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Determining efficient extraction procedure of phytochemicals from the fruit paste of *Ziziphus abyssinica* and *Tamarindus indica*.

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ABSTRACT

Objective: To determine the most effective method of extracting metabolites from the two herbs *Ziziphus abyssinica* and *Tamaridus. Indicus*.

Methodology and results: The methods used included cold and soxhlet extraction using methanol as the solvent and hot extraction using distilled water. To determine the efficiency in which compounds are extracted TLC was performed on silica gel aluminium plates using ethyl acetate: formic acid: glacial acetic acid: water (100:11:11:27). To determine the quantity of phenolic compounds in the extract, the Folin and Ciocalteau's method (1927) was used, using Gallic acid in various concentrations. For the total quantity of flavonoid compounds, the method of Miliauskas *et al*. (2004) was used. To determine the Proanthocyanidin content in the extract a method previously reported by Ayoola *et al*, 2006 was used. To measure the antioxidant capacity of the extracts the hydrogen donating or free radical scavenging activity, was measured using the stable radical DPPH. The compounds extracted by all the methods were about seven but the difference was noted when the individual compounds were analysed. The cold extraction on the herb extract of *Z. abyssinica* had significantly high amount of total phenols 1.99g/100g of sample than both soxhlet and water extraction with 1.51g and 0.61g/100g of sample respectively. The results of *T. indicus* indicate that the extracts from the soxhlet and cold extraction methods contained a significantly low amount of all the three compounds compared to the water extracts

Conclusion and application of results: The method best suited for obtaining extracts from the two herbs *T. indicus* and *Z. abyssinica* is, cold method of extraction with methanol as the solvent for *Z. abyssinica* and hot extraction using distilled water for *T. indicus.* The results obtained give guidance to the fact that using both herbs would result in a better preservative than using one herb since the identified compounds would complement each other.

Key words: Antimicrobial, antioxidant, phytochemicals and Radical scavenging activity.

INTRODUCTION

Traditional herbs have been used for a long time in the history of humankind for preservative and curative purposes (Cowan, 1999). Their crude extracts were mainly obtained from boiling the herbs in water for specific periods and then filtering off the solid matter. The liquid extract obtained was later used to preserve mainly meat products by mixing the herbs and air-drying. Unfortunately, the amount and effectiveness of the extract realized was limited by the inefficient methods of extraction. This led to very small amounts of food being preserved relative to the amount of meat that needed preservation (Sasidharan *et al*., 2011). This preservation activity has been practiced for a long time but unfortunately, scientists never took consideration of it until recently after it was proved that herbal plants in their crude form have preservative capabilities that are brought about by their antioxidant and antimicrobial activity. The Antioxidant and antimicrobial activity of herbs is brought about by the presence of phenolic compounds such as flavonoids, phenolic acids, tannins and phenolic diterpenes that are present (Ayoola *et al*., 2008) Presently about 80% of the world's population relies on traditional herbs for various needs including food preservation (According to the World Health Organization (WHO)). This results from the fact that herbs are culturally acceptable, known to have fewer side effects (Cushnie, *et al*., 2005; Kaur *et al*.,2009; Mahesh *et al*., 2000; Arunkumar and Muthuselvam, 2009). Research that has preceded this findings has indicated that fruit paste of the herbs *Ziziphus abyssinica*, locally known as *Angau* of family *Rhamnaceae* and *Tamarindus indicus* locally known as *Oron* or *Ukwaju* of family *Caesallpinoideae* are both found in tropical Africa. These herbs are known to have been used for various purposes including preservation of meat by the pastoralists of West Pokot County. These herbs were analysed and found to contain phytochemicals such as saponins, sterol and steroids, alkaloids, tannins, flavonoids and reducing compounds, which may have given them various properties including antioxidant, and antimicrobial characteristics (Nyaberi, 2010). The antioxidant activity tends to deter lipid-containing foods such as meat from producing rancid odours and flavours during storage as a result decreasing the nutritional quality and food safety by forming secondary, potentially toxic compounds. As spoilage in meat is associated with off flavours and off odours, addition of herbs that contain antioxidants such as *Z. abyssinica* would be able to increase the shelf life of meat (Zainol *et al*., 2003). On the other hand, meat spoilage is also associated with spoilage microbial proliferation; if this is not checked in meat, food poisoning can occur, as is the case when the microorganism *C. botulinum* produces its toxins in meat. Antimicrobial activity of *T. indicus* would be handy to check microbial progress. This two herbs can therefore, be used to control spoilage of meat (Nyaberi 2009). In order to get sufficient quantities of the herbs extract and use it for the purpose of preservation of meat, several methods were tested and evaluated to determine the best possible method of extraction. They included cold and soxhlet extraction using methanol as the solvent and hot extraction using distilled water, as the pastoralists used in early times.

