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Methods for Extraction and Characterization of Tannins from Some *Acacia* Species of Sudan

Isam Eldin Hussein Elgailani* and Christina Yacoub Ishak

Department of Chemistry, Faculty of Science, University of Khartoum, Khartoum, Sudan

*Corresponding Author Email: gailani23@hotmail.com

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Abstract

The study is aimed to analyze and compare extraction methods of tannins from three common *Acacia* species of Sudan. The *Acacia* species selected were *Acacia nilotica*, *Acacia seyal* and *Acacia senegal*. Bark samples from bulk collections of the three *Acacia* species were extracted with water, 80% methanol and 70% acetone. Two sets of extraction were made, one by boiling and a second by shaking the samples in the respective solvents for eight hours at room temperature. Although the amount of material extracted by these two procedures did not differ greatly ($P > 0.05$), 70% acetone was a more efficient solvent than either water or 80% methanol. The tannins of mature fruits extract of *Acacia nilotica* were identified by using Thin Layer Chromatography (TLC), Ultraviolet and Infrared spectroscopy. Comparisons of absorption spectra and TLC of the reference tannins and some phenolics with that of *Acacia nilotica* extracts revealed the presence of both condensed and hydrolyzable tannins, since it consists of catechin, tannic and gallic acids. Catechin considered to be the phenolic precursor of condensed tannins. Hydrolysis of *Acacia nilotica* extract, tannic and gallic acids by butanolic-hydrochloric acid yielded gallic acid which is considered to be a chemical precursor of hydrolyzable tannins.

***Keywords*:** Tannins; *Acacia* species; Extraction; Characterization; Sudan

Introduction

Tannins are polymeric phenolic compounds with numerous hydroxyl groups and quite diverse in chemical structure [1-2]. Hydrolysis of some of tannins yields the simple, seven-carbon gallic acid, others give ellagic acid or other phenolic acids [3-4]. Tannins are generally divided into the hydrolyzable and condensed tannins. *Acacia* species are found in different climate areas, there are even a few aquatic legumes [5]. In Sudan, *Acacia* species are widespread and are of medicinally and economically valuable [6]. *Acacia nilotica* fruits have been used as one of the traditional medicine and as an antimicrobial agent in many countries around the world. Various parts of the plants are selected especially roots, young shoots and stem. The extract of *Acacia nilotica* leaves is play an important role in antibacterial processes [7].

Tannins complexes with sorghum proteins, this complex is hard to digest by human and hence lower the protein value [8]. The tannin- protein precipitation behaviors confirmed complexity and differences in their nature and potentiality for tanning or other uses [9]. Polyphenols and related structures are responsible for the antioxidant processes in the human body system [10]. Tannic acid with low levels affords protection against polycyclic aromatic hydrocarbon-induced forestomach and lung [11]. Tannins of *Agrimonia japonica* has been used as antidiarrheic and a hemostatic in Japan and China [12].

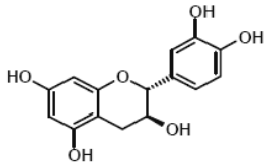
Literature shows that tannins were extracted by different procedures and techniques. In guava leaves, comparison for solvent extraction for tannins by using ethanol and acetone which resulted that ethanol 30% (v/v) is the best solvent

[13]. Tannins also extracted from the bark of *Pinus oocarpa* with sodium carbonate and sodium bisulfite [14]. Tannins of *Galium tunetanum* were extracted by two methods by ethanol 30% for 2 hours and the other by acetone for 24 hours [15].

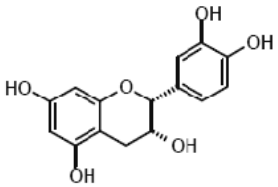
The vegetable tannins may be classified into two categories based on the nature of combination to achieve the molecular size and reactivity required. The first class is condensed tannins which are not hydrolyzed in the presence of acid, bases or appropriate enzymes, and the second class of tannins is hydrolyzable tannins which are hydrolyzed in the presence of acids, bases or appropriate enzymes to give either gallic acid or ellagic acid [3, 16-17]. Condensed tannins contain only phenolic nuclei, most tannins of this type are formed by the condensation of two or more of flavanols, such as catechin(I) and epicatechin(II) and leucocyanidin(III), or can be mixture of these [17-19].

