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RESEARCH ARTICLE

COMPLEMENTARY ANALYTICAL TECHNIQUES PAPER, THIN-LAYER, HIDE-POWER, AND COMBINED METHODS FOR CHARACTERIZATIONOF TANNIN IN PLANTS

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Abstract

The polyphenolic compoundsextract rich in gallo-catechol tannins submitted to complementary analytical techniqueswas evaluated. The whole plantspecies screened were of the condensed type *except Acacia seyal var. fistuala*, Acacia*seyal var. seyal*, *Casuarina equistifolia*, and *Pithecellobium dulce*were of mixedhydrolysable-condensed(gallo-catechol) type. The quantitative data indicated that 5 parts (bark) out of 12 species, when extracted, contained more than 10% tannins (oven-dry basis), the level of commercial interest. The catechin numbers indicated that all the studied species contained condensed tannin in varying amounts (0.6-45.7), while the presence of both gallic acid and catechin means that the tannin is of mixed type. Thin-layer and paper chromatography with different solvent systems confirmed the presence of catechin and gallic acid, and showed that tannic acid, fisetin, epicatechin and some unidentified phenolics were present. However, dihydrofisetin and robinetin, which were used as standards, were not detected. Astringency values shows that the *Acacia mellifera*(0.18), *Acacia seyalvar.fistuala*(0.18), *Pithecellobium dulce* (0.15), *Acacia senegal* (0.14), *Acacia farnesiana* (0.13), *Calotropis procera* (0.13)barks could be used in place of *A. mearnsii*(international commercial tannin materials) (0.16) because the degree of relative astringency or the ability of their tannin to combine with protein is close to that of *A. mearnsii*; in other words these six species can give leather with characteristics comparable with that of *A. mearnsii*.

Introduction:-

Vegetable tannins are polyphenolic compounds widely distributed in plants which have the property to precipitate proteins (Vermerris and Nicholson, 2006; Khanbabaee and van Ree, 2001). Since ancient times, this property has been empirically discovered to convert animal skins, a proteinaceous biomaterial, into leather (Goffer, 2007; Covington, 2009). The process, termed vegetable tanning, is one of the oldest known leathers making processes and it can be succinctly described as a treatment of hides/skins with powdered barks, leaves, wood, fruits, pods or galls, or their extracts, obtained from different vegetable sources (Thomson, 2006). With this treatment, traditionally performed in pits, a chemical interaction between collagen protein (the main constituent of dermis) and tannins present in vegetable materials is slowly established, generating a very useful and remarkably non-putrescible material under moist and warm conditions, termed vegetable tanned leather (Covington, 2009; Haslam, 1997). It was

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the main material of a wide range of artefacts and adapted to very diverse functional needs such as footwear, bookbinding, saddles, harness, liquid vessels, cases and caskets coverings or seating furniture and carriages upholstery. Beyond its utilitarian function, it was also used as support material for artistic and decorative paintings, wall hangings and screen coverings.

Different indigenous plants materials have been traditionally used in Europe: barks from birch (Betula spp.), willow (Salix spp.), larch (Larix spp.) and spruce (Picea spp.) were used in northern Europe and Russia; barks from various species of oaks (Quercus spp.) widely used throughout Western Europe; leaves from sumac shrub (Rhus coriaria), valonia (Quercus Aegilops) oak galls from Quercus infectoria in Mediterranean (Novak et.al, 2008; Gülcin et.al, 2010).

Condensed tannins, or proanthocyanins, are natural polyphenolic oligomers made of flavan-3-ol units. They are recognized as suitable natural substitutes in the formulation of wood adhesives (Yazaki and Collins, 1994; Roffael et al. 2000; 2006; Pizzi, 2008), foamed resins (Lacoste et al., 2013) and heavy metal removal systems. Industrially used tannins are mostly extracted from the bark of black wattle (*Acacia mearnsii* [De Wild.]) and the heartwood of Quebracho (*Schinopsislorentzii* [Engl.]). The bark of softwood species has also been reported as a valuable source of condensed tannins (Krogell et al., 2012). In Switzerland, 425,000 m^3 of bark was produced in 2013, the majority of which was burned for energy production (Lacoste et al., 2013). Thus, softwood bark represents an important source of condensed tannins in Switzerland. In particular, silver fir (*Abies alba* [Mill.]), Norway spruce (*Piceaabies* [Karst.]), Scot's pine (*Pinus sylvestris* [L], European larch (*Larix decidua* [Mill.]) and Douglas fir (*Pseudo Sugamenziesii* [Mirb.]) are species of special interest, representing more than 95% of the total Swiss softwood growing stock (Lacoste et al., 2013).

