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Application of Chemometrics and HPLC Fingerprint for Species Differentiation and Authentication of the Genus *Pterocarpus*

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ABSTRACT

Pterocarpus species are well known for their nutritional and medicinal values, in order to examine the similarities or differences in the chemical profile of some common *Pterocarpus* species, four species of the genus namely; *Pterocarpus erinaceus*, *P. mildbraedii*, *P. osun* and *P. santalinoides* were analyzed using HPLC combined with Principal component analysis (PCA) and Hierarchical clustering analysis (HCA). This study aims to investigate the chemical fingerprints of the species and compare them in order to highlight the similarities in their chemical constituents. The ethanol extract of each sample was taken and filtered through a 0.45 µm millipore membrane filter and then transferred into the HPLC vial before injecting it into the HPLC machine. PCA and HCA were performed on the relative retention times and percentage peak composition. The species were chemically similar with nine (9) peaks in common, the most prominent peaks in all samples appeared at 3.26 min which corresponds to gallic acid (a known compound). Cluster analysis revealed some similar chemical variables with Gallic acid being the major compound in the *Pterocarpus* species and could be used as a marker compound for taxonomic purposes.

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INTRODUCTION

Species of *Pterocarpus* are popularly known as "Oha" in Nigeria. The genus *Pterocarpus* Jacq (Fabaceae) which comprises about 60 species widely distributed across the tropics, with around twenty (20) of them well documented in Africa (The plant list, 2013), is represented by four species in Nigeria (Gabriel & Onigbanjo, 2010). The fresh leaves sourced as vegetables; are highly recommended in any diet virtually without quantitative restriction (Osuagwe, 2008). The leaves of the *Pterocarpus* species are good sources of alkaloids, flavonoids and tannins, and can serve as antispasmodic, analgesic, anti-inflammatory and anti-oxidant agents according to previous reports. The tender leaves are eaten as vegetables in the soup while the stem bark is used in making pepper soup and the root helps in erosion control and nitrogen fixation (Ujowundu *et al.*, 2013). Fresh leaves of *P. mildbraedii* Harms contains adequate minerals and high amounts of vitamins A, B1, B2, B5, and B6 (Uchegbu & Okwu, 2012), they are used as vegetables for human, and livestock feed for goats and are recommended for consistent use for diabetics (Durugbo, 2013) while its decoction is used in the treatment of fever accompanied by diarrhea (Uchegbu *et al.*, 2013).

There is a long history and wide reports on the consumption of members of the genus *Pterocarpus* as vegetables; the leaves of *P. soyauxii* Taub. and *P. santalinoides* DC. are widely consumed as soup in the South Eastern part of Nigeria. Some tribes in the Eastern and Southern Nigeria use leaf extracts in the treatment of headaches, pains, fever, convulsions, and respiratory disorders (Ogukwe *et al.*, 2004). According to Emebu and Anyika (2011), the leaves, barks, roots and sap of *P. erinaceus* Poir. are used in the treatment of more than thirty-three (33) diseases. The bark of *P. erinaceus* is used for the treatment of gonorrhea, dysentery, tooth and mouth complications (Aladesanmi, 2007), as well as in the treatment of dysentery, menstrual disorders, anemia, gonorrhea, ringworm infections, leprosy, wounds, tumors and ulcers (Aja *et al.*, 2015). The grated root when combined with tobacco is used to treat coughs. The stem is used as fuel wood, as a woodworking material, and is useful as a nitrogen-fixing plant to improve nutrient-depleted farming land (Bosu, 2014). The crude extract of *P. osun* Craib. has been found to be useful in the treatment of chickenpox in children in the eastern part of Nigeria (Ezeokonkwo & Okoro, 2012). The antioxidant potential and the attenuation of acetaminophen-induced redox imbalance by *P. osun* were reported by Iroanya *et al.* (2010).

Adewuyi *et al.* (2014) revealed that the leaves ethanolic extract of *P. osun* has antibacterial activity. The powdered stem is used in the prevention of infections of freshly severed umbilical cord; it is also used in the treatment of rheumatism, eczema, gonorrhea, candidiasis, acne and as traditional medicines against sickle-cell disorder and amenorrhea (Durugbo, 2013). The dry leaf is also an ingredient of traditional black soap that is based on the ash of burnt cocoa pods and palm oil while the heartwood, bark and roots are pounded into a paste and used as skin cosmetics (Durugbo, 2013).

