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PHYTOCHEMICAL AND MICROSCOPICAL EVALUATION OF *DESMODIUM VELUTINUM* (P. BEAUV.) DC (PAPILIONACEAE) USED FOR PAIN MANAGEMENT IN LOKOJA, KOGI STATE, NIGERIA

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ABSTRACT
Microscopical examination was conducted using powdered samples, transverse and longitudinal sections of the leaves of Desmodiumvelutinum. Both quantitative and qualitative studies were carried out on the leaves using standard methods.The leaf powder of D.velutinum was cleared using chloral hydrate and little quantity of it was mounted on a clean slide using dilute glycerol and observed under the compound microscope for the presence of cell inclusions such as cellulose, starch, oil, tannins, and calcium oxalate crystals. Thin layer chromatography was conducted using prepared silica gel plates.The methanol extract was spotted at about 1cm from the bottom end of the plate using a capillary tube. The plates were developed in the glass tank while closed. Thereafter, the plates were removed, the solvent front marked and dried in an oven at 105^{0}C for about 3mins. Each chamber contained plates, some for ultraviolet examination and others sprayed with different reagents, observed, and sometimes heated before observation.The microscopic examination of the leaf powder of Desmodiumvelutinum(Papilionaceae) and thin section revealed various anatomical features of the plant. The epidermal cells were cuboidal in form. Anomocytic type of stomata which range from 10-12µm long and 6-8µm wide were observed only on the lower epidermis. Calcium oxalate crystals were observed in between veins in fresh leaf and numerous unicellular covering trichomes were observed all over the epidermis. The thin layer chromatography(TLC) of the leaf powder of the plant revealed the presence of tannins, steroids, flavonoids and terpenes but absence of alkaloid.

INTRODUCTION

Man has used plant drugs for health care delivery over the centuries; disease remedies from plants sources for mankind are as old as human history and are still in use to date. It is estimated that about 75% of useful bioactive plant derived pharmaceuticals used globally are discovered by systemic investigation (Tomoko *et al*., 2002). Traditional medicine is defined by the World Health Organization (WHO) as "the total combination of methods and practice, whether explicable or inexplicable, used in the diagnosis, prevention or elimination of mental, physical or even social diseases (WHO, 2001). The use of traditional medicine in various therapies by indigenous populations all over the world cannot be over emphasized as 80% of the world's population depends on it (WHO, 2002). With the poor economic status of many underdeveloped countries, this form of medical practice is worthy of consideration. This is because it is cheaper, more affordable, more acceptable and easily accessible when compared to orthodox form (Sofowora, 2008).However, it has the following challenges in the form of criticisms, shortcomings and inadequacies leveled against traditional medicine such as bad record keeping as well as its vulnerability to medical error in the areas of diagnosis and prescription as stated by the orthodox practitioners (Elujoba, 2003). Many herbal preparations are being prescribed as analgesic in traditional literature. The search for new analgesic agents from the huge array of medicinal plants is intensifying. Such taxa may hold potential for the discovery of novel therapeutic agents capable of suppressing, reducing or relieving pain (Anilkumar, 2010). The need for a systematic approach to the use of the locally available medicinal plants as tools in drug development has been recognized. It is obvious that the plant kingdom offers a better opportunity of providing useful medicinal compounds (Gill, 1992). In modern times, the active ingredients and curative actions of medicinal plants were first investigated through the use of European scientific methods. The most important ingredients present in plants include alkaloids, terpenoids, steroids, phenols, glycosides, tannins etc (Iauk*et al*., 1993). The aim of this research was to conduct phytochemical andpharmacognostic evaluation of the leaf of *D. velutinum*.

MATERIALS AND METHODS

Collection and Preparation of Herbarium Specimen

The method described by Francis (2005) was adopted. The plant sample was collected by the herbalist who uses it for pain remedy. The plant was thereafter prepared according to standard procedures of herbarium specimen preparation and preservation as follows; both flowers and fruits were left intact, clean cut of the stem was made using a secateurs and the specimen was tagged using Jeweller's tag,name or initials and a unique collection number on one side and date and site number on the other side were recorded, unnecessary twiggy shoots were cut away, the specimen was flattened out using a press and straps, the site, habitat, habit and flower colour were recorded, the sample was made pressed and dried quickly. Photographs of the plant species was also made so as to facilitate its identification process. Final identification and authentication with voucher's numbers were made at the Herbarium unit, Department of Biological Sciences, Ahmadu Bello University, Zaria with the help of the Taxonomistof the unit, MrMusa Muhammad. Voucher specimen was deposited for future reference. The plant parts prescribed for therapy were collected in considerable quantity for preliminary screening and biological studies.

