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Application of Chemical Fingerprinting in resolving nomenclatural ambiguities between *Citrus sinensis* and *Citrus reticulata*

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Abstract: Morphological authentication of closely related taxa using vegetative parts has been challenging for taxonomist. Present study aimed to applying phytochemical fingerprinting techniques in distinguishing *Citrus sinensis* and *Citrus reticulata* individuals in their non-fruiting seasons. Fresh leaf samples obtained were prepared using standard phytochemical protocols to determine colour change discrimination and thereafter subjected to Gas Chromatography-Mass Spectrophotometer (GCMS) analysis to characterize the active ingredients and possibly infer compound (s) that influenced discrimination between both samples. The results of study revealed that flavonoid, terpenoids, and triterpene tests showed differential colorations each, thus these tests are potent in discriminating sample of *C. sinensis* from *C. reticulata*. Although, GCMS study for both samples showed the presence of thirty-six common active ingredients from both species, among these some common are lycoxanthin, carotenoid, hemiporphyrin, and flavonoid. This implied that these two species shared some common compounds which showed relation between these two species. However, the presence of some unique compounds to each sample could be the underlying sources of discrimination observed in the flavonoid, terpenoid and triterpene tests. Putative reactions of the twelve compounds/functional groups and reagents used for flavonoid or terpenoids tests aligned the likely products with the distinctive colours observed. Nonetheless some of the discriminatory compounds have complex structures that proved challenging to react. Conclusively, the study revealed that morphologically similar specimens of *C. sinensis* and *C. reticulata* can be authenticated by flavonoid, terpenoids and triterpene on their extracts. Hence, the study validates the use of chemical fingerprinting of plants in resolving taxonomic intricacies.
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Keywords: Chemotaxonomy, Phytochemical screening, Gas Chromatography-Mass Spectrophotometer (GCMS) analyses, Citrus species.

1. Introduction

Plants identifications have been carried out over the years by using their morphological characters (Rocha *et al*., 2015). Plants are biosynthetic laboratories for multitude of compounds like alkaloids, glycosides, Saponins, steroids, resins, tannins, flavonoids, Sesquiterpenes, lactones which exert physiological and therapeutic effects (Correa *et al*., 2011). These compounds are known as secondary metabolites (Pagare *et al*., 2015). Each group of plant is known to produce unique type of secondary metabolites. This specificity is utilized in delimiting plant taxa (Funk and Seaman, 2000).

Researchers in the field of phyto-taxonomy had established terpenes, and allied phenolic, sulphur and nitrogen containing alkaloids as the three broad groups of secondary metabolites of taxonomic importance (Lin et al., 2014). Though plant taxonomists may resort to using macro- and micro-morphological methods, this may however be time consuming, error prone and requires the services of an expert which may not be available when needed (Lin et al., 2014). In this light, phytochemical analyses have become a very useful tool in examining relationships between plant taxa (Dutkiewicz et al., 2014). This has gone a long way to complimenting morphology as the conventional tool of plant identification.

Plants are undoubtedly reservoirs of chemical information. The chemical bank produced and stored by each species is unique and uniform over all ages and clime (Tao and Yu-Ping, 2008). This principle of species specificity has found usage in delimitating plant taxa.

Accurate species nomenclature is the bedrock of most scientific, agricultural, medical and industrial enterprises (Ebigwai *et al*., 2019). Several taxonomic

lines of evidences such as anatomy, biogeography, cytology, molecular, morphology, phytochemistry, pollen and spore are variously applied for species identification and authentication. Although each has its limitations, the Expert Recognition Method (ERM) is widely practiced in the third world countries due in parts to dearth of equipment and poorly trained personnel.

It is inconceivable that the nomenclatural identity of some taxa could not be determined morphologically due to its life cycle stage and/or an individual of a taxon is not at its fruiting sequence. Put other ways, it is implausible while employing the ERM, that the nomenclature of an individual should only be determined using floral characters (Wäldchen et al., 2018). The implication posed by this limitation is grave. First, it is unimaginable for one to wait for the individual of a species to commence fruiting before its nomenclatural identity could be determined. Second, since leaves are always present in any plant individual and it exhibits varied but unique characters, it is imperative that the ERM system of determining the nomenclatural identity of an individual should be leaf – centered and not florally- centered. Third, it implies that the nomenclatural identities of such individuals could not be ascertained when and if sterility, which is a frequent occurrence in these taxa is observed (Ebigwai and Ngele, 2020).

