PHYTOCHEMICAL EVALUATION OF "HERB 25" A HERBAL ANTI-MALARIAL PRODUCT BY GAS CHROMATOGRAPHIC-MASS SPECTROMETRIC ANALYSIS (GC-MS).

By

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'Herb 25' is an anti-malarial herbal product containing dried powdered leaves of *Azadirachta indica* (Neem), as the major constituent. The product is packaged in tea bags and orally taken in hot water as an infusion for prevention and treatment of malaria. It represents the first-herbal drug to be registered from any Nigerian University by the Nigerian food and drug regulatory agency, NAFDAC. This study was carried out to evaluate the composition of the aqueous and the ethanolic extracts of 'Herb-25 using the Gas Chromatographic-Mass Spectrometric (GC-MS) analysis. The aqueous and ethanolic extracts presented 9 and 14 identified phytochemical components respectively. The aqueous extract of the "Herb 25" contained more of terpinoids and fatty acids than the ethanolic extract . Major terpinoid in the aqueous extract was 7-Oxabicyclo (4.1.0) heptanes, 1-methyl-4-(2-methyloxiranyl)- (40.98%) (Retention time 29.808) whilst in the ethanolic extract Naphtho(2,1-b)furan-2,8-dione,decahydro-3a,6,6,9a-tetramethyl-,(3aS,5aS,9aS,9Br)- (23.11%) (Retention time 29.839). Major fatty acid in the aqueous extract was n-Hexadecanoic acid (28.21%) (Retention time 28.235) and Oleic Acid (17.02%) (Retention time 29.413), whilst in the ethanolic extract was n-Hexadecanoic acid (32.05%)(Retention time 28.309). "Herb 25" is therefore, a combined therapy based on the numerous compounds identified.

Key words: 'Herb 25', Malaria, GC-MS, Azadirachta indica

INTRODUCTION

"Herb 25" is an anti-malarial herbal product containing dried leaves of *Azadirachta indica* (Neem tree), as the major constituent. It is packaged as a tea bag and it represents the first-herbal drug to be registered from any Nigerian University by the Nigerian National Agency for the regulation of Food and Drug Administration and Control (NAFDAC).

Malaria affects millions of people around the globe. It devastates lives and affects communities and their economies (UNICEF, 2012). Malaria control is one of the key Millennium Development Goals.

The high malaria morbidity cases and the mortality rates caused by malaria are issues of serious concern. Although malaria is a grave global problem, its epidemic in Sub-Saharan Africa (SSA) is more severe and causes much higher damage. Sub-Saharan Africa is home to the most efficient vector, the *Anopheles*

gambiae and the prevalent form of the parasite, *Plasmodium falciparium* has developed resistance to conventional drug therapy (Anonymous, 2005).

According to the WHO (2011), there were 216 million cases of malaria and an estimated 655 000 deaths in 2010. Malaria mortality rates have fallen by more than 25% globally since the year 2000 and by 33% in the WHO African Region. Most deaths occur among children living in Africa where a child dies every thirty seconds of malaria and it accounts for more than a million child deaths yearly (UN Development Group 2013). Twenty-five million pregnant women are currently at risk for malaria, and, according to the WHO (2009), malaria accounts for over 10,000 maternal deaths.

It is estimated that approximately US$ 3 billion is required annually to effectively

prevent and control malaria worldwide (WHO, 2005). Malaria presents a huge burden to Africa and continues to cripple the economic development of the continent.

In Nigeria, the disease is responsible for 60% of outpatient visits to health facilities, 30% of childhood deaths, 25% of deaths under one year and 11% of maternal deaths (WHO, 2010a). In financial terms, the disease is estimated to cost the country about 132 billion naira (US$ 862.4 million) every year, taking into account treatment and prevention cost, and loss of working hours (WHO, 2010b). Poor people in Nigeria face several health issues as they lack basic health amenities and competent medical practitioners (Ucha, 2010).

The high cost of anti-malarial drugs and cases of resistance to orthodox medicine makes it necessary for the development of alternative drugs that are efficient and cost effective in the treatment of malaria.

Although Traditional Medicine (TM) is widely used to treat malaria and is often more available and affordable than orthodox medicine, there is little clinical data on the safety and efficacy of such drugs (Balogun *et al.*, 2009 and Razak *et al.*, 2011)

As a contribution to the process of understanding the natural constituents of alternative drug therapies, this study was conducted to evaluate the phytochemical constituents that may be the bioactive ingredients in "Herb 25", which could be responsible for its action in the prevention and treatment of malaria.

