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Identification of Volatile Compounds in Sweetened *Hibiscus Sabdariffa* Drink

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Abstract:
Background: *Hibiscus sabdariffa drink ("zobo") is a sweetened thirst-quenching beverage with high nutritional value and health benefits. The drink is made from the hot water extract of H. sabdariffa calyx, and consumed popularly in Nigeria, and other West African countries. Although much is known about the nutritional profile of "zobo," and volatile constituents of H. sabdariffa parts, there is paucity of information on volatile compounds in the drink.*
Materials and Methods*: The drink was prepared from hot water extract of dried red H. sabdariffa calyx, mixed with freshly prepared juice of pineapple, watermelon, carrot, cucumber, beetroot, ginger, cloves, and dates. The essential oil from the sample was gotten using hydrodistillation. Fatty acids extraction was done with Soxhlet apparatus. The extracted fatty acids were converted to fatty acid methyl extract and analysed using gas chromatography coupled to mass spectrometer.*
Results*: Thirty-one metabolites: ketones (32.917%), sesquiterpenes (26.57%), aldehyde (13.98%), monounsaturated fatty acids (9.763%), saturated fatty acids (9.37%), steroids (4.35%), acids (2.32%), and alcohol (0.31%) were identified. The bioactivities of the detected compounds were also identified.*
Conclusion*: H. sabdariffa drink contains bioactive compounds with potential medicinal value for pharmaceutical importance. The different compounds identified share similar biological functions. The synergism of these principles validates the medicinal claims of "zobo."*
Key Word*: Hibiscus sabdariffa; Volatile compounds; Gas chromatography mass spectrometry; Fatty acids; "Zobo"*

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I. Introduction

Many plants are utilized in preparing food drinks in Nigeria, one of which is *Hibiscus sabdariffa* [1,2]. Other names for *H. sabdariffa* from different parts of the world are "'Roselle' in Australia, 'Tengamoran' in Asam, 'Gongura' in Hindi, 'Krajeab' in Thailand, 'Bissap' in Senegal, Guinea Bissau, Mali, Burkina Faso, Ghana, Benin, Niger, Congo and France. 'Wonjo' in Gambia, 'Torosh' in Iran, 'Sorrel' in Carribean, 'Karkade' in Egypt, 'Asam Paya' in Malaysia, 'Luoshen Hua' in Chinese, 'Lamanda' in Zambia and 'Zobo' in Nigeria" [3]. According to the authors, other names for the plant include "Roses of anthea, African mallow, Roselle, Rose mallow, Indian sorrel, Flore de Jamaica and Jamaica tea."

An extract of dry calyces of this plant with hot water produces a popular thirst quenching "zobo" drink [4]. This non-alcoholic beverage is red in colour, with a fruity punch taste [5]. The taste is attributed to addition of fruits [6]. The nutritional value of *H. sabdariffa,* and "zobo" has severally been documented [7–10]. Bioactive components of *H. sabdariffa,* and other hibiscus species were also reported [11,12]. According to Inikpi et al. [13], *H. sabdariffa* has "antihypertensive, hepatoprotective, antihyperlipidemic, anticancer, antidiabetic, cytotoxicity, antibacterial, immunomodulatory, antinociceptive, anti-inflammatory, antidiarrheal and antioxidant activities." The anti-hypertensive properties of *H. sabdariffa* were also reported [3,14].

In addition to *H. sabdariffa,* fruits, spices, sweeteners, and other additives are usually added to improve the nutritional value, flavour, and taste of "zobo" beverage [1,6]. Fruits, and spices are food sources of volatile constituents. These compounds have been identified in the leaves of Hibiscus specie (*Hibiscus surattensis*) [2]. Different parts of *H. sabdariffa* have also shown rich distribution of volatile compounds [13]. These components contribute to the sensory property, and acceptability of food [15]. Mixture of several "low molecular-weight volatile compounds" impact flavour, and aroma to fruits [16,17] and food [18]. Essential oils found in spices have

antioxidant, and kidney protecting properties [19]. According to Bonilla and Sobral [20], essential oils in plants are important because of their high antimicrobial, and antioxidant properties.

