Application of Phytochemical Protocols in Authenticating Six Morphologically Identical Mimoisoidea Members

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Abstract
Species authentication is fast becoming an issue of concern to researchers in biological, medical and chemical sciences. Over reliance of expert recognition and the use of voucher specimens in herbaria is fraught with inconsistencies and avoidable pitfalls. Like in every human endeavor, standardization is essential. Since plant species act as natural sink for chemical products, it is imperative that given species will elicit specific responses when subjected to standard phytochemical test. *Parkia biglobosa* (Jacq.), *Tetrapleura tetraptera* (Schum. &Thonn.), *Albizia adianthifolia* (Schum.), *Pentaclethra macrophylla* (Benth.), *Leucaena leucocephala* (Lam.),and *Prosopis africana* are six morphologically identical members of mimoisoidea with huge and varied indigenous uses but whose taxonomical authentication has been extremely challenging. These wild species were collected in triplicates at three ecologically distinct areas in Cross River State over a three year period and subjected to eleven standard phytochemical tests. The result showed only flavonoid and triterpene tests as discriminatory enough for the authentication of the six species. The expression of a yellow orange coloration within minutes authenticates *Parkia biglobosa* while a yellowish brown colour authenticated *Prosopis africana.* Triterpene tests yielded brown coloration with *P. macrophylla* and green colour with *L leucophylla*. The formation of a clear layer above a brown one using triterpene test authenticates *Tetrapleura tetraptera*, while a red ring in-between two layers authenticates *Albizia adiantifolia.* The results were same for all individuals of same species analyzed regardless of ecological sites where the species were collected despite statistical significant differences in soil conditions. The study showed the use of phytochemical protocols as potent enough for species authentication and recommends concerted research efforts in this direction.

Key words: Fabaceae, Nomenclature, Phytochemical test, Plant species authentication.

1.1**Introduction**

Plant identification has been done over the years by plant taxonomists, using their morphological characters.[1, 2 &3]. But, the use of morphological characteristics is not reliable as widely diverge taxons may be morphologically identical. Worst still, environmental factors may mimic otherwise similar species to exhibit obvious variations. This poses challenges to accurate species authentication. Though plant taxonomists may adopt other taxonomic lines of evidence, these may either be time consuming, expensive or unavailable when the need arises. Where one is available and affordable, it maybe time consuming and error prone [4 & 5]

Authenticating six species of mimoisoidea with huge indigenous uses in Nigeria using vegetative characters has been fraught with grave difficulties. Leaf morphologies of *Parkia biglobosa* (Jacq.),

Tetrapleura tetraptera (Schum. &Thonn.), *Albizia adianthifolia* (Schum.), *Pentaclethra macrophylla* (Benth.), *Leucaena leucocephala* (Lam.),and *Prosopis africana* (Guill. &Perr.)) are so morphologically identical for even well trained taxonomist to authenticate, often relying on floral characters which may or may not be available all the time. Worst still, all do not enjoy same flowering and fruiting sequence. Some that do, also share similar inflorescences and fruit types.