Chemotaxonomy refers to the scientific investigation of the potentialities of chemical characters for the study of circumscribed groups of plants (biologydiscussion.com). Earlier reports of the phytochemical constituents of this highly important genus have consistently focused on either one or two of the species; Gabriel and Onigbanjo (2010) screened the phytochemical properties of the stem bark extracts of *P. erinaceus*, Udaysing and Gaikwad (2011) examined the phytochemicals activity of stem bark of *P. marsupium* Roxburgh. Ndukwe and Ikpeama (2013) did a comparative evaluation of the phytochemical and proximate analysis of leaves of *P. soyauxii* and *P. santalinoides*. Nwokorie *et al.* (2015) screened the phytochemical and nutritional compositions of *P. santalinoides*, Phytochemical screening, mineral composition and in vitro antioxidant activities of *P. mildbraedii* leaves was done by Usunobun and Chinwe (2016), Raj *et al.* (2017), screened the phytochemicals and studied the antioxidant, antimicrobial, antidiabetic, anti-inflammatory and analgesic activities of extracts from stem wood of *P. marsupium* among others.

The medicinal values of the plants center on the bioactive phytochemicals they possess; these phytochemicals are responsible for the therapeutic effects of plants. Owing to the numerous therapeutic values of *Pterocarpus* species, there is a need to examine the similarities or differences in the phytochemicals of the common species. In this present study, the fingerprint pattern of four *Pterocarpus* species (*Pterocarpus erinaceus*, *P. mildbraedii*, *P. osun* and *P. santalinoides)* were not developed using the HPLC-DAD, to establish the similarities and differences in the chemical profile of the species. Marker compound(s) in the four species were not identified by cross-referencing them with a known compound. This study aims to shed more light on the phytochemistry of *Pterocarpus* species and provide a basis for the identification of *Pterocarpus* species based on their chemical compositions.

MATERIALS AND METHODS

Plant materials

Matured, freshly collected leaf specimens were used for the work. The fresh sample of *P. erinaceus* was collected from the National Parks Headquarters, Abuja, *P. santalinoides* was collected from the Millennium Park, Abuja, while *P. mildbraedii* and *P. osun* were collected from the Botanical Gardens, University of Ibadan, Ibadan, Nigeria. The plants were identified and voucher specimens were deposited in the National Institute for Pharmaceutical Research and Development Herbarium (NIPRDH), Abuja where voucher numbers were obtained for the species (Table 1).

Extraction

Cold maceration method of extraction was employed, about 0.2 g, of the dried and pulverized leaves of *P. erinaceus*, *P. santalinoides*, *P. osun* and *P. mildbraedii* were weighed into four (4) clean and well-labelled sample bottles, 10 mL of ethanol (70%) was added to each sample bottle and allowed to stand for 24 hours, the mixture was filtered into clean bottled. An aliquot of each sample was taken with the aid of a 2 mL syringe and filtered through a 0.45 µm Millipore membrane filter and then transferred into the HPLC vial before injecting into the HPLC machine.

High Performance Liquid Chromatography Analysis

The chromatographic system used includes the Shimadzu HPLC system consisting of Ultra-Fast LC-20AB prominence equipped with SIL- 20AC auto-sampler; DGU-20A3 degasser; SPD-M20A UV diode array detector (UV-DAD); column oven CTO-20AC, system controller CBM- 20Alite and Windows LC solution software (Shimadzu Corporation, Kyoto Japan); column, VP-ODS 5 µm and dimensions (150 x 4.6 mm).

The method described by Adamu *et al.* (2018) was employed. The chromatographic conditions included mobile phase solvent A: 0.2% v/v formic acid in HPLC grade water and solvent B: HPLC grade acetonitrile; mode: isocratic; injection volume 10 µL of extracts solution in the mobile phase; detection was at UV 254 nm wavelength. Gallic acid reference standard was also injected with the same method as the samples. The HPLC operating conditions were programmed to give the following: solvent B: 20% at a flow rate of 0.6 mL/min; and column oven was set to 40°C temperature. The total run time was 20 minutes.