Characterization of fatty acids present in food is important for experimental, medical, and industrial purposes [21]. Fatty acids have been identified in the leaves of Hibiscus specie (*Hibiscus surattensis*) [2]. Different parts of *H. sabdariffa* have also shown rich distribution of fatty acids [13]. "Zobo" drink is popularly taken traditionally for reduction of total cholesterol, and management of high blood pressure [5,22]. Meanwhile, the onset and progression of hypertension, and various cardiovascular related diseases can be tied to excessive metabolism, and subsequent elevated serum fatty acid levels [23]. On the other hand, oleic acid, a non-essential monounsaturated fatty acid is cardio-protective, and is involved in metabolism of cholesterol [24]. Also, α-linolenic acid, an essential polyunsaturated fatty acid (PUFA) lowers blood pressure, and its cardio-protective mechanism has been documented [25]. Other authors have recorded the health benefits of PUFA [26–30].

Although, much is known about the nutritive value of "zobo" drink, there is sparse documentation on its volatile constituents. Considering the role fatty acids, and volatile compounds play in life, it is imperative to investigate their levels in "zobo." This study is aimed at bridging the divide, by characterizing the metabolites in sweetened "zobo" drink using GC-MS. Pineapple and dried dates were used as a natural sweetener.

II. Materials and Methods

Experimental material collection: Dried calyces of red *H. sabdariffa*, pineapple, watermelon, carrot, cucumber, beetroot, ginger, cloves, and dates were bought from Tomato market, Lafia, Nasarawa State-Northern Nigeria. They were immediately transported to the Department of Biochemistry, Federal University of Lafia, where laboratory experiments took place. Preparation of samples started immediately raw materials arrived at the laboratory.

"Zobo" drink production: To produce sweetened "zobo" drink, 400 g of *H. sabdariffa* calyces, 400 g cloves, and 200 g ginger were washed, and boiled in an aluminium pot for 15 minutes before filtering. Exactly 800 g pineapple, 500 g watermelon (rind/pulp), 500 g cucumber, 400 g dates, 300 g carrot, 120 g beetroot were separately washed, chopped, blended, and filtered. The filtered fruit blend was then mixed with the extract of boiled *H. sabdariffa* calyces, ginger, and cloves to give "zobo" drink. This beverage was packed in airtight bottles, and refrigerated at 4 ^{o}C for analyses.

Extraction of oils: Exactly 350 g of "zobo" drink was hydrodistilled for 3 hours with a Clevenger-type apparatus to get essential oil from the sample. The distilled oils were collected, and stored in clean bottles at 4 ^{o}C for analyses.

Extraction of fatty acids: Fatty acids were extracted with Soxhlet apparatus 1000 ml Pyrex extractor. The solvent used was hexane. The extraction conditions were 8 hr at 80 °C. The extracts were then evaporated with a rotary vacuum evaporator.

Extraction of Fatty Acid Methyl Ester for GC-MS Analysis: Precisely 50 mg extracted oil, and fatty acids were saponified at 95 ^{o}C for 5 mins using 3.4 ml of 0.5 M KOH in dry methanol. Approximately 0.7 M HCl was used to neutralize the mixture. Exactly 3 ml of 14 % boron trifluoride in methanol was then added to the mixture. To achieve complete methylation, the mixture was heated at 90 ^{o}C for 4 mins. Redistilled n-hexane was used three times to extract fatty acid methyl esters (FAME) from this mixture. Concentrated extracted FAME (1 ml) was used for GC analysis; with 1 µL injected into the injection port of same instrument.

GCMS conditions for analyses of Fatty Acid Methyl Esters (FAME): GC-MS analyses of FAME was carried out according to manufacturer's instruction. The gas chromatography was from Agilent USA. It was hypherated to a mass spectrophotometer model 5975C. The spectrophotometer has triple axis detector with 10 µL syringe auto injector. The carrier gas was helium. Chromatographic separation was done on capillary column treated with phenyl methyl silox. The column specifications are: length (30 m), internal diameter (0.2 µm), and thickness (250 µm). Other GC-MS conditions were interface temperature (300 ^{o}C), ion source temperature (EI) (250 ^{o}C), out time (1.8 mm), pressure (16.2 psia), 1µl injector in Split mode (ratio 1:50), injection temperature (300 ^{o}C). Temperature of the column began at 35 ^{o}C for 5 mins. It was then increased to 150 ^{o}C at 4 ^{o}C/min. The temperature was again increased to 250 ^{o}C at 20 ^{o}C/min for 5 mins. The total elution took place for 47.5 mins. This system was controlled, and data acquired using MS solution software made available by the supplier. Machine model used was 7890A GC system, 5675C Inert MSD with triple-axis detector. Column was Agilent 19091-433HP-5Ms 5% phenyl methyl silox.

