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ARE THE PODS AND LEAVES OF *PROSOPIS JULIFLORA* GROWING IN BARINGO KENYA TOXIC TO LIVESTOCK? CHEMICAL ANALYSIS PERSPECTIVES.

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ABSTRACT

Studies were carried out to understand the reasons of the reported toxicity of Prosopis juliflora pods and leaves to livestock in Baringo, Kenya. Pods and leaves were extracted with acetone, dichloromethane, water and toluene/ethanol separately using soxhlet method (10-12 cycles per hour for 12 hours) to evaluate *% crude extracts. Gas Chromatography – Mass Spectrometry (GC-MS), Fourier- Transform Infrared Spectroscopy (FTIR), ^{1}H NMR(Proton nuclear magnetic resonance) analysis of crude extracts, NIST(National Institute of Standards and Technology) library and literature information indicated that P.Juliflora pods contain upto 6.3% unsaturated fatty acids such as hexadecanoic acid, octadecanoic acid, palmitic acid, dehydroabietic acid, oxyhydroabietic acid, free sugars such as sucrose and glucose, mannose, galactomanans and traces of aromatic compounds. Extracts from the leaves of P. juliflora contain upto 8.5% alkaloids such as tryptamine, piperidine,phenethylamine and juliprosopine described in literature as having antifungal and plant growth inhibiting properties as well as capable of inducing neuronal damages in animals. GC-MS analysis further indicates the presence of an important quantity of fatty acids such as hexadecanoic and octadecanoic acids, glucopyranose, hydroquinone, glucopyranosides and galactose sugar in leaves extractives. Such amount and diverse chemical products in edible parts of one plant species may work in a synergistic manner in ruminants and livestock to induce the reported toxicity.*

Keywords: chemical constituents, leaves, *Prosopis juliflora*, synergism, pods

INTRODUCTION

Prosopis juliflora, an exogenous wood species native from South and Central America was introduced in Kenya in the early 1970's in order to remedy environmental problems, improve biomass cover and rehabilitate disused quarries (sirmah *et al*., 2009). Over the years it spread rapidly and colonized agricultural lands and pasture. Its suckering ability after cutting made it difficult to control (sirmah *et al*., 2009). Today it has overgrown road networks, diverted the flow of water, formed impenetrable thickets and caused death to livestock feeding on its pods and leaves, justifying chemical analysis.

Despite the negative effects witnessed in Kenya, valorisation of *P. juliflora* pod flour, beans and gums as food supplement and medicine in animals and humans has been reported from other parts of the world (Barba *et al.*, 2006; Choge *et al.*, 2007). Chemical characterization of gums by Lopez *et al*. (2006) indicated an arabinogalactan protein with potential uses for beverages and pharmaceutical products. Fatty acids and free sugar such as glucose, sucrose, rhamnose, fructose, glucose, galactose and galactouronic acid in the seeds and pods enhances its use as a food supplement (Fatma *et al.*, 1991; Sawal *et al.*, 2004; Silva *et al.*, 2002). Saturated and unsaturated fatty acids including caproic, lauric, myristic, palmitic, stearic,

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oleic, linoleic and linolenic acids and amino acids such as valine, leucine, tyrosine, and phenylalanine have been isolated from the pods of *P. Juliflora* (Marangoni *et al.*, 1988; Fatma *et al.*, 1991; Liu *et al.*, 2008). Mixtures have been reported to posses some antifungal and antibacterial properties.

Malhotra and Misra (1981) isolated from the fresh pods of *P. juliflora* ellagic acid glycosides such as rhamnosylgentiobiosyl, while Wassel *et al.* (1972) identified the flavonoïd patulitrin. Galactomannan present in the seed gum of *P. juliflora* has excellent thickening properties useful for textile, pharmaceutical and food industries (Chaires-martinez *et al.*, 2008). Galactomannans have the fundamental structure consisting of a main chain of β-(1-4)-D-mannopyranose units substituted by a single α- (1- 6) galactopyranose units, although there are few deviations (Vieira *et al*, 2007). Choge *et al*. (2007) analysed the food quality and safety of flour produced from *P.juliflora* pods in Kenya and found to be rich in sugar, carbohydrates and proteins therefore can be useful as a supplement in human and animal feeds.