Chemometrics and Statistical Analysis

Data transformation, mean, standard deviation and charts were performed using MS Excel. Principal component analysis PCA and Hierarchical clustering analysis HCA were carried out using the Chemometrics software package, The Math Works Incorporations' MATLAB version 4.0 for Windows and the Eigenvectors Research Incorporations' PLS_Toolbox version 6.2.

RESULTS

The result of the HPLC analysis of *P. erinaceus*, *P. santalinoides*, *P. osun* and *P. mildbraedii* were presented in Table 2 and the

Table 1: List of Species studied and their voucher numbers with GPS coordinates

S. No.	Species	Voucher number
01	*Pterocarpus erinaceus*	NIPRD/H/7064
02	*Pterotcarpus mildbraedii*	NIPRD/H/7063
03	*Pterocarpus osun*	NIPRD/H/7065
04	*Pterocarpus santalinoides*	NIPRD/H/7244

Table 2: Retention times of common peaks observed in the *Pterocarpus* species

S. No.	Retention Time (Mean±S.D)	Composition (%) *P. erinaceus*	Composition (%) *P. mildbraedii*	Composition (%) *P. osun*	Composition (%) *P. santalinoides*
01	1.48±0	-	-	-	3.991304
02	2.79±0.002	3.89743	2.565108	5.153552	3.848693
03	3.1±0	9.683219	-	-	-
04	3.26±0.21	24.82782	25.40221	35.09347	37.92676
05	3.63±0.007	1.929749	-	2.024243	2.399666
06	3.82±0.1	20.291147	17.839563	18.50369	19.6588
07	4.64±0.02	12.48146	15.87714	24.04044	5.975077
08	4.99±0.03	-	17.40495	-	5.136853
09	5.42±0.2	9.845795	-	-	9.030285
10	6.29±0.09	5.422468	8.157798	7.991483	3.0439
11	6.77±0	-	-	-	5.635496
12	6.96±0	7.253267	-	-	-
13	7.28±0.03	-	6.856103	2.90277	-
14	7.89±0.1	2.859378	-	1.337167	0.870087
15	8.32±0	-	-	0.580717	2.028273
16	9.33±0.01	-	4.27769	0.518687	0.454807
17	10.33±0.01	0.242683	1.521275	0.139777	-
18	11.44±0.2	0.01528	-	0.063071	-
19	12.83±0	1.250306	-	-	-
20	16.96±0	-	-	0.704976	-
21	17.99±0	-	-	0.945948	-
22	18.42±0	-	0.02732	-	-
23	18.6±0	-	0.033152	-	-
24	18.78±0	-	0.013414	-	-
25	18.94±0	-	0.024279	-	-

chromatograms are presented in Figures 1 to 4. The ethanol leafs extract showed the species chemical similarities. They had nine (9) peaks in common, five (5) of these peaks were major peaks present in all the samples. They appeared at retention times of 2.79 min, 3.26 min, 3.82 min, 4.64 min and 6.29 min respectively (Table 2 and Figures 1 - 4).

In addition to these peaks highlighted above, four (4) other important peaks were seen in three of the four samples analyzed. These peaks appeared at 3.63 min, 7.89 min, 9.33 min and 10.33 min respectively (Table 2). The most prominent peak in all the samples appeared at 3.26 min which when cross-referenced, corresponds to a known compound; Gallic acid (Figure 5).

Although *P. erinaceus*, *P. santalinoides*, *P. osun* and *P. mildbraedii* showed a high degree of similarities in their chemical profiles, each of the samples retained a certain level of individual uniqueness. There were some peaks peculiar to each species, in this context, the unique peaks are termed "characteristic peak" of each of the samples. *P. mildbraedii* had four (4) characteristic peaks (18.42, 18.6, 18.78 and 18.94 minutes), *P. erinaceus*, had three (3) characteristic peaks appearing at 3.1, 6.96 and 12.83 minutes, while *P. osun* and *P. santalinoides* had two (2) of such peaks (16.96 and 17.99 minutes & 1.48 and 6.77 minutes respectively). This "characteristic peak" represented a particular compound found across these species, which could be used in the authentication of the genus.

