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Resolving Morphological Nomenclatural Conflict between *Musa sapientum* L and *Musa paradisiaca* L using qualitative Flavonoid and Anthocyanin tests

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Abstract: The challenge in the use of morphological characters in the authentication of species possessing similar vegetative morphology has stretched the integrity of the Expert Recognition Method (ERM). Chemotaxonomy is one of the offshoots of the limitations imposed on the scientific enterprise by the ERM. This study was conducted to test the applicability of routine phytochemical tests by the use of color and precipitate in the authentication of *Musa sapientum* and *Musa paradisica* using their frond. Gas Chromatography-Mass Spectrophotometer (GCMS) analyses was conducted to possibly infer the mechanisms of action responsible for the observations obtained using the unique chemical signatures in each sample. Fresh leaf samples were obtained, processed and analyzed following standard phytochemical and GCMS protocols. The result obtained from phytochemical analyses showed that the two specimens can be differentiated based on color change when their extracts are subjected to either flavonoid test (basic) or Anthocyanin test. Eight other tests did not prove useful in discriminating between them. When the extracts were exposed to GCMS analyses, the result revealed the existence of six flavonoid compounds unique to *M. paradisica* and one alcoholic compound unique to *M. sapientum*. Conclusively, a taxonomic key was constructed for to be used to authenticating both species at any time across all phases of the taxa life cycle. The cost affordability, rapidity, potency and its application whenever and wherever the need arises makes this approach novel and interesting. However, further research utilizing frond samples obtained across different habitats, different times of the day backed with soil analyses needed be conducted before a statement on its universal application could be authoritatively made.

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Keywords: Gas Chromatography-Mass Spectrophotometer (GCMS), *Musa species,* Nomenclature, Phytochemical, Calabar.

1. Introduction

Expert Recognition Method (ERM) employing vegetative and floral morphological traits in species authentication remain the most used technique in spite of its huge limitations, because it does not require extensive laboratory processes and equipment (Ebigwai *et al*., 2019). This renders the method quick and easy to apply. It only requires an 'expert' and in some difficult cases, a voucher specimen in an herbarium complimented by Identification keys (Ebigwai *et al* 2019).

The real time authentication process ERM offers using vegetative characters is untenable in all taxa owing largely traits homology (Ebigwai *et al*., 2019).

Musa parasidiaca, L. and *Musa sapientum* L., are two taxa with indistinguishable vegetative characters (Osuji *et al*., 1995; Karamura *et al*., 2011). The root, leaf and stem architectures of one cannot be differentiated from the other. This renders it practically impossible for the nomenclatural identity of the two taxa to be determined in non-fruiting seasons and in non-fruiting individuals. This is a challenge that requires taxonomic attention.

Plants we know, are undoubtedly, reservoirs of natural products (Ebigwai and Enudi, 2019). The differential expression of their individual fruit types is underlain by their specific DNA traits and in turn by their unique chemical signatures and spectral. It is unarguable that the chemical information in the frond of *Musa sapientum* and that in *Musa paradisica* is different.

It is this basic principle that is applied in this study to determine the nomenclatural identity of the two taxa using basic qualitative phytochemical protocols and using the analyzed GC MS signatures to explain the chemical compounds responsible for the observed color change.

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 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 simply 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. 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. Secondly, 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.

These challenge call for the development of a technique that combines the cheap and fast advantages of the ERM with the reliability and unlimited authentication process of all taxa.

The fruits of *Musa sapientum* (Banana) and *Musa paradisiaca* (Plantain) serves as important food sources to man and some primates and have been applied to several industrial concerns (Sharrock, *et al*., 1998). The fronds are excellent fodder for ruminants. The leaves and sap of each has variously been showed as possessing antibacterial (Ememobong, *et al.,* 2016), anti-fungus (Priya, *et al.,* 2014). and anti-viral (Priya, *et al.,* 2014). properties. It is thus imperative that a reliable system for differentiating all individuals of the two taxa at any time across any phase of their life cycle to be developed.