Authenticating species using secondary metabolites could prove helpful. Plants as we are aware, are biosynthetic laboratories for multitude of compounds like alkaloids, glycosides, saponins, steroids, resiannins, flavonoids, sesquiterpenes and lactones . Application of this knowledge in species delimitation technically referred to as chemotaxonomy is premised to a large extent on the specificity of a taxon exhibiting distinct secondary metabolites. This specificity is utilized in delimiting plant taxa [6]The chemical structure of secondary metabolites and their biosynthetic pathways is often specific and restricted to taxonomically related organisms and hence useful in classification as it relies on the [7]chemical similarity of taxon. It has found wide usage in plant systematics as evident in several works.
discriminated between *Prosopis africana* and *Anacardium occidentale* using saponins, flavonoids, alkaloids and anthraquinones contents while[8] utilized alkaloid as a discriminatory evidence among members of *Uncaria*. [9] and [10]; applied phenolic, tannin and saponin compositions to establish differences among members of *Penthacletra.* Members of Urticaceae, Fagaceae, Anarcadiaceae, Plumbaginaceae, Linaceae, Polygonaceae, and Adoxaceae were delimited using phenols and flavonoids[11] just as[12] applied finger printings of flavonoids, saponins, tannins, alkaloids and steroides, among members of *Ficus*.[13] applied Diterpene alkaloids profiling in screening seven (7) species of *Spiraea.*[14] on the other hand expressed the usefulness of alkaloid, saponin and glycoside in delimiting members of *Leucaena* while[15] separated *Erythrina* and *Prosopis* members using tannin, saponin and anthraquinone and [16] applied the chemical structure of secondary metabolites in species classification. [17] expressed the usefulness of phytochemicals delineating species of *Loranthus* leaves from those of *Parkia* as [18] established taxonomic relationships between *Tetrapleura tetraptera* and *Piper quineense* using phenol, glycoside, alkaloide and flavonoids compositions. [19] used Anthraquinones, triterpenes, indole alkaloids, flavonoids, phenolics and terpenoids as chemotaxonomic markers to delineate members of Rubiaceae. [20] applied steroids and flavonoids characters to establish taxonomic evidence among three (3) species of *Stachytarpheta* while [21]established chemotaxonomic relationships among members of Loranthaceae using alkaloids, flavonoids, phenolics, saponins, tannins, glycosides, terpenes, volatile oils, resins, phytobetalins, sterols, and Anthraquinones. [22] demarcated five species of *Rauvolfia* with the use of flavonoids as chemotaxonomic marker. Furthermore, [23], established boundaries among members of *Hedychium* using phenolics, flavonoids, reducing sugars, proteins, steroides, triterpenoids, cardiac glycosides, tannins and saponins as chemotaxonomic markers. [24] *established* 36 taxonomic sections, from 469 species of the genus *Hypericum* L using various secondary metabolites. [25] utilized content and concentrations of tannins, flavonoids, saponins, steroides, and essential oils to differentiate species of *Chamaerop*. [26] utilize glycoside and flavonoid contents in establishing relationships among members of *Albizia* while [27] applied flavonoid types in discriminating members of cucurbitaceae. [28], showed the importance of tannins, flavonoid, anthraquinone alkaloid and saponin while [29] included phenols as discriminatory compounds among members of *Penthacletra*. [30] used variations in flavonoid contents in the taxonomy of *Pteridium* aquilinum (L) Kuhn. Taxonomic boundaries among members of *Solanum nigrum* were established using alkaloid composition while anthraquinones was used in delimiting members of Asphodeloideae [31]. [32] used terpenoids to establish taxonomic affiliation among members of *Cannabis*. [33] used alkaloids as

discriminatory character to catalogued 229 herbarium samples of Rubiaceae. [34] used phenol to established demarcation between *Citrullus lanatus*, *Cucumeropsis mannii* and *Lageneria siceraria*. [35] applied Flavonoids and phenolics in delimiting several members of Asteraceae, Solanaceae, Boranginaceae, Onagraceae, Rubiaceae, Gentianaceae, Rosaceae, Fabaceae and Lamiaceae.

Regrettably, the huge knowledge of phytochemistry espoused here is yet to be applied to establishing periodicity (akin to periodicity in Chemistry) and authenticate plant taxa. This draw back fuels assignment of wrong scientific names made possible by poorly trained taxonomists, over reliance on expert recognition and valuable man hour lost in searching herbaria that may not contain species of interest. Since each active ingredient is chemically distinct, it infers that a characteristic reaction is possible for each also. The reactions can be visible in form of colour and rate of colour change and precipitates, among other qualitative observations. This novel approach would standardize species authentication process, reduce taxonomic revisions and misplaced taxon. It is in the light of taxonomic challenges so expounsed herein and the inherent benefit to science and the allied medical profession that this research aims at establishing periodicity within six morphological identical species in mimoisoidae sub family.

2.1 Materials/Equipment

Beakers, Blender, Burette, Conical flask, Cotton wool, Electronic weighing balance, Filter paper, Forceps, Funnel, Glass Petri dishes, Glass rod stirrer, Hand gloves, Laboratory coat, Light source, measuring cylinder, nose mask, note pad/biro, Paper masking tape, Plant specimens, Reagent bottles, Stop watch timer, Test-tube and test tube racks. Reagents: Alcohol, chloroform, acetic anhydride, glacial acetic acid, sulphuric acid (H_2SO_4), distilled water, hydrochloric acid (HCL), ammonia (NH_3), sodium hydroxide (NaOH), Feric chloride and cupper acetate.

2.2 Collection of Plant Materials

The vegetative parts of the candidate plant species were collected in triplicates from the wild from different habitats within Cross River State as shown in Table 1. The samples were authenticated in the taxonomy unit of the Department of Plant and Ecological Studies (PES), University of Calabar. A voucher specimen for each was deposited in the Herbarium. Plate A – F are pictorial illustrations of the investigated species.

Plate A: *Pentaclethra macrophylla* Benth

Plate B: *Tetrapleura tetraptera* (Schum & Thonn) Taub

Plate C: *Leucaena leucocephala* (Lam) DE WIT.