Identification of compounds: Compounds were identified by comparing the mass spectra gotten with that of standard mass spectra from NSIT library (NISTII).

III. Results

GC-chromatogram for sweetened "zobo" is presented in Figure no 1. Thirty-one components were identified from the number of peaks using GC-MS. The chemical constituents, their retention time (RT), molecular formula (MF), molecular weight (MW), peak area (%), classification, and activities are itemized

(Table no 1). The compounds were identified by matching their retention time, and percentage concentration with those of "National Institute Standard and Technology (NIST) library." The constituents identified include fatty acids, alcohol, ketones, aldehydes, acids, sesquiterpenes, and steroids. The first constituent identified was 2-furanmethanol, while the last was 2-[4-methyl-6-92,6,6-trimethylcyclohex-1-enyl)hexa-1,3,5-trienyl]cyclohex-1-en-1-carboxaldehyde with RT of 5.31, and 43.35 minutes respectively.

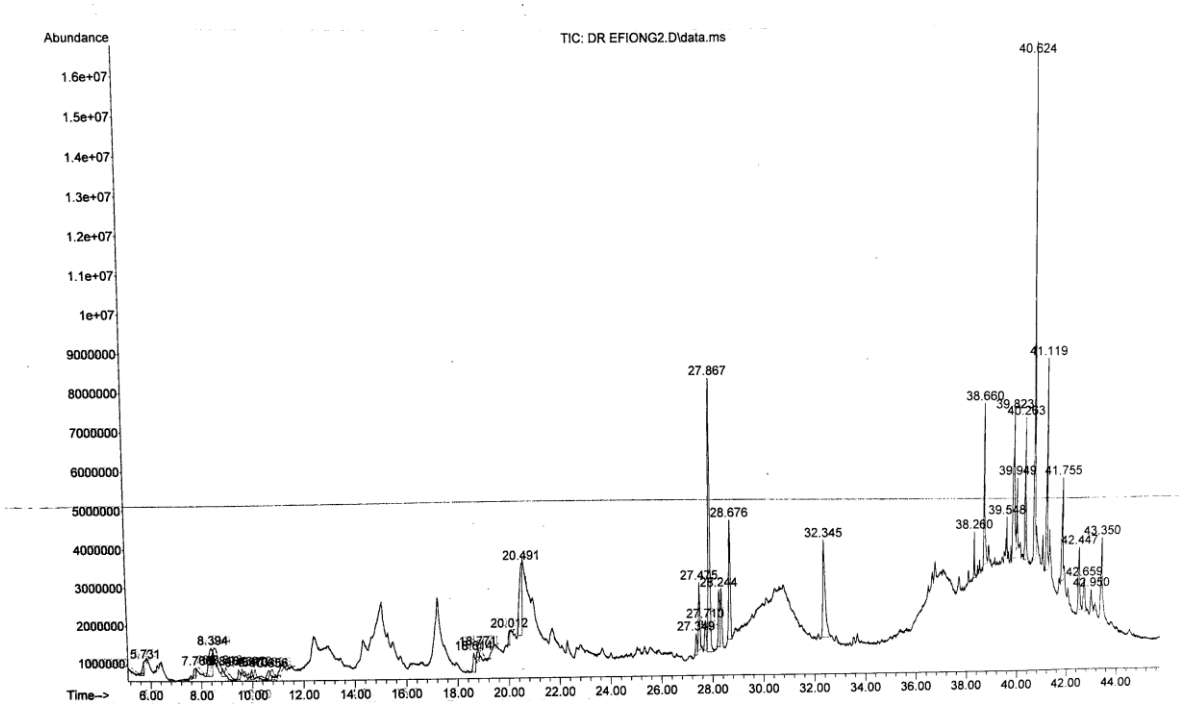

Figure no 1: GC-MS chromatogram for fatty acids and volatile principles in sweetened "zobo" drink