The present study was undertaken to develop a process of authenticating *C. sinensis* and *C. reticulata* individuals in their non-fruiting seasons, in a bid to determine the chemical differences in the two species and also the type of phytochemical test that could be used in the field for authenticating the two species at sapling or at non-fruiting stages, and possibly develop a chemical and leaf morphological taxonomic keys for the two species, and finally, explain the chemistry behind the mechanism of action governing the expression of differential color change that promoted discrimination between them.

Methodology

Sample collection and preparation

The plant sample was obtained from the University of Calabar Botanical Garden, Calabar, Southern Nigeria (N4.95252^0, E8.34309^0). The identities of samples were confirmed by obtaining leaves from fruiting individuals. The harvested leaves were washed under running water, air-dried at room temperature (27^{o}C) for 15 days, then grinded in to powder using electric blender. The powdered plant material was packed in zip-lock bag and transported to Mifor Consult Laboratory, Calabar for extraction and GC-MS analysis (Kumar and Mathew, 2014; Yusuf et al., 2014).

GCMS Analysis

An Agilent 5890N gas chromatography equipped with an auto sampler connected to an Agilent Mass Spectrophotometric Detector was used. One (1) µl of the sample was injected in the pulsed spitless mode onto a 30 m x 0.25 mm id DB 5MS coated fused silica column with a film thickness of 0.15micrometer. Helium gas was used as carrier gas and the column head pressure was maintained at 20psi to give a constant of 1ml/min. Other operating conditions were present. The column temperature was initially held at 55^{o}C for 4 min, increased to 200^{o}C at a rate of 25^{o}C/mins, then to 280^{o}C at a rate of 8^{o}C/mins and to final temperature of 300^{o}C at a rate of 25^{o}C/mins, for 2 mins. The identification of phytochemical constituents in the extract was based on Retention Time (RT) since each of the active ingredients has its unique RT in the column.

Preparation of extracts for preliminary phytochemical test

Ten (10) g of the candidate plant powder were weighed using a chemical balance (110C), transferred to a rubber bottle and soaked with 150ml absolute ethanol and incubated for 24 Hrs. for maximum extraction and filtered first through a Whatman filter paper No.4, and then through cotton wool to obtain a clear solution. The solutions were stored and used for qualitative test.

Phytochemical Test

Preliminary phytochemical tests were conducted on the plant extracts following the standard methods for phytochemical screening described by (Ermias et al., 2011; Tariq and Reyaz, 2012; Vijisaral and Arumugam, 2013; Thilagavathi et al., 2015; Amita and Shalini, 2014; Vimalkumar et al., 2014).

Tests for the presence of flavonoids (basic), Coumarin, flavonoids (acidic), tannins, phenols, anthocyanins, glycosides, triterpene, steroids and Phytosterol were carried out. Resultant colour change, formation of precipitates and foam, characteristic smell and time required for colour to change in the extracts were observed and noted. The different tests conducted were reviewed to be a characteristic of the different families or genera to which candidate species belong.

Results

Basic phytochemical test

Results of basic phytochemical tests on the ethanol extract of the specimens were shown in Table 1. As shown in the table, the two plant extracts produced different results when tested for flavonoid, phenol and triterpene, making them the differentiable from each other. All other tests could not discriminate both species. Based on the result, three (3) taxonomic keys were proposed for the authentication of *C. sinensis* and *C. reticulata.*

Table 1. Phytochemical profile of *C. sinensis* and *C. reticulata* based on Basic phytochemical Test

Basic test	Colour change in the extracts	
	C. sinensis	*C. reticulate*
Initial	Green	Green
Flavonoid (Basic)	Light green	Light green
Coumarin	Light green	Light green
Flavonoids (Acidic)	Emerald	Blackish green
Tannins	Emerald	Emerald
Phenols	Gold	Dark green
Anthocyanin	Dark green	Dark green
Glycosides	Antique gold	Antique gold
Triterpenes (Lieberman Burckhardt)	Blackish green	Emerald
Steroids and Phytosterol	Light green	Light green
Saponins	Lemon green	Lemon green

***Initial colour were green for all tests**

Taxonomic keys:

Key 1: Extract plus phenol

(a) A gold colour observed in the solution *Citrus sinensis* authenticated (1)

(b) A dark green colour observed *Citrus reticulata* authenticated (2)

Key 2: Extract plus flavonoid test (Sulphuric acid test)

(a) Colored observed is emerald; *C. sinensis* authenticated (1)

(b) Colored observed is blackish green; *C. reticulata* authenticated (2)

Key 3: Extract plus triterpene test

(a) Colour observed is blackish green colour observed; *C. sinensis* authenticated (1)

(b) Colored observed is emerald; *C. reticulata* authenticated (2)

Gas Chromatography-Mass Spectrophotometer (GCMS) Analysis

The results of the GCMS analysis are presented in Figs. 1 and 2, which showed the chromatograms, indicating the retention time (mins) and the abundance of each components. Table 2 represented the phytochemical profile of *C. sinesis* and *C. reticulata* based on GCMS result.

