RESEARCH ARTICLE

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

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Manuscript Info

Abstract

The polyphenolic compounds extract rich in gallo-catechol tannins submitted to complementary analytical techniques was evaluated. The whole plant species screened were of the condensed type except Acacia seyal var. fistuala, Acacia seyal var. seyal, Casuarina equistifolia, and Pithecellobium dulce were of mixed hydrolysable-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 seyal var. 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; Khanbabae 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
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 (Schinopsis lorentzii [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 (Picea abies [Karst.]), Scot’s pine (Pinus sylvestris [L]), European larch (Larix decidua [Mill.]) and Douglas fir (Pseudotsuga menziesii [Mirb.]) are species of special interest, representing more than 95% of the total Swiss softwood growing stock (Lacoste et al., 2013).

This investigation purposes to expand the knowledge about vegetable tanning materials that had 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 choose suitable 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.

| 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 with forestal 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 FeCl₃ in 98 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, dihydrosin and robinetin were used as standard compounds (Rᵢ ×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 acetate then 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’s method (Folin and 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 polyhydralcohol (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; Gülcin et 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 seyal var. fistuala, and Acacia seyal var. 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 seyal* var. *fistuala*, *Acacia seyal* var. *seyal*, *Acacia Senegal*, *Pithecellobium dulce*, and *Casuarina equistifolia*) (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, dihydrofisetin and androbinetin, 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 seyal* var. *seyal* and *Acacia seyal* var. *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 by different 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 seyal* var. *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 seyal* var. *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*tannin, but much higher values were obtained for *Acacia mellifera* and *Acacia seyal* var. *Fistuala*bark. However, the *Acacia mellifera*bark has low tannin contents (17.9%) (Table 4).

**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*, *Cassia siamea*, *Albizia amara*, bark reflected their different nature and relative astringency (Figure 1). The fairly gradual solubilization of *A. mearnsii*tannins (wattle) and *Cassia siamea*, *Albizia amara*, *Acacia senegal*, and *Acacia seyal* var. *fistuala*bartannins 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 siameabark 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.
Table 2: Analysis of the tannin cold aqueous extracts (% oven-dry part extracted).

| 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.fistula | 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            |
| Species              | Part      | Extracted with                                      | Gallic acid TLC | Tannic acid TLC | Catechin TLC | Epicatechin TLC | Fisetin TLC | Unknown TLC |
|----------------------|-----------|-----------------------------------------------------|-----------------|-----------------|--------------|----------------|-------------|-------------|
|                      |           |                                                     | PC 82 63        | PC 56 32        | PC 78 64     | PC 66 64       | PC 66 15    | PC 64 -     |
| **Acacia albida**    | Bark      | Amyl alcohol Ethyl acetate                          | -               | -               | 77 67        | 66 66          | 65 15       | -           |
|                      |           |                                                     |                 |                 |              |                |             |             |
| **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 67        | -              | -           | -           |
|                      |           |                                                     |                 |                 |              |                |             |             |
| **Acacia seyalvar.fistuala** | Bark | Amyl alcohol Ethyl acetate                      | - 62           | -               | 77 67        | -              | -           | -           |
|                      |           |                                                     |                 |                 |              |                |             |             |
| **Acacia seyalvar.seyal** | Bark | Amyl alcohol Ethyl acetate                      | - 62           | -               | 78 64        | -              | -           | -           |
|                      |           |                                                     |                 |                 |              |                |             |             |
| **Acacia senegal**   | Bark      | Amyl alcohol Ethyl acetate                          | 82 62           | -               | 77 67        | -              | -           | -           |
|                      |           |                                                     |                 |                 |              |                |             |             |
| **Albizia amara**    | Bark      | Amyl alcohol Ethyl acetate                          | - 62           | -               | 78 64        | -              | -           | -           |
|                      |           |                                                     |                 |                 |              |                |             |             |
| **Calotropis procera** | Bark | Amyl alcohol Ethyl acetate                      | - 65           | -               | 78 62        | 63            | -           | -           |
|                      |           |                                                     |                 |                 |              |                |             |             |
| **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        | -              | -           | -           |

**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; FeCl₃.

**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 | Relative Stringency |
|------------------|-------|---------------------------------------------|-------------------------------------|---------------------------------------------|---------------------|
| *Acacia albida*  | Bark  | 14.2                                        | 14.3                                | 1.1                                         | 60.0                | 13.8                | 7.0                | 0.12               |
| *Acacia farnesiana* | Bark  | 14.0                                        | 14.3                                | 1.9                                         | 49.2                | 14.2                | 6.8                | 0.13               |
| *Acacia mearnsii* | Bark  | 39.8                                        | 38.1                                | 4.5                                         | 72.8                | 35.6                | 17.8               | 0.16               |
| *Acacia mellifera* | Bark  | 17.9                                        | 15.5                                | 2.2                                         | 46.8                | 16.0                | 8.0                | 0.18               |
| *Acacia seyal var. fistula* | Bark  | 23.7                                        | 15.5                                | 1.4                                         | 46.8                | 16.0                | 8.0                | 0.18               |
| *Acacia seyal var. seyal* | Bark  | 24.8                                        | 10.2                                | 2.1                                         | 47.1                | 10.0                | 5.1                | 0.12               |
| *Acacia senegal* | Bark  | 19.3                                        | 19.2                                | 2.2                                         | 45.6                | 18.6                | 9.3                | 0.14               |
| *Albizzia amara* | Bark  | 14.2                                        | 14.3                                | 1.1                                         | 60.0                | 13.8                | 7.0                | 0.12               |
| *Calotropis procera* | Bark  | 10.5                                        | 10.6                                | 1.6                                         | 43.8                | 10.1                | 5.0                | 0.13               |
| *Cassia siamea* | Bark  | 10.4                                        | 10.3                                | 1.7                                         | 50.4                | 10.3                | 5.3                | 0.12               |
| *Casuarina equistifolia* | Bark  | 10.2                                        | 10.2                                | 2.2                                         | 47.1                | 10.0                | 5.1                | 0.12               |
| *Pithecellobium dulce* | Bark  | 28.8                                        | 27.9                                | 4.2                                         | 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 seyal* var. *fistula* bark (24.8%), *Acacia seyal* 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. fistula*, *Acacia seyal* 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.

Acknowledgement:
The authors are grateful to forest National cooperation, for financial support and national Centre for research for giving the chance to use the resources and facilities of their laboratory.

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