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Research Article

Anatomical and Phytochemical Studies of the Leaves of *Acacia nilotica* Subspecies Kraussiana

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Abstract

Yemen has a rich culture of medicinal herbs, but only very few have been studied pharmacognostically, phytochemically and pharmacologically for their potential medicinal value. *Acacia nilotica* subspecies kraussiana, family: Fabaceae (alt. Leguminosae) is a one of the most widespread plant in Yemen. The leaves were selected for the study with the aims to establish microscopical characteristics and phytochemical parameters towards its standardization. The whole leaves and the powders of dry leaves were used for microscopical examinations and the results showed that the paracytic stomata, non-glandular, unicellular, straight, or curve trichomes, some of them are slightly bended near the base, and cluster of calcium oxalate crystals are characterized for the leaves and their powders. The leaves were subjected to phytochemical screening by soxhlet extraction with 70% methanol and distilled water after defatting with petroleum ether. Phytoconstituets like carbohydrates, glycosides, triterpenes, sterols, saponins, flavonoids, coumarins and tannins were identified in studied leaves. Sterols and triterpenes were identified in all extracts, saponins in methanol 70% extract only, while alkaloids were not observed in all extracts. The total content of flavonoids (2.20%), tannins (22.78%) and saponins (7.50%) were high in studied leaves. The fluorescence characteristics of powdered drug were studied. Thin layer chromatography of the extracts yielded 7 sports for petroleum ether extract, 6 for methanol 70% extract was and 6 spots for water extract. The results of the present study may confirm the pharmacological properties of the drug and explain partially the uses of the drug in traditional medicine for many illnesses. This study is a substantial step and it further requires a long term phytochemical and pharmacological studies.

1. INTRODUCTION

Therapeutic efficacy of medicinal plants depends upon the quality, quantity of chemical constituents and purity of drugs. All these problems can be solved by using different techniques and methodology e.g. pharmacognostic and phytochemical studies. These steps and processes are helpful in identification and standardization of the plant material. Correct characterization and quality assurance of starting material is an essential step to ensure reproducible quality of herbal medicine which will help to justify its safety and efficacy. Qualitative chemical examination employing different analytical techniques is conducted to detect and isolate the active constituents[1, 2, 3]. The macroscopic and microscopic description of medicinal plants is the first step towards the establishing the identity and degree of purity of such materials and should be carried out before any tests are undertaken[4]. In Yemen the use of Medicinal and Aromatic plant species goes back thousands of years[5]. Thus, phytotherapy is practiced by a large proportion of Yemen population for the treatment of several physical, physiological, mental and social ailments[6]. Nevertheless, little scientific research was done to investigate the plants of Yemen used in herbal medicine[7]. *Acacia nilotica* subspecies kraussiana, family: Fabaceae (alt. Leguminosae) is a one of the most widespread plants in Yemen[8]. Traditionally in Yemen the leaves of plant are used to reduce stomach pain[9]. Very few works has been carried out on the leaves of this plant toward documenting its ethnomedicinal uses and establishing its standardization. No pharmacognostic or phytochemical studies have been reported for *Acacia nilotica* subspecies kraussiana. The leaves were selected for the study with the aims to establish microscopical characteristics and phytochemical parameters towards its standardization. The present study is a substantial step and it further requires a long term phytochemical and pharmacological studies.

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2. MATERIALS AND METHODS

2.1 Plant material

Acacia nilotica subspecies kraussiana leaves (Fabaceae) were collected in October 2013 from Dhala, Yemen, dried in the shaded area and then manually grinded and stored at room temperature for further analysis. The plant sample was identified by a taxonomist, Professor Abdul Nasser Algifri, of the department of Biology, of University of Aden, Yemen. The leaves were stored under the normal environmental conditions for further analysis.