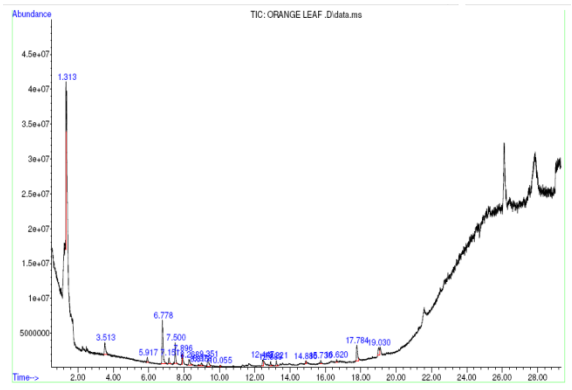
When the peaks were subjected to the NIST library, twenty compounds were identified for each species. The results showed that (5_)Pregnane-3,20_-diol, 14_,18_-[4-methyl-3-oxo-(1-oxa-4-azabutane-1,4-diyl)]-, diacetate (33.36 %), Benzene methanol, 2-nitro-, 4-methylbenzenesulfonate (ester) (19.57 %) and Phorbol (7.33 %) were the dominant compounds in *C. sinensis*, while 4-(2,5-Dihydro-3-methoxyphenyl)butylamine (46.32) 5H-Cyclopropa [3,4] benz [1,2-e]azulen-5-one, 3,9,9a-tris (acetyloxy)-3-[(acetyloxy)methyl]-2-chloro-1,1a,1b,2,3,4,4a,7a,7b,8,9,9a-dodecahydro-4a,7b-dihydroxy-1,1,6,8-tetramethyl-, [1aR (1a,1b,2,3,4a,7a,7b,8,9,9a)]-, (13.90%) and Tungsten, dicarbonyl-(_-4-pinocarvone) [1,2-bis (dimethylphosphino)ethane] (12.81%) were dominant in *C. reticulata*.

Of the individual compounds identified in the two plant extracts, Hematoporphyrin, (5_) Pregnane-3, 20_-diol, 14_, 18_-[4-methyl-3-oxo-(1-oxa-4-azabutane-1, 4-diyl)]-, diacetate and Lycoxanthin. However, (5_) Pregnane-3, 20_-diol, 14_, 18_-[4-methyl-3-oxo-(1-oxa-4-azabutane-1, 4-diyl)] - was significantly higher (33.36 %) in *C. sinensis* compared to 1.55 % in *C. reticulata.*

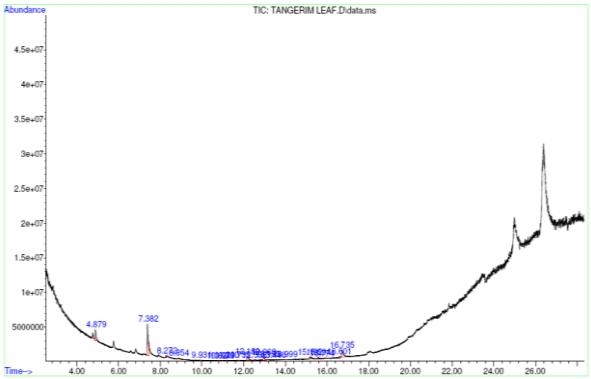
When the compounds were grouped according to their functional groups, results showed that Terpenoid and fatty acid/ester were the most frequent in *C. sinensis* with four (4) members each. Similarly, Terpenoid followed by flavonoid was the most frequent compounds in *C. reticulata* with four (4) and three (3) respectively.

Quantitatively, steroid (33.36 %) followed by fatty acid/ester (31.89 %) were the dominant compounds in *C. sinensis* while amine (46.33 %) followed by Terpenoid (27.28 %) were the dominant compounds in *C. reticulata.*

Furthermore, Terpenoid, Carotenoid, Alkaloid, Ester/fatty acid and Steroids were common to the two plants. But alcohol, alkene and hexene were limited to *C. sinensis* while amine was specific to *C. reticulata.*



a Fig 1: GCMS chromatogram of *C. sinensis*.



b Fig 2: GCMS chromatogram of *C. reticulata*.