Furthermore, some of the species had some phytochemicals in common. *P. mildbraedii* and *P. santalinoides* had a similar compound occurring at the same retention time of 4.99 min. While *P. erinaceus* and *P. santalinoides* shared a peak seen at 5.42 min, *P. osun* and *P. mildbraedii* had a common peak at

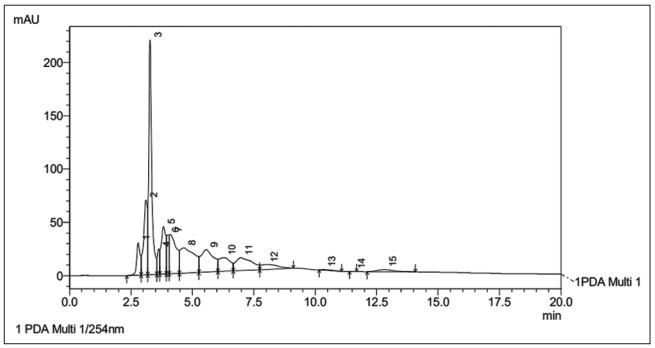

Figure 1: HPLC Chromatographic fingerprint of *P. erinaceus* showing retention times of all peaks at 254 nm. Peak 3 corresponds to Gallic acid

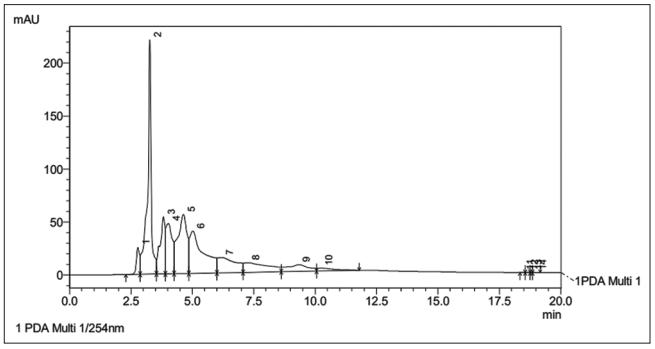

Figure 2: HPLC Chromatographic fingerprint of *P. mildbraedii* showing retention times of all peaks at 254 nm. Peak 2 corresponds to Gallic acid

7.28 min, *P. osun* and *P. santalinoides* as well had a conjoint peak at 8.32 min. However, in *P. erinaceus* and *P. osun*, a common peak was observed at 11.44 min (Table 2 and Figures 1 - 4).

Principal Component Analysis (PCA)

PCA was employed using the relative retention times and percentage compositions of the peak areas as input data to strengthen the relationship among the species in the genus (Figure 6). The data matrix for the *Pterocarpus* species was of dimension; 4 rows by 26 columns. In an auto-scaled PCA processing outcome, the dimensions were reduced with the eigenvalue suggesting two (2) principal components that retain the maximum variability of the data. The first principal component (PC1) and second principal component (PC2) accounted for 51.05 % and 26.23% cumulative variance captured by the PCA model. The first two principal components PC1 and PC2 provided a convenient visual aid for identifying homogeneity in the data sets.

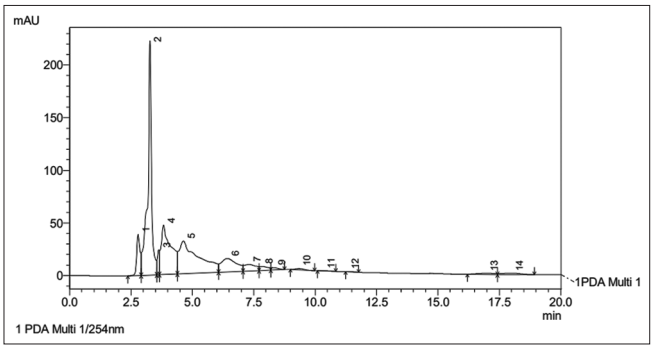

Figure 3: HPLC Chromatographic fingerprint of *P. osun* showing retention times of all peaks at 254 nm. Peak 2 corresponds to Gallic acid