Antifungal, plant growth inhibiting and DNA binding alkaloids such as tryptamine, piperidine, phenethylamine and juliprosopine and their isomers have been isolated from the leaves of *P. juliflora* (Tapia *et al.*, 2000; Ahmed *et al.*, 1989). *P. juliflora* leaf alkaloids and their different isomers have the ability to inhibit growth of surrounding plants (Nakano *et al*., 2004; Kishore and Pande, 2005; Pasiecnik *et al.*, 2001). Fractionated alkaloids isolated from leaves have been shown to induce cytotoxicity leading to neuronal damages, neuromuscular alterations and gliosis in animals (Silva *et al.*, 2007). The leaves are reported to contain crude protein levels of 14-22%, crude fibre 21-23%, nitrogen free extract 43-50%, calcium 1.5% and phosphorus 0.2% while mineral content is directly related to the levels of minerals in the soil (Pasiecznik *et al.*, 2001).

MATERIAL AND METHODS

Collection and Solvent Extraction of *P. Juliflora* Pods and Leaves

Eight year old *Prosopis juliflora* were selected randomly from Baringo forest (latitude 0°, 20'N, longitude 35°, 57'E), Kenya. Leaves and pods from each tree were picked separately, transported while fresh to University of Kabianga Botany laboratory, identified, conditioned for two months before grinding to pass through a 115-mesh sieve in a vibrating hammer mill. The resulting powder was then dried at 60°C to constant weights before solvent extraction. The voucher specimens are kept at the Botany laboratory, University of Kabianga.

Soxhlet extraction was done using hexane, dichloromethane, acetone, toluene/ethanol (2/1 v/v) and or water. 10gm of sample powder were extracted with 180ml of the solvent for 15 hours at the rate of 10 to 12 cycles per hour. Three replicate extractions were done for each sample and test.

Quantification of Extractives in P. Juliflora Pods and Leaves

After each extraction, the solvent was evaporated under reduced pressure in a Büchi rotavapor and the residue dried over P_2O_5 under vacuum before weighing. Two methods based either on direct determination of extractives after solvent evaporation (direct methods, DM) or on the difference between dry weight of powder before and after extraction (indirect method, IM) were used to evaluate extractive contents.

The percentage of extractives was evaluated according to the formula:

$$\%\,\mathrm{DM} = \frac{m_e}{m_s} \times 100$$

$$\%\,\mathrm{IM} = \frac{(m_s - m_d)}{m_s} \times 100$$

where m_e is the weighed mass of extracts after solvent evaporation m_s is the dry mass of the powder before extraction, and (m_d) is the dry mass of extracted powder.

^{1}H NMR, ^{13}C NMR and FTIR Analysis of *P. Juliflora* Pods and Leaves Extractives

All compounds and reagents used unless stated were purchased from Fluka-Sigma-Aldrich Chimie SARL (St Quentin Fallavier, France). ^{1}H and ^{13}C NMR spectra were recorded in chloroform-D, methanol-D_4, acetone-D_6 or dimethylsulfoxide-D_6 as required on a Brüker DRX 400 spectrometer. Chemical shifts were expressed in ppm and calculated relative to tetramethylsilane (TMS). FTIR spectra were recorded as KBr disks on a Perkin Elmer FTIR spectrometer SPECTRUM 2000 between wave number ranges of 4000-500 cm^{-1}. Finely divided extractive samples were dispersed in a matrix of KBr and pressed to form disks.