Microscopical Examination of Leaf

The powdered samples, transverse and longitudinal sections of the leaves of *Desmodiumvelutinum* were used for this study. Both quantitative and qualitative studies were carried out on the leaves using standard methods.

Qualitative Leaf Microscopy

Thin longitudinal and transverse sections of the leaf were cut and cleared using chloral hydrate solution. The section was placed on a clean slide. A few drops of dilute glycerol was added and covered with a clean cover slip. The slide was observed under a compound microscope. The nature of epidermal cells, types of stomata, veins and trichomes as well as the arrangement of the various plant tissues were noted (Evans, 2009).

Quantitative Leaf Microscopy

Quantitative microscopy was carried out on the anatomical section and powdered sample of the leaves based on the method outlined in Evans (2009).

a) Stomatal Number

Fragments of leaf from the middle of the lamina were cleared with chloral hydrate solution. Using a camera lucida and a stage micrometer, a clean white paper was divided into squares of 1mm x 1mm using (x10) objective. The stage micrometer was then removed and replaced by the cleared preparation of the leaf. The number of stomata was counted by indicating it on the paper by a small cross. The total number of stomata in one square of 1mmx1mm was then determined (Evans, 2009).

b) Stomatal Index

Pieces of leaf were cleared with chloral hydrate solution, mounted in dilute glycerol and viewed under the microscope using 4mm (x40) objective. The number of stomata divided by summation of epidermal cells and stomata multiplied by 100% gives the stomata index.

(c)Veinlet Number, Veinlet Termination Number, Veinislet Number

Fragments of leaf from the middle of the lamina were cleared with chloral hydrate solution. Using a camera lucida and a stage micrometer, a clean white paper was divided into squares of 1mm x 1mm using (x10) objective. The stage micrometer was then removed and replaced by the slide of the cleared preparation of the leaf. The number of veinlet, veinlet termination and veinislet were counted and recorded accordingly.

Chemomicroscopic Examination

The leaf powder of *Desmodiumvelutinum* was cleared using chloral hydrate and little quantity of it was mounted on a clean slide using dilute glycerol and observed under the compound microscope for the presence of cell inclusions such as cellulose, starch, oil, tannins, and calcium oxalate crystals according to the method outlined in Evans (2009).

(a)Test for Cellulose

Cleared powdered sample of leaf (0.1g) was mounted in iodine solution followed by 66% sulphuric acid. A blue colour indicates the presence of cellulose.

(b) Test for Tannins

Cleared powdered sample of leaf (0.1g) was mounted in ferric chloride solution on a clean slide. A dark blue or dark green colour indicates the presence of tannins.

(c) Test for Starch

Cleared powdered sample of leaf (0.1g) was mounted in iodine solution. A blue colour indicates the presence of starch.

(d) Test for Calcium Oxalate Crystals

Cleared powdered sample of leaf(0.1g) was mounted on a clean slide using dilute glycerol. Calcium oxalate crystals, if present, are seen as bright structures of definite shapes and sizes. After addition of a few drops of concentrated hydrochloric acid and viewing under the microscope, the disappearance of the crystals without effervescence confirms the presence of calcium oxalate crystals.

(e) Test for Oil

Cleared powdered sample of leaf (0.1g) was mounted in Sudan (IV) reagent on a slide and warmed. A pink colour in any of the structures indicates the presence of oils.

Thin Layer Chromatography (TLC) of *D.velutinum* Leaf

Extraction of the leaf of *D.velutinum* for chromatographic analysis

The method described by Eloff, (1998) was adopted. The leaf powder of the plant (0.5g) was extracted using 5ml methanol under vigorous shaking and the insoluble residue was removed by centrifugation for five minutes. The procedure was repeated twice and the acetone was removed from the combined extracts under air stream at room temperature. This fresh extract was used for thin layer chromatographic (TLC) analysis.

Solvent systems and chromatography

Enough solvent system was prepared and poured into a glass tank and allowed to equilibrate for 1-2 hours (Kotz and Eloff, 2002).

