

Phyto-Chemical Screening of Wild Types and Tissue Cultured Yellow Oleander *Thevetia Peruviana* Pers.K.Schum in Kenya

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Abstract

Search for lesser known crops or plant species with potential for human food, animal feed, and medicine or as bio-fuel has continued to receive greater attention in recent times due to increasing costs of food, fuel prices and climatic change. *Thevetia peruviana* Pers. K.Schum (Yellow Oleander), family Apocynaceae is one out of 250,000 species of plant endowed with seed oil, proteins and biologically active principles which can be harnessed for food, bio-fuel and medicinal purposes in Kenya. However its utilization has been hampered by the coexistence of the oil, proteins and the biologically active principles in the seed kernels of the plant.

Phyto-chemical screening for biologically active principles was done by solvent extraction, thin layer chromatography (TLC) for detection of the biologically active principles and Gas Chromatography (GC) for fatty acid determination. Extracts of the biologically active principles were obtained from fermented and non fermented samples from mature *T. peruviana* plants, young germinated seedlings and tissue cultured materials. Authentic sample for TLC of peruvoside was bought from SIGMA-ALDRICH Inc. of United States of America. The plant species has high potential for production of seed oil, pharmacologically active principles and proteins especially in the dry land areas along the Lake Victoria basin region in Kenya.

Keywords: *Thevetia peruviana*; Peruvoside; Seed oil; Glycosides; Fatty acids

Introduction

The search for lesser known crops or plant species with potential for human food, animal feed, and medicine or as bio-fuel has continued to receive greater attention in recent times due to increasing costs of food, fuel prices and climatic change.

Thevetia peruviana Pers. K. Schum (Yellow Oleander), family Apocynaceae is one out of 250,000 species of plant endowed with seed oil, proteins and biologically active principles which can be harnessed for food, bio-fuel and medicinal purposes in Kenya.

However the coexistence of cardio-active glycosides together with proteins and lipids in the seed kernel hinders the direct utilization of the kernels for edible purposes before the removal of the glycosides. This study focused on screening for the active principles in various parts of the plant using conventional phyto-chemical techniques such as solvent extraction and thin layer chromatography (TLC) of the extracts from various parts of the plant.

The seeds contain about 62% pale yellow non drying oil, 30% proteins and about 8% of biologically active cardiac glycosides. The oil hydrolyses to give 64% oleic, 6.3% linoleic, 17% palmitic, 11.8% stearic and 0.4% arachidonic acids. The oil has a density of 0.9108, refractive index of 0.10682 at 210°C, acid value of 0.6, saponification value of 191, esterification value of 190 and an iodine value of 73 [1,2]. The n-hexane oil extract is free from the cardio-active glycosides and had been recommended as a suitable replacement for peanut and almond oils [2]. Hydrogenation studies using cobalt chloride-molybdenum oxide catalyst supported on activated charcoal has also been reported, Kareem and Kadiri, [3]. The crude oil is also suitable for industrial applications in bio-diesel manufacture and soaps [4].

Eighteen amino acids including essential and non essential amino acids have been isolated from the leaves from Indian cultivars.

These include glutamic acid, leucine, glucine, isoleucine which are predominant over arginine, valine, alanine, praline, phenylalanine, aspartic acid, cysteine, lysine, serine, tyrosine, histidine, threonine, methionine and tryptophan [5]. Attempt by Atteh *et al.* [6] to detoxify the *T. peruviana* cake using hydrolysis and solvent extraction failed as reflected by the mortality recorded in the broilers placed on the detoxified seed cake-based diet.

Usman (2009) [7] modified the method of Atteh *et al.* [6] by using 0.1–0.5 M HCl, NaOH and Ca(OH)₂ solutions.

The detoxification was monitored by the level of bitterness of the cake. HCl and NaOH treated cake were devoid of bitter taste irrespective of concentrations of de-toxicants. However, only the cakes treated with 0.4 and 0.5 M Ca(OH)₂ were freed of bitter principles. The effect of these treatments on the crude protein, albumin and globulin content were monitored. The nutrient estimated in detoxified cake compared favorably well with Nigerian grown Groundnut cake. The leucine to lysine ratio in the raw, ethanol treated and acid treated cake are 1.23, 0.99 and 1.23 respectively. This compares favorably well with soybean with a value of 1.23. The leucine/lysine ratio that is lower than 4.6 will enhance the utilization of iso-leucine and lysine in the diet. This shows that raw and treated seed cake has better leucine/lysine ratio than most

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Nigerian grown cereals and Usman *et al.*, The crude protein content of the defatted and de-toxified seed cake is 66% third to soya 70% and sesame 68%.

The following steroidal (cardiac) glycosides have been isolated from the seed kernels, thevetin A, thevetin B, peruvoside and nerifolin. Of all the glycosides, peruvoside is the most important as it is already used in Germany as a cardio-tonic drug despite its natural occurrence in the plant in trace amounts. Flavanone and flavonol glycosides from the leaves of *Thevetia peruviana* and their HIV-1 reverse transcriptase and HIV-1 integrase inhibitory activities have been reported [8].