2.2 Microscopic studies

The microscopic characters of leaves and powdered materials were studies as per the produce given in WHO guidelines[4]. Free hand sections of leaflet, petiole, rachis and rachilla were taken. Sections were cleared by heating with chloral hydrate solution, transferred onto slides, mounted in 50% v/v glycerol in water and then examined under microscope. The powder of the shade dried leaves was cleared by heating with chloral hydrate solution then a small amount of the powder was taken onto a microscopic slide, mounted in 50% v/v glycerol in water and observed under microscope to study the characteristic features. Various identifying characters, such as type of trichomes, type of stomata and epidermal cells were recorde[10, 11]. Photomicrographs were taken with Leica USA model 2000ATC (ocular: CPL W10X; objective: 4X, 10X and

40X). Photographs were taken with the help of digital camera (Sony 16 MP).

2.3 Preparation of extracts

Preparation of extracts was achieved by standard method[12, 13]. The dried powdered leaves were extracted successively extracted in a Soxhlet's apparatus with petroleum ether (boiling point 60-80 ^{0}C), 70% methanol (80-90°C) and water. The solvents in the extracts were removed by distillation and the concentrated extracts so obtained were further dried at a temperature not exceeding 40 0C in water bath and then stored at 4°C in refrigerator till further use. The yield values were calculated.

2.4 Qualitative Phytochemical analysis

Preliminary chemical tests were carried out for petroleum ether, 70% methanol and water extracts to identify different phyto-constituents as per the standard methods[14, 15, 16, 17, 18, 19].

2.5 Quantitative phytochemical analysis

2.5.1 Flavonoid Determination

The flavonoid content of the leaves of the plant was determined by the gravimetric method as was described by Harborne[20]. 5g of the powdered sample was placed into a conical flask and 50ml of water and 2ml HCl solution was added. The solution was allowed to boil for 30 minutes. The boiled mixture was allowed to cool before it was filtered through Whatman filter paper (No 42). 10ml of ethyl acetate extract which contained flavonoid was recovered, while the aqueous layer was discarded. A pre weighed Whatman filter paper was used to filter second (ethyl-acetate layer), the residue was then placed in an oven to dry at 60 ^{0}C. It was cooled in a desiccator and weighed. The quantity of flavonoid was determined using the formular.

$$\% \text{ Flavonoid} = \frac{W2 - W1}{\text{Weight of sample}} \text{ x } 100$$

Where:
W1= Weight of empty filter paper
W2= Weight of paper + Flavonoid extract

2.5.2 Determination of Tannin

Five grams of each part (leaves, stems) was milled into powder. The powder was extracted with 100 ml acetone–water (70/30, V/V), and the mixture was stirred continuously for 72 h at room temperature. Then, the mixture was filtrated and evaporated under vacuum at 40 8C to remove acetone. The washed with 30 ml dichloromethane to remove lipid soluble remaining solution was substances. After that, the solution was further extracted with ethyl acetate at a ratio of 30/30 (V/V). The water layer was separated and extracted twice more similarly. Then the resulting water layer was evaporated to dryness, and the resulting substance was weighed[21].

2.5.3 Determination of Saponin

Saponin content of the sample was determined by double solvent extraction gravimetric method[20]. 2g of the powdered sample was mixed with 50mls of 20% aqueous ethanol solution. The mixture was heated with periodic agitation in water bath for 90 mins at 55 ^{0}C. It was filtered through filter paper through Whatman filter paper. The residue was extracted with 50mls of the 20% ethanol and both extracts were pooled together. The combined extract was reduced to about 40mls at 90 ^{0}C and transferred to a separating funnel where 40mls of diethyl ether was added and shaken vigorously. Separation was by partition during which the ether layer was discarded and the aqueous layer reserved. Re-extraction by partition was done repeatedly until the aqueous layer become clear in colour. The saponins were extracted with 60mls of normal butanol. The combined extracts were washed twice with 10ml of 5% aqueous NaCl solution and evaporated to dryness in a pre-weighed evaporating dish. It was dried at 60 ^{0}C in the oven and reweighed. The experiment was repeated two more times to get an average.