MATERIALS AND METHODS

Extraction

Twenty kilograms (20kg) of "Herb 25" was extracted by maceration with 70% ethanol and distilled water for 24 hours. The extracts obtained were evaporated to dryness on a water bath. The dried extracts were allowed to cool to room temperature in a dessicator (Trease and Evans, 1989).

Isolation and characterization of chemical compounds by GC-MS analysis

Fractions of the aqueous and the ethanolic extract of "Herb 25" were subjected to Gas Chromatogram- Mass spectrometry (GC-MS) analysison a GC-MS Clarus 500 Perkin Elmer system comprising a AOC- 20i autosampler and gas chromatograph. This was interfaced to a mass spectrometer (GC-MS) employing the following conditions: column Elite-1 fused silica capillary column (30mm x 0.25mm ID x 1 µMdf, composed of 100 % Dimethyl poly siloxane), operating in electron impact mode at 70 eV; helium

(99. 999%) was used as carrier gas at a constant flow of 1ml/min. An injection volume of 0.5 µl was employed (split ratio of 10:1); injector temperature 250°C. The oven temperature was programmed from 110°C (isothermal for 2 min), with an increase of 10°C/min, to 200°C, then 5 °C / min to 280°C, ending with a 9 min isothermal at 280°C. Mass spectra were taken at 70 eV; a scan interval of 0.5 seconds and fragments from 40 to 550 Da. Interpretation on mass spectra of GC-MS was conducted using the database of National Institute of Standards and Technology (NIST). The mass spectrum of the unknown components were compared with that of the known components stored in the NIST library. The name, molecular weight and structure of the components of the test materials were ascertained.

Data Analysis

Cluster analysis was used to determine the level of similarity between the aqueous and ethanolic extracts of "Herb 25" using paleontological statistics (PAST) version 18 (Hammer *et al.*, 2008).

RESULTS

The results obtained from GC-MS analysis showed that the aqueous extract of "Herb 25" is represented by 9 chemical components which included 7-Oxabicyclo (4.1.0) heptanes, 1-methyl-4-(2-methyloxiranyl) (40.98%); n-Hexadecanoic acid (22.81%), Oleic Acid (17.02%); 2-cyclopenten-1-one,2-hydroxy- (2.46%); 4H-pyran-4-one,2,3-dihydro-3,5-dihydroxy-6-methyl(6.24%); 2-methoxy-4-vinylphenol (2.28%); Lilac aldehyde B (4.10%); 2-cyclohexen-1-one,4-hydroxy-3-methyl-6-(1-methylethyl)-,trans (1.12%) and 10-methyl-8-tetradecen-1-olacetate (2.29%)(Table 1).

The ethanolic extract was represented by 14 chemical components which included: n-Hexadecanoic acid (32.05%), Naphtho(2,1-b)furan-2,8-dione,decahydro-3a,6,6,9a-tetramethyl ,(3aS,5aS,9aS,9Br)-(23.11%), Cis-9-Hexadecenal (22.12%), Benzoic acid, 2-hydroxyl-, methyl ester (0.91%), Cyclohexanol, 5-methyl-2-(1-methyl)-, (1.alpha2.beta, 5.alpha.)- (+/-) - (2.37%), Benzoic acid, 2-hydroxyl,methyl ester (1.63%), Lilac aldehyde C (1.61%), Phenol,3,5-bis(1,1-dimethylethyl)- (0.64%), Lilac alcohol B (4.42%), Tetradecanoic acid(1.44%), 2-pentadecanone,6,10,14-trimethyl-

(0.74%), Hexadecanoic acid, methyl ester (1.77%), 9-octadecenoic acid(Z)-,methyl ester (2.29%), 2H-Pyran-2-one,tetrahydro-6-tridecyl- (4.94%)(Table 2).

A Cluster Analysis between the two extracts show that they had a Euclidean Similarity index of -0.98 (Fig.1).

DISCUSSION

The aqueous extract of the "Herb 25" accumulates more of terpinoids and fatty acids than the ethanolic. 7-Oxabicyclo (4.1.0) heptanes, 1-methyl-4-(2-methyloxiranyl)- (40.98%) is the major terpinoid in the aqueous extract and is also known as limonene diepoxide (a monoterpenes). Limonene has been reported to arrest the malaria parasite development and inhibits isoprenylation of proteins in *Plasmodium falciparium* (Moura *et al.*, 2001).