Table no 1: Retention time (RT), compounds, molecular formula (MF), molecular weight (MW), class, biological activity, and area % of constituents in sweetened "zobo" drink

S/No	RT (min)	Compound	MF	MW	Class	Biological activity	Area %
1	5.731	2-Furanmethanol	$C_5H_6O_2$	98.09	Alcohol	Antimicrobial activity [31,32]	0.731
2	7.766	9-Hexadecenoic acid (palmitoleic acid)	$C_{16}H_{30}O_2$	254.41	MUFA	Prevention/amelioration of insulin resistance and diabetes [33,34], anti-inflammatory [35], reduces LDL-C and increases HDL-C [36], prevents apoptosis of beta-cells [34]	0.613
3	8.394	Cyclopentanone, 2-methyl-	$C_6H_{10}O$	98.14	Ketone	Flavouring agent [16,32]	3.187
4	8.818	5-Octadecenal	$C_{18}H_{34}O$	266.46	Aldehyde	Flavouring agent [16,37], antimicrobial [32]	0.49
5	9.454	2-Furancarboxaldehyde,5-methyl-	$C_6H_6O_2$	110.11		Flavouring agent [16], antimicrobial [32]	0.86
6	9.973	2,4-Dihydroxy-2,5-dimethyl-3(2H)-furan-3-one	$C_6H_8O_4$	144.12	Ketone	Flavouring agent [16,32]	0.45
7	10.656	1,2-Cyclohexanedione	$C_6H_8O_2$	142.16		Flavouring agent [16,32]	1.13
8	18.614	Octadecanal,2-bromo	$C_{18}H_{36}O$	347.37	Aldehyde	Flavouring agent [16,37], antimicrobial [32]	1.16
9	18.771	Benzoic acid	C_6H_5COOH	122.12	Acid	Antibacterial [38], antioxidant properties of its derivatives [39]	0.77
10	20.012	Catechol	$C_6H_6O_2$	110.11		Flavouring agent, anti-cancer, antioxidant, and pesticide properties [40]	0.52
11	20.491	5-Hydroxymethylfurfural	$C_6H_6O_3$	126.11	Aldehyde	Anagestic, antipyretic anticoagulant, flavouring agent [16,37], antimicrobial [32]	5.81
12	27.349	7-epi-cis-sesquisabinene hydrate	$C_{15}H_{26}O$	222.36	Sesquiterpene	Anti-cancer [41,42]	0.91