GC-MS Analysis

Test extractive samples were analyzed as trimethyl derivatives using the following procedure. In a screw-capped vial, a sample of approximately 1 mg of dry extract sample was dissolved in 0.5 ml of anhydrous acetonitrile (Acros Organics) and 0.4 ml of *N,O*-bis-trimethylsilyl etamide) containing 1% of trimethylchlorosilane (BSTFA / 1%TMCS) (Acros Organics) was added. The solution was sonicated for about 1 min and heated at 60°C for 60 min. After evaporation of the solvent in a stream of dry nitrogen, the residue was diluted in 1 ml of anhydrous acetonitrile.

GC-MS analysis was performed on a Clarus® 500 GC gas chromatograph (Perkin Elmer Inc., USA) coupled to a Clarus® 500 MS quadrupole mass spectrometer (Perkin Elmer Inc., USA). Gas chromatography was carried out on a 5 % diphenyl / 95 % dimethyl polysiloxane fused-silica capillary column (Elite-5ms, 60 m x 0.25 mm, 0.25 mm film thickness, Perkin Elmer Inc, USA). The gas chromatograph was equipped with an electronically controlled split / splitless injection port. The injection (injection volume of 1 µl) was performed at 250°C in the split mode (split flow of 20 ml/min). Helium was used as carrier gas, with a constant flow of 1.2 ml/min.

The oven temperature program was as follows: 200°C constant for 4 min, 200°C to 330°C at a rate of 5°C/min and then constant for 330°C. Ionization was achieved under the electron impact mode (ionization energy of 70 eV). The source and transfer line temperatures were 250°C and 330°C, respectively. Detection was carried out in scan mode: m/z 35 to m/z 700 a.m.u. The detector was switched off in the initial 10 min (solvent delay).

HPLC Analysis

HPLC analysis was performed using a Supercosil ^{TM} LC-18 column (250 mm x 4.6 mm i.d,) at 35°C on a waters liquid chromatograph (Waters SAS, Saint Quentin-en Yvelines, France) equipped with a system controller 600E, a manual injector system with a 20µl loop and a Waters 2996 photo diode array (PDA) detector. The data were recorded on a 210 nm to 400 nm range with the Empower software.

Solvents used for elution were solvent A (water containing 0.05% of trifluroacetic acid) and solvent B (methanol (HPLC grade) containing 0.05% of TFA) at a flow rate of 1ml/min. Elution was achieved with a binary gradient starting at 95% A and 5% B for 1 min followed by a linear ramp to 50% A and 50% B at 10 min to finish at 100% B after 20 min.

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RESULTS AND DISCUSSION

Extractive Content of *P. Juliflora* Leaves and Pods

The quantities of extractives in pods and leaves of *P. juliflora* by soxhlet extraction are reported in table 1. Water yielded the highest (8.5%) dry extract in leaves and (6.3%) in pods. The amounts of extractives in the pods and leaves are however less than those of bark and wood as reported by (sirmah *et al.*, 2009; Toshiaki, 2001). For all cases the yield of extractives increases with increasing solvent polarity.

Table 1. Percentage extractives from *P. juliflora* leaves and pods (n = 3)

	Yield of extractives (%)							
Solvent	Dichloromethane		Acetone		Toluene/ethanol		Water	
	IM	DM	IM	DM	IM	DM	IM	DM
Leaves	3.2	3.1	5.1	4.9	6.1	5.6	8.5	7.8
Sd±	0.45	0.47	0.13	0.12	0.14	0.33	0.16	0.12
Pods	2.6	2.1	3.8	3.2	4.3	4.1	6.3	6.2
Sd±	0.16	0.28	0.24	0.41	0.21	0.27	0.09	0.13

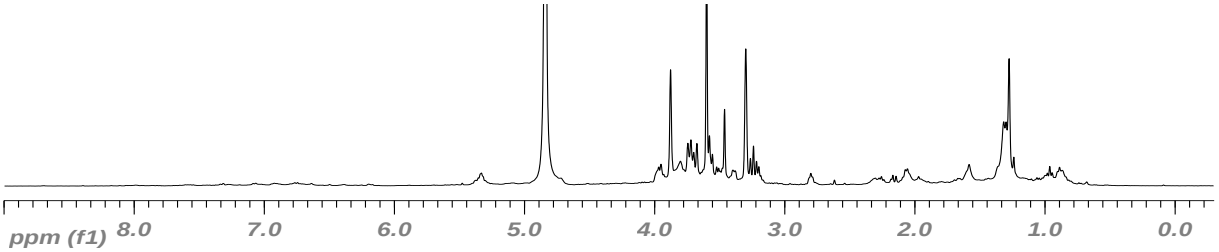
DM: Direct method, IM: Indirect method, Sd±: Standard deviation