Plate D*: Albizia adianthifolia* (Schum.) W.F. Wit

Plate E: *Parkia biglobosa* (Jacq.)G. Don

Plate F: *Prosopis africana* (Guill & Perr.) Taub

Plate A - F : Images of investigated species

2.3 Preparation of Plant Material

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 sample leaves were grinded to uniform powder using an electric blender [36]. The fine powders were then packed separately in zip-lock bags to avoid the effect of humidity and then stored at room temperature [37].

2.4 Preparation of Extracts for Preliminary Phytochemical Test

Ten (10) g of the candidate plant powder was weighed using a chemical balance (110c), transferred to a rubber bottle and socked with 150 ml absolute ethanol and incubated for about 24 Hrs. for maximum extraction [38] and filtered, first through a Whatmann filter paper No. 41 and then through cotton wool to obtain a clear solution [39]. The solutions were stored and used for qualitative test.

2.5 Phytochemical Test

Preliminary phytochemical test was conducted on the plant extracts following the standard methods for phytochemical screening described by [40],[41],[42] ; [43]; [44]; [45]; [46] were carried out on the candidate plant extracts and the resultant colour change, formation of precipitates, and foam, characteristic smell and time required for colour change in the extracts were observed and noted. The test conducted were reviewed to be present in the families or genus to which the candidate species belong.

Test for Flavonoids (Basic)

Three (3) ml of extracts were treated with 3 drops of aqueous NaOH followed by 3 drops of 40% HCl and observed for changes.

Test for Coumarins

Three (3) ml of the extract was treated with 3 ml of 10% NaOH and observed for changes.

Test for Flavonoids (Acidic)

2 ml of plant extract was treated with 4 drops of concentrated sulphuric acid and observed for changes.

Test for Tannins

To 1 ml of plant extract, 2 ml of 5 % ferric chloride was added.

Test for Phenols

To 1 ml of the extract, 2 ml of distilled water followed by 5 drops of 10% ferric chloride was added and observed for changes.

Anthocyanins

To 4 ml of extract was treated with 2 ml of HCl followed by 2 ml of Ammonia and observed for changes.

Test for glycosides

To 2 ml of plant extract, 1 ml of glacial acetic acid and 5% ferric chloride was added, 3 drops of concentrated sulphuric acid was added and observed for colour change.

Tests for Triterpenes

Chloroform solution of the extract with few drops of acetic acid and one ml concentrated sulphuric acid.

Tests for Diterpenes

To 2 ml of extracts, 1 ml of aqueous solution of copper acetate was added and observed for colour change.

Test for steroids and phytosterols

To 1 ml of plant extract, equal volume of chloroform and 3 drops of concentrated sulphuric acid were added and colour changes were noted

Test for saponins

To 1 ml of plant extract, 5-10 ml of distilled water was added and shaken for 2 min observing the formation of foams. Tableau Colour Palettes was used for observation

3.0 Results and Discussion

The results of phytochemical tests conducted to authenticate one species from the other are presented in Table 1.

Table 1 : Results of Phytochemical tests on plant samples

S/N	Tests	Species	Initial colour	Final colour	Duration for colour change (Secs.)
1	**Flavonoid test (basic)**	*P. biglobosa*	Green	Yellowish orange	3
		T. tetraptera		Green	16
		A. adiantifolia		Green	4
		P. macrophylla		Green	3
		L. leucocephala		Green	2
		P. Africana		Yellowish brown	5
2	**Coumarins test**	*P. biglobosa*	Green	Yellow	3
		T. tetraptera		Green	10
		A. adiantifolia		Green	5
		P. macrophylla		Yellowish green	10
		L. leucocephala		Green	2
		P. Africana		Dark brown	6
	Flavonoids test (acidic)	*P. biglobosa*	Green	Orange	2
		T. tetraptera		Yellowish orange	4
		A. adiantifolia		Yellowish green	4
		P. macrophylla		Yellowish green	6
		L. leucocephala		Yellowish green	2
		P. Africana		Yellow	4
	Tannin test	*P. biglobosa*	Green	Greenish black	2
		T. tetraptera		Greenish black	60
		A. adiantifolia		Greenish black	6
		P. macrophylla		Greenish black	3
		L. leucocephala		Green	5
		P. Africana		Greenish black	3
	Phenol test	*P. biglobosa*	Green	Dark green	1
		T. tetraptera		Light green	5
		A. adiantifolia		Green	5
		P. macrophylla		Greenish black	5
		L. leucocephala		Green	3
		P. Africana		Greenish black	4
	Anthocyanin test	*P. biglobosa*	Green	Brown	2
		T. tetraptera		Brown	2
		A. adiantifolia		Brown	4
		P. macrophylla		Brown	10
		L. leucocephala		Brown	2
		P. Africana		Dark brown	8
7	**Glycoside**	*P. biglobosa*	Green	Green	2