The present study seeks to address this through the use of basic phytochemical protocols and subsequent development of morphological and chemical identification keys for future users.

Methodology

Collection and preparation of plant materials

The plant samples were obtained from the University of Calabar Botanical Garden, Calabar Southern Nigeria (N4.95252^0, E8.34309^0). The identity of the samples was confirmed by obtaining fronds from fruiting individuals. The phytochemical extraction and GC-MS analyses were carried out at Mifor Consult Laboratory, Calabar.

The leaves of the candidate plant samples were washed under running tap, to remove impurities and air dried at room temperature (25°C) in order to rupture the cells and cause them to release active ingredients in them. The dried samples leaves were grinded to uniform powder using an electric blender (Kumar and Mathew 2014). The fine powders were then packed separately in 3 zip-lock bags to avoid the effect of humidity and then stored at room temperature according to method described by Yusuf *et al*. (2014)

GC-MS 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, held 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 gotten wool to obtain a clear solution. The solutions were stored and used for qualitative test.

Phytochemical test

Preliminary phytochemical test was 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, 2014), Amita and Shalini, 2014), Vimalkumar *et al.,* 2014).

The test was carried out with the candidate plant extracts and the resultant color change, formation of precipitates and foam, Characteristic smell and time required for color to change in the extracts were observed and noted. The test conducted were reviewed to be present in the families or genus to which candidate species belong.

Results

Basic phytochemical test

Result of basic phytochemical tests on the ethanol extract of the specimens is shown in Table1. As shown in the Table, the two plant extracts showed different actions when tested for flavonoid and anthocyanin respectively. All other tests could not discriminate both species.

Table 1. Phytochemical profile of *M. sapientum* and *M. paradisica* based on Basic phytochemical test

Basic test	Final color change upon basic test	
	M. sapientum	*M. paradisiaca*
Flavonoid (Basic)	Orange	Brown
Coumarin	Orange yellow	Orange yellow
Flavonoids (Acidic)	Brown	Brown
Tannins	Brown	Brown
Phenols	Dark brown	Dark brown
Anthocyanin	Orange	Brown
Glycosides	Brown	Brown
Steroids and Phytosterol	Brownish orange	Brownish orange
Saponins	Cream	Cream

**All initial colors were green for both samples across all tests*

GC-MS analysis

The result of the GCMS analysis showing the chromatograms indicating the retention time (mins) and the abundance of each component as well as the percentage (%) composition of components in the sample was presented per studied plant taxon as shown in Figs 1 and 2 and Table 2 respectively.

When the peaks were subjected to the NIST library, twenty compounds were identified for each species. The result showed that no one chemical compound was common to both species. 2,6-di-tert-Butyl-4-(dimethylaminomethyl)phenol (17.15 %), Per methyl 2"-O-xylosylvitexin (7.95 %) and 10,10-Dichloro-9-oxa-10-sila-9,10-dihydrophenanthrene (7.35 %) were the dominant compounds in *M. sapientum* against the Androstan-3-one, 17-methoxy-, 3-methoxime, (5_,17_)- (25.20) 4,6-Androstadien-3_-ol-17-one, acetate (13.40%) and Rapamycin (7.70 %) that were the dominant type in *M. paradisiaca*.

When the identified compounds were grouped according to their functional groups, result for *M. sapientum* showed that alcohol had a single member, alkaloid had 8 members, associated hydrocarbon had 3 members and fatty acid had 8 members. The result for *M. paradisiaca* revealed the presence of alkaloid (with 4 members), associated hydrocarbon (with 6 members), fatty acid (4 members) and flavonoid (with 6 members).

Generally, it was observed that while alkaloid compounds were the dominant members in *M. sapientum,* flavonoids were observed exclusively in *M. paradisiaca*, as against alcoholic compound that was limited to *M. sapientum*.