Benzene - ethanol - ammonia (9:1:0.1)

Chloroform - ethylacetate - formic acid (5:4:1)

Ethylacetate - methanol - water (10:1.35:1).

The second solvent system gave the best separation, and so was finally used.On a prepared silica gel plate, the methanol extract was spotted at about 1cm from the bottom end of the plate using a capillary tube. The plates were developed in the glass tank while closed.Thereafter, the plates were removed, the solvent front marked and dried in an oven at 105^0C for about 3mins. Each chamber contained plates, some for ultraviolet examination and others sprayed with different reagents, observed, and sometimes heated before observation.

Spraying

The methods described by Touchstone and Wiley (1992) and Harborne (1998) were adopted. Some plates were viewed under 360nm ultraviolet light the different colours that developed were observed. Spraying reagents for visualizing components were also prepared fresh. Chromatograms were carefully heated at 105^0C until optimal colour development.Recording chromatogram under UV light was done with the aid of a digital camera.

1. Test for Alkaloids:

The chromatograms were sprayed with Dragendorff's reagent (0.11g KI+0.18g Bismuth subnitrate dissolved in 20ml acetic acid, made up to 100ml with distilled water). Appearance of a red or orange colour indicates the presence of alkaloids.

2. Test for Tannins:

(a) The chromatograms were sprayed with ferric chloride solution. Appearance of a dark blue or dark green colour indicates the presence of phenolic compounds including tannins.

(b) The chromatograms were sprayed with bromine water. A dull yellow spot indicates the presence of tannins.

3. Test for Steroids:

The chromatograms were sprayed with 0.5g vanillin in 20ml ethanol and 80ml sulphuric acid. Presence of steroids would be indicated by a purple colour.

4. Test for Flavonoids:

The chromatograms were sprayed with 1% ethanolic solution of aluminum chloride and viewed under ultraviolet (UV) light (360nm). The presence of flavonoids would be indicated by a yellow fluorescence.

5. Test for Terpenes:

The chromatograms were sprayed with 0.5ml P-anisaldehyde in 50ml ethanol and 1mlsulphuric acid. Appearance of a green colourcomfirmed the presence of terpenes.