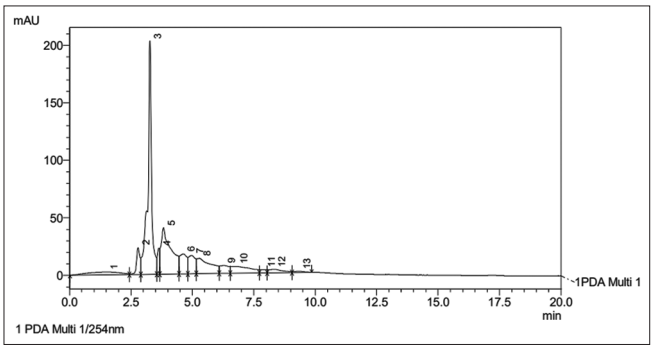

Figure 4: HPLC Chromatographic fingerprint of *P. santalinoides* showing retention times of all peaks at 254 nm. Peak 3 corresponds to Gallic acid

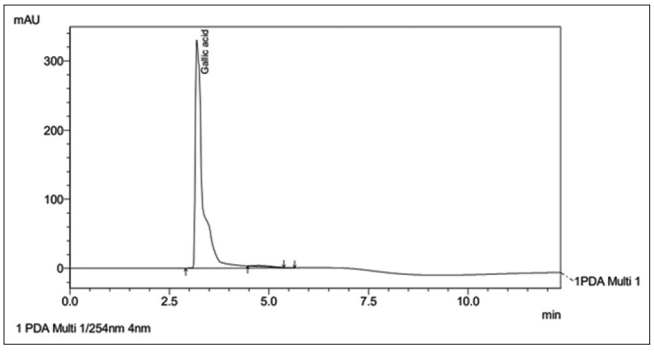

Figure 5: HPLC Chromatogram of Gallic acid reference compound at 254 nm

The scatter points diagram showed one main group and three subgroups, these were marked as group A (*P. osun)*, group B (*P. mildbraedii)* and as a separate category, group C contained *P. erinaceus*, and *P. santalinoides* (Figure 7).

The PCA showed a high degree of similarity in the chemical profiles of the species *Pterocarpus*, with a little degree of variation (Figure 7). *P. erinaceus* and *P. santalinoides* were very similar, therefore they could be substituted and sometimes misidentified. In fact PCA score plot of PC1 versus Q Residuals (Figure 8) recognised *P. erinaceus* and *P. santalinoides* as more or less the same species.

Hierarchical Clustering Analysis (HCA)

A hierarchical clustering analysis of the four *Pterocarpus* species was carried out to study the degree of resemblances and differences based on the relative retention times and percentage (%) peak area compositions of all peaks observed in the chromatographic analysis (Figure 9). The samples were again classified as one main cluster (Figure 9) with three groups, further establishing a chemical variable relationship in all the examined *Pterocarpus* species. At a high phenon level of 70% similarity, the taxa splitted into two main groups. Group A comprised of *P. osun* and *P. milbraedii*, while *P. erinaceus*, & *P. santalinoides* made up the group B.

At a 10% average phenon level of similarity, group B was further divided to contain *P. erinaceus*, & *P. santalinoides* (cluster). This cluster shared the closest resemblances in chemical compositions than the other species, followed by a single cluster *P. osun* which showed differences in chemical variables, (70% phenon level). *P. mildbraedii* and *P. osun* were slightly different in chemical composition compared to *P. erinaceus*, and *P. santalinoides* clusters.

The kinship in the cluster of *P. erinaceus*, and *P. santalinoides* (Figure 9) affirmed their use as a close substitute for each other in terms of their chemical composition and substitutability for herbal medicine preparations.

The results of PCA were similar to that of HCA. Both results validated each other and provided more references for the quality evaluation and authentication of the genus *Pterocarpus*.

DISCUSSION

Phytochemical screening has been used in all levels of classification. A comprehensive chemical analysis of the flower buds of five Lonicera species was carried out by Li *et al.* (2018) and Ibrahim *et al.* (2014) established it as a useful tool to chemically profile Loranthaceae species. They cautioned against uncontrolled use of the taxa in phytomedicine as all species irrespective of the host and locality exhibit chemical variations, by implication; same species of Loranthaceae from different hosts will exhibit different phytochemicals.