This investigationpurposes to expand the knowledge aboutvegetable tanning materialsthathad significant concentrations of these compounds. This information is important to recognize tannin structure, knowledge, deprivation susceptibility or state in demand to bring out suitable techniques, and if required, to choosesuitable one for tannin vegetable materials.

Materials and Methods:-

Preparation of sample

Fresh plant parts (bark) (0.3–2.0 kg) from different species growing in Khartoum area, Blue Nile, and South Kordofan (Dalang), were used for this study (Table 1). The conformation of the identity of the plant species is done by Soba Forestry Research Center Herbarium. The samples were air-dried and reduced to powder with a star mill. The fractions passing through 40-mesh and retained on 85-mesh sieve were collected, thoroughly mixed and kept in airtight containers.

Table 1:- Collection data for the tanniferous plant species studies.

Species	Part	Age	Collection site	Air-dried Material
Acacia albida	Bark	15	Obeid	2.0
Acacia farnesiana	Bark	10	Soba	1.0
Acacia mearnsii	Bark	25	Jebel Marra	2.0
Acacia mellifera	Bark	20	Soba	0.5
Acacia seyalvar. fistuala	Bark	9	Blue Nile	1.0
Acacia seyalvar.seyal	Bark	10	Soba	0.3
Acacia senegal	Bark	10	Soba	0.3
Albizzia amara	Bark	5	Blue Nile	0.3
Calotropis procera	Bark	4	Soba	0.3
Cassia siamea	Bark	7	Shambat	1.0
Casuarina equistifolia	Bark	18	Blue Nile	0.3
Pithecellobium dulce	Bark	30	Soba	0.3

Analysis of Tannins

Extraction Using ALCA-Palsy Method

Cold water extracts (2 litres) were obtained with an ALCA (American Leather Chemist Association)-Palsy apparatus (Doat, 1978). The presence of tannins was detected by the gelatin salt test and their types were identified using the iron-alum and formaldehyde-HCl test (SLTC. 1965).

Qualitative Analysis

Paper chromatography was done on Whatman No. 1 paper withforestal solvent system (concentrated acetic acid: HCl: water, 10:3:30) (Harborne,1998). The chromatography was developed by ascending method at room temperature (30–36 °C) to a height of 7–15 cm. Spots were detected first under UV light (254 nm) and then by spraying with ferric chloride reagent (2 g $FeCl_3$ in98 ml methanol) or exposing to ammonia vapour (Stahl,1969). Thin layer chromatography was done with sheets (20 × 20 cm) precoated with polyamide six layer (thickness 0.1 mm). The solvent system used was acetone-propanol-water (5:4:1) (Stahl,1969).

Tannic acid, catechin, gallic acid, epicatechin, fisetin, dihydrofisetin and robinetin were used as standard compounds (R_f ×100) for the above chromatographic analyses. Samples were prepared by hydrolyzing 5 g raw materials with 2M HCl using reflux for 30 min. The effluent was then cooled and filtered; ethyl acetatethen used to extract the produced filtrate. The aqueous layer was heated to remove any trace of solvent and extracted with a small volume of amyl alcohol. The solvent extracts were concentrated to thick syrup under vacuum (Harborne,1998).