METHODS

Study Area and Design: The study involved determining the extracting efficiency when using soxhlet apparatus, as compared to cold and hot water extraction processes on *Z. abyssinica* A. Rich and *T. indicus* herbs. Sampling was done thrice from Chepararia and Kongelai sub Counties of West Pokot County. Trees were randomly identified within a given sub county and samples collected. The fruits where then harvested and taken to the JKUAT food science and technology laboratory for analysis.

Preparation of samples: A 500g fresh portion of each of the fruits of *Z. abyssinica* A. Rich (AZA) and *T. indicus* was gently cleaned using running tap water to remove soil, and then dried at ambient temperatures of 25 ± 2°C in a room for 20days. The fruits and the shell of *T. indicus* were removed, while only the seeds of *Z. abyssinica* were removed. The total weight of the resulting sample was expressed as a percentage against the original weight of the fresh fruit samples with their seeds and their shell. This percentage, in the context of this paper is referred to as the percentage yield. The yield realized from *Z. abyssinica* A. Rich and *T. indicus* was ground into moderately coarse powder using an electric grinder

(model M10R Japan) and stored at 4-±2°C until needed for use (Onoruvwe and Olorunfemi, 1998).

Moisture content: Moisture content of the different herbs samples was determined according to the AOAC method 950.46 (AOAC, 1996) using forced air conventional oven set at 100°C for 3 hours. 10gms of each of the herbs sample was weighed in triplicate, placed in crucibles and then transferred into a hot air oven set at 100°C. After 3hrs the crucibles were removed and placed in a dessicator to cool for one hour, the weight was recorded and percentage weight loss determined.

Extraction: The ground herbs were divided into three 45g portions. One portion was extracted with distilled water therefore referred to as aqueous extraction, another portion through cold extraction with methanol while the last portion was extracted using the soxhlet extraction with methanol as the solvent. The 45g portion of each of the herb extracted using distilled water was to simulate the traditional practice by the pastoralists (Bautista-Banos *et al*., 2003). The water mixture was boiled for one hour and then left to cool for one day. The mixture was later centrifuged at 40,000rpm for 10 minutes at a temperature of 4°C using a Kokusan Centrifuge from Kokusan Corporation (Model 2000C, Tokyo Japan). The supernatant was filtered using No. 1 Whatman filter paper and the filtrate evaporated to dryness at about 80±2°C. The other two 45g portions were extracted using the cold and soxhlet extraction methods with methanol as the solvent. In cold extraction, the herbs were immersed in the extracting solvent and placed in opaque glass containers. The containers were shaken for 30 minutes to ensure sufficient contact using a Kika Labortechnik Shaker, (Model KS 250 Basic, Staufen, Germany). The mixtures were left to stand for four days at 25 ± 2°C and then filtered. The filtrates obtained from the product of cold extraction and soxhlet extractions were evaporated to dryness under vacuum at 80 ± 2°C using a rotary evaporator (Model RE 100, Staffordshire, England). All the dried samples were put in labelled and tightly corked opaque glass containers and store at 4 ± 2°C. This process was repeated three times. Further qualification on the efficiency of the extraction methods was obtained by identifying the number of chemical compounds isolated using TLC.

Thin Layer Chromatography: This is the simplest technique used to determine the overall picture of the number and type of metabolites in a plant extract (Dlederchs, 2006). The stationary phase consisted of a thin layer of silica gel adsorbent on a flat, thick aluminium plate carrier (Fried and Sherma, 1986). For chemical variation, aluminium backed silica gel F254 plates (Machery-Nagel 20x 20cm, 0.25 mm) were used. TLC was performed on silica gel aluminium plates using ethyl acetate: formic acid: glacial acetic acid: water (100:11:11:27), (Silva *et al.,* 2004). The dry methanol extracts were re-dissolved in 2cm^3 methanol. These were loaded into well-labelled TLC plates 2cm from the bottom of the plate and 2cm apart. These solvents were left in the developing tanks with filter paper for 10minutes to allow for saturation of the atmosphere in the tanks with solvent vapours before developing the well-labelled TLC plates. Visualization of compounds was done using iodine vapour (Wagner *et al* 1983).