Hydrolyzable tannins yield on hydrolysis by acids, bases or appropriate enzymes glucose core (IV) together with gallic acid (V) or its congeners such as hexahydroxydiphenic acid (VI). Also ellagic acid (VII) which is obtained by aerial oxidation of gallic acid [1, 16, 20]. Tannic acids belong to the hydrolyzable group of tannins [21- 22].

In this work, we compare the efficiency of various solvents for extraction of tannins from bark of the three common *Acacia* species of Sudan. The effect of temperature and shaking on the extraction efficiency is also compared. TLC, Infrared and Ultraviolet spectrometry were used for identification of compounds which are found in mature fruits extract of *Acacia nilotica* and compared with the reference of tannins and related phenolics.



Catechin (I)



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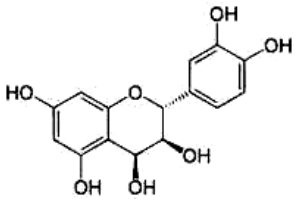
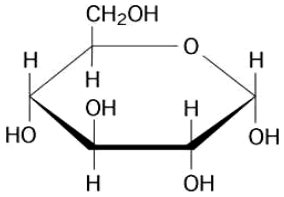
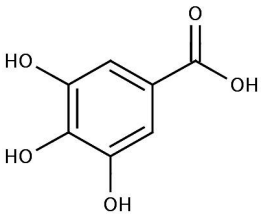


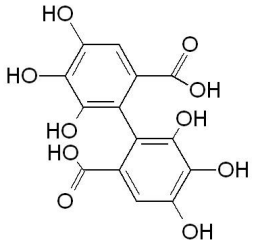
Leucocyanidin (III)



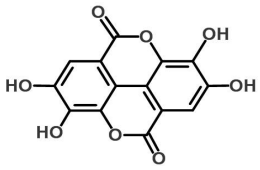
Glucose core (IV)



Gallic acid (V)



Hexahydroxydiphenic acid (VI)



Ellagic acid (VII)

Materials and Methods

Sampling

Samples of bark of *Acacia nilotica, Acacia seyal* and *Acacia senegal* from individual collections were used for the study extraction efficiency. Mature fruits of *Acacia nilotica* were collected and used to identify the tannins. Bark was removed from wood before drying. Plant Materials were taken from several trees in each instance from the Sunt Industrial and Tourism Centre (Sunt Forest) at Khartoum and Debabat Forest at South Kordofan State at West of Sudan. Three individuals for each samples were used for the analysis (n = 3).

Chemicals and reagents

The chemical materials used for the analysis in this work were of high grade.

Extraction of bark samples

Air-dried bark samples (from bulk collections) were ground in a Wiley mill (2 mm screen). A portion (40 g) was extracted with water, another with 80% methanol, and a third with 70% acetone (200 ml) by shaking at room temperature for 8 hours and another series by boiling for 10 minutes. The samples were filtered (Whatman 1 paper, 18.5 cm disc) and the residual material rinsed with additional solvent (two portions each of 50 ml). Extracts were transferred to a tared, round-bottomed flask and concentrated under vacuum by rotary evaporator to form a thick extract. The sample extracts were then dried in a vacuum oven at 60°C until a solid material was obtained. The amount of extract was determined by weight difference Table 1.

Characterization of acacia nilotica tannins

Samples of mature fruits from individual collections of *Acacia nilotica* were used to determine the tannins. Material was taken from several trees in each instance. Air-dried sample was ground in a Wiley mill (2 mm screen). In order to determine the composition of tannins of the sample, it was characterized by TLC, UV, and IR spectrophotometer and was compared with the references (standards) of tannins and related phenolics.

Identification of tannins by TLC

Dried and powdered fruits of *Acacia nilotica* (100 g) were shaken with water (500 ml) for 24 hours at 25°C by using the mechanical stirrer, the solution filtered through glass wool. 100 ml of extract were put into a 250 ml beaker, and the pH adjusted to 6.2 by addition of ortho-potassium dihydrogen phosphate (10 ml) and sodium hydroxide solution (2N, approximately 5 ml) by using pH-meter before extraction with 50 ml of ethyl acetate for 10 times. Heat at 30°C for elimination of solvent, then a bright brown amorphous powder will be obtained.