Naphtho(2,1-b)furan-2,8-dione,decahydro-3a,6,6,9a-tetramethyl-,(3aS,5aS,9aS,9Br)- (23.11%) is the major terpinoid in the ethanolic extract and it is a derivate of artemisinin. **Artemisinin** and its derivatives are a group of drugs that possess the most rapid action against *Plasmodium falciparium* malaria. Treatments containing an

artemisinin derivative (artemisinin-combination therapies, ACTs) are now standard treatment worldwide for *P. falciparum* malaria (Krishna *et al.*, 2004, Hsu, 2006, Benoit-Vical *et al.*, 2007, Li and Zhou, 2010, Crespo-Ortiz1 and Wei, 2011).

Oleic acid is one of the major fatty acids of the aqueous extract of 'Herb 25' and it has been reported that polyunsaturated Another bioactive compound found in the ethanol extracts was Hexadecanoic acid, a methyl ester. The methyl esters of the fatty acids were reported to be as potent as the free acids in killing the parasite (Kumaratilake *et al.*, 1992). The authors also pointed out that these fatty acids were not toxic to either normal red blood cells (RBC) or parasitized red blood cells (PRBC) cells and did not induce hemolysis (Kumaratilake *et al.*, 1992).

Another bioactive component found in the ethanolic extract was Cyclohexanol, 5-methyl-2- (1-methyl)-, (1.alpha.,2.beta.,5.alpha)-(+/-)- (2.37%), **(Menthol)**, also known as peppermint camphor, is a monoterpene (10 carbons); topical pain reliever and antipuretic (relieves itching) obtained from mint oils (mainly peppermint) or made synthetically from coal tar. Goulart *et al.*, (2004) reported that the constituent fatty acids (oleic acid included) showed marked growth inhibition of *P. falciparum* (Kumaratilake *et al.*, 1992). These authors also noted that the monodiglycerides of oleic acid did not cause any inhibition of the parasite, thus implying that the free fatty acid was critical for the anti-plasmodial activity. Krugliak *et al.* (1995) reported that oleic acid has more inhibitory properties than linoleic and linolenic acids.

terpenes (like menthol, carvone and thujone) or phenols (like eugenol and myristicin) work against parasitic infection, by either causing paralysis of the worms or disrupting the parasitic life cycle.

CONCLUSION

The aqueous and ethanolic extracts of "Herb 25" contain significant amounts of phytochemicals with known antimalarial properties, serving as inhibitors or scavengers of malaria parasites. "Herb 25" is therefore, a combined therapy based on the many compounds identified. Combination therapy appears to offer a highly effective treatment for malaria and, at the individual level, may reduce the likelihood of resistance of the parasites.

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Table 1. Summary of GC-MS results for peaks in aqueous extract of "Herb 25" spectrum

Peak No	C (%)	Compound	MF	M W	RT (sec)	Mass peak	Fragmentation Peaks
1	2.46	2-cyclopenten-1-one,2-hydroxy-	$C_5H_6O_2$	98	8.308	19	40(15%), 41(20%), 55(60%) 69(30%), 71(15%), 98(100%)
2	6.24	4H-pyran-4-one,2,3-dihydro-3,5-dihydroxy-6-methyl	$C_6H_8O_4$	144	15.710	27	38(1%), 43(100%), 58(4%), 72(20%), 85(1%), 101(32%), 115(1%), 130(1%), 144(35%)
3	2.28	2-Methoxy-4-vinylphenol	$C_9H_{10}O_2$	150	20.405	35	14(2%), 15(24%), 39(22%), 51(30%), 63(15%), 77(70%), 89(10%), 107(65%), 118(1%), 135((100%), 150(80%)
4	4.10	Lilac aldehyde B	$C_{10}H_{16}O_2$	168	22.022	45	41(68%), 55(100%), 67(40%), 71(40%), 93(32%), 111(30%) 125(5%), 153(6%), 154(1%)
5	1.12	2-cyclohexen-1-one,4-hydroxy-3-methyl-6-(1-methylethyl)-,trans	$C_{10}H_{16}O_2$	168	23.152	40	31(1%), 41(35%), 42(15%), 69(30%), 97(10%), 98(100%), 124(50%), 126(60%), 53(5%), 168(20%)
6	2.99	10-methyl-8-tetradecen-1-ol acetate	$C_{17}H_{32}O_2$	268	26.423	70	41(40%), 43(100%), 67(30%), 81(30%), 97(20%), 111(50%), 125(20%), 151(5%), 165(4%), 170(4%), 211(4%)
7	22.81	n-Hexadecanoic acid	$C_{16}H_{32}O_2$	256	28.235	103	27(18%), 41(75%), 43(100%), 60(90%), 73(98%), 85(20%), 115(15%), 129(30%), 143(4%), 157(10%), 171(10%), 185(8%), 213(20%), 227(4%), 256(40%)
8	17.02	Oleic Acid	$C_{10}H_{16}O_2$	282	29.413	109	27(15%), 41(75%), 55(100%), 69(75%), 83(60%), 97(45%), 98(30%), 125(10%), 151(5%), 264(15%)
9	40.98	7-Oxabicyclo (4.1.0) heptane, 1-methyl-4-(2-methyloxiranyl)-	$C_{10}H_{16}O_2$	168	29.808	182	27(18%), 41(30%), 43(100%), 67(30%), 79(28%), 93(28%), 107(25%), 123(10%), 137(8%)