Quantitative Analysis

The extracts were quantitatively analyzed for total and soluble solids, non-tannins and tannins by the official hide-powder method (Jamet,2000) (hide-powder batch C28). A modification of the hide-powder method, i.e., the combined method (Swain and Goldstein,1964) was also used. Total phenolic materials in the extract were measured using the Folin-Denis'smethod (Folinand Denis, 1915). Freshly hydrated chromated hide-powder equivalent to 3.0 g oven-dried was prepared. Tannin was then allowed to absorb onto the hide powder, after which the remaining phenolic materials were determined. The catechin number (Stiasny number) was determined according to the method by Yazaki and Hillis (Folin and Denis, 1915). For this 100 ml extract were filtered through a glass fritted funnel (G4) and poured into a conical flask. Stiasny reagent (5 ml of HCl + 10 ml of 37% formaldehyde) was added into the flask and then the mixture was allowed to stand for 24 hours at room temperature (30–35 °C). Then the precipitate was filtered on a tared crucible (G4) before being dried to constant weight at about 100 ± 5 °C to obtain the weight of catechin (Folin and Denis, 1915).

Results and Discussions:-

Tannins are phenolic compounds of relatively high molecular weight. They are classified as condensed and hydrolysable tannins. The hydrolysable tannins are readily hydrolyzed by acids, alkalis or enzymes (tannases) into a sugar or a related polyhydricalcohol (polyol) and a phenolic carboxylic acid (Pizzi, 2008). Depending on the nature of the phenolic carboxylic acid, hydrolysable tannins are subdivided into gallotannins and ellagitannins. Hydrolysis of gallotannins yields gallic acid while hydrolysis ofellagitannins yields hexahydroxy diphenic acid which is isolated asellagicacid (Pizzi, 2008). Hydrolysable tannins are considered as one of the most potentantioxidants from plant sources. They are ready to form complexes with reactive metals, avoiding free radical generation whichresults in oxidative damage of cellular membranes and DNA (Lacoste et al., 2013). Hydrolysable tannins, in addition, clean free radicals within the body by neutralizing them before cellular damage occurs (Hagerman, 1998; Gülcinet al., 2010).

Formaldehyde-HCl and Iron Alum Test

From the formaldehyde-HCl and iron alum test, the whole twelve species screened were of the condensed type except *Acacia seyalvar. fistuala*, and *Acaciaseyalvar. seyal*, *Casuarina equistifolia*, and *Pithecellobium dulce* were of mixed hydrolysable-condensed (gallo-catechol) type. The gallic acid and catechin number test results supported these assignments (Table 2). The quantitative data indicated that fiveparts (bark) of twelve species, when extracted, contained more than 10% (oven-dry basis) of tannins, the level of commercial interest. Of these 12 species, 6species had an acceptable extraction ratio (tannin to non-tannin) of 1.0-4.5. The tannin purity or the ratio of tannin/soluble solids was good, >0.5, for 7species of the twelve species studied (Table 2). However, the type of tannin present and the part extracted are also important.

Different parts of species bark, leaves, and fruits had the same type of tannin but in different proportions. Usually, the tannin content was higher in the barks (*Acacia mearnsii, Acacia seyalvar. fistuala, Acacia seyalvarseyal, Acacia Senegal, Pithecellobium dulce,* and*Casuarinaequistifolia)* (Table 2). The catchin numbers indicated that all the studied species contained condensed tannin in varying amounts (0.6-45.7), while the presence of both gallic acid and catechin means that the tannin is of mixed type (hydrolysable-condensed) (gallo-catechol) (Table 2).

Thin-layer and paper Chromatography

Thin-layer and paper chromatography with different solvent systems confirmed the presence of catechin and gallic acid, and showed that tannic acid, fisetin, epicatechin and some unidentified phenolics were present. However, dihydrofisetinandrobinetin, which were used as standards, were not detected (Table 3).