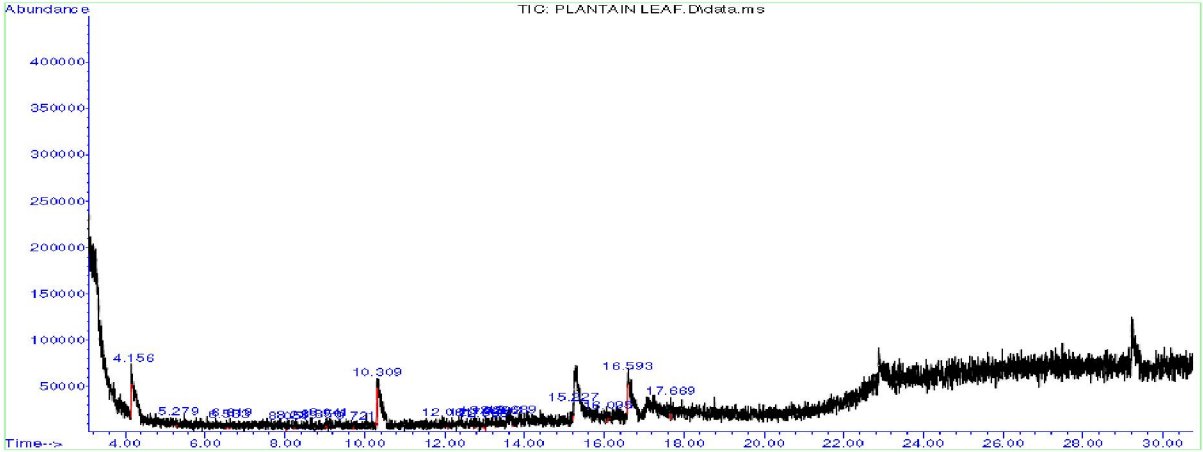


Fig 1. GCMS chromatogram of *M. paradisica*

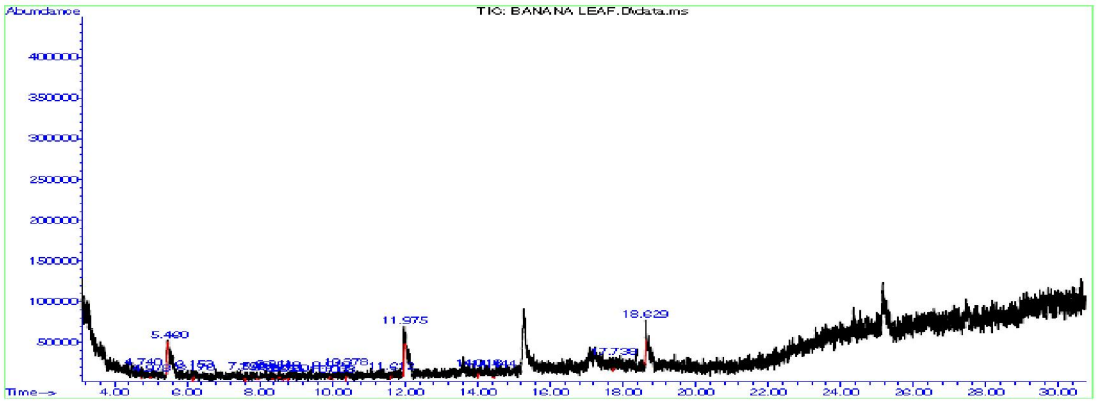
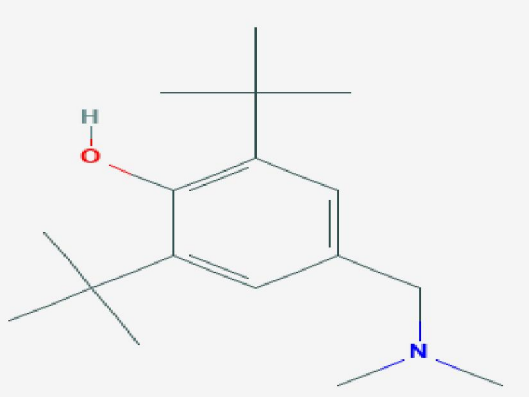