RESULTS

Microscopical Examination of *D.velutinum*Leaf

Pates of Photomicrographs

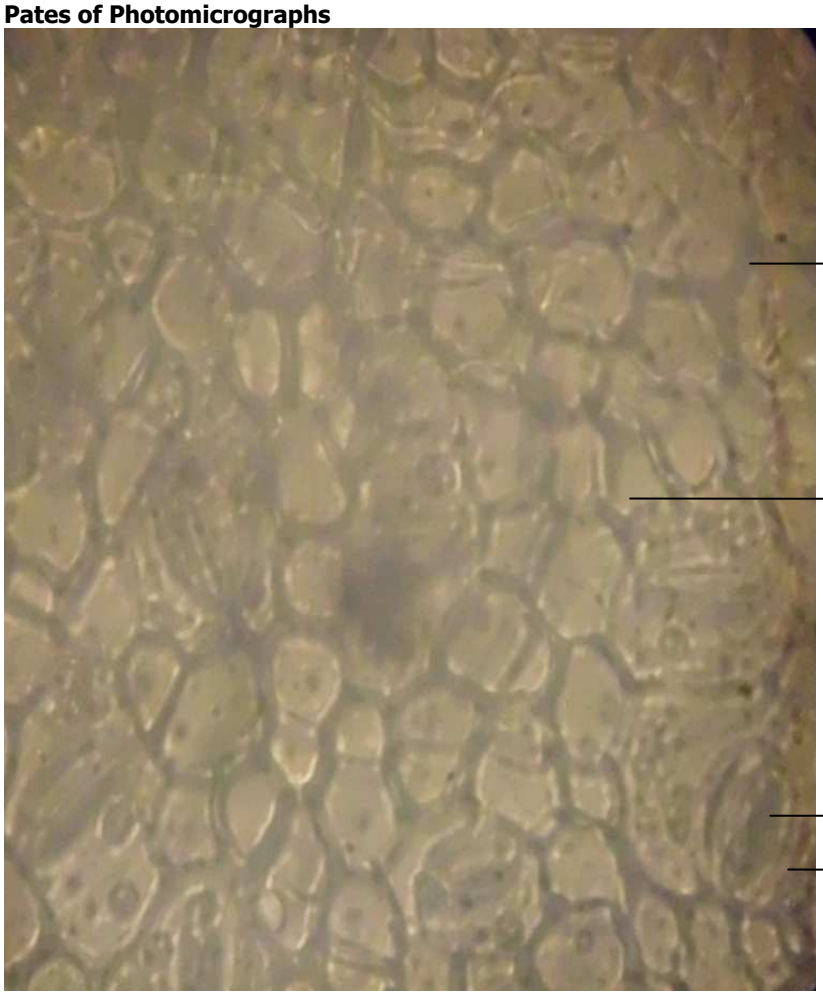

Plate I:**Cross section of the Lower epidermis of *D. velutinum*leaf viewed under compound microscope (X40)**

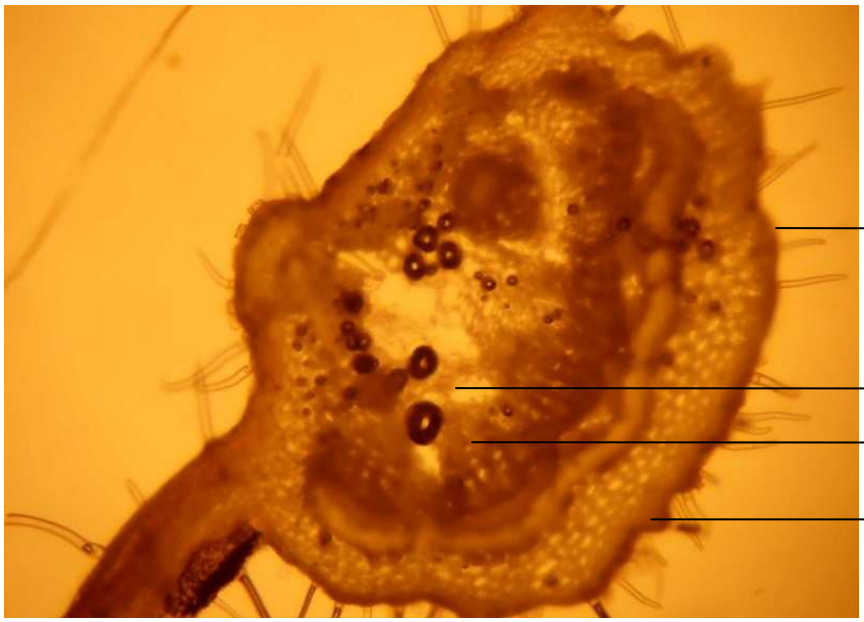

Plate II: **Transverse section of *Desmodiumvelutinum*mid Leaf viewed under microscope (X40)**