$$\% \text{ Saponins} = \frac{W2 - W1}{\text{Weight of sample}} \text{x } 100$$

Where W1 = Weight of evaporating dish;
W2 = Weight of dish + sample

2.6 Fluorescence analysis of powdered drug

A fine powder of the leaves was placed on a grease free clean microscopic slide and added 1-2 drops of the freshly prepared reagent solutions mixed properly and waited for 1-2 minutes. Then the slide was viewed in day light and inside the UV viewer chamber short (254 nm) and long (365 nm) ultraviolet radiations. The colors observed by application of different reagents in different radiations were recorded[19].

2.7 Thin layer chromatography studies

Thin Layer Chromatography (silica gel G 60 F254 TLC plates of layer thickness 0.2mm) of petroleum ether, 70% methanol and water extracts was performed to determine the number of spots and Rf values[22, 23]. Various solvent systems were tested to obtain best results. TLC plates were first viewed in day light then in UV chamber and *Rf* values were calculated. Different solvent systems were found to be effective to get maximum no. of spots for various extracts.

3. RESULTS AND DISCUSSION

3.1 Microscopic studies

Microscopic analysis is needed to determine the correct species and/or that the correct part of the species is present, and the presence of certain microscopic structures such as leaf stomata can be used to identify the plant part used[1].

Leaflets: In surface view of upper and lower surfaces showed the presence of epidermal cells exhibiting a polygonal or rectangular shape at the center of the lamina. Rectangular cells were observed in the edge (3 to 4 layers). Paracytic stomata and large number of cluster of calcium oxalate crystals were found on both surfaces (Fig. 1, 2). Unicellular, non-glandular trichomes, having thick cell walls were presented rarely in the edge of lamina. They are an acute apex and bended near the base, sometimes they are erect (Fig. 3).

Venation pattern: The densely reticulate venation was observed. It comprise of vein islets and vein terminations. The large vein islets are well defined, they are variable in shape and contain small islets that including vein terminations. The vein terminations are branched, sometimes simple, and slender (Fig. 4).

Petiole, rachis and rachilla: In surface view of studied surfaces, showed the presence of epidermal cells with a polygonal or rectangular shape. Paracytic stomata were observed in petiole and rachilla, but in rachis not found. Unicellular, non-glandular trichomes, having thick cell walls, an acute apex and bended near the base were presented abundantly in all surfaces. Some trichomes are wavy or curved, rare erect (Fig. 5, 6, 7, 8).

Powdered leaves microscopy: Microscopical study of powder showed the following characters: fragment of epidermis with paracytic stomata and unicellular, non-glandular curved trichome (Fig. 9a); cluster of calcium oxalate crystals and unicellular, non-glandular trichome with acute apex and bended near the base (Fig. 9b); spiral vessels (Fig. 9c); pitted vessels (Fig. 9d); fragment of midrib with spiral vessel (Fig. 9e) and venation pattern (Fig. 9f).
Microscopy is an important tool for authentification of crude drugs and study of powdered drugs[24]. It is important to interpret morphological and anatomical descriptions of crude drugs as well as characteristic features of drugs and adulterants of commercial significance[25]. Paracytic stomata and non-glandular, unicellular, straight or curve trichomes have been found in Fabaceae members[26], including *A. auriculiformis* and *A. etbaica*[27, 28], and as seen in the studied species.

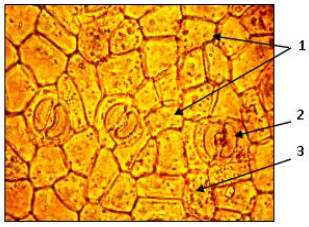

Fig. 1: Surface view of epidermal cells of leaflet (10x40): 1- Cells with polygonal shape, 2- Paracytic stomata, 3- Calcium oxalate crystals.