Determination of total phenol content: To determine the quantity of phenolic compounds in the extract, the Folin and Ciocalteau's method (1927) was used, using Gallic acid in the concentrations of 0.01, 0.02, 0.03, 0.04 and 0.05 mg/ml was prepared in methanol as the standard. Concentrations of 0.1 and 1 mg/ml of plant extracts were prepared in methanol and 0.5 ml of each sample was mixed with 2.5 ml of a ten-fold diluted Folin-Ciocalteau's reagent and 2 ml of 7.5% sodium carbonate. The mixture was left to stand for 30 min at room temperature before the absorbance was read at 760nm spectrophotometrically in triplicates. The total phenolic content was obtained from the regression equation of the calibration curve of Gallic acid ($y = 10.454x = 0.0201$, $R^2 = 0.97$), expressed as garlic acid equivalent (GAE).

Determination of total flavonoid content: To determine the total quantity of flavonoid compounds, the method of Miliauskas *et al*., (2004) was used. To 2 ml of sample 2 ml of 2% $AlCl_3$ in ethanol was added, and left at room temperature for 1hr. Concentrations of 0.1 mg/ml to 1 mg/ml of the extract in methanol were constituted. Rutin concentrations of 0.01, 0.02, 0.04, 0.08 and 0.10 mg/ml were prepared in methanol and used to obtain the calibration curve. UV absorption was measured at 420 nm. Total flavonoid content was calculated as rutin equivalent (RE) from the concentration rutin equivalent reading were obtained from the calibration curve in triplicates. The Total flavonoid content was obtained from the regression equation of the calibration curve of rutin ($y = 2.9215x + 0.3292$, $R^2 = 0.93$), and expressed as rutin equivalents (RE).

Proanthocyanidin content: To determine the Proanthocyanidin content in the extract a method previously reported by Ayoola *et al*, 2006 was used. The concentrations of 0.1 to 1 mg/ml of the sample extract were prepared, 0.025, 0.05, 0.1, 0.2 and 0.4mg/ml of catechin were prepared as the standard solutions for the calibration curve. Solutions were prepared in methanol and 0.5 ml HCl was added to each test tube and the

solutions allowed to stand for 15 min. The absorbance was measured at 500 nm. Proanthocyanidin content was measured as catechin equivalent (CE) from the concentration of catechin obtained from the calibration curve. All chemicals and reagents were obtained from Sigma-Aldrich, UK. All determinations were carried out in triplicates. Proanthocyanidin contents were determined from the regression equation of the calibration curve of catechin ($y = 2.1145x + 0.0145$, $r^2 = 1.0$) and expressed as catechin equivalents (CE).

Determination of the free radical scavenging activity (FRSA) of plant extracts: To measure the antioxidant capacity of the extracts the hydrogen donating or free radical scavenging activity, was measured using the stable radical DPPH. To extracts of various concentrations (0.02 – 0.1 mg/ml) 1ml methanol was added together with 0.5 ml of 1 mM DPPH solution in methanol. A blank solution was prepared containing 1 ml of methanol and 0.5 ml of 1mM DPPH in methanol. The experiments were carried out in triplicates. The test tubes were incubated for 15 min, methanol was used to zero the spectrophotometer and the absorbance was read at 517 nm. The radical scavenging activity was calculated using the following formula:

% inhibition of DPPH = {(AB – AA)/AB} x100

Where:

- AB is the absorption of blank sample and
- AA is the absorption of tested extract solution.

The results are expressed as percentage inhibition of DPPH and mean inhibitory concentrations (IC_{50}) determined from a plot of absorbance of DPPH versus concentration of extract.

Statistical Analysis: All the data will be analyzed for variance (ANOVA) using SAS computer program version 9.1. The comparison of the means, standard error and standard deviations at 5% level of significance will be done using Duncan's multiple range tests (Steel and Torrie, 1980).

RESULTS AND DISCUSSION

Percentage yield and Moisture content: The fruits of *T. indicus* realized more yield than those of *Z. abyssinica* (Table 3). This indicates that *T. indicus* has more material to be used for extraction than *Z. abyssinica*. This can be attributed to the fact that the seeds of *T. indicus* are smaller with more flesh around it than *Z. abyssinica*, which have bigger seeds, and less flesh. The moisture content of *T. indicus* was found to be more than that of *Z. abyssinica*. This is attributed to the fact the *T. indicus* being of gummy texture retains more water than *Z. abyssinica* which attains a powdered texture. Therefore, there may be more water activity a_w with regard to *T. indicus* than *Z. abyssinica* after twenty days of exposure to room temperature.