The two methods of extract quantification based either on the weight of extracts (DM) after evaporation of the solvent or on the mass loss of extracted powder (IM) gave close values indicating that practically no products were lost during vacuum evaporation.These high amounts may play an important effect on the ability of this species to colonize arid and semiarid lands, cause livestock toxicity and may offer more generally different possibilities of chemical valorization (Neya *et al.*, 2004).

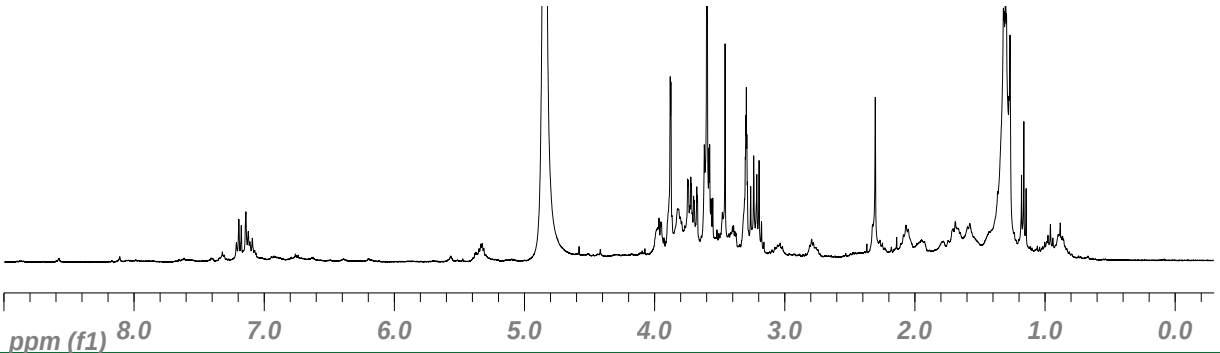
Chemical analysis of P. Juliflora leaves extractives

^{1}H NMR analysis of *P. Juliflora* leaves crude extractives by different solvents is reported in figure 1.