Fig 2. GCMS chromatogram of *M. sapientum* Table 2. Phytochemical profile of *M. sapientum* and *M. paradisica* based on GC-MS results

Chemical group	Compounds	Molecular Formula	*M. sapientum*	*M. paradisica*
Alcohol	Bacchotricuneatindaldehyde alcohol	$C_{20}H_{28}O_3$	2.28	
Alkaloid	2-Oxo-3-[4-nitrophenyl] propanoic acid	$C_9H_7NO_5$	4.33	
	Rapamycin	$C_{51}H_{79}NO_{13}$	7.70	
	1H-Isoindol-1-one, 3-(dimethylamino)-3a,4,5,7a-tetrahydro-3a-methyl-	$C_{11}H_{16}N_2O$	3.29	
	4-(1-Methylpropylamino) pyrido [3,2-c] pyridazine	$C_{11}H_{14}N_4$		3.02
	Pyridine-3-carboxylic acid, 1,2-dihydro-5-benzylaminocarbonyl-6-methyl-2-oxo-, methyl ester	$C_{16}H_{16}N_2O_4$	2.44	
	N-(p-Chlorophenyl)-p-chloro-benzenesulfinylamide	$C_{12}H_9C_{l2}NOS$	4.60	
	Anthraquinone, 1-(o-bromophenyl)-	$C_{20}H_{11}BrO_2$	2.74	
	Sarcosine, N-(2,6-difluorobenzoyl)-, hexyl ester	$C_{16}H_{21}F_2NO_3$	3.21	
	Oxazole, 5-(4-chlorophenyl)-2,5-dihydro-2,4-diphenyl-	$C_{21}H_{16}CINO$	2.32	
	Phenethylamine, N-hexyl-	$C_{14}H_{23}N$		4.50
	7H-1,4-Oxathiepin-7-one, tetrahydro-	$C_5H_8O_2S$		3.91
	5-Benzofurazancarboxylic acid, 1-oxide	$C_7H_4N_2O_4$		4.16
Associated hydrocarbon	1-(2-Hydroxyethylthio)-2-(vinylthio)ethane	$C_6H_{12}OS_2$	2.45	
	2-cyclohexen-1-ol, 1-phenyl-	$C_{12}H_{14}O$		4.31
	2,2,4,4-Tetrafluoro-1,3-dimethyl-2,4-bis (2,2-dimethylhydrazino)-1,3,2,4-diazadiphosphetidine	$C_6H_{20}F_4N_6P_2$	4.17	
	2-cyclohexen-1-ol, 1-phenyl-	$C_{12}H_{14}O$		3.61
	1,2-Naphthoquinone, 4,6-dimethoxy-	$C_{12}H_{10}O_4$		4.20
	3-Chloro-2,6-diethylaniline	$C_{10}H_{14}ClN$		6.25
	trans-O-Dithiane-4,5-diol	$C_4H_8O_2S_2$		3.16
	1,1-Dimethyl-1-sila-3-thia-cyclopentane	$C_5H_{12}SS$	4.38	
	Permethyl 2"-O-xylosylvitexin	$C_{35}H_{46}O_{14}$		7.59
Fatty acid (ester)	Benzoic acid, 4-methyl-3-nitro-	$C_8H_7NO_4$		3.77
	8-Chloro-3-methylpyrimido [1,2-a] benzimidazol-1-ol	$C_{11}H_8ClN_3O$		3.18
	Isopropyl butyrate	$C_7H_{14}O_2$	3.27	
	Acetic acid, (2,4,5-trichlorophenoxy)-, 2-methylpropyl ester	$C_{12}H_{13}C_{l3}O_3$		3.32
	Pyrrole-2,5-dicarboxylic acid, 4-(2-diethylamino) ethyl-3-methyl-, 2-ethyl ester	$C_{15}H_{24}N_2O_4$	2.34	
	8-[3-Oxo-2-(pent-2-en-1-yl) cyclopent-1-enyl] octanoic acid	$C_{18}H_{28}O_3$	2.89	
	Androstan-3-one, 17-methoxy-, 3-methoxime, (5_,17_)-	$C_{21}H_{35}NO_2$	25.20	
	2-[2-(2-Ethoxyethoxy) ethoxy] ethyl 2,2,3,3,4,4,4-heptafluorobutanoate	$C_{12}H_{17}F_7O_5$	2.53	
	9H-Pyrido [3,4-B] indole-1-propanoic acid	$C_{14}H_{12}N_2O_2$	2.84	
	4,6-Androstadien-3_-ol-17-one, acetate	$C_{21}H_{28}O_3$	13.40	
	1,2-Hydrazinedicarboxylic acid, diethyl ester	$C_6H_{12}N_2O_4$		5.30
	Benzeneethanol, 3-nitro-, acetate (ester)	$C_{10}H_{11}NO_4$	3.66	
Flavonoid	2,6-di-tert-Butyl-4-(dimethylaminomethyl)phenol	$C_{17}H_{29}NO$		17.15
	Thiirane	C_2H_4S		4.01
	Imidazole, 4-bromo-2-trifluoromethyl	$C_4H_2BrF_3N_2$		2.99
	Vomitoxin, triacetoxy derive	$C_{21}H_{26}O_9$		4.31
	9,10-Anthracenedione, 1,8-diethoxy	$C_{18}H_{16}O_4$		3.92
	10,10-Dichloro-9-oxa-10-sila-9,10-dihydrophenanthrene	$C_{12}H_8C_{l2}OSi$		7.35