Thin-layer plates (size 20 cm length and 20 cm width) and another (20 cm length 5 cm width) were prepared with cellulose. 6% aqueous acetic acid being used as developing solvent. The extract was dissolved in acetone (100 ml) and separated by TLC on cellulose, it gives three fractions when run with two dimensional TLC Fig. 1. Comparison of standards Tables 2 and the three isolated fractions gave the patterns shown in Tables 3.

Degradation of tannins with alcoholic-hydrochloric acid

In order to determine the composition of tannins of *Acacia nilotica* whether it is hydrolyzable or not, the sample of mature fruits was hydrolyzed by alcoholic hydrochloric acid according to the following procedure: 0.5 ml of extract was heated for 2 hours at 95°C with 5 ml 5% butanol-HCl [23], the product of acid hydrolysis were characterized by TLC Table 3.

Detection reagents

After the development of the chromato-gram, tannins and related phenolics were detected by iodine vapours. Tannins and related phenolics appear as brown spot after exposure to iodine fumes in a closed tank.

Chemical tests of tannins extract
Test with ferric chloride solution

To a 1 ml portion of the extract was taken in a test tube and 5 drops of $FeCl_3$ solution in methanol were added. A green to black precipitate appears in the presence of tannins Table 4.

Test with gelatin solution

1 ml portion of the extract was taken in a test tube and added 1ml of gelatin (1% solution) and NaCl. The formation of a white precipitate will show that tannins were exist [24] Table 4.

Test with ferrous sulphate solution

1 ml portion of the extract was taken in a test tube and 2 ml of 0.1% $FeSO_4$ and 0.5% sodium potassium tartrate were added. The appearance of violet colour indicates the presence of tannins.

Chemical tests of tannins were applied for the isolated fractions of *Acacia nilotica* mature

fruits extract obtained from TLC for identification of tannins Table 4.

UV Spectrophotometry of tannins and related phenolics

100 mg of the water extract, (dried) of mature fruits of *Acacia nilotica* were dissolved in 25 ml of methanol. Standards of tannins and related phenolics were prepared by dissolving 10 mg in 25 ml of methanol. After that the solutions were diluted with the same solvent (1:100), and the spectrophotometric measurements were recorded as seen in Table 5. On the other hand, the acid hydrolyzed tannins was also determined spectrophotometrically as in Table 6.

IR Spectrophotometry of tannins and related phenolics

About 1.0 g of the dried water extract of mature fruits of *Acacia nilotica* was dissolved in 5 ml of methanol. Standards of tannins and related phenolics were dissolved by the same manner. The sample and standards were subjected to IR measurements, Table 7. On the hand, the sample was dissolved in acetone by the same manner as previously described, (1.0 g in 5 ml), and subjected to IR measurements and reveal the same IR spectra as in Table 7.

Statistical analysis

Each treatment was carried in replicates and each sample was analyzed three time The results are expressed as mean (n = 3), by using one-tail analysis of variance. Testing hypothesis for the comparison of the two procedures were carried out by using the following relations:

$$\text{For } S_{Pooled}, S_{pooled} = \sqrt{\frac{S_1^{\,2}(N_1-1)+S_2^{\,2}(N_2-1)}{(N_1-1)+(N_2-1)}}$$

where S_1 and S_2 ar standard deviations, N_1 and N_2 are replicates (= 3 for each), and S_{pooled} is pooled standard deviations.

and for the comparison of the two procedures:

$$\bar{x}_1 - \bar{x}_2 = \pm t s_{pooled}\sqrt{\frac{N_1+N_2}{N_1N_2}}$$

where $\bar{x}_1$ and $\bar{x}_2$ are the means, and t for t-value (at 95% confidence level, t-test table, $P < 0.05$). The null hypothesis is accepted if the calculated value (left side) is less than the tabulated value (right side) [25-26].