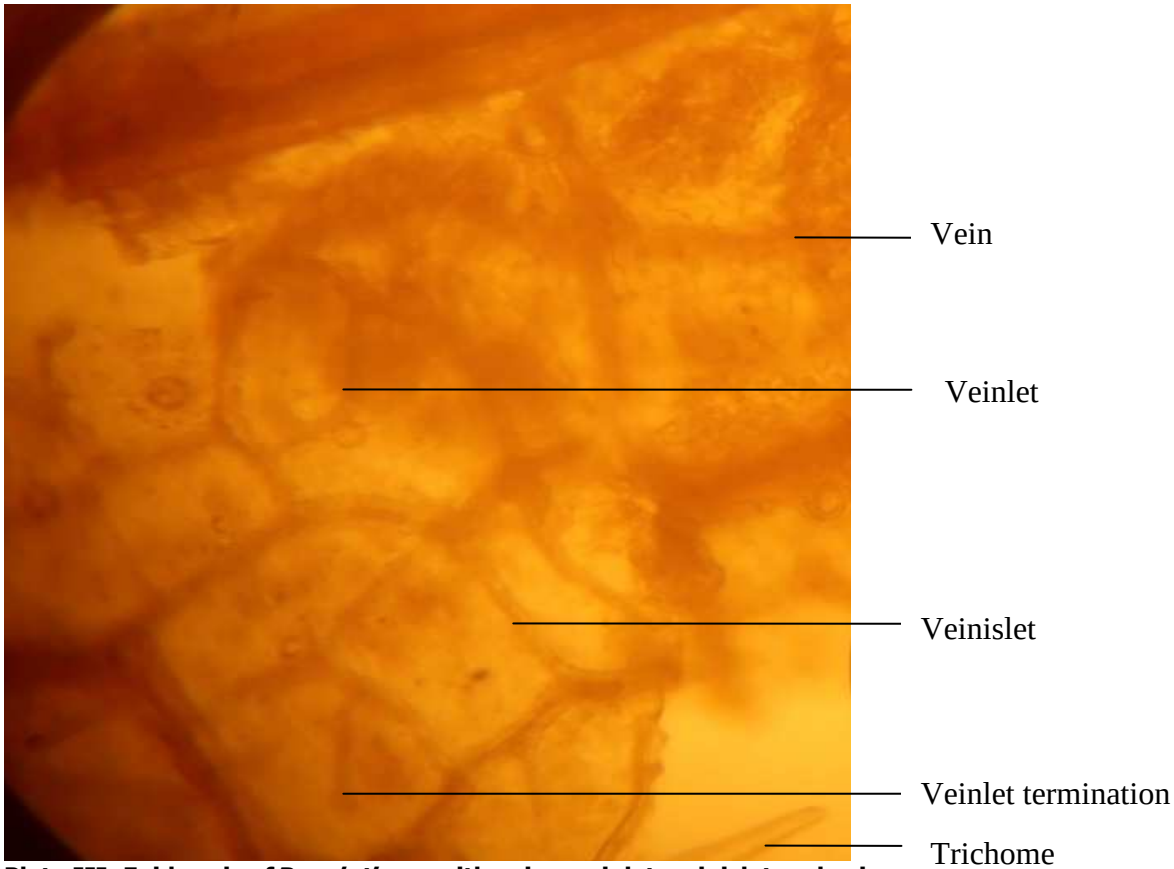

Plate III: Epidermis of *D. velutinum* with veins, veinlet, veinislet and vein as microscope (X40)

Qualitative Leaf Microscopy

The microscopic examination of the leaf powder and section of the leaf revealed various anatomical features of the plant. The epidermal cells are cuboidal forms. Anomocytic type of stomata ranging from 10-12µm long and 6-8µm wide were observed only on the lower epidermis. Calcium oxalate crystals were observed in between veins in fresh leaf and numerous unicellular covering trichomes were observed all over the epidermis.

Quantitative Leaf Microscopy

The result of the average stomatal number, veinlet number, veinlet termination number and veinislet number for upper and lower epidermis were determinedunder microscope. The results are as shown in table 1.

Table 1: The results of quantitative leaf microscopy

S/N	Parameter	Value (Mean ± SEM)
1.	Stomatal number	4.3 ± 0.23
2.	Stomatal index	9.09 ± 0.31
3.	Veinlet number	4.7± 0.06
4.	Veinlet termination number	2.40 ± 0.26
5.	Vein islet number	3.20 ± 0.60

n = 4

1. Trichomes

The trichomes were seen as unicellular, non-glandular structures, covering in nature. They are numerous and are found on both upper and lower epidermis.

2. Calcium Oxalate Crystal

These were seen as acicula structures and are few in numbers and scattered along the veins of the leaf.The transverse section of the leaf through the midrib revealed a network venation as well as open and markedly differentiated vascular bundles which are characterisctic features of dicotyledonous plants. Spongy parenchyma cells are conspicuous with much air space in between. The xylem vessels are located just above the phloem without cambium. The petiole had a single layer of epidermis and parenchyma cells with a set of vascular bundle in the middle. Trichomes are also observed along the tip.

Table 2: Summary of the Chemomicroscopical Examination of the Leaf of *D. velutinum*

S/N	Constituents/Reagents	Observation	Inference
1	Cellulose + N/50 iodine + 66% H_2SO_4	bluish colouration	present
2	Starch + N/50 iodine	blue-black colouration	present
3	Tannin + $FeCl_3$	greenish colouration	present
4	Oils + Sudan (IV) reagent	no pink colouration	absent
5	Calcium oxalate crystal + Chlorahydrate solution + Conc. HCl	Disappeared on addition of HCl	present

n = 4

Results of Thin Layer Chromatography of *D. Velutinum* Leaf
Plates of thin layer chromatograms developed from methanolic extract of *D. Velutinum* Leaf.