Pterocarpus species showed a high degree of morphological similarities and relationships that either of them is easily

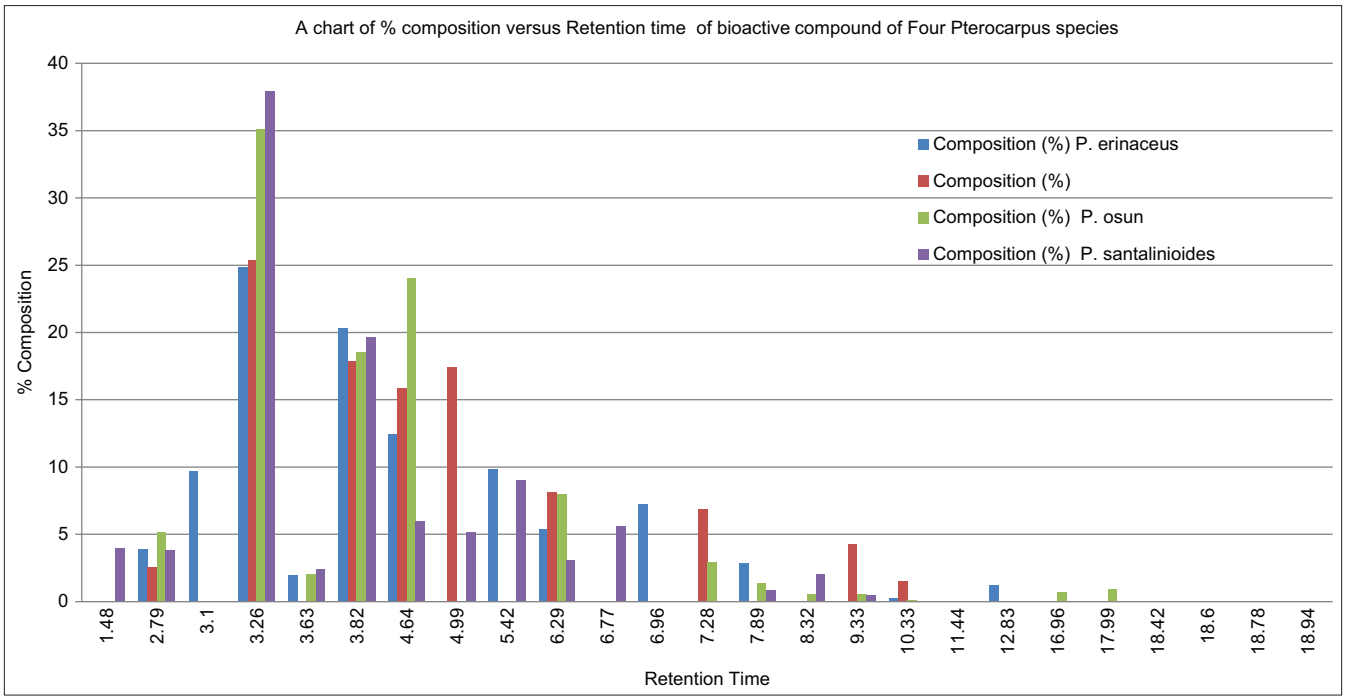

Figure 6: Bar chart showing percentage peak composition in *P. erinaceus, P. santalinoides, P. osun* and *P. mildbraedii*