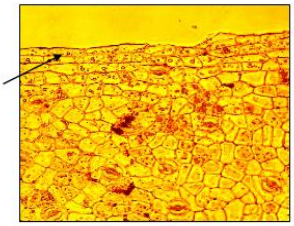

Fig. 2: Surface view of epidermal cells of leaflet (10x10): 1- Cells with rectangular shape.

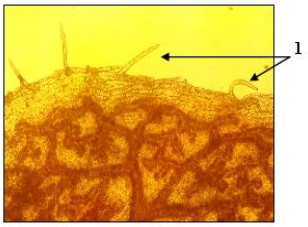

Fig. 3: Surface view of epidermal cells of leaflet (10x10): 1- Unicellular non-glandular trichome.

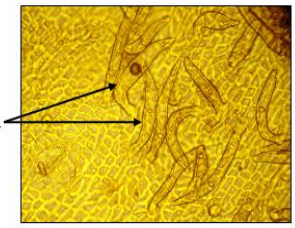

Fig. 6: Surface view of epidermal cells of petiole (10x40): 1- Unicellular non-glandular trichomes.

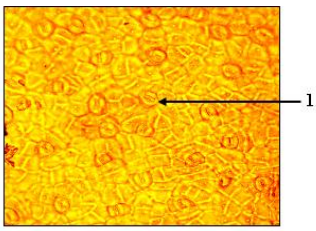

Fig. 5: Surface view of epidermal cells of petiole (10x10): 1- Paracytic stomata.

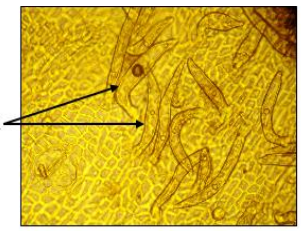

Fig. 6: Surface view of epidermal cells of petiole (10x40): 1- Unicellular non-glandular trichomes.

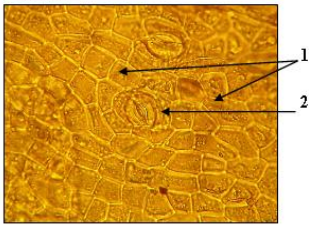

Fig. 7: Surface view of epidermal cells of rachilla (10x40): 1- Epidermal cells, 2- Paracytic stomata.

Fig. 8: Surface view of epidermal cells of rachilla (10x10): 1- Unicellular non-glandular trichome.

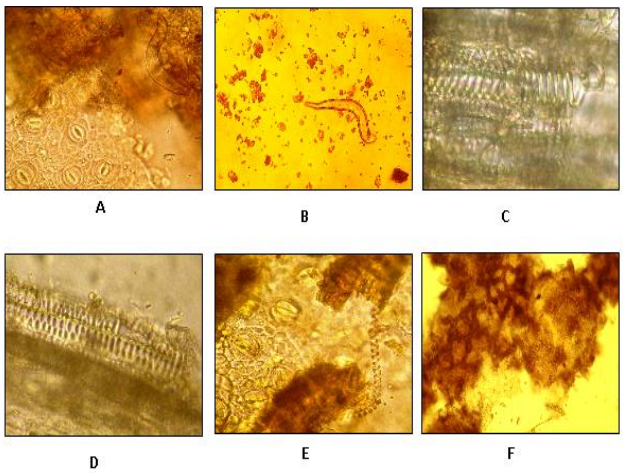

Fig. 9: Powder microscopy: (a) Paracytic stomata, (b) Non-glandular trichome with acute apex and curved near the base, and calcium oxalate crystals, (c) Spiral vessels, (d) Pitted vessels, (e) Fragment of midrib with spiral vessels, (f) Fragment of venation pattern.