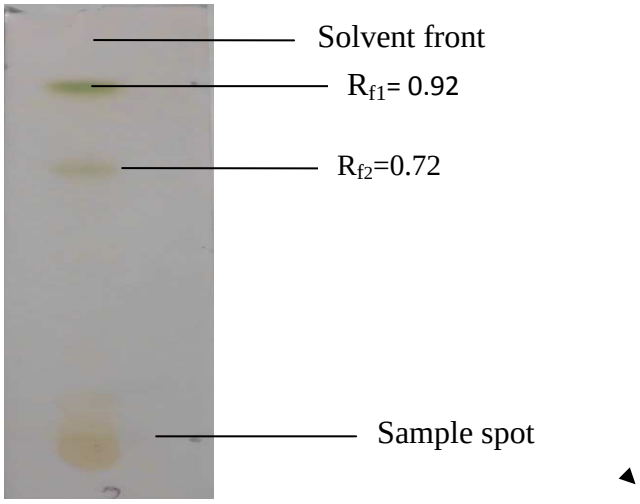

Plate IV: Chromatogram developed in chloroform - ethylacetate - formic acid (5:4:1), sprayed with 1% Ethanolic solution of aluminum chloride and viewed under ultra violet light (360nm), yellow fluorescence. R_{f2} indicates the presence of flavonoids.

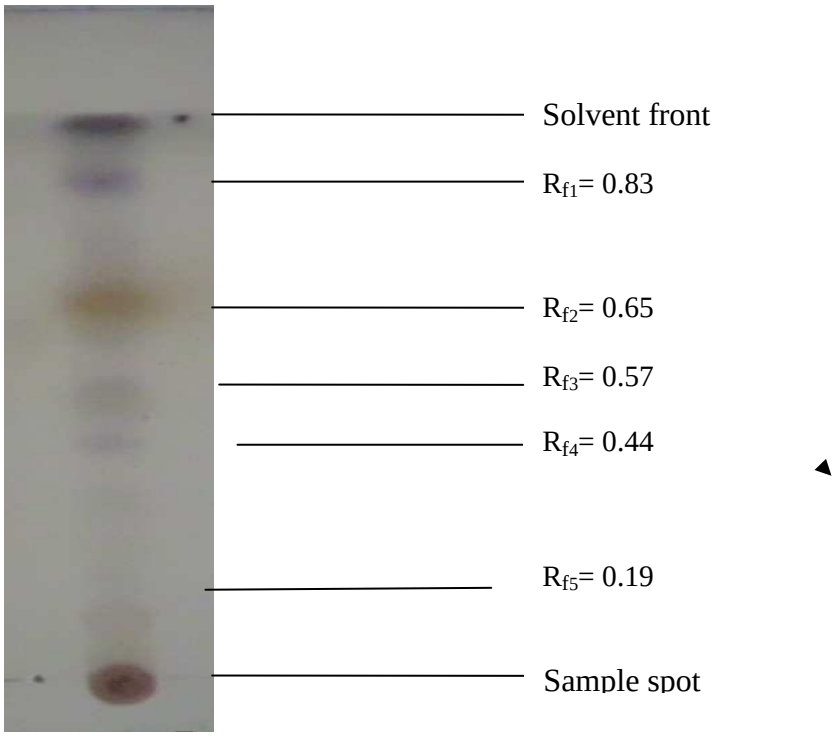

Plate V: Chromatogram developed in chloroform - ethylacetate - formic acid (5:4:1) and sprayed with vanillin ethanolicsulphuric acid (purple-R_{f3}colour forsteroidal presence)

Plate VI: Chromatogram developed in chloroform - ethylacetate - formic acid (5:4:1) and sprayed with p-anisaldehyde in ethanloicsulphuric acid (green/grey-R_{f1}colour for terpenepresence) respectively.

Plate VII: Chromatogram developed in chloroform - ethylacetate - formic acid (5:4:1) and sprayed with ferric chloride solution (dark green colour at R_{f2} shows the presence of phenolic compounds). Further

treatment with bromine water shows a dull yellow colour at R_{f1} which indicates the presence of tannins.