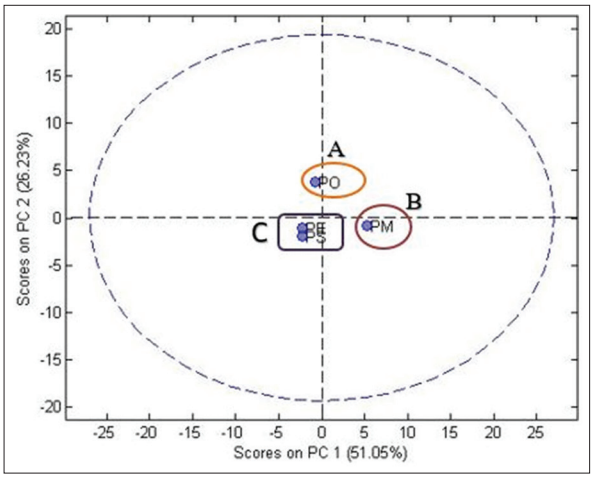

Figure 7: Score plot of all data set showing scatter points of PC1 versus PC2

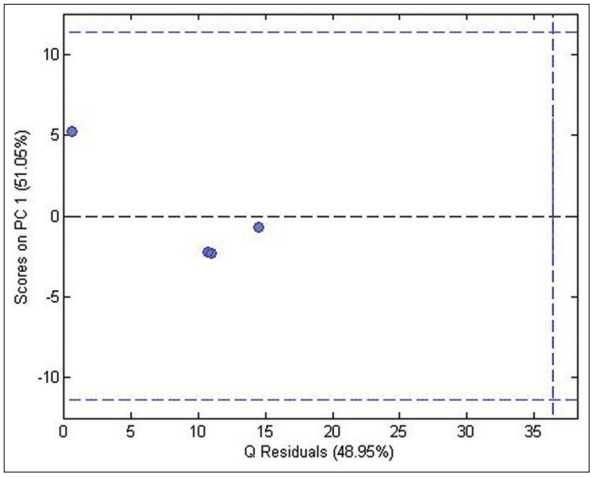

Figure 8: Score plot showing PC1 versus Residual of all data set

substituted for use in vegetables and herbal medicine recipes. Without reproductive parts, they are frequently misidentified. Therefore, a simple and reliable chemical analytical method was employed to profile the phytochemical similarities that made them an easy substitute for one another either as food, ethnobotanical preparations, misidentification in the field, or the herbarium. HPLC in combination with the Chemometric technique was used to bring out taxonomic information in four (4) species of *Pterocarpus*. The method was very simple, highly precise, accurate, and proved reliable in the identification and authentication of phytochemicals in *Pterocarpus* species.

Chromatographic observation of the species showed a great degree of resemblance, close examination was required to distinguish the chromatographs from one another. This probably confirms further the observed similarities in morphologies. The most prominent compound in *P. erinaceus*, *P. santalinoides*, *P. osun* and *P. mildbraedii* is gallic acid. Hence can be referred to as a marker compound for *Pterocarpus* species. Deepa *et al.* (2015) isolated gallic acid from the stem bark of *Pterocarpus marsupium* Roxb., Manish *et al.* (2009) and Su *et al.* (2014), reported the isolation of several other phenolics, flavonoids and other compounds from *P. marsupium* and *P. soyauxii*. However, this is the first time gallic acid is being reported across the taxa (*P. mildbraedii*, *P. osun*, *P. erinaceus*, and *P. santalinoides*).

The study proved that the four species are quite phytochemically similar. However, despite the high degree of similarities among the samples, each sample still maintained their individualities possessing phytochemicals which were peculiar to each species.

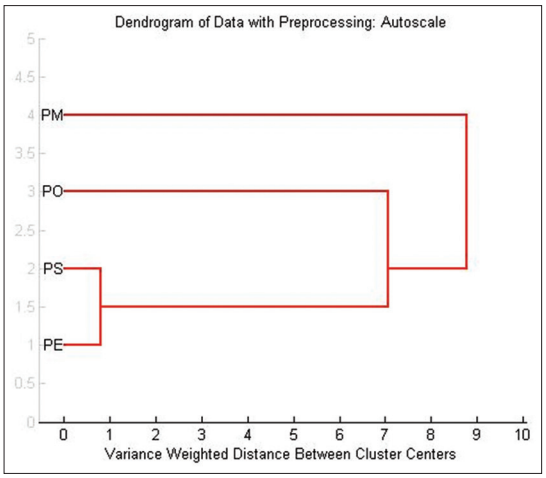

Figure 9: Dendrogram of *Pterocarpus* species

Both PCA and HCA confirmed our observation that *P. erinaceus* and *P. santalinoides* could be used as a close substitutes for each other.

CONCLUSION

From the results obtained from this work, chromatograms of the leaves of the examined species showed the chemical profiles were quite similar, with gallic acid being the most prominent phytochemical occurring alongside some other trace elements. These abundant phytochemicals confirm the medicinal relevance of these species in ethnomedicine. Furthermore, owing to their wide consumption as vegetables, the leaves contain nutrients and minerals important in the human diet.

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