Table 2. Phytochemical profile of *Citrus senesis* and *Citrus reticulata* based on GCMS result

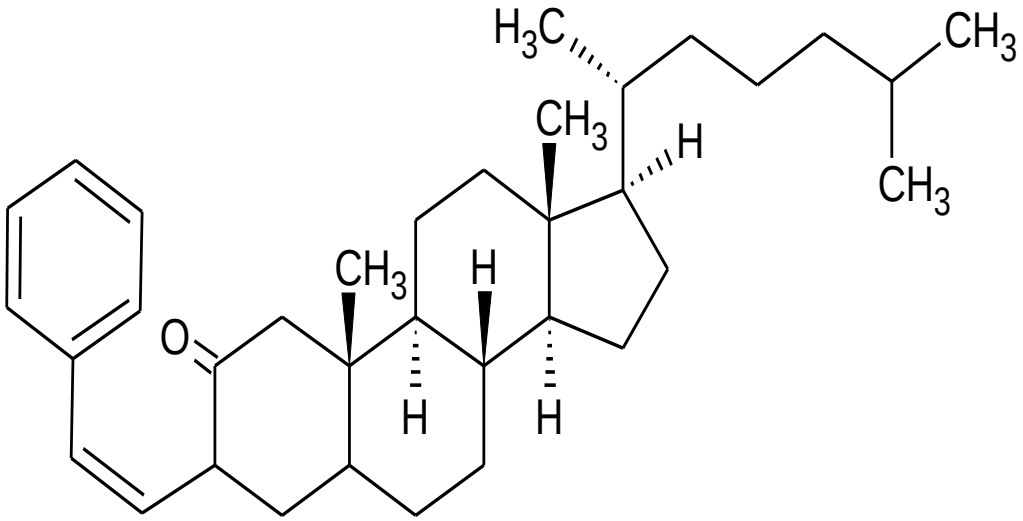
Chemical group	Compound	Molecule	% Peak Area	
			C. sinensis	*C. reticulata*
Alcohol	5-Methyl-2-nitrobenzyl alcohol, methyl ether	$C_9H_{11}NO_3$	6.327	
	5-Methyl-2-nitrobenzyl alcohol	$C_8H_9NO_3$	1.937	
	1-Heptatriacotanol	$C_{37}H_{76}O$	0.574	
Alkaloid	Delsoline	$C_{25}H_{41}NO_7$		0.318
	Zearalenone, bis (pentafluoropropionate)	$C_{24}H_{20}F_{10}O_7$		0.215
	Formamide, N-methyl-N-4-[1-(pyrrolidinyl)-2-butynyl]-	$C_{10}H_{16}N_2O$	0.473	
	Benzeneacetamide, _-ethyl-	$C_{10}H_{13}NO$	0.992	
	Pyrimidine-2,4(1H,3H)-dione, 1,3-dimethyl-6-[2-(4-morpholyl) ethenyl]-5-nitro-	$C_{12}H_{16}N_4O_5$	0.480	
Alkene	9-Borabicyclo [3.3.1]nonane, 9-(1-ethyl-1-butenyl)-, (E)-	$C_{14}H_{25}B$	3.400	
Amine	4-(2,5-Dihydro-3-methoxyphenyl)butylamine	$C_{11}H_{19}NO$		46.329
Carotenoid	psi.,.psi.-Carotene, 1,1',2,2'-tetrahydro-1,1'-dimethoxy-	$C_{42}H_{64}O_2$		0.298
	Lycoxanthin	$C_{40}H_{56}O$	1.486	1.401
Fatty acid/ ester	Doconexent	$C_{22}H_{32}O_2$	1.261	
	Trilinolein	$C_{57}H_{98}O_6$	4.219	
	10,13-Octadecadiynoic acid, methyl ester	$C_{19}H_{30}O_2$		8.133
	Benzenemethanol, 2-nitro-, 4-methylbenzenesulfonate (ester)	$C_{14}H_{13}NO_5S$	19.566	
	N,N'-Bis (Carbobenzyloxy)-lysine methyl (ester)	$C_{23}H_{28}N_2O_6$	2.371	
Flavonoid	Hematoporphyrin	$C_{34}H_{38}N_4O_6$	0.299	0.877
	17-(1,5-Dimethylhexyl)-10,13-dimethyl-3-styrylhexadecahydrocyclopenta [a]phenanthren-2-one	$C_{35}H_{52}O$		6.725
	2-(5-(5-[Cyano-(9,9-dimethyl-1,4-dioxa-7-aza-spiro [4.4]non-7-en-8-yl)-methylene]-3,3-dimethylpyrrolidin-2-ylidenemethyl)-3,3-dimethyl-_1-pyrrolin-5-ylidenemethyl-4,4,5-trimethyl-_1-pyrroline-5-carbonitrile]	$C_{32}H_{42}N_6O_2$		0.254
	2-(16-Acetoxy-11-hydroxy-4,8,10,14-tetramethyl-3-oxohexadecahydrocyclopenta [a]phenanthren-17-ylidene)	$C_{32}H_{48}O_6$		0.274
	7,8-Epoxylanostan-11-ol, 3-acetoxy-	$C_{32}H_{54}O_4$		0.215
Hexene	3-Cyclohexene-1-propanal	$C_9H_{14}O$	3.747	
	1,7-Dimethyl-3-phenyltricyclo [4.1.0.0(2,7)]hept-3-ene	$C_{15}H_{16}$	0.848	
	1H-2,8a-Methanocyclopenta [a]cyclopropa [e]cyclodecen-11-one, 1a,2,5,5a,6,9,10,10a-octahydro-5,5a,6-trihydroxy-1,4-bis	$C_{20}H_{28}O_6$	0.878	