Discussion

The results of the basic phytochemical test revealed the potency of phytochemical screening and GCMS tests 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 et al 2019, Amita and Shalini (2014), Vimalkumar *et al.* (2014) and Idu, *et al*., (2007).



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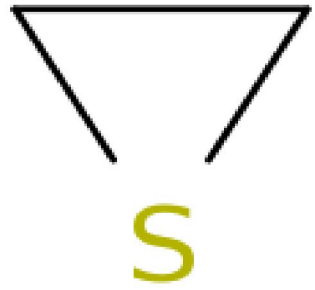

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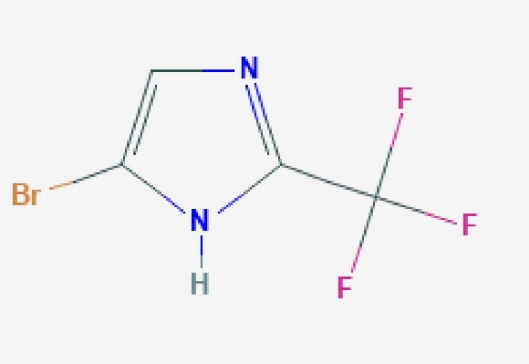

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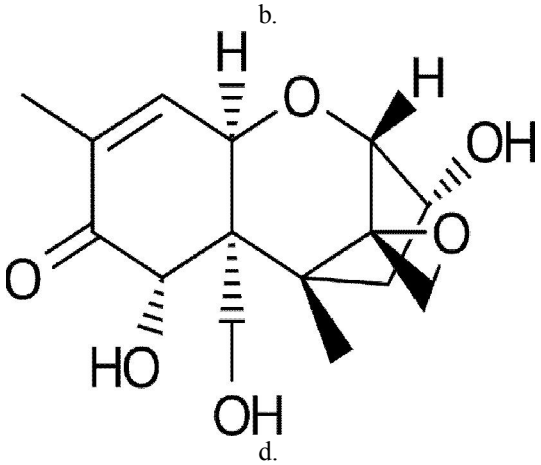

