ISSN: 0975-8585

Research Journal of Pharmaceutical, Biological and Chemical Sciences

Anatomical and Phytochemical Studies of the Leaves of *Acacia etbaica* subspecies Etbaica.

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ABSTRACT

Yemen has a rich culture of medicinal herbs, but only very few have been studied chemically and pharmacologically for their potential medicinal value. *Acacia etbaica* Schweinf, subspecies etbaica, family: *Leguminosae* is a one of the most widespread plant in Yemen. The aims of present study were to establish identity and quality of leaves microscopically and phytochemically. The whole leaves and the powders of dry leaves were used for micro-characterisation and the results showed that the paracytic stomata and non-glandular, unicellular, straight or curve trichomes are characterized for the leaves and their powders. The leaves were subjected to phytochemical screening by soxhlet extraction with petroleum ether, chloroform, 96% ethanol and distilled water and different compounds such as carbohydrates, glycosides, saponins, flavonoids, coumarins, tannins, triterpenes, sterols, amino acids and protein were presented while alkaloids were not present. The fluorescence characteristics of powdered drug were studied. Thin layer chromatographic examination of the extracts yielded 8 discrete sports for petroleum ether extract, 9 sports for chloroform extract, 5 sports for ethanol extract and 3 sports for the water extract. This study could be useful to set some diagnostic parameters for preparation of monograph, standardization as well as for confirming identity of plant.

Keywords: *Acacia etbaica*, leaves, anatomical, phytochemical.

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ISSN: 0975-8585

INTRODUCTION

The use of medicinal plants in traditional medicine is well known in rural areas of many developing countries [1]. An estimated 80% of the population in much of the developing world relies on traditional systems of medicine, and 70-80% of the populations in developed countries have used some form of alternative or complementary medicine [2]. Medicines obtained from plants are relatively safer than synthetic alternative [3]. Yemen has a rich culture of medicinal herbs, but only very few have been studied chemically and pharmacologically for their potential medicinal value. *Acacia etbaica* Schweinf subspecies etbaica, family: *Leguminosae* is a one of the most widespread plant in Yemen, locally known as 'Qarad' [4], traditionally the leaves are crushed, mixed with water and taking orally to reduce stomach pain [5]. In East Africa the bark of this plant is chewed as a stimulant and is also used in the treatment of gonorrhea [6]. Very few works has been carried out on the leaves of this plant toward documenting its ethnomedicinal uses and establishing its chemical constituents. No pharmacognostic or phytochemical studies have been reported for this plant. Keeping these points in mind, the plant is selected for the study with the aims: to establish identity and quality of leaves microscopically and phytochemically for the standardization of the drug; to set up standard anatomical and phytochemical parametres for further refefrece of the plant.

MATERIAL AND METHODS

Plant material

The leaves of *Acacia etbaica* subspecies etbaica were collected in September 2012 from Yaffa, Republic of Yemen and were authenticated by a taxonomist, Professor Abdul Nasser Algifri, of the department of biology, faculty of Education, University of Aden. The leaves were stored under the normal environmental conditions for further analysis.

Microscopic studies

The microscopic characters of compound leaves and powdered materials were studies as per the procedure given in WHO guidelines [7]. 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 shade dried leaves of the plant were powdered and powder was passed through 100# sieve. A small amount of powder was taken onto a microscopic slide, cleared from chlorophyll by heating with chloral hydrate solution and was mounted in 50% v/v glycerol in water, then observed under microscope to study the characteristic features. Photomicrographs were taken with Leica USA model 2000ATC (ocular: CPL W10X; objective: 4X, 10X and 40X). Various identifying characters, such as type of trichomes [8], type of stomata and epidermal cells were recorded, and then photomicrography was done. Photographs were taken with the help of digital camera (Sony 10 MP).

Preliminary phytochemical Screening

Powdered plant material was successively extracted in a Soxhlet's apparatus with petroleum ether, chloroform, ethanol and water for 18 hours in the order of increasing polarity of solvents [9]. The solvent in the extracts were removed by distillation and the concentrated extracts so obtained were further dried at a temperature not exceeding 40 ^{0}C in water bath and then stored at 4°C in refrigerator till further use. The yield values and other physical properties were observed. The extracts obtained were subjected to following chemical tests for identification of various phytoconstituents as per the methods given by Harborne [10], Khandewal K.R. [11], A Sofowara [12] and GE Trease, WC Evans [13].

Fluorescence analysis of powdered drug

A finely powdered plant material 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 [14].