13	27.475	Benzene,1-(1,5-dimethyl-4-hexnyl)-4-methyl	$C_{15}H_{22}$	202.33		No reported activity	2.82
14	27.710	β-copaene	$C_{15}H_{24}$	204.35		Antioxidative [41]	1.36
15	27.867	1,3-cyclohexadiene,5-(1,5-dimethyl-4-hexenyl)-2-methyl-,[S-(R*,S*)]-	$C_{15}H_{24}$	204.35		Antioxidant, antinociceptic, anti-inflammatory[42], anti-fertility, antiviral, anti-ulcer [43]	13.08
16	28.244	7-epi-trans-sesquisabinene hydrate	$C_{15}H_{26}O$	222.36		Anti-cancer [41,42]	2.72
17	28.676	Cedrene	$C_{15}H_{24}$	204.35		Antimicrobial [44]	5.68
18	32.345	2-Butanone,4-(4-hydroxy-3-methoxyphenyl)-(zingerone)	$C_{11}H_{14}O_3$	194.2	ketone	Antioxidant, anti-inflammatory, flavouring agent [16,32], anti-diabetic, hypolipidemic [45], and hepato-protective [46].	7.17
19	38.260	Hexadecanoic acid, methyl ester	$C_{17}H_{34}O_2$	270.45	SFA ester	Flavouring agent, antioxidant and hypocholesterolemic [32]. Anti-inflammatory, hepato-protective, anti-cancer, anti-androgenic, anti-acne, anti-eczemic, insectifuge, nematicide, antihistaminic, alpha reductase inhibitor, anti-arthritic, cardio-protective [47].	1.04
20	38.660	n-Hexadecanoic acid	$C_{16}H_{32}O_2$	256.42	SFA	Anti-oxidant, hypocholesterolemic, nematicide, hemolytic, anti-androgenic, pesticide, lubricant, antipsychotic, 5-alpha reductase inhibitor [48], anti-inflammatory, antibacterial [49], hypercholesterolemic, antiandrogenic [40], insulin promoter, anti-eczamatic, anti-mutagenic, anti-secratoric, anti-hipoxic [50].	4.76
21	39.548	Oleic acid (Octadecanoic acid)	$C_{18}H_{34}O_2$	282.5	MUFA	Antioxidant, anti-inflammatory, anti-hypertensive, prevents obesity and ulcerative colitis [24,51,52] [24], anti-atherogenic, and anti-thrombotic [53,54].	0.71
22	39.823	Oleic acid (Octadecanoic acid)	$C_{18}H_{34}O_2$	282.5			7.01
23	39.949	Oleic acid (Octadecanoic acid) acid	$C_{18}H_{34}O_2$	282.5			1.43
24	40.263	Cyclohexanone,2-(2-nitro-2-propenyl)-	C_9H_{13}	183.2	Ketone	Flavouring agent [16,32].	3.17
25	40.624	Gingerol	$C_{17}H_{26}O_4$	294.4		Flavouring agent, antioxidant, anti-inflammatory [16,32], analgesic, antipyretic, antibacterial, anti-cancer, sedative [55].	11.73
26	41.119	Gingerol	$C_{17}H_{26}O_4$	294.4			6.08
27	41.755	15-Hydroxypentadecanoic acid	$C_{15}H_{30}O_3$	258.4	SFA	Lactonization and study of ω-hydroxylase [56]	3.57
28	42.447	6β-Hydroxytestosterone	$C_{19}H_{28}O_3$	304.4	Steroid	"Contributed to vascular changes in angiotensin II-induced hypertension in male mice" [57].	2.65
29	42.659	11.α-Hydroxy-17.α-methyl testosterone	$C_{20}H_{30}O_3$	318.45		Anti-microbial, anti-inflammatory [58].	1.70
30	42.950	Methyl 6-methyl-5-(4 methylphenyl)sulfonyloxy-11-[(Z)-prop-1-enyl]-12,13-dioxatricyclo[7.3.1.01,6]tridecane-8-carboxylate	$C_{24}H_{32}O_7S$	464.6	Acid	No activity recorded	1.03
31	43.350	2-[4-methyl-6-92,6,6-trimethylcyclohex-1-enyl)hexa-1,3,5-trienyl]cyclohex-1-en-1-carboxaldehyde	$C_{23}H_{32}O$	324.5	Aldehyde	Flavouring agent [16,37], anti-inflammatory, antimicrobial [32]	5.66