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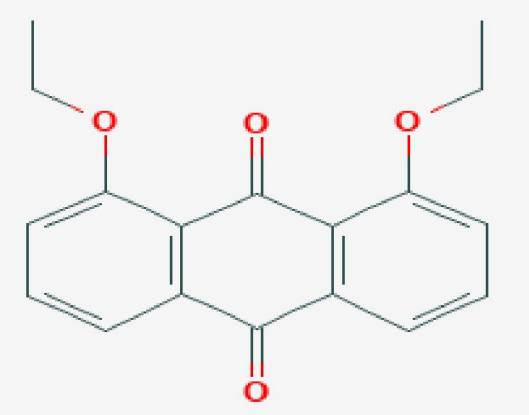

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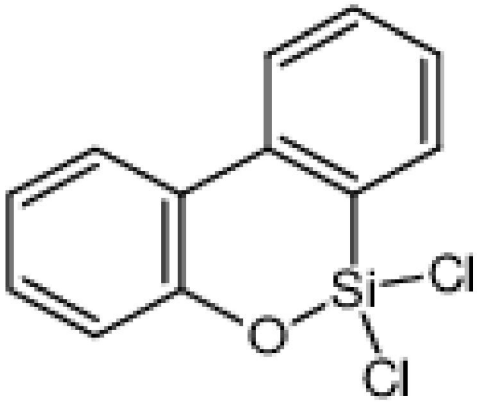

f.

a. Chemical structure of 2,6-di-tert-Butyl-4-(dimethylaminomethyl) phenol. **b.** Chemical structure of Thiirane. **c.** Chemical Structure of 4-bromo-2-trifluoromethyl Imidazole. **d.** Chemical Structure if Vomitoxin. **e.** Chemical Structure 1,8-diethoxy-9,10 anthracenedione. **f.** Chemical Structure of 10,10-Dichloro-9-oxa-10-sila-9,10-dihydrophenanthrene

As evident in Table 2, the presence of the six flavonoid compounds present in *Musa paradisiaca* and the unique alcohol in *Musa sapientum* could have been responsible for categorizing them as two separate species. In the same vein, the inability of the other tests to discriminate between the two taxa could be owed to the occurrences of Alkaloids, Hydrocarbons and Fatty acids in both species as expressed in the GCMS spectra. Fig 3 is a constructed key for the identification of both species.

1a Extract plus flavonoid or Anthocyanin tests turned green color to orange – *Musa sapientum.*

1b Extract plus flavonoid or Anthocyanin test turned green color to brown –*Musa paradisiaca.*

Fig 3 Constructed key for differentiating *Musa sapientum* and *Musa paradisiaca* using flavonoid test or Anthocyanin test.

The chemistry of the six flavonoid compounds that acted discriminately between both taxa is discussed in relation to industrial applications of each taxon under study.

2,6-di-tert-Butyl-4- (dimethylaminomethyl)

2,6-di-tert-Butyl-4-(dimethylaminomethyl) phenol possess both the amino and hydroxyl functionality which infers diverse properties on the compound. It is an important hindered phenol derivative, its molecules interact through O—H----N hydrogen bonds to form a tetramer arranged around a twofold rotation axis (Zeng and Hou, 2007). It is toxic to aquatic organism, and also gives irritation to human skin on contact. It has been applied industrially as anti-oxidant and in lubricants and greases as additive. It is also used as vulcanizing agent in rubber processing.

Thiirane (C_2H_4S)

Thiirane also known as episulphide or ethylene sulphide is a three membered ring heterocyclic compound that belong to the organic sulphide family. In the plastic industry, anionic polymerizations of thiiranes are propagated by attack of a sulfhydryl anion from a ring-opened thiirane on another molecule of thiirane. The initiation of this process can occur by attack of an external thiolate anion. For instance, cadmium thiolates of esters of cysteine initiate the polymerization of racemic 2-methylthiirane to give optically active and highly isotactic polymer (Dittmer, 1984). Derivatives from thiirane have been incorporated into epoxy-resin composites to reduce their curing time and metal bonding strength (Dutkiewicz *et al*., 2014). Derivatives of thiirane possess mainly antimicrobial and cytotoxic activities (Mohammad, 2014).