Table 2. Summary of GC-MS results for peaks in ethanolic extract of "Herb 25" spectrum

Peak No	C (%)	COMPOUND NAME	MF	MW	RT (sec)	Mass peak	Fragmentation Peaks
1	0.91	Benzoic acid, 2-hydroxyl,methyl ester	$C_8H_8O_3$	152	13.215	25	37(5%), 39(15%), 53(10%), 61(12%), 81(2%), 92(72%), 120(100%), 137(1%), 152(50%), 152(4%)
2	2.37	Cyclohexanol, 5-methyl-2- (1-methylethyl)- ,(1.alpha.,2.beta.,5.alpha)-(+/-)-	$C_{10}H_{20}O$	156	16.061	49	27(12%), 41(55%), 55(55%), 67(40%), 71(100%), 95(80%), 109(20%), 123(32%), 138(20%)
3	1.63	Benzoic acid, 2-hydroxyl,methyl ester	$C_8H_8O_3$	152	16.777	49	37(5%), 39(20%), 53(10%), 65(22%), 92(72%), 109(1%), 120(100%), 137(1%), 152(50%), 154(2%)
4	1.61	Lilac aldehyde C	$C_{10}H_{16}O_2$	168	22.052	54	41(58%), 55(100%), 67(32%), 71(35%), 93(40%), 111(22%), 125(5%), 153(10%), 154(1%)
5	0.64	Phenol,3,5-bis(1,1-dimethylethyl)-	$C_{14}H_{22}O$	206	24.230	85	27(2%), 41(20%), 55(5%), 74(5%), 91(8%), 107(5%), 115(4%), 135(5%), 147(2%), 163(4%), 191(100%), 206(25%)
6	4.42	Lilac alcohol B	$C_{10}H_{18}O_2$	170	26.425	101	41(62%), 55(100%), 67(45%), 71(28%), 93(72%), 111(71%), 112(5%), 137(2%), 143(1%), 155(20%), 170(1%)
7	1.44	Tetradecanoic acid	$C_{14}H_{28}O_2$	228	26.740	99	27(25%), 41(92%), 60(92%), 73(100%), 87(22%), 98(14%), 115(15%), 129(50%), 228(10%)
8	0.74	2-pentadecanone,6,10,14-trimethyl-	$C_{18}H_{36}O$	268	27.237	81	27(8%), 41(36%), 43(100%), 58(90%), 71(45%), 85(22%), 109(!5%), 124(10%), 140(2%), 165(4%), 179(4%), 120(4%), 250(10%),
9	1.77	Hexadecanoic acid, methyl ester	$C_{17}H_{34}O_2$	270	27.820	104	27(5%), 41(32%), 57(15%), 74(100%), 87(70%), 101(8%), 115(4%), 129(8%), 143(20%), 199(5%), 213(2%), 227(15%), 239(5%), 270(15%)
10	32.05	n-Hexadecanoic acid	$C_{16}H_{32}O_2$	256	28.309	167	27(8%), 41(75%), 43(100%), 60(90%), 78(98%), 85(25%), 98(18%), 115(5%), 129(4%), 143(5%), 157(10%), 17(8%), 185(8%), 213(18%), 227(5%), 256(42%)
11	2.29	9-octadecenoic acid(Z)-,methyl ester	$C_{19}H_{36}O_2$	296	29.027	145	27(4%), 41(58%), 55(100%), 69(60%), 74(4%), 97(45%), 123(15%), 137(10%), 166(5%), 180(10%), 222(12%), 264(20%)
12	22.12	Cis-9-Hexadecenal	$C_{16}H_{30}O$	238	29.460	193	27(8%), 41(72%), 55(100%), 69(60%), 81(48%), 95(35%), 149(5%), 220(4%)
13	23.11	Naphtho(2,1-b)furan-2,8-dione,decahydro-3a,6,6,9a-tetramethyl-,(3aS,5aS,9aS,9Br)-	$C_{16}H_{24}O_3$	264	29.839	225	41(38%), 43(50%), 68(25%), 83(48%), 95(45%), 107(40%), 121(22%), 135(60%), 150(62%), 165(18%), 179(8%), 191(10%), 221(15%), 231(10%), 249(85), 264(100%),
14	4.94	2H-Pyran-2-one,tetrahydro-6-tridecyl-	$C_{18}H_{34}O_2$	282	30.647	171	27(5%), 41(45%), 55(58%), 69(35), 70(42%), 97(30%), 99(100%), 114(22%), 134(55), 151(4%), 220(10%), 264(25%), 282(4%)