	(hydroxymethyl)-1,7,9-trimethyl-, [1S-(1_,1a_,2_,5_,5a_,6_,8a_,9_,10a_)]-			
Steroid	Pregn-4-ene-3,20-dione, 11,21-bis [(trimethylsilyl)oxy]-, bis (O-methyloxime), (11_)-	$C_{29}H_{52}N2O_4Si_2$		2.486
	(5_)Pregnane-3,20_-diol, 14_,18_-[4-methyl-3-oxo-(1-oxa-4-azabutane-1,4-diyl)]-, diacetate	$C_{28}H_{43}NO_6$	33.361	
	(5_)Pregnane-3,20_-diol, 14_,18_-[4-methyl-3-oxo-(1-oxa-4-azabutane-1,4-diyl)]-, diacetate	$C_{28}H_{43}NO_6$		1.548
Terpenoid	Chloro-decaborane,	$B_{10}ClH_{13}$	3.199	
	Tungsten, Decaborane, chloro-dicarbonyl-(_-4-pinocarvone) [1,2-bis (dimethylphosphino)ethane]	$C_{18}H_{30}O_3P_2W$		12.817
	2,5-Bis (1-naphthyl)-1,5-hexadiene	$C_{26}H_{22}$	5.945	
	4,5-di-epi-aristolochene	$C_{15}H_{24}$	1.602	
	Tungsten, dicarbonyl-(_-4-2-methylenecycloheptanone) [1,2-bis (dimethylphosphino)ethane]			0.335
	5H-Cyclopropa [3,4]benz [1,2-e]azulen-5-one, 3,9,9a-tris (acetyloxy)-3-[(acetyloxy)methyl]-2-chloro-1,1a,1b,2,3, 4,4a,7a,7b,8,9,9a-dodecahydro-4a,7b-dihydroxy-1,1,6,8-tetramethyl-, [1aR-(1a_,1b_,2_,3_,4a_,7a_,7b_,8_,9_,9a_)]-	$C_{28}H_{37}ClO_{11}$		13.908
	Phorbol	$C_{20}H_{28}O_6$	7.333	