Thin layer chromatography

Thin Layer Chromatography of prepared extracts was performed to determine Rf values [15]. Various solvent systems were tested to obtain best results. TLC plates were first viewed in day light then in UV chamber before keeping in iodine chamber and Rf of all were noted. Different solvent systems were found to be effective to get maximum number of spots for various extracts.

RESULTS

Microscopic studies of leaves

The leaves are bipinnate, with 3-9 pairs pinnulae and 10-30 pairs of leaflets. Hand sections of leaflets, petiole, rachis and rachilla were used for micro-characterisation.

Leaflets: The blade, in surface view of both surfaces, showed the presence of epidermal cells exhibiting a polygonal or rectangular shape, coated with a striate and thick cuticle. Rectangular cells were present in the edges of the blade. Paracytic stomata were present on both surfaces of epidermis (figure 1). Numerous unicellular, non-glandular trichomes with an acute apex, curved near the base and thick cell walls were found on the edge of lamina, sometimes they are erect (figure 2), rare with cap (figure 3).

Venation pattern: Cleaned lamina was slidied for the venation pattern. The venation is densely reticulate comprising thick lateral vein which gradually reduce in thickness cultivating into distinct and prominent vein termination. The vein islets are well defined which are variable in shape: they are polygonal in outline. The vein terminations also vary. They are prominent, branched, sometimes simple, and slender, (figure 4).

ISSN: 0975-8585

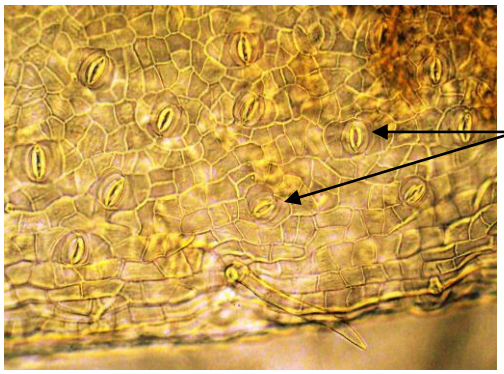

Figure 1: Surface view of epidermal cells (10x10): 1- Paracytic stomata.

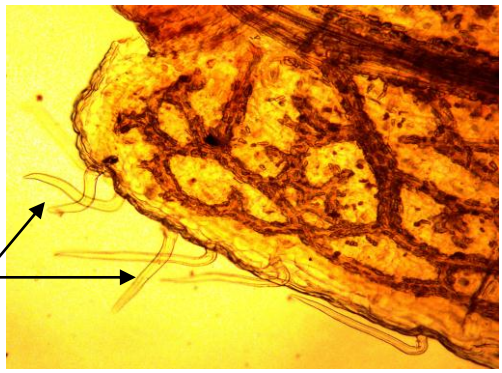

Figure 2: Surface view of epidermal cells (10x10): 1- Unicellular trichomes

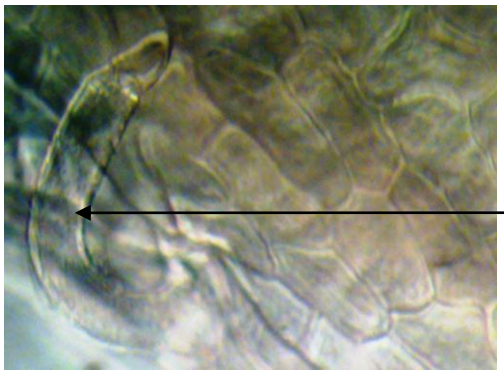

Figure 3: Surface view of epidermis (10x40): 1-Unicellular trichome with cap.

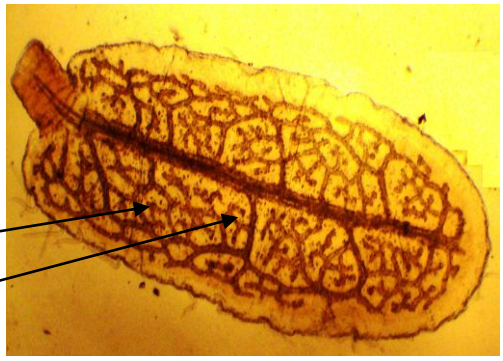

Figure 4: Cleared leaflet showing vein-islets and vein-termination (10x10): 1-vein-islet 2- vein-termination.

Figure 5: Surface view of epidermal cells of petiole (10x40): 1- Paracytic stomata.