3.2 Qualitative Phytochemical analysis

Extractive value of petroleum ether, methanol 70% and water extracts were determined and the result showed in the Table-1. Phytochemical screening of the studied extracts was represented in Table-2. The medicinal values of the plants are due to the chemical substances that produce a definite physiological action on human body[29, 30]. Phytoconstituets like carbohydrates, glycosides, triterpenes, sterols, saponins, flavonoids, coumarins and tannins were identified in studied leaves. Sterols and triterpenes were identified in all studied extracts, saponins in methanol 70% extract only, while alkaloids were not observed in all extracts.

Table 1: Percent extractives and colors of successive extracts of the leaves of *acacia nilotica* subspecies kraussiana

S. No.	Solvent	Weight of plant material (gm)	Percentage of Yield (%)	Colors of extracts
1	Petroleum Ether	50	5.30	Green
2	70% methanol	50	46.88	Reddish brown
3	Water	50	5.00	Brown

Table 2: Results of phytochemical screening of deferent extracts of the leaves of *acacia nilotica* subspecies kraussiana

Phytochemical Screening		Petroleum Ether extract	70% Methanol extract	Water extract
Alkaloids	Wagner's test	-	-	-
	Mayer's test	-	-	-
	Dragendorff's reagent	-	-	-
Carbohydrates	Molisch's test	-	++	++
	Fehling's test	-	++	++
Glycosides	Kellar Kiliani test	-	+	+
	Conc. H_2SO_4 test	-	+	+
Saponins	Foam test	-	++	-
	Haemolysis test	-	++	-
Sterols/ Triterpenes	Salkowski test	++	+++	+++
	Liebermann-Burchard test	++	+++	+++
Polyphenols	Ferric chloride test	-	++	++
Tannins	Ferric chloride test	-	++	++
	Gelatin test	-	++	++
	Conc. HCL test	-	++	++
	Lead acetate test		++	++
Flavonoids	Shinoda test	-	+	+
	NaOH Test	-	+	+
Coumarins		+	++	-

+++ = Most intense, ++ = moderately intense, + = Least intense, - = absent.

3.3 Fluorescence analysis of powdered drug

Fluorescence study powdered drug under ultra violet light is very distinctive and helpful in establishing the purity of the drug[11]. The fluorescence analysis of the powder drug was done. The powdered drug was treated separately with different reagents then observed under short UV (254 nm), long UV (365 nm) and visible light. Results are given in Table-3.

Table 3: Fluorescence analysis of leaves powder of *acacia nilotica* subspecies kraussiana

S. No.	Treatments	Observations		
		Day light	Short UV	Long UV
1.	Powder as such	Greenish	Dark brown	Dark brown
2.	Powder + 1N NaOH (aqueous)	Brown	Yellowish green	Green
3.	Powder + 1N NaOH (alcoholic)	Orange	Orange	Green
4.	Powder + conc H2SO4	Brown	Dark brown	Dark brown
5.	Powder + 50% H2SO4	Greenish yellow	Greenish yellow	Green
6.	Powder + 50% N HNO3	Brown	Reddish brown	Dark brown
7.	Powder + conc.HNO3	Reddish brown	Dark brown	Reddish brown
8.	Powder + dil HNO3 10%	Brown	Yellowish brown	Brown
9.	Powder + 1N HCl	Green	Green	Green
10.	Powder + 10% Ammonia	Orange	Orange	Greenish yellow
11.	Powder + Acetic acid	Brown	Dark brown	Brown
12.	Powder + 5% Iodine	Reddish brown	Dark brown	Dark brown
13.	Powder + 5% FeCl3	Dark green	Florescent green	Green
14.	Powder + Methanol	Green	Dark green	Green
15.	Powder + 50% Methanol	Brown	Dark brown	Dark brown
16.	Powder + water	Green	Dark green	Green