Materials and Methods

Sample collection and preparation

Calli tissues were obtained from the *in vitro* regenerates and leaves harvested from young germinated seedlings. Seeds were harvested from mature plants growing in Kenya. 100 g of the seeds were de-shelled, pestled and extracted by soxhlet method using n-hexane. The defatted seed kernels were fermented at 37°C for two days then extracted with ethanol and chloroform. Calli from tissue cultured materials and leaves from young seedlings were also extracted with ethanol.

Thin layer chromatography

The crude ethanol and chloroform extracts from the fermented kernels, and leaf extracts were spotted onto a TLC plate at about 1 cm apart. Peruvoside from SIGMA Aldrich inc. was the standard glycoside. The solvent system used in elution was (ethyl acetate-methanol-water in the ratio of 81:11:8) and detection was by spraying with concentrated sulphuric acid. The TLC plate was 20cm×20cm, Polygram silica gel/UV254, 0.25 mm Silica gel with fluorescent indicator UV 254 made by Machinery-Nagel (MN), Germany.

The T.L.C. Plate was photographed and scanned below.

Fatty acid analysis

Sample preparation: I. Derivatisation: 10.0 mg of the oil extract was weighed into a 50 ml conical flask, hydrolyzed by adding 2 ml of 1% of hydrochloric acid in methanol and refluxed for 1 hour in a fume chamber. After cooling, the methyl esters were extracted thrice with 2 ml of n-hexane. The combined n-hexane extract was purified by addition of brine and sodium hydrogen carbonate to remove traces of hydrochloric acid.

Anhydrous sodium sulphate (Na_2SO_4) was added in excess to finally remove all the water molecules from the methylated samples. The n-hexane fraction was then eluted through silica gel in a column to remove other polar impurities prior to gas chromatographic analysis. The eluted samples were then concentrated to about 2 ml in a water bath and kept in a deep freezer awaiting GC analysis.

II. Gas chromatography: 1 μl of each the methylated oil sample was injected separately into gas chromatograph equipment (Shimadzu 9A) equipped with flame ionization detector (FID) maintained at 220°C through a glass column diameter of 5 mm and 1meter long packed with diethylene glucosuccinate (DEGS) at 170°C. Nitrogen at flow rate of 50 ml per minute was used as the carrier gas. A standard sample containing methyl esters of oleic, linoleic, stearic, palmitic, myristic, and linolenic fatty acids were injected for comparative analysis. The fatty acids content of the oil sample were identified by comparing the retention times of the sample peaks with the retention times of peaks for the standard runs.

Results

Phytochemical screening of the seed oil and glycosides

The cardio-active glycosides stains dark blue to brownish on T.L.C plate after spraying with concentrated sulphuric acid. The naturally occurring glycosides were extracted with 95% ethanol from defatted non-fermented seed kernels, young leaf extracts from the wildtype and tissue cultured samples and separated by TLC. Peruvoside was separated by TLC from the defatted-fermented seed kernels extracted by ethanol and chloroform. The natural (three sugar glycosides) from leaf extracts had low RF values ranging from 0.1-0.2 while the peruvoside (mono-glycoside) had high RF values ranging from 0.6-0.7. Peruvoside was detected in fermented-defatted seed kernels but not in non fermented defatted seed kernel extracts and crude leaf extracts. The non fermented kernel extracts and the leaves showed presence of the natural cardiac glycosides (tri-sugar glycosides). The oil and calli showed no glycosides presence. The chloroform extract had larger spots indicating that the glycoside were more soluble in chloroform than ethanol (Figure 1 and 2).

TLC Results summary

TLC Results summary mentioned in table 1



Figure 1: Defatted seed kernel (L) and n-hexane extracted seed oil®.

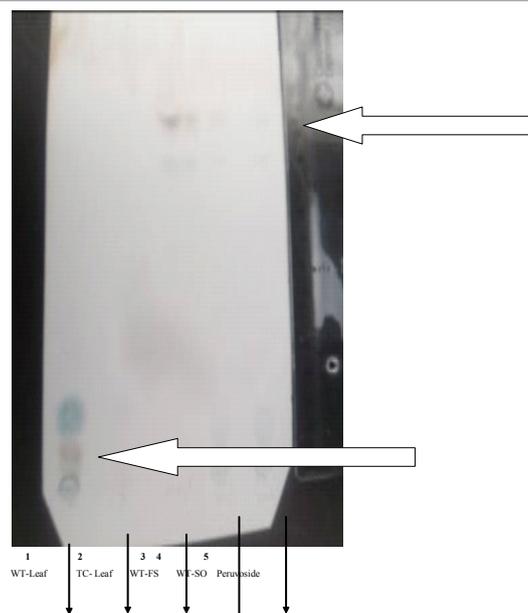


Figure 2: TLC plate.