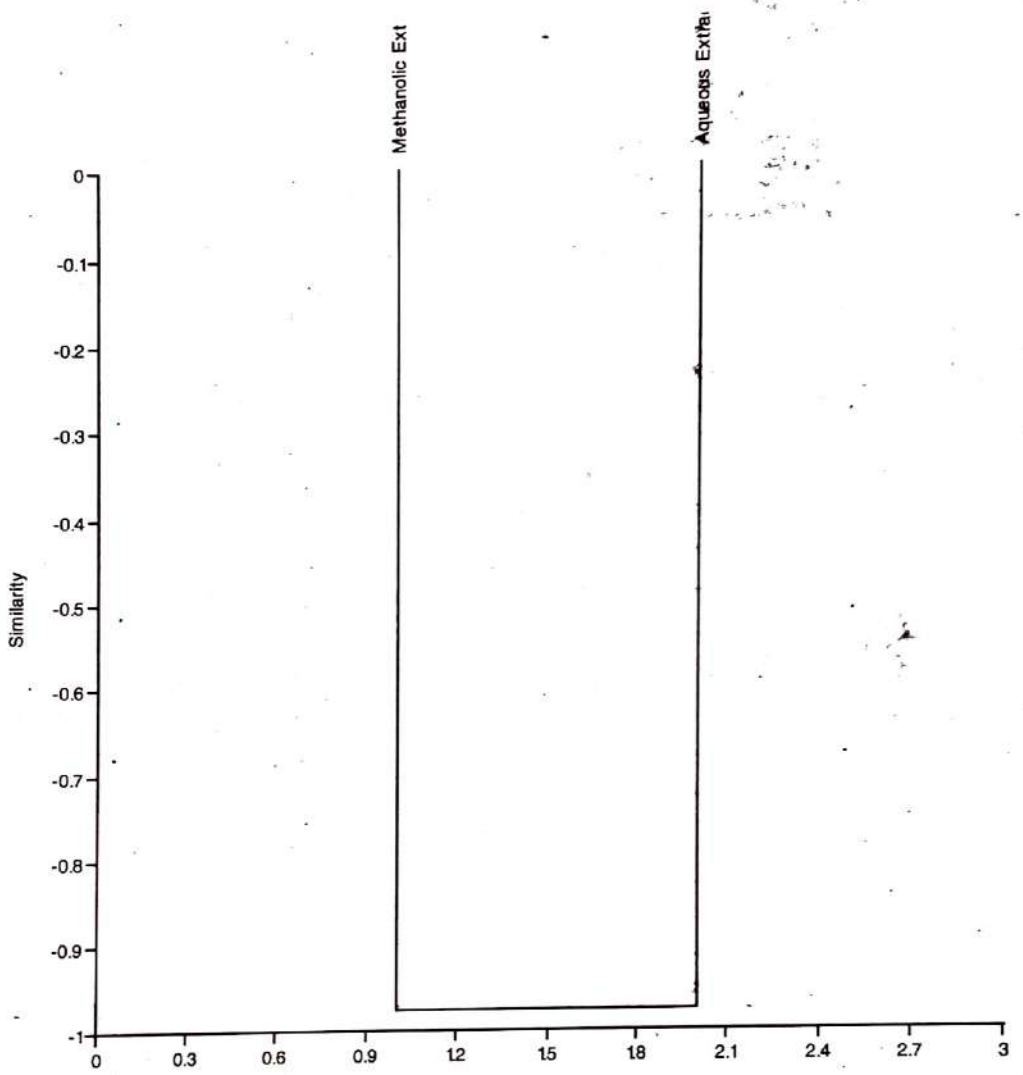

Fig 1: Cluster Analysis showing Euclidean Similarity Index for Aqueous and Ethanolic Extracts of "Herb 25"