Methods of Determination of Tannins

The tannin content determined by the hide-powder method was highest (39.8) for*Acacia mearnsii* followed by (28.8%) for *Pithecellobium dulce* bark, and for *Acacia seyalvar. seyal*and*Acacia seyalvar. fistuala*, *Casuarina equistifolia*bark (24.8,23.7,10.2% respectively) (Table 2). These data were compared with those obtained from the spectroscopic method of Swain and Goldstein (Hagerman,1998) and also with two methods for total phenolic (Yazaki and Hillis,1998; Hagerman and Butler 1978) (Table 4). In the first comparison, the correlation between total phenolics and tannin content was high (r^2= 98.7%, n = 24, $p < 0.01$). In the second case, the phenolic content by the Hagerman and Butler method (Judd, et al., 2007; Talhouk et al., 2007) was approximately half that of Folin-Denis's assay, but the correlation between the two assays was still high (r^2= 70.9%, n = 24, $p < 0.01$). The combined method also gave slightly lower values of tannin content and extraction rates (Table 4). Care should be taken when comparing tannin content determined bydifferent methods as the isolation procedures may affect the proportion and types of phenolic present (this due to different method have different ways of determination and isolation). The relative astringency values for most of these tannins were quite close to that of *A. mearnsii* tannin, but much higher values were obtained for *Acacia mellifera* and *Acacia seyalvar. Fistuala*bark. However, the*Acacia mellifera*bark has low tannin contents (17.9%) (Table 4).

Stringency Factor

Astringency values shows that the *Acacia mellifera*(0.18), *Acacia seyalvar. Fistuala*(0.18), *Pithecellobium dulce* (0.15), *Acacia senegal*(0.14), *Acacia farnesiana*(0.13), *Calotropis procera*(0.13)barks could be used in place of *A. mearnsii*(international commercial tannin materials) (0.16) because the degree of relative astringency or the ability of their tannin to combine with protein is close to that of A. mearnsii; in other wards thesesix species can give leather with characteristics comparable with that of A. mearnsii.

Precipitation of Protein

The protein precipitation curve for the tannins from *A. mearnsii* bark (international commercial tannin materials) and*Acacia senegal, Acacia seyal var. fistuala, Cassiasiamea, Albizzia amara*, bark reflected their different nature and relative astringency (Figure 1). The fairly gradual solubilization of *A. mearnsii*tannins (wattle) and *Cassia siamea*, *Albizzia amara*, *Acacia senegal*, and *Acacia seyal var. fistuala*barktannins indicated greater reactivity. It seemed probable that the highly astringent and strongly binding tannin would react with animal hide protein so firmly and rapidly that the penetration of the materials would have to be controlled by selection of pH and concentration. Thus, the resulting leather might be hard and coarse. In contrast the less astringent tannin (mixed type) obtained from the *Acacia seyal var. fistuala*bark and *Cassia siamea*bark mixed with *Calotropis procera*barkshould penetrate the hide more extensively and the reaction should not be weaker in terms of poorer tanning or greater vulnerability to microbiological damage.

Species	Part	Total solids (TS)%	Soluble solids (SS) %	pH	Tannins, (T)%	Non-Tannins, (NT)%	Extraction Ratio (T/NT)	Catechin number	Gallic acid	Tannin type	Purity (T/SS) %
Acacia albida	**Bark**	**10.3**	**9.5**	**6**	**4.6**	**4.9**	**0.9**	**4.6**	**-**	**C**	**0.5**
Acacia farnesiana	Bark	13.7	12.4	6	3.6	8.8	0.4	2.1	-	C	0.3
Acacia mearnsii	**Bark**	**51.8**	**48.7**	**6**	**39.8**	**8.9**	**4.5**	**45.7**	**-**	**C**	**0.8**
Acacia mellifera	Bark	12.7	10.9	6	4.3	6.7	0.6	2.0	-	C	0.4
Acacia seyalvar.fistuala	Bark	40.9	40.4	6	23.7	16.7	1.4	30.5	+	HC	0.6
Acacia seyalvar.seyal	Bark	39.0	36.6	6	24.8	11.7	2.1	32.4	+	HC	0.7
Acacia senegal	Bark	16.2	15.7	5	6.8	8.9	0.8	8.6	-	C	0.4
Albizzia amara	Bark	13.4	13.4	6	7.2	6.1	1.2	6.5	-	C	0.5
Calotropis procera	Bark	15.3	13.2	6	2.5	10.7	0.2	4.5	-	C	0.2
Cassia siamea	Bark	5.6	5.4	6	2.2	3.2	0.7	0.6	-	C	0.4
Casuarina equistifolia	Bark	16.7	14.9	6	10.2	4.7	2.2	12.3	+	HC	0.7
Pithecellobium dulce	Bark	38.9	35.7	6	28.8	6.9	4.2	26.6	+	HC	0.8

Table 2:- Analysis of the tannin cold aqueous extracts (% oven-dry part extracted).