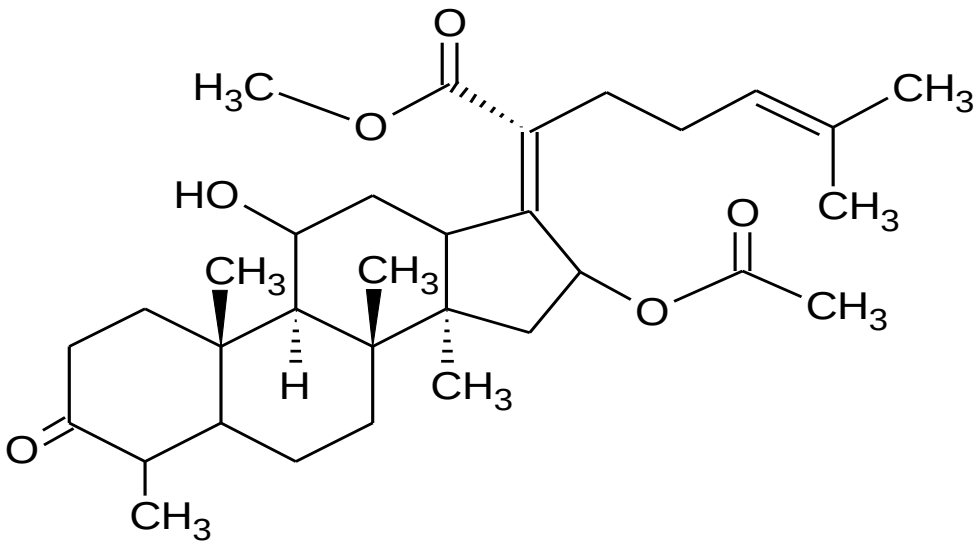
Discussion

Reactions between compounds used in preparing the phytochemical tests and the plant extract resulted in change in physical and chemical properties and hence a valuable chemotaxonomic marker for species authentication (Ebigwai and Enudi, 2019; Savithramma et al., 2012). The results of the basic phytochemical test revealed the potency of flavonoid (acidic test), phenol and triterpene test to discriminate between the two taxa and hence could offer cheaper, reliable and quick methods of authenticating both species during field studies. These findings are in line with Ebigwai and Ngele, 2020, Amita and Shalini, 2014, Vimalkumar et al., 2014, Ebigwai and Akomaye, 2014. Although the GCMS spectra revealed several other compounds that are unique to each taxon, discussion shall focus on the Twelve (12) unique flavonoids and terpenoids compounds (see Table 2) that would have been responsible for the discriminatory colour change exhibited when extracts of the two species was subjected to flavonoid and terpenoids test as shown in Table 1. In the same vein, the inability of the other tests to discriminate between the two taxa could be owed to the presence of same phytochemical groups observed in the GCMS spectra of both test samples.

The four flavonoid compounds that are unique to the *C. reticulata* species are 17-(1,5-Dimethylhexyl)-10,13-dimethyl-3-styrylhexadecahydrocyclopenta [a]phenanthren-2-one, 2-(16-Acetoxy-11-hydroxy-4,8,10,14-tetramethyl-3-oxohexadecahydrocyclopenta [a]phenanthren-17-ylidene), 3-acetoxy-7,8-Epoxylanostan-11-ol and 2-(5-(5-[Cyano-(9,9-dimethyl-1,4-dioxa-7-aza-spiro [4.4]non-7-en-8-yl)-methylene]-3,3-dimethylpyrrolidin-2-ylidenemethyl)-3,3-dimethyl-1-pyrrolin-5-ylidenemethyl-4,4,5-trimethyl-1-pyrroline-5-carbonitrile).



(a)



(b)

Fig. 3. Chemical Structures of (a) 17-(1, 5-Dimethylhexyl)-10, 13-dimethyl-3-styrylhexadecahydrocyclopenta [a]phenanthren-2-one, (b) 2-(16-Acetoxy-11-hydroxy-4, 8, 10, 14-tetramethyl-3-oxohexadecahydrocyclopenta [a]phenanthren-17-ylidene)

17-(1,5-Dimethylhexyl)-10,13-dimethyl-3-styrylhexadecahydrocyclopenta [a]phenanthren-2-one and 2-(16-Acetoxy-11-hydroxy-4, 8, 10, 14-tetramethyl-3-oxohexadecahydrocyclopenta [a]phenanthren-17-ylidene) are steroid derivative, which are reactive in a Sulphuric acid environment, resulting in products exhibiting varied colorations as observed with flavonoid test with *C. reticulata*. Discriminatory ability of steroids and its derivatives among plant species have been reported (Widmer et al., 2005; Hamdan et al., 2016; Gan et al., 2003; Ahlgren et al., 1996; Splinter and Rhine, 1998). The presence of oxygen atoms in the form of oxo- and hydroxyl- groups reacts with the dissociated hydroxyl group in the NaOH solution resulting in formation of aklyl and carbonyl groups. This reaction pathway is peculiar to *C reticulata*.

The electron deficient carbonyl carbon present in 17-(1,5-Dimethylhexyl)-10,13-dimethyl-3-styrylhexadecahydrocyclopenta [a]phenanthren-2-one

and 2-(16-Acetoxy-11-hydroxy-4,8,10,14-tetramethyl-3-oxohexadecahydrocyclopenta [a]phenanthren-17-ylidene) in acidic phytochemical test solution is attacked by sulphate ions (SO_4^{2-}) which introduces an auxochromatic to the compound, thereby changing the optical properties of the reaction system. Colour change of *C reticulata* with flavonoid test is owed in part to this mechanism of action.