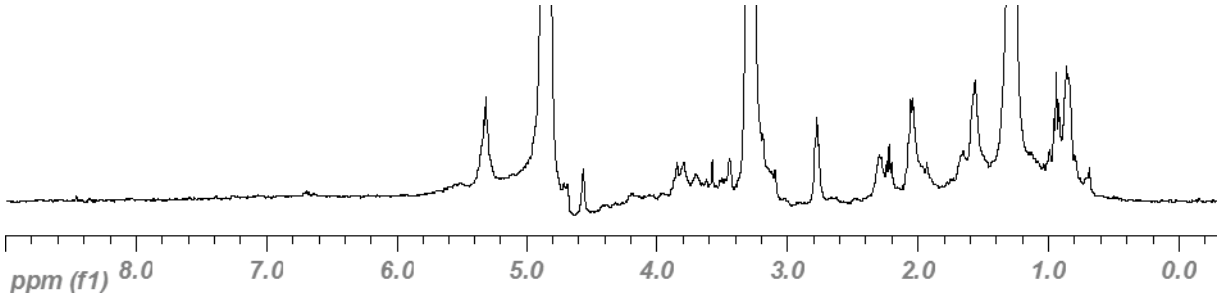
Acetone



Toluene/ethanol



Dichloromethane



Water

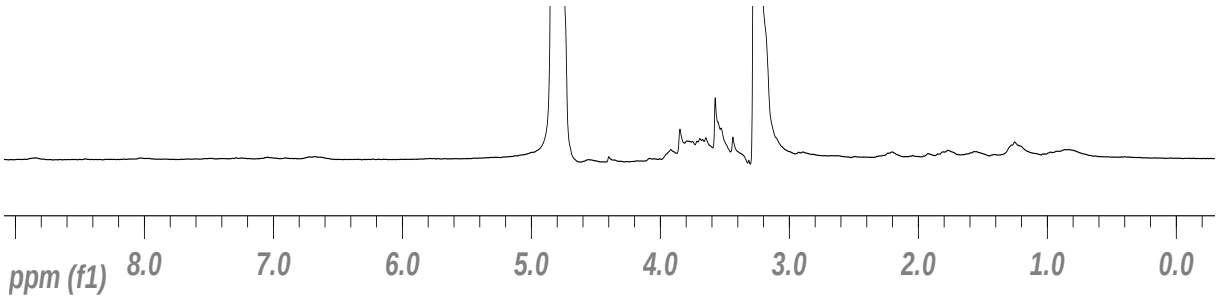
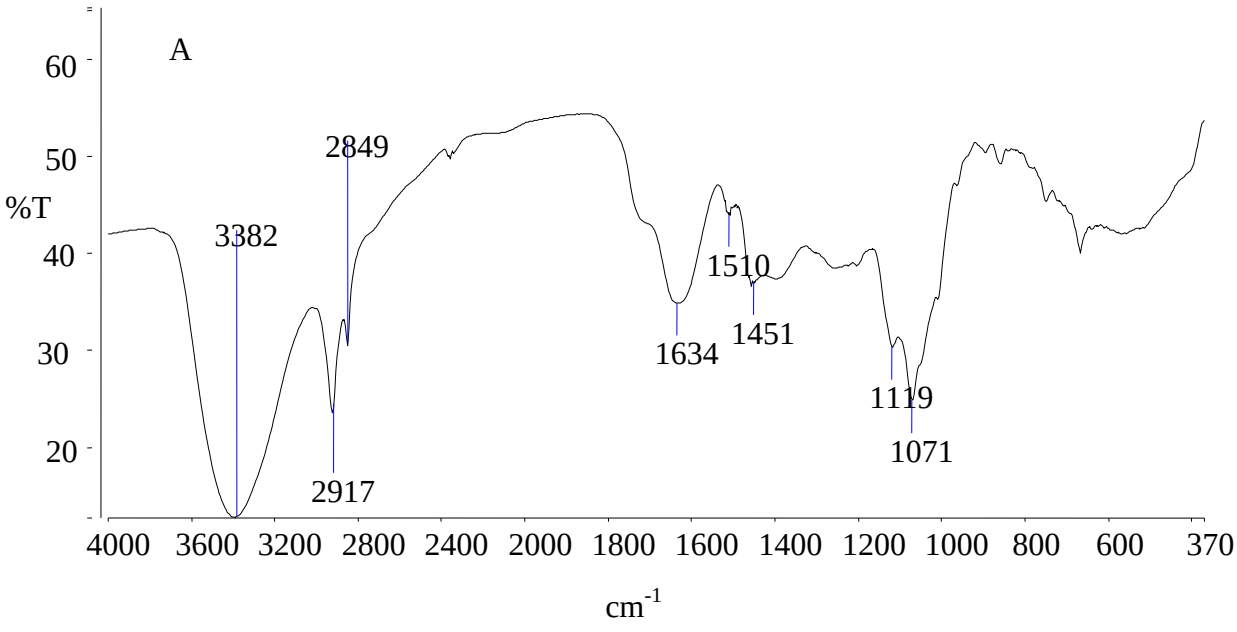


Figure 1. [1]H NMR analysis for *P. Juliflora* leaves extractives by different solvents

[1]H NMR analysis of leaves crude extractives by different organic solvents indicates mainly the presence of signals between 0.8 and 2.0 ppm typical of fatty alkyl chains of fatty acids, fats or waxes and signals between 3.0 and 4.0 ppm ascribable to sugars or glycerol moiety of fats. Presence of allylic protons between 2.0 and 2.5 ppm and vinyl protons at 5.4 ppm is characteristic of unsaturated fatty alkyl chains. In their study on the leaf extracts of *P. juliflora*, Ahmed *et al.* (1998) showed the presence of alkaloids. These latter ones are characterized by different signals at 0.8, 1.3, 3.1, 3.4 and 3.6 ppm. According to our NMR analysis, such products cannot be totally excluded, even if they do not represent the main components. FTIR spectra of leaves extracts are shown in figure 2.