3.4 TLC of petroleum ether, methanol 70% and water extracts

The presence of phytoconstituents was further confirmed by thin layer chromatography. Thin Layer Chromatography is still the basic tool for the separation and identification of natural compounds. It is often used to provide the first characteristic fingerprints of herbs[31]. In addition, TLC technique is constantly improving[32, 33]. Thin Layer Chromatography (silica gel G 60 F254 TLC plates of layer thickness 0.2mm) of prepared extracts was performed to separate and determine Rf values. Various solvent systems were tested to obtain best results. The best solvent systems for petroleum ether extract was Benzene- Chloroform (1:1), where detected 7 spots; for methanol 70% extract was Ethyl acetate- Toluene- Formic acid (4:4:1), where detected 6 spots. In solvent system Ethyl acetate-Butanol-Water-Formic acid (10:10:4:2) methanol 70% extract reveal 5 spots, while water extract reveal 6 spots. *Rf* values of developed spots of different extracts were calculated as well as the colour of spots were observed, which is mentioned in Table-4.

Table 4: Observations of thin layer chromatographic studies of the leaves of *acacia nilotica* subspecies kraussiana

Extracts	Mobile phase	No. of spots	Rf values	Spot Colour At 254 nm	Spot Colour At 254 nm
Petroleum Ether	Benzene: Chloroform (1:1)	7	0.14	Light green	Not visible
			0.16	Green	Deep purple
			0.27	Dark green	Deep purple
			0,32	Dark purple	Not visible
			0.56	Dark green	Deep purple
			0.63	Dark purple	Not visible
			0.71	Green	Not visible
Methanol 70%	Ethyl acetate- Toluene- Formic acid (4:4:1)	6	0.08	Light dark green	Purple
			0.18	Light dark green	Purple
			0.24	Dark green	Purple
			0.33	Light dark green	Purple
			0.38	Dark green	Deep purple
			0.46	Dark green	Deep purple
	Ethyl acetate-Butanol-Water-Formic acid (10:10:4:2)	5	0.11	Light dark brown	Purple
			0.45	Dark brown	Purple
			0.53	Light dark brown	Purple
			0.63	Light dark brown	Purple
			0.73	Dark brown	Deep purple
Water	Ethyl acetate-Butanol-Water-Formic acid (10:10:4:2)	6	0,08	Not visible	Deep blue
			0.22	Not visible	Blue
			0.38	Light dark brown	Blue
			0.50	Light dark brown	Violet
			0.61	Light dark brown	Violet
			0.66	Light dark brown	Violet

3.5 Quantitative Determination of the Phytochemical Constituents

The percentage concentration of flavonoids, tannins and saponins in the leaves of this plant are determined gravimetric methods. The phytochemical content of the leaves of the plant are as follows: flavonoids 2.20%, tannins 22.78% and saponins 7.50%. All measures were performed in triplicate. The results indicate that the leaves of this plant have appreciable amount of these phytochemicals, hence their medicinal value.

4. CONCLUSIONS

The leaves of *Acacia nilotica* subspecies kraussiana appear rich in secondary metabolites. It is why; these leaves are used extensively in traditional medicine to heal different health problems. The leaves were selected for the study with the aims to establish identity and quality microscopically, to identify and determine the chemical constituents. Result of microscopic examination showed that the paracytic stomata, unicellular, non-glandular trichomes and cluster of calcium oxalate crystals are characterized for the leaves and their powders. Phytoconstituets like carbohydrates, glycosides, triterpenes, sterols, saponins, flavonoids, coumarins and tannins were identified in studied leaves. Sterols and triterpenes were identified in all studied extracts, saponins in methanol 70% extract only, while alkaloids were not observed in all extracts. The total content of flavonoids (2.20%), tannins (22.78%) and saponins (7.50%) are high in studied leaves. In the present study, the microscopical and phytochemical studies of the leaves of *Acacia nilotica* subspecies kraussiana have been reported for the first time. The results may confirm the pharmacological properties of the drug and explain partially the uses of the drug in traditional medicine for many illnesses. Moreover this study is a substantial step and it further requires a long term phytochemical and pharmacological

studies. More detailed study must be done for further isolation leading to the pure compounds and establishment pharmacological activities of this drug.

5. ACKNOWLEDGEMENT

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