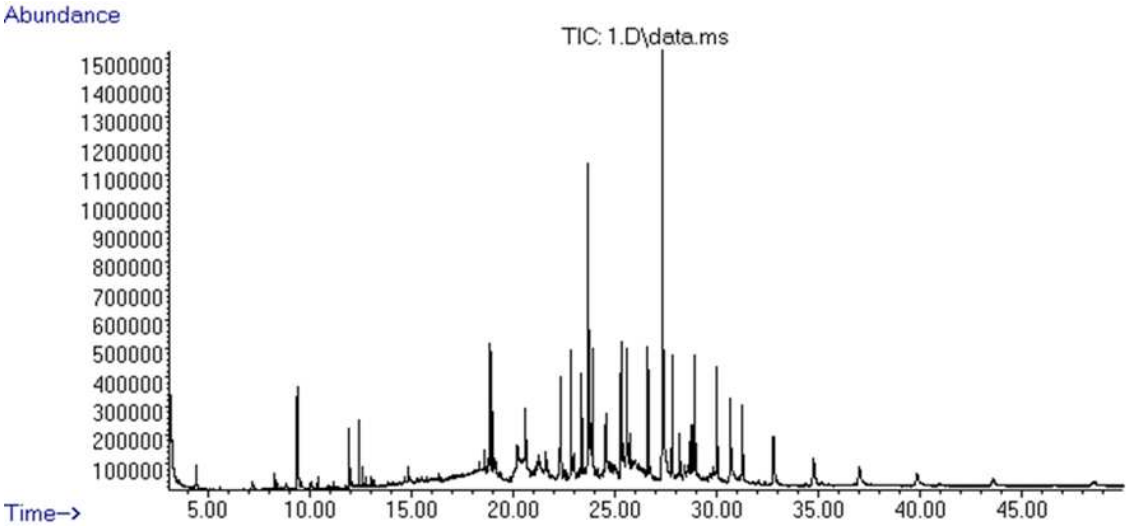

Figure 1: GC-MS product distribution of the AZA sample that showed antioxidant activity in the TLC plate analysis

Table: 1: Compounds identified by the GC-MS from the AZA sample that had antioxidant activity

Library/ID	MW	RT	Peak height	Corr area	Corr % max	% of total
2-Hexanol, 5-methyl- (Aroma-Herbaceous)	116.2	8.299	30,520	819,615	3.4	0.54
Oxime-, methoxy-phenyl-_	151.2	9.32	299,536	8,837,459	36.9	5.819
Nickel, nitrosyl [(1,2,3,4,5-.eta.) -1,2,3,4,5-pentamethyl-2,4-cyclopentadien-1-yl]-	223.9	9.586	23,432	1,121,702	4.7	0.739
2-Pyrrolidinone, 1-methyl-	99.13	11.932	209,106	4,921,262	10.3	0.973
Acetophenone	120.1	12.418	70,644	1,357,525	5.7	0.894
1,2-Benzenediol, 3,5-bis(1,1-dimethylethyl)-	222.3	12.576	69,705	1,804,883	3.8	0.357
Benzenamine, 3,4-dimethyl-	121.17	12.757	38,237	990,231	2.1	0.196
Phenol, 2,5-bis(1,1-dimethylethyl)-	206.32	18.806	61,312	1,350,856	5.6	0.890
Butylated hydroxytoluene	220.35	18.870	466,536	10,493,086	21.9	2.074
Benzoic acid, 4-ethoxy-, ethyl ester	194.23	19.005	112,622	3,686,869	15.4	2.428
Pyridine, 4-(phenylmethyl)-	169.24	20.198	48,315	1,274,126	5.3	0.839
Methyl tetradecanoate (floral)	242.4	21.245	59,386	2,509,443	10.5	1.652
Methyl hexadecanoate	270.46	23.345	859,033	23,716,207	99.1	15.617
Methyl decanoate	186.29	23.345	362,059	7,331,195	15.3	1.449
Hexadecanoic acid	256.43	23.685	1,076,883	27,587,042	57.6	5.452
Methyl octadecanoate	298.50	25.258	874,734	23,941,074	100.0	15.765
Hexadecanamide	255.44	25.756	159,159	5,866,074	12.3	1.159
9-Octadecenamide, (Z)-	281.48	27.34	1,484,338	47,871,541	100.0	9.461
Phenol, 2,2'-methylenebis[6-(1,1-dimethylethyl)-4-ethyl-	368.55	28.616	33,441	935,093	3.9	0.616

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From the 21 compounds identified by GC-MS, four compounds were identified to have the most antioxidant activity they are Benzenamine, 3,4-dimethyl-, Phenol, 2,5-bis(1,1-dimethylethyl)-, Butylated hydroxytoluene, Methyl decanoate, Hexadecanoic acid / Palmitic acid and Phenol, 2,2'-methylenebis[6-(1,1-dimethylethyl)-4-ethyl-. it can be noted that Butylated hydroxytoluene may be the compound largely responsible for the antimicrobial activity exhibited by the extract. The compounds were found to be rich in long chain hydrocarbons as listed on table