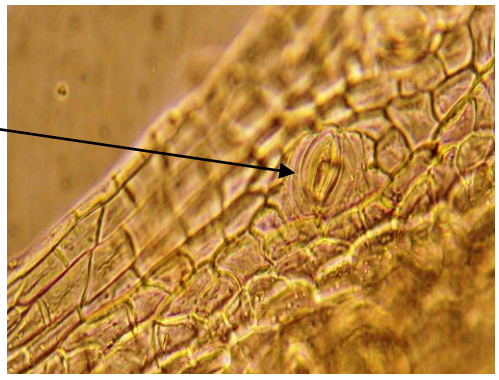

Figure 6: Surface view of epidermal cells of rachis (10x40): 1- Paracytic stomata.

Petiole, rachis and rachilla: In surface view of all surfaces, showed the presence of epidermal cells exhibiting a polygonal or rectangular shape, coated with a thick cuticle and paracytic stomata (figures 5,6,7). All surfaces covered with unicellular, conical, swollen, non glandular, trichomes, with thick wall, curved near the base and rounded or an acute at apex, sometimes with cap. Some trichomes have enlarged base. (figures 8,9,10).

ISSN: 0975-8585

Powder microscopy

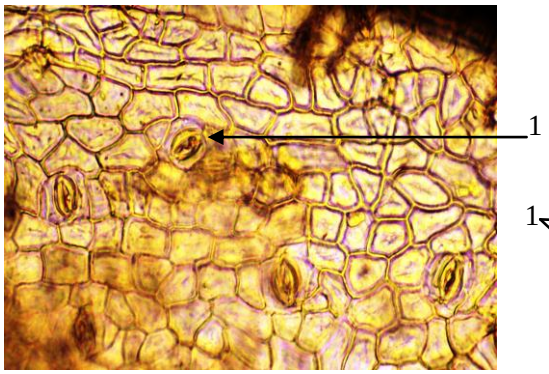

Figure 7: Surface view of epidermal cells of rachilla (10x40): 1- Paracytic stomata.

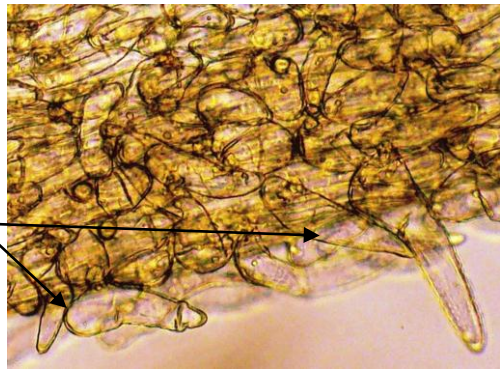

Figure 8: Surface view of epidermal cells of petiole (10x10): 1- Unicellular trichomes.

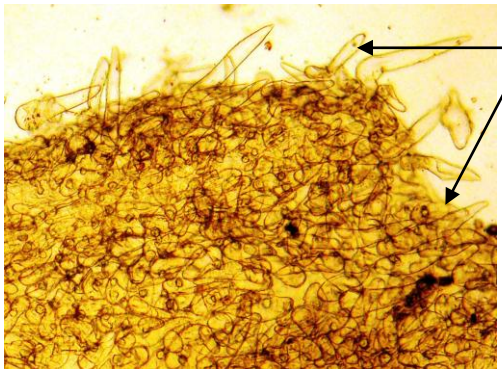

Figure 9: Surface view of epidermal cells of rachis (10x10): 1- Unicellular trichomes.

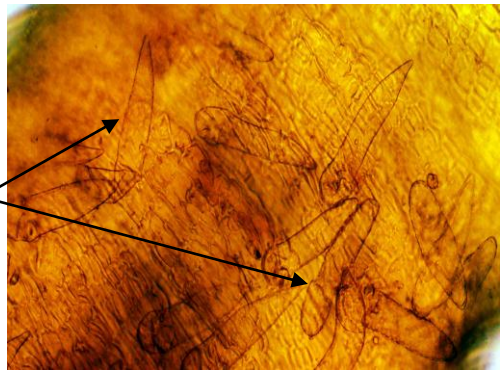

Figure 10: Surface view of epidermal cells of rachilla (10x10): 1- Unicellular trichomes.