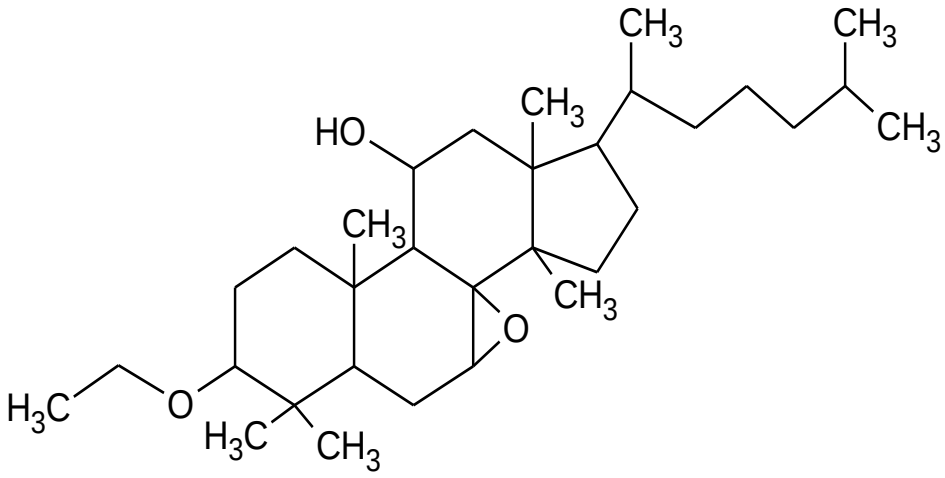


Fig. 4. Molecular Structure of 3-acetoxy-7, 8-Epoxylanostan-11-ol

7, 8-epoxylanostan-11-ol, 3-acetoxy- which was confirmed to be present only in the *reticulata* species is an epoxy-alcoholic ester compound. The structure is built on the basic steroid, have three (3) fused cyclohexane rings, and a cyclopentane fused to ring C of the molecule. The chemical reactivity of 7, 8-epoxylanostan-11-ol, is attributed the presence of the hydroxyl functionality. Also, the epoxy group that is fused between C7 and C8 can react through a ring opening mechanism to yield unique chemicals (Lee et al., 2013).

In acidic solutions, 3-acetoxy-7, 8-epoxylanostan-11-ol due to the presence of amphoteric hydroxyl group will react as a base by accepting protons from the solution to give oxonium ion. This gives a positive test to acidic test solution for flavonoids.

2-(5-(5-[Cyano-(9,9-dimethyl-1,4-dioxa-7-aza-spiro [4.4]non-7-en-8-yl)-methylene]-3,3 dimethylpyrrolidin-2-ylidenemethyl)-3,3-dimethyl-1-pyrrolin-5-ylidenemethyl-4,4,5-trimethyl-1-pyrroline-5-carbonitrile) is one of the phytochemicals identified to be present only in the C. *reticulata* species. Not much chemistry of this compound is recorded (Qian et al., 2019), but the presence of basic nitrogen (which possess a lone pair of electron) makes it reactive to an acidic proton, hence a positive test with Sulphuric acid.

Discriminatory Terpenoid Compounds
Chloro-Decaborane

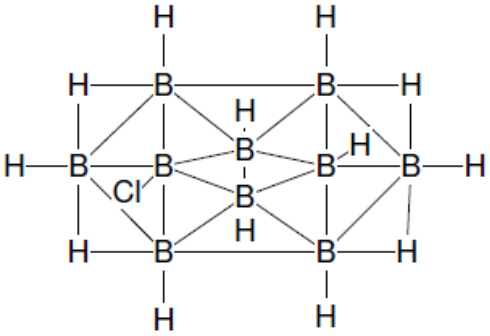


Fig. 5: Molecular structure of Chloro-decaborane

In the presence of water, decaborane is rapidly transformed into one or more polar intermediate products that are eventually degraded to boric acid with its resulting coloration and distinctive reactions.**4, 5-di-epi-aristolochene**

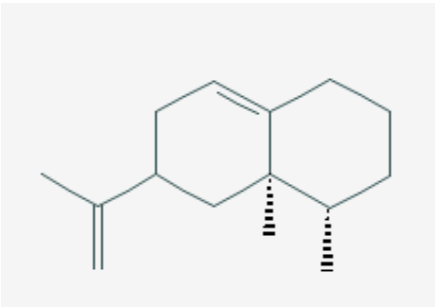


Fig. 6: Molecular structure of 4,5-di-epi-aristolochene

4,5-di-epi-aristolochene involved in the biosynthesis of capsidiol catalyzes the successive and

independent hydroxylations at the C1 and C3 positions of 5-epiaristolochene. The second hydroxylation step is 8-fold more efficient than the first hydroxylation reaction. This initiates a colour change as observed with flavonoid, terpenoid and triterpene tests.