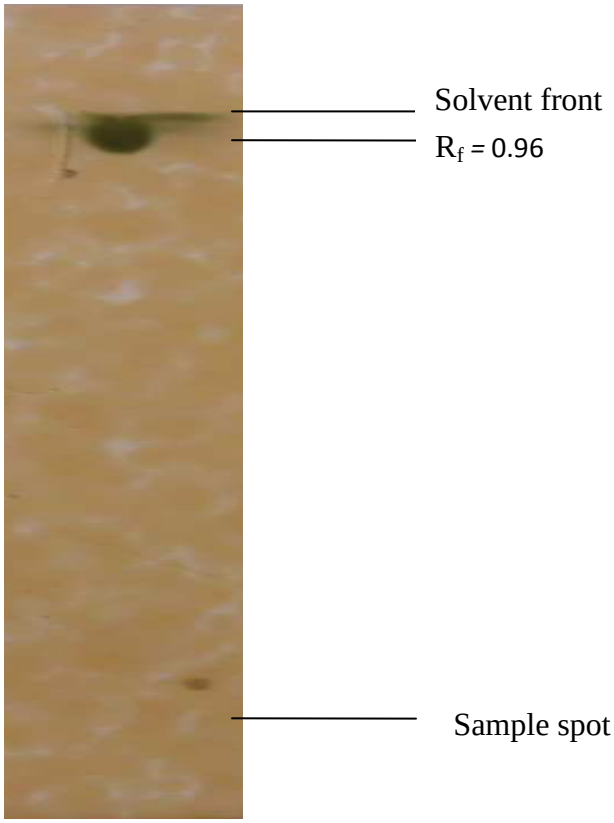

Plate VIII: Chromatogram developed in chloroform – ethylacetate - formic acid (5:4:1) and sprayed with Dragendorff's reagent (no orange or red colour indicating alkaloids are absent).

Table 3: Thin layer chromatographic analysis of phytochemical constituents of the leaf of *Desmodiumvelutinum*

S/N.	**CONSTITUENTS**	**SOLVENTS**	**REAGENTS**	**OBSERVATION**	**R_f**	**INFERENCE**
1	Alkaloids	Chloroform, Ethylacetate, Formic acid(5:4:1)	Dragendorff's reagent	No red or orange spot	-	Absent
2	Terpenes	,,	P-anisaldehyde	Green spot	0.65	Present
3	Flavonoid	,,	Aluminum Chloride and viewed at UV (360nm)	Yellow fluorescence	0.72	Present
4	Steroids	,,	Vanillin sulphuric acid	Purple spot	0.57	Present
5	Tannins	,,	(a)Ferric chloride (b)Bromine water	Dark green spot Dull yellow	0.87	Present

DISCUSSION

The unicellular non glandular trichomes observed from the microscopy of the powder and thin sections of the leaf could serve as a diagnostic feature since the presence of a particular type of trichomes are normally employed in the taxonomy of some families, genera and species (Evans, 2009). Trichomes The chemomicroscopic examination of the leaf powder and thin sections showed calcium oxalate crystals (acicula type)which are found along the veins of the leaves and this could serve as a diagnostic feature of this plant species.

maintain a layer of still air on the leaf surface which prevents excess water loss by transpiration (Evans, 2009). The absence of stomata on the upper epidermis of the leaf and their few numbers on the lower epidermis could be a mechanism by which *D.velutinum* reduces the amount of water loss through transpiration as it is a tropical plant.

Generally, information from phytochemical test of *D.velutinum*(table 4) as well as those of other plants obtained from literature survey can be used to study a possible pattern of the chemosystematics of these surveyed plant species, judging from fact that these phytochemical constituents reported or detected in different plants can be compared across species for similarities and differences. In this way, species sharing same compounds can be presumed to be more closely related by descent. This information would play a leading role towards the bioassays of these plants for the discovery and development of novel drugs.

The results of phytochemical screening of *D.velutinum* showed the presence of tannins, flavonoids, terpenes and steroids. This is in line with the investigation made by Anowi*et al.,*(2012). Similarly, Frank and Buckingham, (2009) and Bojaxa*et al*., (2010) studies on thephytochemical screening of species belonging to the Fabaceae showed that they possess tannins, flavonoids, phenols and steroids in common.

CONCLUSION

The microscopic,chemomicroscopic and phytochemical results in this research could serve as vital tools in the preparation of the monograph of the plant. The vein islet number, vein termination number and other parameters evaluated in the quantitative microscopy are useful and can help to differentiate closely related species of the plants. The findings in this research have provided useful information for a monograph of the plant and have validated the traditional use of the plant for pain management/treatment.

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