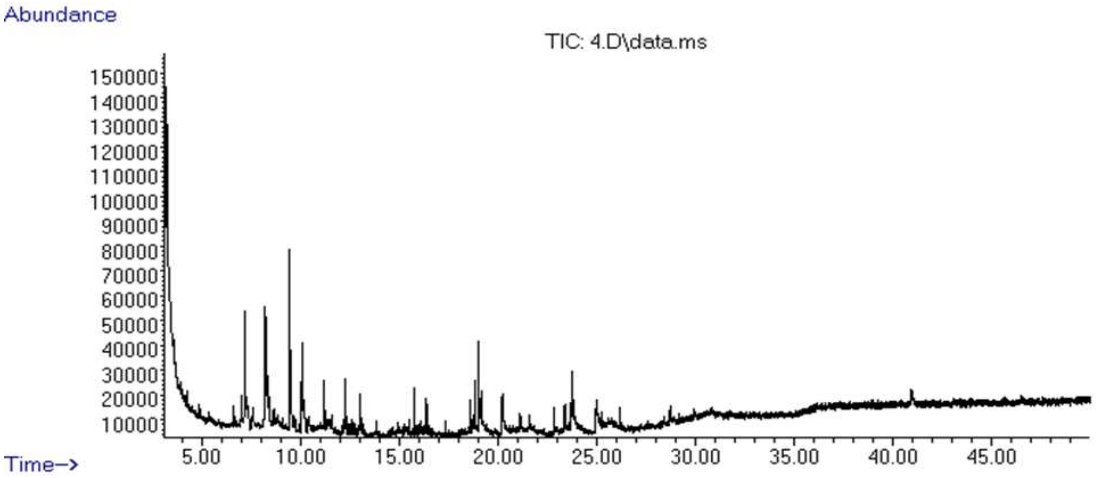

Figure 2: GC-MS product distribution of the *AZA* sample that showed antimicrobial activity in the TLC analysis

Table 2: List of compounds generated by the GC-MS

Library/ID	MW	RT	Peak height	Corr area	Corr % max	% of total
4-Methyl-2-hexanol	116	8.1999	43521	1108747	59.86	9.201
Heptanol<2->	116.2	8.2759	20946	506327	27.33	4.202
2-Butenal, 3-methyl-	84	9.4167	70549	1473593	79.55	12.23
1-Hexene, 3,5,5-trimethyl-	126	9.4693	31947	1001989	53.83	4.185
1-Dodecanone, 1-cyclopropyl-		9.4634	30479	994324	53.68	8.252
Pentane, 2,3,4-trimethyl-	224	10.0368	19150	365085	19.71	3.030
Sulphurous acid, cyclohexylmethyl undecyl ester	114	10.0953	31381	735388	39.70	6.103
Cyclotetrasiloxane, octamethyl-	332	11.1775	18993	466242	25.17	3.869
Hexacosane	297	12.2246	21140	439653	23.74	3.649
Heptadecane (C17)		12.991	15206	304334	16.43	2.526
Hexadecane, 2,6,10,14-tetramethyl-	282.6	16.3664	14529	336404	18.16	2.792
Tetradecane (C14)	198	17.3492	110287	1861272	100.0	7.775
Methyl p-tert-butylphenyl acetate	206	18.8	19500	366576	19.79	3.042
Anisyl propanoate	180	19.0106	35041	1004803	54.25	8.339
Docosane	310.6	19.1335	13903	352438	19.03	2.925
Hydrazinecarboxamide, N,N-diphenyl-	227.27	20.2098	15252	605010	32.66	5.021
Decanoic acid	172	23.6613	28704	1082150	58.14	4.520
9-Acridinamine	194	24.9659	11485	658024	35.52	5.461

Eicosane (C20) Alkane	282.55	28.3881	23119	821454	44.13	3.431
Hexacosane	366.7	29.1603	23580	619178	33.27	2.586
Untriacontane	436.8	29.9208	33283	932324	50.09	3.894
Octacosane	394.8	30.7749	19307	927876	49.85	3.876
Tetracosane	338	31.7752	30327	1604838	86.22	6.704

(MW- Molecular Weight, RT- Retention Time)

The GC-MS analysis of sample that exhibited antimicrobial activity yielded 24 compounds (Table 2). These compounds were analysed from literature for their previous records on antimicrobial activity. It was noted that out of the 24 compounds isolated 2-Furancarboxaldehyde $C_5H_4O_2$, 4-Methyl-2-hexanol, Sulphurous acid, cyclohexylmethyl undecyl ester, Decanoic acid (capric acid), Hydrazinecarboxamide, and N,N-diphenyl Aldehyde compounds had diverse Antimicrobial and Antifungal activity. Due to their antimicrobial, an antifungal and antiseptic effect, have an extensive ethnobotanical use in many areas (Shankar *et al.*, 2005).

5 CONCLUSION

Various compounds from the extract may be contributing to the overall antioxidant and antimicrobial effects in AZA. Some of the compounds identified to be responsible are Benzenamine, 3,4-dimethyl-, Phenol, 2,5-bis(1,1-dimethylethyl)-, Butylated hydroxytoluene, Methyl decanoate, Hexadecanoic acid / Palmitic acid and Phenol, 2,2'-methylenebis[6-(1,1-dimethylethyl)-4-ethyl- while the compounds identified to be responsible for antimicrobial effects are 4-Methyl-2-hexanol, Sulphurous acid, cyclohexylmethyl undecyl ester, Decanoic acid (capric acid) and Hydrazinecarboxamide, N,N-diphenyl. It is therefore worth to note that AZA has antioxidant and antimicrobial properties that can qualify it to be a natural preservative.

6 ACKNOWLEDGEMENT

The authors thank the National Council of Science and Technology for funding this research. Jomo Kenyatta University for Agriculture and Technology for facilitating the research.

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