Species	Part	Extracted with	Gallic acid		Tannic acid		Catechin		Epicatechin		Fisetin		Unknown	
			TLC 82	PC 63	TLC 56	PC 32	TLC 78	PC 64	TLC 66	PC 64	TLC 66	PC 15	TLC	PC
Acacia albida	Bark	Amyl alcohol Ethyl acetate	- -	- -	- -	- -	77 -	67 -	66 -	66 -	65 -	15 -	- 64	- -
Acacia farnesiana	Bark	Amyl alcohol Ethyl acetate	- 83	- 62	- -	- -	77 -	67 -	- -	- -	- -	- -	- -	- -
Acacia mearnsii	Bark	Amyl alcohol Ethyl acetate	- 83	- 62	- -	- -	77 -	67 -	- -	- -	- -	- -	- -	- -
Acacia mellifera	Bark	Amyl alcohol Ethyl acetate	- -	- 62	- -	- -	77 78	67 -	- -	- -	- -	- -	- -	- -
Acacia seyalvar.fistuala	Bark	Amyl alcohol Ethyl acetate	- -	- 62	- -	- -	77 78	67 -	- -	- -	- -	- -	- -	- -
Acacia seyalvar.seyal	Bark	Amyl alcohol Ethyl acetate	- -	- 62	- -	- -	78 -	64 -	- -	- -	- -	- -	- -	- -
Acacia senegal	Bark	Amyl alcohol Ethyl acetate	- 82	- 62	- -	- -	77 -	67 -	- -	- -	- -	- -	- -	- -
Albizzia amara	Bark	Amyl alcohol Ethyl acetate	- -	- 62	- -	- -	78 -	64 -	- -	- -	- -	- -	- -	- -
Calotropis procera	Bark	Amyl alcohol Ethyl acetate	- -	- 65	- -	- -	78 -	62 -	- -	63 -	- -	- -	- -	51 -
Cassia siamea	Bark	Amyl alcohol Ethyl acetate	- -	- 63	- -	- -	78 -	67 -	- -	- -	- -	- -	- -	- -
Casuarina equistifolia	Bark	Amyl alcohol Ethyl acetate	- 83	- 65	- -	- -	78 -	62 -	- -	- -	- -	- -	- -	- -
Pithecellobium dulce	Bark	Amyl alcohol Ethyl acetate	- 82	- 64	- -	- -	77 -	66 -	- -	- -	- -	- -	- 64	- -

Table 3:- Thin layer (TLC)* and paper (PC) ** chromatography of hydrolyzed bark extracts.

* Adsorbent: Polyamide precoated plate (10x10 cm); solvent system: acetone- propanol- water (5/4/1); detection: UV/254nm; $FeCl_3$.
**Adsorbent: Whatman paper no.2; solvent system: acetic acid-conc. HCl- water (10/3/30); detection: UV/254nm; strong ammonia vapor.

Table 4:- Determination of total phenolics content and astringency factor in tannin extract by different methods.

Species	**Part**	**Tannin content, % in oven-dry part extracted**		**Extraction Ratio (Tannin/non-tannin)**		**Total phenols, % in oven-dry part extracted**			
		Hide Powder Method	**Combined Method**	**Hide Powder Method**	**Combined Method**	**Combined Method**	**Folin Denis Method**	**Hagerman Butler Method**	**Relative Stringency**
Acacia albida	Bark	14.2	14.3	1.1	1.2	60.0	13.8	7.0	0.12
Acacia farnesiana	Bark	14.0	14.3	1.9	0.4	49.2	14.2	6.8	0.13
Acacia mearnsii	Bark	39.8	38.1	4.5	2.7	72.8	35.6	17.8	0.16
Acacia mellifera	Bark	17.9	15.5	2.2	0.6	46.8	16.0	8.0	0.18
Acacia seyalvar.fistula	Bark	23.7	15.5	1.4	0.6	46.8	16.0	8.0	0.18
Acacia seyalvar.seyal	Bark	24.8	10.2	2.1	1.0	47.1	10.0	5.1	0.12
Acacia senegal	Bark	19.3	19.2	2.2	3.2	45.6	18.6	9.3	0.14
Albizzia amara	Bark	14.2	14.3	1.1	1.2	60.0	13.8	7.0	0.12
Calotropis procera	Bark	10.5	10.6	1.6	1.0	43.8	10.1	5.0	0.13
Cassia siamea	Bark	10.4	10.3	1.7	1.5	50.4	10.3	5.3	0.12
Casuarina equistifolia	Bark	10.2	10.2	2.2	1.0	47.1	10.0	5.1	0.12
Pithecellobium dulce	Bark	28.8	27.9	4.2	1.4	39.6	27.3	14.5	0.15