Results and Discussions

Numerous studies have examined the solubility of tannins in solvents, but no solvent system has been found to be completely satisfactory. Solubility of tannins depend on many factors including the structure of the tannins themselves. Aqueous acetone solutions are generally most effective in removing both condensed and hydrolyzable tannins. Pure solvents were insufficient extraction media for the recovery of phenolics and particularly tannins.

In this study, two sets of extractions were made, one by boiling and the other by shaking the samples in the respective solvents for 8 hours at room temperature Table 1. The solvents used for extraction were distilled water, 80% methanol and 70% acetone. Although the amount of material extracted by these two procedures did not differ greatly ($P > 0.05$), 70% acetone was a more efficient solvent than either water or 80% methanol Table 1 in terms of the weight of material extracted from a given weight of *Acacia* species material and in the percentage of tannins in the phenolic materials extracted, and the percentage of tannins exracted from the bark samples.

Also in this work, the tannins of mature fruits extract of *Acacia nilotica* were identified by TLC, UV and IR spectrometry. Comparison of the absorption spectra and TLC chromatograms of the reference tannins and some related phenolics with that of *Acacia nilotica* extract revealed the presence of both condensed and hydrolyzable tannins.

The results presented in our work, show that when the extract was run with two dimensional TLC, it give three fractions Fig. 1. Comparison of standards with the extract gave the pattern shown in Table 2 by TLC. The three fractions of the extract on TLC were scratched and taken separately, the composition of the pigment

fractions was investigated by TLC Table 3. Some of the chemical tests were applied for each of the three fractions in order to identify the nature or property of the fractions, fraction I and III, Table 4 did not show tannin properties.

Table 1. **Total extractives from a*cacia* bark samples with water, 80% methanol and 70% acetone.**

Species	Solvent	Boiled (g)	% Extracted	Unboiled (g)	% Extracted
A. nilotia	water	6.2	15.5	6.1	15.3
	80% MeOH	10.2	25.5	10.0	25.0
	70% acetone	10.5	26.3	11.2	28.0
	Std. dev. (S)	0.2		0.1	
	(S_{pooled})		0.1580		
A. seyal	water	7.7	19.3	8.1	20.3
	80% MeOH	10.3	25.8	11.1	27.8
	70% acetone	11.0	27.5	11.8	29.5
	Std. dev. (S)	0.05		0.1	
	S_{pooled}		0.0791		
A. senegal	water	3.4	8.5	3.7	9.3
	80% MeOH	4.4	11.0	4.3	10.8
	70% acetone	4.8	12.0	4.5	11.3
	Std. dev. (S)	0.1		0.1	
	(S_{pooled})		0.2270		

** Values are the mean of three determinations (n=3), (std. dev. is the standard deviation),(P < 0.05).*

Table 2. **TLC of some standards of tannins and related phenolics.**

Tannins and Related Phenolics	Retardation Factor (R_f) in 6% acetic acid as developing system
1. Gallic acid (standard)	0.47
2. Tannic acid (standard)	0.47
3. Catechin (standard)	0.47
4. Catechol (standard)	0.73
5. m-hydroxybenzoic acid (standard)	0.71

Table 3. **TLC, "Cellulose", 6% aqueous acetic acid for the three fractions isolated from *acacia nilotica* (mature fruits).**

Fration	Retardation Factor (R_f) in 6% acetic acid as developing system
I	0.11
II	0.47
III	0.71

Table 4. **Chemical tests of the isolated fractions of *acacia nilotica* (mature fruits).**

Property of Test	Fraction (I)	Fraction (II)	Fraction (III)
1. Ferric chloride	negative test	positive test	positive test
2. Gelatin in the presence of sodium chloride	negative test	positive test	negative test
3. Ferrous sulphate in the presence of sodium tartrate	negative test	positive test	negative test

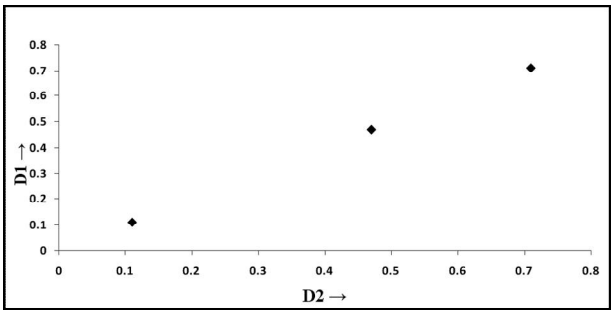


Figure 1. **Diagram of the two dimensional (D1 & D2) TLC of *Acacia nilotica* (mature fruits). Stationary phase: Cellulose. mobile phase: 6% acetic acid. Detection reagent: Iodine vapours**