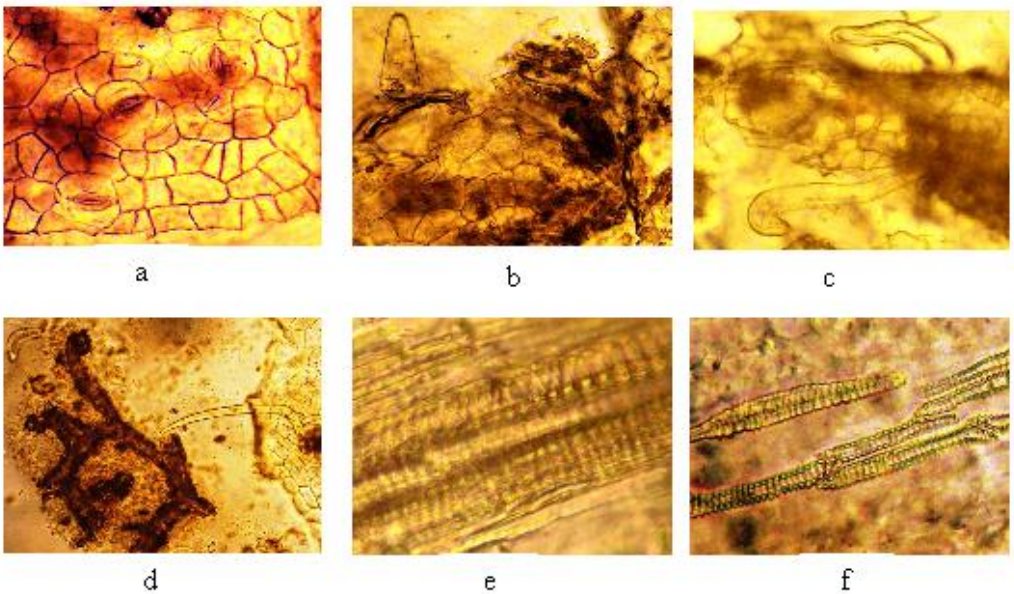

Figure 11a, b, c, d, e, and f: Powder microscopy

The powder microscopy study showed the presence of epidermis in surface view with polygonal and rectangular cells with paracytic stomata (figure 11a), unicellular, conical trichomes (figure 11b), unicellular, thicked wall trichomes with acute apex and curved near the base (figure 11c), fragment of midrib and lateral vein (figure 11d), and spiral, annulate, scalariform vessels (figures 11,e,f).

Preliminary phytochemical Screening

The extractive value of petroleum ether, chloroform, ethanol and water extracts were determined (Table 1). The results of the phytochemical screening of leave extracts were represented in Table 2. Phytoconstituets like carbohydrates, glycosides, saponins, flavonoids, coumarins, tannins, triterpenes, sterols, amino acid and protein were presented, while alkaloids were not present. Sterols/ triterpenes, carbohydrates and glycosides found in all extracts.

Table 1: Percent extractives and colors of successive extracts of the leaves of *Acacia etbaica* subspecies etbaica

S. No.	Solvent	Weight of plant material (gm)	Percentage of Yield (%)	Colors of extracts
1	Petroleum Ether	75	2.60	Yellowish-green
2	Chloroform	75	4.42	Green
3	Ethanol	75	9.52	Reddish Brown
4	Water	75	10.50	Brown

Table 2: Results of phytochemical screenings of successive extracts of the leaves of *Acacia etbaica* subspecies etbaica

Phytochemical Screening		Petroleum Ether extract	Chloroform extract	95% Ethanol extract	Water extract
Alkaloids	Wagner's test	-	-	-	-
	Mayer's test	-	-	-	-
	Dragendorff's reagent	-	-	-	-
Flavonoids	Shinoda test	-	+	+	+
	NaOH Test	-	+	+	+
Saponins	Foam test	-	-	++	++
	Haemolysis test	-	-	++	++
Glycosides	Kellar Kiliani test	+	+	+	+
	Conc. H_2SO_4 test	+	+	+	+
Sterols/ Triterpenes	Salkowski test	++	++	+++	+++
	Liebermann-Burchard test	++	+++	+++	+++
Carbohydrates	Molisch's test	+	+	++	++
	Fehling's test	+	+	++	++
Tannins	Ferric chloride test	-	+	++	++
	Gelatin test	-	+	++	++
	Conc. HCL test	-	+	++	++
Amino acid/ Protein	Ninhydrin test	-	-	++	++
	Biuret test	-	-	++	++
Coumarins		+	+	++	-

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

ISSN: 0975-8585

Fluorescence analysis of powdered drug

The fluorescence analysis of the powder drug was done and results were represented in Table 3. The powder was treated with various reagents and the mixture was observed under day light and under UV light to see the type of fluorescence.

Table 3: Fluorescence analysis of powdered *Acacia etbaica* subspecies etbaica leaves.