Table 3: Percentage yield of *Z. abyssinica* and *T. indicus* plant material for extraction and their percentage moisture levels

Item	% Moisture	% Yield
Z. abyssinica	6.5±0.51	18.9±2.5
T. indicus	9.88±0.41	35.7±3.45

When the yields from *T. indicus* and *Z. abyssinica* were subjected to extraction, the amount of extract realized is stipulated in table 4. The water extract of *T. indicus* produced significantly high amount of extract 35.52±0.7gms from 45gms of ground product compared to cold and water extractions, which produced 32.49±1.63gms and 29.6±0.83gms respectively from 45gms of ground product. While the extract from soxhlet extraction of *Z. abyssinica* produced, significantly, high amount of extract amounting to 21.3±0.96gms compared to cold and water extracts which produced 11.04±1.73gms and 13.89±0.93gms respectively from 45gms of ground product. Therefore, this may be an indicator that for *Z. abyssinica*, the compounds extracted were more soluble in methanol than those in *T. indicus*, **which** dissolve better in water. It also may indicate that the extract in *Z. abyssinica* needs more contact with the solvent a property that is there when using the soxhlet method of extraction.

Table 4: Amount of extract realized in both weight and percentage yield from the two herbs AZA and *T. indicus* using various extraction techniques and solvents

Sample	Solvent	Extraction method	Amount of Extract (gm)	% Extract
Z. abyssinica	Methanol	Soxhlet	21.3±0.96^{d}	47.29±2.13
Z. abyssinica	Methanol	Cold	11.04±1.73^{f}	24.52±3.85
Z. abyssinica	Water	Water	13.89±0.93^{e}	30.86±2.07
T. indicus	Methanol	Soxhlet	32.49±1.63^{b}	72.21±3.61
T. indicus	Methanol	Cold	29.6±0.83^{c}	65.77±1.84
T. indicus	Water	Water	35.52±0.7^{a}	78.93±1.55

Values bearing the same small letter within the same line are not significantly different (P>0.05). All the figures are in milligrams, values on mean ± SD of n = 4

Thin Layer Chromatography: The TLC was undertaken to determine the number of compounds each method of extraction was able to extract. This method was used on the basis that different compounds have different Rf values, compounds with the same Rf value turn out to be the same compound. (Sherma, & Fried, 2003). The compounds extracted by soxhlet and cold extraction using methanol from *Z. abyssinica* were similar with seven compounds identified for each. This meant that despite the difference for extract obtained (table 4) the compounds extracted might have been the same. The water extraction similarly, had seven compounds identified but the Rf values of three compounds differed Rf 0.18, 0.47 and 0.76 (table 5). This therefore gives the possibilities that some other compounds may be soluble in water and not in methanol and may not have been affected by heating. The compounds with Rf values 0.03, 0.14 and 0.28 were present in the extract from water extraction while absent in the extract from soxhlet and cold extraction. This may have been due to either the compounds were insoluble in water or affected by high temperatures or both insoluble and destroyed by high temperatures. In the case of *T., indicus* the compounds extracted were seven for water and soxhlet while cold extraction realized only six. The absence of the two compounds with Rf values 0.03 and 0.14 in the extracts of soxhlet and cold extraction may be attributed to their inability to dissolve in methanol. (Simon *et al.,* 2015) This behaviour is also seen with the extracts of *Z. abyssinica*. The properties of the compound with Rf value of 0.03 may not be attributed with solubility or effect of heat since it is present in *Z. abyssinica* water and *T. indicus* soxhlet and *T. indicus* cold extracts. Therefore, taking TLC into consideration *T. indicus* cold extraction method may not be a good method of extraction since it extracts less compounds compared to all the other methods (Table 5).