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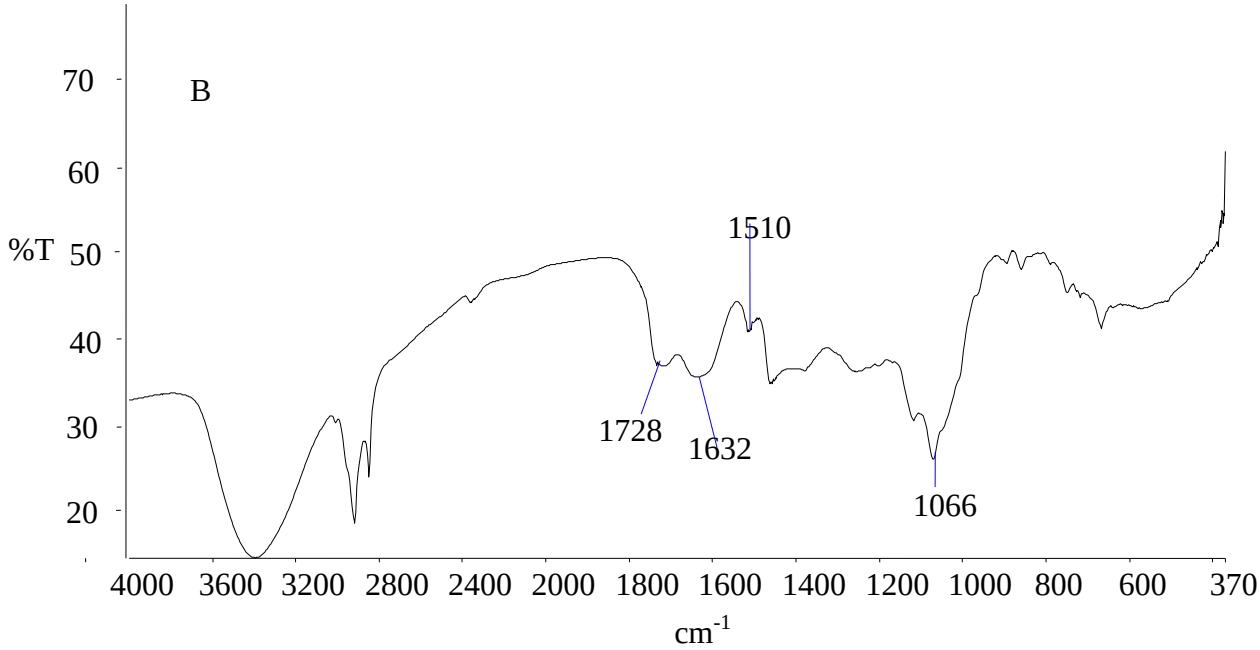

Figure 2. FTIR spectrum of (A) toluene/ethanol and (B) acetone extractives of *P. juliflora* leaves

FTIR analyses indicated absorption bands at 3400 cm^{-1} characteristic of OH group and bands at 1119 and 1071 cm^{-1}, which could be ascribable to sugars detected by NMR. Strong absorption bands at 2917 and 2849 cm^{-1} characteristic of C-H vibrations and 1728 cm^{-1} characteristic of C=O vibrations can be attributed to the presence of fatty acids in their free or esterified form, while bands at 1510 and 1450 cm^{-1} are ascribable to aromatic structures.

Identification of products based on GC-MS chromatograms, NIST library and other literature information indicate that leaves are constituted of fatty acids such hexadecanoic acid or linolenic acid, different sugar units and alkaloids as reported in figure 3.