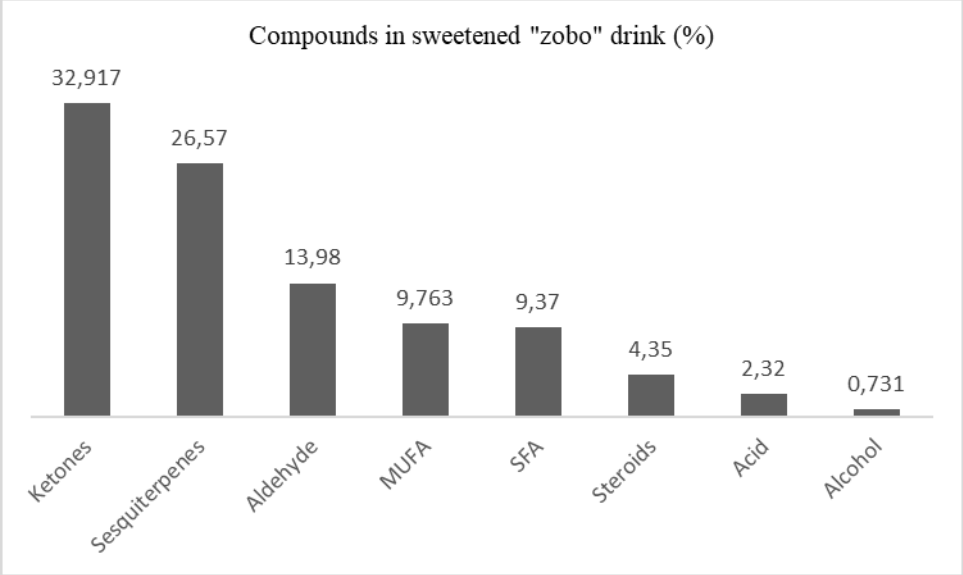

Figure no 2. Compounds in sweetened "zobo" drink

IV. Discussion

Ketone was the most abundant (32.917 %) compound detected in the sample. Seven ketones ranging from 0.45 to 11.73 % were observed. The ketones and their concentrations were: gingerol (11.73 %), 2-Butanone,4-(4-hydroxy-3-methoxyphenyl)- (zingerone) (7.17 %), gingerol (6.08 %), cyclopentanone, 2-methyl- (3.187 %), cyclohexanone,2-(2-nitro-2-propenyl)- (3.17 %), 1,2-cyclohexanedione (1.13 %), and 2,4-Dihydroxy-2,5-dimethyl-3(2H)-furan-3-one (0.45 %). In some other study by Da-Costa-Rocha et al. [14], five ketones were identified in *H. sabdariffa* calyx. Twelve ketones were equally detected in hot Mexican tea of *H. sabdariffa* [37]. Ketones were identified as flavouring agents by [16]. According to Alghamdi et al. [32], "ketones might be formed by beta-oxidation of fatty acids," thus, generating flavour constituents. The most abundant ketone- gingerol is a flavouring agent with antioxidant [16], anti-inflammatory [32], analgesic, antipyretic, antibacterial, anti-cancer, and sedative [55] properties. Zingerone, the second most abundant ketone has hypolipidemic, anti-diabetic, antioxidant [45], and hepato-protective properties [46].

The next most abundant volatile compound was sesquiterpenes (26.57 %). Six different sesquiterpenes identified were 1,3-cyclohexadiene,5-(1,5-dimethyl-4-hexenyl)-2-methyl-,[S-(R*,S*)]- (13.38 %), cedrene (5.68 %), benzene,1-(1,5-dimethyl-4-hexnyl)-4-methyl (2.82 %), 7-epi-trans-sesquisabinene hydrate (2.72, 0.91 %), and β-copaene (1.36 %). These compounds are important for production of steroid hormones, cholesterol, vitamin D, cosmetics, and pharmaceutical agents [47]. Anti-microbial property of sesquiterpenes have also been reported [59].

Aldehyde (13.98 %) occupied the third place. Five aldehydes were identified, and they included: 5-hydroxymethylfurfural (5.81 %), 2-[4-methyl-6-(2,6,6-trimethylcyclohex-1-enyl) hexa-1,3,5-trienyl]cyclohex-1-en-1-carboxaldehyde (5.66 %), octadecanal,2-bromo (1.16 %), 2-Furancarboxaldehyde,5-methyl- (0.86 %), and 5-Octadecenal (0.49 %). Aldehydes were observed in the oil of *H. sabdariffa* seed [2]. Twenty-three aldehydes were reported in hot tea made from *H. sabdariffa* calyces, with furfural compounds being the most abundant [37]. 5-hydroxymethylfurfural was also the most abundant volatile principle identified in dates [49], which was one of the sweeteners in this study. 5-hydroxymethylfurfural has antibacterial, anti-inflammatory, and antioxidant activities [49]. Based on a study by Avalos-Martínez et al. [37], the sweet/caramel aroma of *H. sabdariffa* tea was attributed to aldehydes. Aldehydes have strong antimicrobial activity owing to the electronegative arrangement of its functional group, which has inhibitory effect on nitrogen moieties of nucleic acids, and proteins of microbes [32].

MUFAs followed next with 9.763 %. The MUFAs identified were oleic acid (octadecanoic acid) (7.01, 1.43, 0.71 %), and 9-hexadecenoic acid (0.613 %). MUFAs are important for several physiological processes, including maintaining optimal fluidity of membrane lipid bilayer [60,61]. MUFAs also have "healthy blood lipid profile, improved blood pressure, insulin sensitivity, and glycaemic control" [33]. Octadecanoic acid elevates high density lipoprotein-cholesterol, and reduces low density lipoprotein-cholesterol levels in blood, thus, it is anti-atherogenic [16,53,54]. The antioxidant and antimicrobial properties of oleic acid have been reported [52]. Palmitoleic acid or 9-hexadecenoic acid is associated with good metabolic profiles, and reduced diabetic incidence [33,35]. Palmitoleic acid also prevents apoptosis of beta-cells [34].