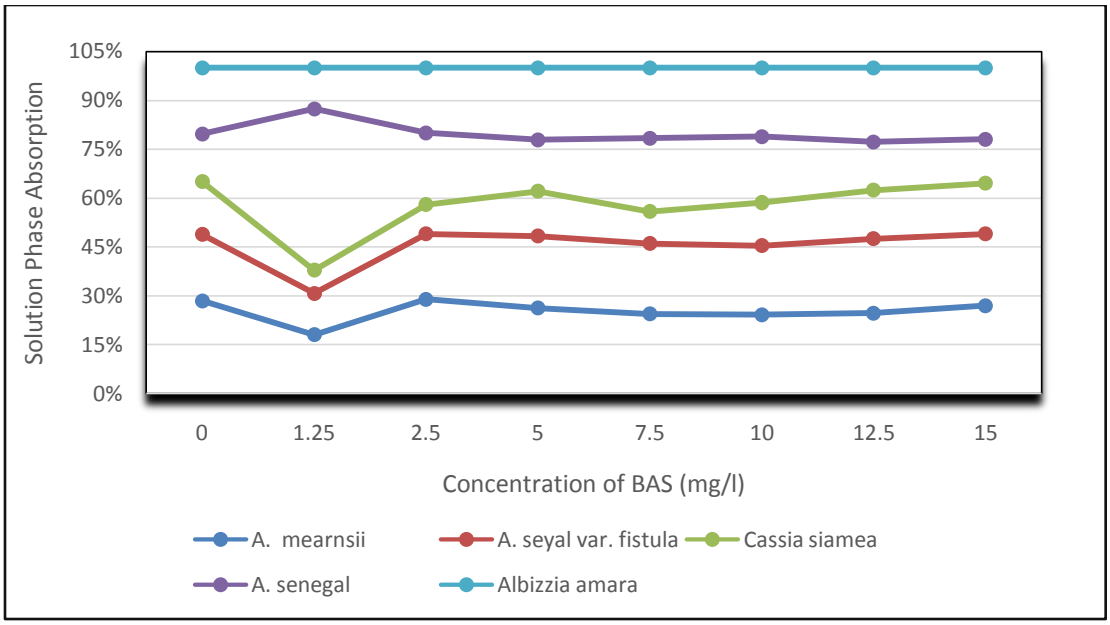

Figure 1:- Tannins phenolics extracts Curve for Protein precipitation.

Conclusions:-

The Complementary analytical technique was shown to be very efficient in the characterization of tannins from plants species. The twelve indigenous and exotic species studied only five contained more than the 10% tannin needed for commercial exploitation. The highest tannin content exotic species, but of limited distribution in Sudan, was *Acacia mearnsii* bark (black wattle) (39.8%) followed by the four indigenous species of *Pithecellobium dulce* bark (28.7%), *Acacia seyalvar.seyal*bark (24.8%), *Acaciaseyal var. fistula*bark (23.7%), and *Casuarina equistifolia*bark (10.2%) (Table 2). All the tannins species studied contained catechin, but four species were of the mixedhydrolysable-condensed(gallo-catechol) type (*Acacia seyal var. fistuala*, *Acaciaseyal var. seyal*, *Casuarina equistifolia*, and *Pithecellobium dulce*). The benefit of the Complementary analytical technique, related to the conventional extraction systems for polyphenols, had similar yield of polyphenols attained with a lesser solvent feeding and a shorter removal time.

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