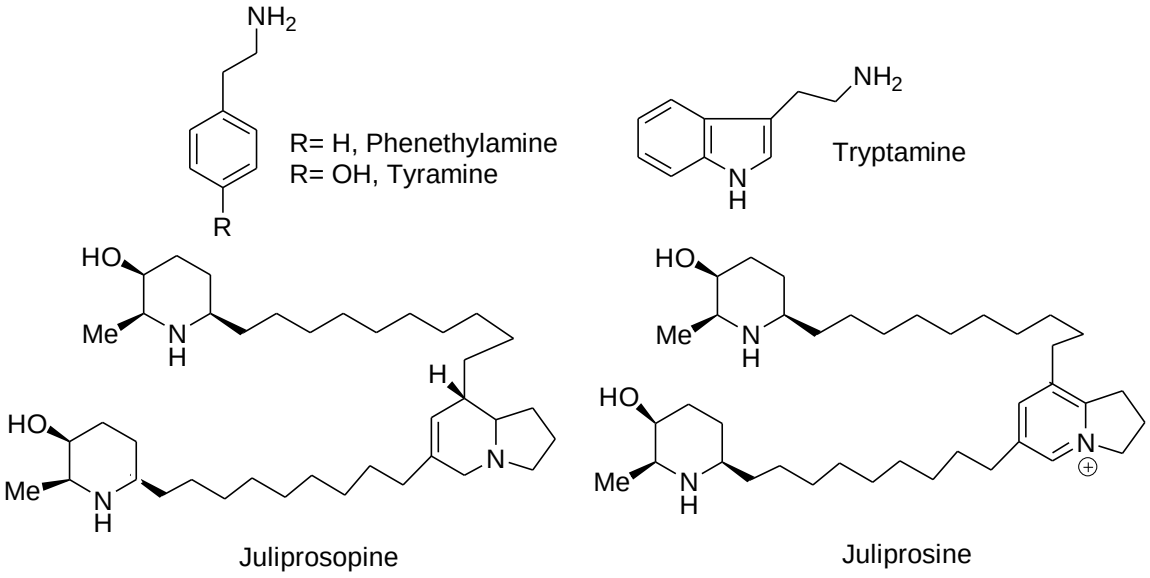

Figure 3. Some components contained in leaf extractives of *P. juliflora*

Chemical Analysis of P. Juliflora Whole Pod Extractives

^{1}H NMR analysis recorded in methanol-D4 of *P. Juliflora* whole pods crude extracts by different solvents is presented in figure 4.