Plant material	Compd. & Color.	R.F
Oil	none	0
Calli	none	0
Seeds	2, Br.	0.1,
	Blu.	0.2,
	1(blu)	0.2,
Leaves	1(blu)	0.2,
Fermented seeds	3, blu.	0.33
	br.	0.63
	br.	0.77
Peruvoside	3, dirty green/br	0.76
	br.	0.64
	br.	0.61

Table 1: TLC Results summary.

Fatty acid composition	Quantity (%)
Myristic	0.83
Palmitic	21.10
Stearic	8.11
Oleic	52.10
Linoleic	17.83

Table 2: Fatty acid composition of *T. peruviana* seed oil of yellow and orange flowering varieties by gas chromatographic analysis.

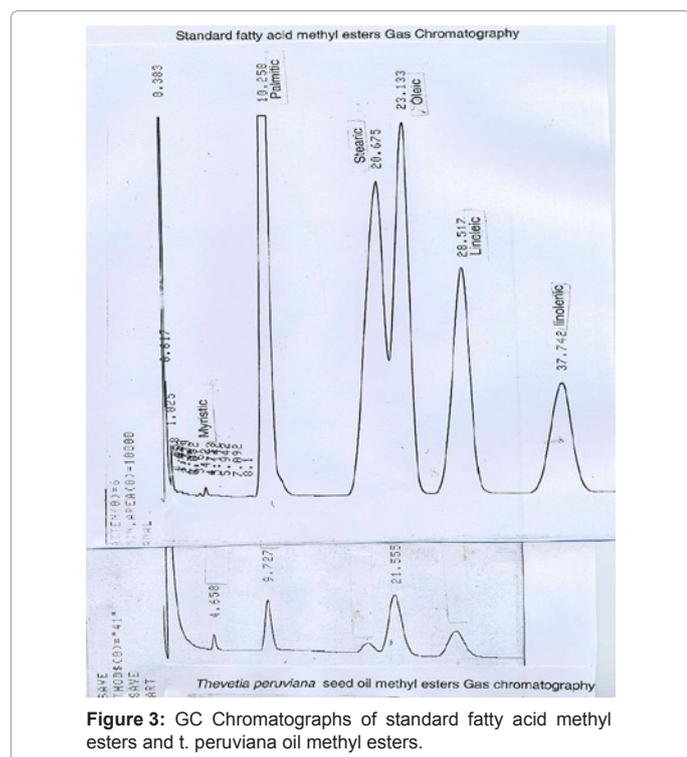


Figure 3: GC Chromatographs of standard fatty acid methyl esters and t. peruviana oil methyl esters.

Fatty acid composition by GLC analysis

The oil was found to contain oleic, linoleic, stearic and palmitic acids, which constituted over 90% of the total fatty acids. Myristic acid was only identified from mechanically extracted oil samples (Table 2).

The oil was found to be rich in unsaturated fatty acids, which comprised over 60% of the total fatty acids present. These mainly included oleic 48–52% and linoleic 16–18% respectively. The short chain fatty acid present is myristic acid as shown in figure 3.

Discussion

The results indicate that peruvoside was only present in the defatted and fermented seed kernels. This indicates that during fermentation three sugar thevetia glycosides such as thevetin are converted to one sugar glycoside (peruvoside). Peruvoside is more potent as a cardio-active therapeutic drug than thevetin which has therapeutic dose near lethal dose. The glycosides are more soluble in chloroform than in ethanol. The absence of glycosides on the calli indicates that in vitro culture conditions does not induce glycoside synthesis, however field growing conditions may elicit glycoside biosynthesis in *Thevetia peruviana*. This helps to explain why Sen and Datta, reported loss of thevetin in dedifferentiating callus in 1981.

The results of *T. peruviana* seed oil compares favorably to the work reported in the literature review and to commonly used vegetable oils such as palm oil, coconut, Soya beans and sunflower in terms of fatty acid composition, physical and other chemical properties. Palm oil constitutes over 90% of the total vegetable oil import into the country from Malaysia and sunflower oils.

The high oleic acid (18:1, mono unsaturated fatty acid) content places the oil as a suitable vegetable oil for human diet, since oleic acid is recommended for alleviation of cardiovascular fat related ailments. The presence of linoleic acid (an essential fatty acid) makes the oil more suitable for dietary purposes. The low acid, value meets the recommended International codex standards for edible oils [9].

Conclusion

Thevetia peruviana growing in Kenya has high potential for peruvoside production. Fermentation is valuable for production of therapeutic cardio-active glycoside (peruvoside) from defatted *T. peruviana*.

Recommendations

- Tissue culture may be used as a tool to develop new varieties of *T. peruviana* free from the toxic glycosides.
- Molecular techniques may be used to identify and suppress the glycoside synthesising gene.

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