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

Table 4: Observations of thin layer chromatographic studies of the leaves of *Acacia etbaica* subspecies etbaica

Extracts	Mobile phase	No. of spots	Rf values	Color	Intensity
Petroleum Ether	Benzene: Chloroform (1:1)	8	0.09 0.11 0.17 0.22 0.31 0.35 0.43 0.48	Gr (V,UV) Gr(V,UV) G(UV) O(I) Y(I) Y (UV) Br(I) Y(V)	+++ + ++ + ++ + +++ ++ + + +
Chloroform	Benzene: Acetic acid (9:1)	9	0.23 0.27 0.30 0.34 0.37 0.42 0.47 0.54 0.57	G(V,UV) G(V,UV) Br(I) Br(I) O(UV) Y(V) Y(V) Br(I) Y(UV)	+++ + ++ + ++ + +++ ++ + + +
Ethanol	Ethyl acetate- formic acid -Acetic acid-water (25:2:2:4)	5	0.07, 0.10, 0.22, 0.37, 0.77	LG(UV) Y(UV) Y(UV) Br(I) Y(UV)	+++ ++ + ++ +
Water	Ethyl acetate- formic acid -Acetic acid-water (25:2:2:4)	3	0.07, 0.30 0.37	G(UV) Y(I) Y(UV)	++ + ++

+++ = Most intense, ++ = moderately intense, + = Least intense, Y=Yellow, G=Green, LG= Light Green, Gr = Grey, B= Blue, Br = Brown, O=Orange, (I) = Iodine, (V) = Visible, (UV) = Ultraviolet 365 nm.

Thin layer chromatography

Thin layer chromatography was used for the separation and identification of various phytochemicals present in petroleum ether, chloroform, ethanol and water extracts. Various solvent systems were tested to obtain best results. The best results were mentioned in Table 4. The solvent system selected for the TLC of petroleum ether extract was Benzene: Chloroform (1:1). TLC resulted in identification of 8 spots. The solvent system selected for the TLC of chloroform extract was Benzene: Acetic acid (9:1). TLC resulted in identification of 9 spots. The solvent system selected for the TLC of ethanol extract was Ethyl acetate- formic acid -Acetic acid-water (25:2:2:4). TLC resulted in identification of 5 spots. The solvent system selected for the TLC of water extract was Ethyl acetate- formic acid -Acetic acid-water (25:2:2:4). TLC resulted in identification of 3 spots. The distances traveled by each spot and Rf values of the constituent compounds or spots were calculated with colour intensity and the results presented on the same table.

DISCUSSION

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 [8]. As a part of standardization study, the microscopic examination of leaf was studied. It is carried out on plant samples in order to establish appropriate data that can be used in identifying crude drugs particularly those supplied in powder form. The occurrence of thick cuticle is usually typical in plants growing in dry habitats [16]. Paracytic stomata and non-glandular trichomes have been found in Fabaceae members [17], as seen in the studied species. Similarly, in *Acacia auriculiformis*, non-glandular trichomes are characterized as unicellular, straight or curve [18]. Paracytic stomata and non-glandular, unicellular, curve trichomes are characterized for *Acacia etbaica* subspecies etbaica.

The phytoconstituents are known to play an important role in bioactivity of medicinal plants. Phytoconstituets like carbohydrates, glycosides, saponins, flavonoids, coumarins, tannins, triterpenes, sterols, amino acid and protein were presented in petroleum ether, chloroform, ethanol and water extracts, while alkaloids were not present; sterols, triterpenes, carbohydrates and glycosides found in all extracts (Table 2). Fluorescence study powdered drug under ultra violet light is very distinctive and helpful in establishing the purity of the drug (Table 3) [19]. The presence of phytoconstituents was further confirmed by thin layer chromatography and their Rf values of developed spots of different extracts were calculated with color intensity (Table 4).

CONCLUSION

The leaves of *Acacia etbaica* subspecies etbaica, locally known as 'Qarad', are generally used as in folk medicine in various parts of Yemen as well as in other counties. In the present study, therefore, the leaves were selected for anatomical and phytochemical studies aimed at established standard parameters. Result of microscopic examination showed that the paracytic stomata and non-glandular, unicellular, curve trichomes are characterized for *Acacia etbaica* subspecies etbaica. Phytoconstituets like carbohydrates,

glycosides, saponins, flavonoids, coumarins, tannins, triterpenes, sterols, amino acid and protein were presented; these compounds are known to have curative activity against several pathogens and therefore can be suggested for the treatment of different diseases. The microscopic and phytochemical studies of the leaves of *Acacia etbaica* subspecies etbaica have been reported for the first time. The present study can serve as an important source of information to ascertain the identity and to determine the quality and purity of the plant material available in market. 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.

ACKNOWLEDGEMENT

We are grateful to Professor Abdul Nasser Algifri, of the department of Biology, of University of Aden, Yemen, for his help to identify and authenticated the plant specimen.

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