Phorbol

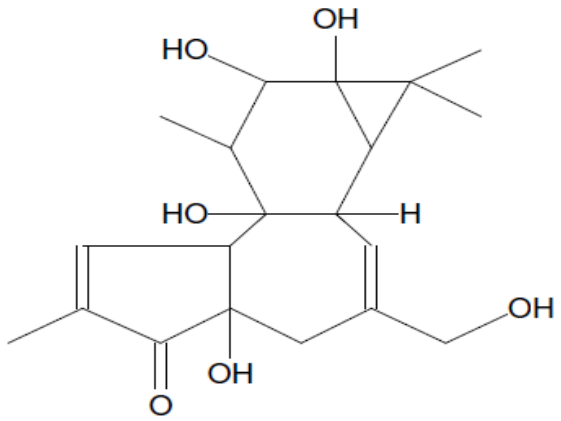


Fig. 7: Molecular structure of Phorbol

Phorbol is a tetracyclic diterpenoid, an enone, a cyclic ketone, a tertiary alcohol and a tertiary alpha-hydroxyl ketone. It is derived from a hydride of a tigliane. Phorbol is a natural product found in many plants, especially those of the Euphorbiaceae and Thymelaeaceae families. Instability of Phorbol on exposure to air, light and ambient temperatures, sensitivity to acids and alkalis and susceptibility to auto oxidation rationalizes exhibition of the positive tests (Pagare et al 2015).

2, 5-Bis (1-naphthyl)-1, 5-hexadiene

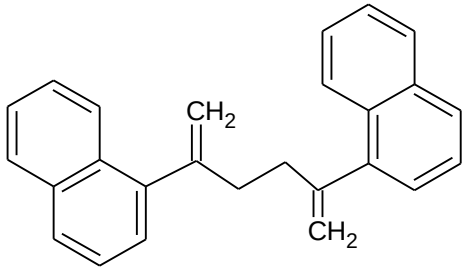


Fig. 8. Molecular Structure of 2,5-Bis (1-naphthyl)-1,5-hexadiene

2,5-Bis (1-naphthyl)-1,5-hexadiene was identified to be present only in *Citrus sinensis*. This hydrocarbon is structurally highly conjugated, which increases the light absorption frequency to the visible region of the electromagnetic spectrum, hence capable of exhibiting colour (Ghaani et al., 2016). Not much of its chemistry have been reported. However, its high melting temperature, optical properties and flexural strength, owing to the presence of aromatic groups around its backbone could possibly explain the chemistry behind the discriminatory colour changes observed.

Sulphuric acid in the presence of chloroform will oxidize the C=C bond of 2,5-Bis (1-naphthyl)-1,5-hexadiene to give a positive test.

Tungsten, Decaborane, chloro-dicarbonyl-(-4-pinocarvone) [1,2-bis (dimethylphosphino)ethane] and Tungsten, dicarbonyl-(-4-2-methylenecycloheptanone) [1,2-bis (dimethylphosphino)ethane] are both Tungsten complexes of 1,2-bis (dimethylphosphino)ethane ligand. Both complexes were identified by the GC-MS of *C. reticulata*. The high affinity of nucleophiles towards the carbonyl group explains the attack of the carbonyl carbon by the surphate ions of the reaction acid to give a colour change in the system.

5H-Cyclopropa [3,4]benz [1,2-e]azulen-5-one, 3,9,9a-tris (acetyloxy)-3-[(acetyloxy)methyl]-2-chloro-1,1a,1b,2,3,4,4a,7a,7b,8,9,9a-dodecahydro-4a,7b-dihydroxy-1,1,6,8-tetramethyl-, [1aR-(1a,1b,2,3,4a,7a,7b,8,9,9a)]-; is another distinguishing compound between the two *Citrus* species that was studied. It was only present in the *reticulata* species. (Nyayiru et al., 2019), identified the presence of this compound in *Origanum majorana*, but not much of its chemistry is known.

Conclusion

The present study revealed that morphologically similar specimens of *Citrus sinensis* and *Citrus reticulata* can be discriminated by carrying out basic phytochemical test for flavonoid, and terpenoids and triterpene on their extracts. The GCMS results revealed twelve flavonoid and terpenoids compound that could have been responsible for the discrimination. Hence, the study validates the use of chemical fingerprint of plants in resolving taxonomic intricacies.

Limitations and Recommendations

It is inexplicable why Triterpenes test that acted discriminately in Table 1 had no representative member in the GCMS spectra shown in Table 2. It is therefore recommended that HPLC analyses be applied in lieu of GCMS peradventure the Triterpene member (s) is/are non -volatile constituent (s). Also, distinguishing flavonoid and Terpenoid compounds should be isolated and studied further for their properties and applicability.

Declaration of Authorship:

We declare that the aforementioned are the authors of this manuscript.

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