SFAs followed closely with 9.37 %. The SFAs identified were n-hexadecanoic acid (4.76 %), 15-hydroxypentadecanoic acid (3.57 %), and hexadecanoic acid, methyl ester (1.04 %). Hexadecanoic acid, the

most abundant SFA is "characterized by a creamy fatty flavour, and dairy nuance" [62]. In addition, it has antibacterial, anti-inflammatory, antioxidant [49], antiandrogenic, and hypercholesterolemic properties [40]. Other properties of hexadecanoic acid were observed [50]. The percentage of hexadecanoic acid, methyl ester was reported to be low also in *H. sabdariffa oil* [13]. An earlier study by the authors stated the antioxidant and hypocholesterolemic potential of hexadecanoic acid, methyl ester [63]. SFAs were observed to be high in different date varieties in the study by Lieb et al [64]. According to Spector [61], SFA are "involved in energy production, energy storage, lipid transport, the synthesis of phospholipids and sphingolipids needed for membrane synthesis, and the covalent modification of many regulatory proteins." Although SFA elevates blood cholesterol levels, and contribute to coronary heart diseases/ high mortality; different researchers have identified beneficial roles of individual SFAs [28,54,65–67].

The next principle identified was steroid (4.35 %). The steroids present were 6β-hydroxytestosterone (2.65%), and 11.α-Hydroxy-17.α-methyl testosterone (1.70 %). The medicinal benefits of steroidal constituents were documented by [52]. Steroids were observed in a study on crude, and phenolic extract of *H. sabdariffa* [31]. A review of hibiscus species by Vasudeva and Sharma [12], also noted the presence of steroids. Steroidal compounds exhibit adaptogenic, anabolic, and other medicinal properties [52]. The anti-inflammatory/anti-microbial activities of steroids were reported respectively by [48,58].

The penultimate constituent present in the sample was acid (2.32 %). Three acids were identified. They were methyl 6-methyl-5-(4 methylphenyl) sulfonyloxy-11-[(Z)-prop-1-enyl]-12, 13-dioxatricyclo [7.3.1.01,6]tridecane-8-carboxylate (1.03 %), benzoic acid (0.77 %), and catechol (0.52 %). Benzoic acid was identified in *H. sabdariffa* tea [37]. The antibacterial property of benzoic acid was reported [38]. Also documented was the antioxidant potential of benzoic acid derivatives [39]. Catechol is an aromatic alcohol with anti-cancer, antioxidant, and pesticide properties [40].

The least compound identified was alcohol: 2-Furanmethanol (0.731 %). Alcohol was confirmed in the oil of *H. sabdariffa* seeds by [2]. The alcohols present can be attributed to bioremediation of unsaturated fatty acids [32]. Antimicrobial activity of alcoholic constituents was reported [31]. In a study by Mozaffarian et al. [68], small amount of alcohol promotes insulin sensitivity, increases serum HDL cholesterol, and decreases systemic inflammation.

The graphical analyses of identified constituents are seen in Figure 2. There is sparse literature on the volatile/fatty acid characterization of sweetened "zobo" drink, as such this study may be the first of this nature. For this reason, too, comparison with the other publications was a challenge, however, the result was compared with those of calyx, and seed oil of *H. sabdariffa.* The results of this study agree with previously documented work on "zobo" drink, and *H. sabdariffa* parts.

V. Conclusion

The GCMS result obtained for sweetened "zobo" revealed the presence of volatile principles, and fatty acids. The flavouring, anti-microbial, anti-inflammatory, anti-hyperglycaemic, hypolipidemic, antioxidant, and anti-cancer properties of these principles were identified. These properties support documented health, and medicinal activities of *H. sabdariffa* calyxes, and beverage. Several identified constituents share similar activities. The synergism of these compounds validates the medicinal effects of "zobo." Drug discovery begins with identification of molecules with desirable activities; thus, these constituents could be exploited by pharmaceutical, and nutraceutical companies for development of drugs/supplements. There is therefore need for further research on the metabolomics of "zobo" drink, and compounds with little or no known activity.

Acknowledgements

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