4-bromo-2-trifluoromethyl Imidazole ($C_4H_2BrF_3N_2$)

This compound is highly halogenated. The present of free NH, Br and F groups makes it highly polar, hence infers solubility in protic solvents like water. Imidazole rings largely exist in the context of natural amino acid histidine. They also occur as a component of unnatural cyclic peptides, hence used as an ester isostere in peptidomimetic studies. Imidazoles are present in lots of alkaloids and natural products, they feature significant biological activities, with potential application as therapeutic agents for thrombosis, cancer, and inflammatory diseases. Highly effective halogen-substituted imidazole-thiosemicarbazides are also known to have anti-*Toxoplasma gondii* effects (Paneth *et al*., 2019). Due to the high presence of halogens, they can be potentially applied in the manufacturing chemical industry as flame retarding agents in products.

Vomitoxin

Vomitoxin, also known as deoxynivalenol (DON), develops when moisture is overabundant during the flowering period of plants. Deoxynivalenol is directly associated with the plant pathogens *Fusarium graminearum* (*Gibberella zeae*) and *F. culmorum*, the causative agents for Fusarium head blight in wheat and Gibberella ear rot in corn (Frobose *et al*., 2010). Vomitoxin possess the carbonyl, hydroxyl, ether and carbon to carbon double bonds functionalities. This polar groups makes the molecule highly soluble in water. There is formation of hydrogen bond between molecules to form large network of molecules with high intermolecular connections. Triacetoxy derivative of vomitoxin is known to be an acute toxic chemical.

1,8-diethoxy-9,10-Anthracenedione

This compound is a derivative of anthraquinone, it possesses the carbonyl, C=C and carboxyl functional groups. Anthraquinones are a class of natural compounds that consists of several hundreds of compounds that differ in the nature and positions of substituent groups (Schripsema et al., 1999). This class of compounds contains derivatives that consist of the basic structure of 9, 10 anthraquinone (Bajaj, 1999). Many anthraquinones are present in plants, and have been applied in various therapeutics (Hemen and Lalita, 2012). 9,10-anthracenedione derivatives inhibits respiratory sulphate reduction by pure cultures of sulphate-reducing bacteria. In the pharmaceutical industry, the natural and synthetic derivatives of 9, 10 anthraquinone are beneficial to mammals and humans as they can display antibacterial, anti-trypanosomal and antineoplastic activities (Heyman *et al*., 2009; Tarus *et al*., 2002; Velez and Osheroff, 2004).

10,10-Dichloro-9-oxa-10-sila-9,10-dihydrophenanthrene

This compound is a derivative of phenanthrene-9,10- dihydrophenantrene derivatives have shown free radical scavenging activities (Guo *et al*., 2007). Phenanthrene, a tricyclic PAH containing three fused benzene rings in angular arrangement, is a major constituent of coal derivatives and fossil fuels. Phenanthrene is one of the 16 PAHs in the list of priority pollutants compiled by the US Environment Protection Agency (Heitkamp *et al*., 1988). This compound being encountered regularly in environmental samples is not genotoxic but has a chemical structure found in several carcinogenic PAHs such as a benzo (a)pyrene.

Conclusion

The study reaffirmed both samples as separate taxon. The two species can either be differentiated using either flavonoid or anthocyanin tests. Both tests using their fronds (leaf) are rapid, cheap and potent and hence could be applied for differentiating both species anytime and across any phase of their life cycle without having to rely on their fruiting seasons. The presence of six flavonoid compounds and one alcoholic member was chemically determined as the fundamental rationale for nesting both organisms as separate taxon. The study concluded by reviewing chemistry of the six flavonoid compounds that acted discriminately between the two taxa.

Recommendation

Further research utilizing samples obtained from various eco-belts and at different times of the day should be conducted for comparison.

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