	test	*T. tetraptera* *A. adiantifolia* *P. macrophylla* *L. leucocephala* *P. Africana*		Yellowish green Brown Brown Yellowish green Light brown	2 4 8 1 5
8	**Tri-terpene test**	*P. biglobosa* *T. tetraptera* *A. adiantifolia* *P. macrophylla* *L. leucocephala* *P. Africana*	Green	Brown band in between two layers A clear layer up and a brown layer under A red ring in between two layers Brown Green Red ring between two layers	7 10 5 7 2 5
9	Diterepene test	*P. biglobosa* *T. tetraptera* *A. adiantifolia* *P. macrophylla* *L. leucocephala* *P. Africana*	Green	Green Green Greenish blue Green Green Blue	5 2 3 5 2 7
10	Steroids and phytosterols test	*P. biglobosa* *T. tetraptera* *A. adiantifolia* *P. macrophylla* *L. leucocephala* *P. Africana*	Green	Brown with ring Bluish green Brown with ring Brown without ring Brown with ring Brown with ring	3 10 60 8 2 5
11	Saponin test	*P. biglobosa* *T. tetraptera* *A. adiantifolia* *P. macrophylla* *L. leucocephala* *P. Africana*	Green	Green + foam	120

The results indicated that *Parkia biglobosa* which yielded a yellow orange between 3-5 minutes can be authenticated by the use of flavonoid test.

P. africana which yielded yellowish brown colour in same duration as P biglobosa can be authenticated using flavonoid test.

In the same vein triterpene test is useful in the authentication of *P. macrophylla* on the production of brown colour.

The production of green coloration in about 5 seconds with triterpene test was discriminatory enough to authenticate L leucophylla.

The formation of a clear layer above a brown one after about 10 seconds for the experimental material using triterpene test authenticates Tetr*apleura tetraptera* while a red ring in-between two layers after about 5 seconds for another plant sample authenticates *Albizia adiantifolia.*

4.0 Discussion

Plants as we are all aware are reservoirs of chemical compounds. The expression of similar vegetative morphological characters in all the species could be due to Gallic and Chlorogenic acid they contain (Ebigwai et al in press). Same reason could also apply to the inability of diterpene, coumarin, saponin, steroid and phytosterol tests to authenticate each species.

However, the capacity of any phytochemical test to discriminate a given species from another could best be explained in part by the occurrence of some active ingredients solely in that species. For instance, profiling of the active ingredients in all six species using HPLC and GCMS (Ebigwai et al in press) revealed the unique occurrence of thirty-six, eighteen, twenty-five, forty-nine, fifty-three and twenty-one phytochemicals in *Parkia biglobosa*, *Tetrapleura tetraptera*, *Albizia adianthifolia*, *Pentaclethra macrophylla*, *Leucaena leucocephala and Prosopis africana respectively.*

The more striking similarities in leaf size and presence of minute hairs in *Parkia biglobosa*, *Prosopis africana and Tetrapleura tetraptera* [47] could be owed to the common occurence of Neophytadiene, a sesquiterpenoids. The occurence of Tetramethyl-2-hexadecen-1-ol in *Parkia biglobosa*, *Leucaena leucocephala and Tetrapleura tetraptera* could best be explained by characters common to these three species which among them is the possession of glabrous leaflets. Similarly, the presence of Trichlorophenyl propenoate in *Leucaena leucocephala, Prosopis africana* and *Pentaclethra macrophylla only* could be among factors governing the expression of glandular structure at the leaf bases. More so, the possession of glabrous/glandular leaflets with rounded apex is a character common to *Albizia adianthifolia* and *parkia biglobosa among the species under investigation. Thus, the occurence of* Naphthoquinone,and tetrahydroxy in these species only could be a factor in the expression of stellate hairs/glandular leaflets while 3-Nonen-1ol may also play a role in the expression of similar characteristics in *Tetrapleura tetraptera* and *Pentaclethra macrophylla.*

5.0 Conclusion

The research has shown that plant authenticating can be standardized using phytochemical test, similar to qualitative tests in chemistry. The study also showed that same result was achieved for a given species regardless of soil types. The ability of only flavonoid and tritepene tests in authenticating these members of Mimosoidea was curious though revealing.

6.0 Recommendations

Similar studies should be encouraged while the discriminatory mechanisms inherent in the flavonoid and triterpene tests is worth further investigation.

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