Table 5: A number of different chemical compounds observed in the various extracts using TLC method, identification were done through their Rf values

Rf Values	AZA Cold extraction	AZA soxhlet extraction	AZA water extraction	*T. indicus* water extraction	*T. indicus* soxhlet extraction	*T. indicus* cold extraction
0.03	nd	nd	x	nd	x	X
0.09	x	x	x	x	x	X
0.14	nd	nd	x	x	nd	Nd
0.18	x	x	nd	nd	nd	Nd
0.28	nd	nd	x	x	nd	Nd
0.47	x	x	nd	nd	x	Nd
0.61	x	x	x	x	x	X
0.68	x	x	x	x	x	X
0.76	x	x	nd	x	x	X
0.86	x	x	x	x	x	X
Total	7	7	7	7	7	6

Nd - Not detected, x- detected

Determination of total phenol, flavonoid and proanthocyanidin content: When the extracts of soxhlet, cold and water extraction methods of both *T. indicus* and *Z. abyssinica* herbs were taken and the total phenol, flavonoid and proanthocyanidins content determined, the soxhlet extraction extracts of *Z. abyssinica* had significantly high (P<0.05) amount of flavonoids 0.84 g/100g of sample extract than the cold and water extraction extracts which had 0.57 and 0.48g/100g of sample extract respectively. The cold extraction herb extract of *Z. abyssinica* had significantly high amount of total phenols 1.99g/100g of sample than both soxhlet and water extraction herbal extracts with 1.51g and 0.61g/100g of sample respectively, and proanthocyanidins 0.09g/100g of sample compared with the extracts from soxhlet and water extraction with 41.72g and 11.00g/100g of sample respectively. The results of *T. indicus* indicate that the extracts from the soxhlet and cold extraction methods contained a significantly low amount of all the three compounds compared to the water extracts (Table 6). These results indicate that with respect to *T. indicus* the water extraction method is the best in extracting the highest amount of phenolic, flavonoids and proanthocyanidin compounds, while with the AZA the cold extraction with methanol may be considered better placed to extract more compounds than the soxhlet and water extraction methods. It was also noted that free radical scavenging activity (FRSA) of various plant extracts (antioxidant capacity) is not significantly different apart from the water extract of *T. indicus* which showed significantly lower activity (P<0.05) at only 54.4% inhibition. This may be attributed to heating during extraction. This may be suggested that, though antioxidation properties were contributed by various phytochemicals, proanthocyanidins may have had the highest contribution, this is attributed to the fact that proanthocyanidins are a type of bioflavonoid that has been shown to have very potent antioxidant activity (Miller 1996).

Table 6: The amount of Total phenols, flavonoids and proanthocyanidins extracted from *Z. abyssinica* and *T. indicus* using different solvents and methods of extraction

Sample	Solvent	Extraction method	Polyphenols	flavanoids	Proanthocyanidins	Antioxidant (% inhibition)
Z. abyssinica	Methanol	Soxhlet	1513.8^{c}	838^{c}	41.72^{b}	89.2^{b}
Z. abyssinica	Methanol	Cold	1991.2^{d}	574^{ab}	87.25^{d}	94.73^{b}
Z. abyssinica	Water	Water	617.2^{b}	483^{a}	11.00^{a}	97.3^{b}
T. indicus	Methanol	Soxhlet	198.0^{a}	789^{bc}	7.03^{a}	96.13^{b}
T. indicus	Methanol	Cold	189.8^{a}	723^{bc}	4.85^{a}	96.65^{b}
T. indicus	Water	Water	572.1^{b}	4578^{d}	54.60^{c}	54.4^{a}
LSD			106.1	205.6	9.6	12.5
C.V			7.9%	9.3%	18%	10.7%

Values are in Mg/100g of sample extract. Differences are separated by Duncan's Multiple Range Test (DMRT). Values with same letter(s) in a column are not significantly different at 95% significance level (P>0.05), N=3.

CONCLUSION

The method best suited for obtaining extracts from the two herbs *T. indicus* and *Z. abyssinica* is clearly seen from the various test carried out. In the case of *Z. abyssinica* the extract of soxhlet and cold extraction methods showed a very close relationship, considering the amount of flavonoids, but the antioxidant and antimicrobial capacities gave a clear indicator that using cold method of extraction with methanol as the solvent may be most efficient method of extraction. In the contrary efforts should be put in place to increase the amount of extract realized. The *T. indicus* extract was consistent in that, the most appropriate method of extraction would be water. The soxhlet and cold extraction methods showed very low antioxidant capacity compared to that of water extract. it is clear that the high antioxidant capacity of *Z. abyssinica* would compensate when the two are mixed together. The study also gave light to the fact that polyphenols and flavonoids while proanthocyanids seem to be partially destroyed by heat.

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