The UV absorption spectra of the extract and standards when using methanol as solvent, they have shown peak maxima at 280 nm, Table 5, indicating the presence of catechin and tannic acid. When the extract and the standards were subjected to hydrolysis by alcoholic-HCl acid, they showed maximum peaks at 272 nm, Table 6 indicating the presence of gallic and tannic acids. Catechin (Flavan-3-ols) is considered to be a monomer of condensed tannins. Hydrolysis of *Acacia nilotica* extract, tannic and gallic acids by butanolic-HCl acid yield gallic acid which is considered to be a chemical precursor of hydrolyzable tannins.

The IR absorption spectra of the extract, show the presence of hydroxyl group (OH^-), aromatic C-H stretch, carbonyl group C=O stretch, C=C ring stretch, C-O stretch and out-of-plane C-H bending when compared with standards, Table 7.

Table 5. **The UV spectra of standards of tannins, related phenolics and the sample *acacia nilotica* (mature fruits) by methanol as solvent.**

Tannins and Related Phenolics	Wavelength (nm)	
	Ethylenic Band (E2-band)	*Benzoic Band (B-band)*
Gallic acid (standard)	216	264
Tannic acid (standard)	220	280
Catechin (standard)	208	280
Catechol (standard)	216	276
m-hydroxybenzoic acid(standard)	207	292
The sample of *A. nilotica* (Mature fruits)	216	280

Table 6. **The UV spectra of standards of tannins, related phenolics and the sample *acacia nilotica* (mature fruits) after hydrolysis by Butanolic-HCl.**

Tannins and Related Phenolics	Wavelength (nm)	
	Ethylenic Band (E2-band)	*Benzoic Band (B-band)*
Gallic acid (standard)	220	272
Tannic acid (standard)	220	272
Catechol (standard)	-	277
Catechin (standard)	-	-
m-hydroxybenzoic acid(standard)	-	-
The sample of *A. nilotica* (Mature fruits)	220	272

Table 7. **The IR spectra of standards of tannins, related phenolics and the sample *acacia nilotica* (mature fruits).**

Tannins and Related Phenolics	Groups (cm^{-1})					
	O-H stretch	*Aromatic C-H stretch*	*C=O stretch*	*C=C ring stretch*	*C-O stretch*	*Out-of-plane C-H bend*
Gallic acid (standard)	3370	2950	1660	1660 1525 1415	1310 1250 1185	-
Tannic acid (standard)	3370	2750	1685	1660 1515 1435	1300 1180 -	-
Catechin (standard)	3370	2900	-	1595 1500 1435	1340 1240 1185	-
Catechol (standard)	3370	3040	-	1590 1500 1465	1355 1235 1185	730
m-hydroxy-benzoic acid (standard)	3370	2820	1670	1595 1500 1455	1300 1230 1160	750
Observed Band of sample *A. nilotica* (Mature fruits)	3370	2920	1670	1595 1520 1435	1315 1190 -	-
Literature Band (cm^{-1})	3200-3550	2880-3030	1650-1670	1500-1660	1080-1300	675-900

Conclusion

This study has shown that the extraction of three *Acacia* species using distilled water, 80% methanol and 70% acetone has been successfully made. The extraction was made once by boiling and the other by shaking the samples in the respective solvent for 8 hours at room temperature. Results showed that the 70% acetone was the most efficient solvent among the three solvent used. In addition, characterization study of *Acacia nilotica* extract revealed the presence of some compounds of both condensed and hydrolyzable tannins.

Recommendations that could be drawn from this study are that further studies will be necessary to identify the tannins in the other *Acacia* species because of the importance and usefulness of the tannins group and its applications in wide range.

Conflicts of interests

The authors declare that there is no conflict of interests regarding the publication of this article.

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