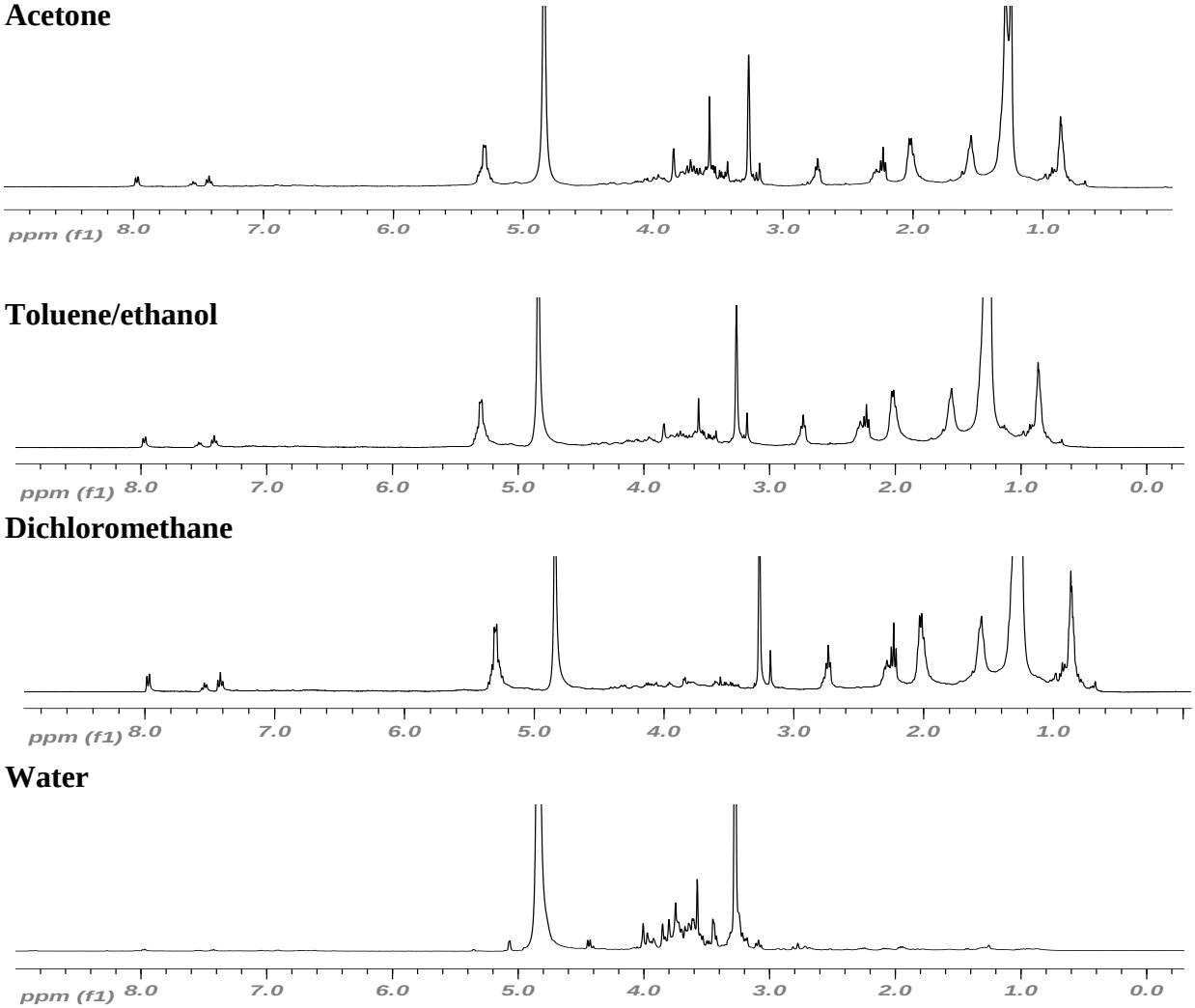

Figure 4. ^{1}H NMR analysis for *P. Juliflora whole pod extractives*

Dichloromethane, acetone and toluene/ethanol extractives present quite similar NMR spectra, while water extractives present completely different signals. Spectrum of water extractives indicates mainly the presence of signals comprised between 3.2 and 4.0 ppm ascribable to sugar units of mono or polysaccharides. Such chemical shifts have been reported from *P. juliflora* pods (Vierira *et al.*, 2007; Gallao *et al.*, 2007). Indeed, *P. juliflora* pods are reported to contain galactomannan: mannan ratio of 1.0: 1.1 (Vierira *et al.*, 2007). Spectra of extractives obtained with other solvents indicate the presence of fats, waxes or fatty acids. Signal at 0.8 ppm is typical of terminal CH_3 group, signals between 1.2 and 1.6 ppm characteristic of methylene groups (CH_2) of the fatty alkyl chain, signals between 1.8 and 2.8 ppm typical of methylene groups in α position of the carbonyl group or allylic position. Vinylic hydrogen atoms appear around 5.2 ppm. Signals comprised between 3 and 4 ppm can be ascribable to hydrogen atoms of glycerol unit of fats or to the presence of some sugar units. These latter ones are more important in acetone and toluene / ethanol extracts comparatively to

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dichloromethane extractives. Aromatic signals present different chemical shifts at 7.4, 7.5 and 8.0 ppm, which can be attributed to alkaloids like juliprosine.

FTIR spectrum is reported in figure 5

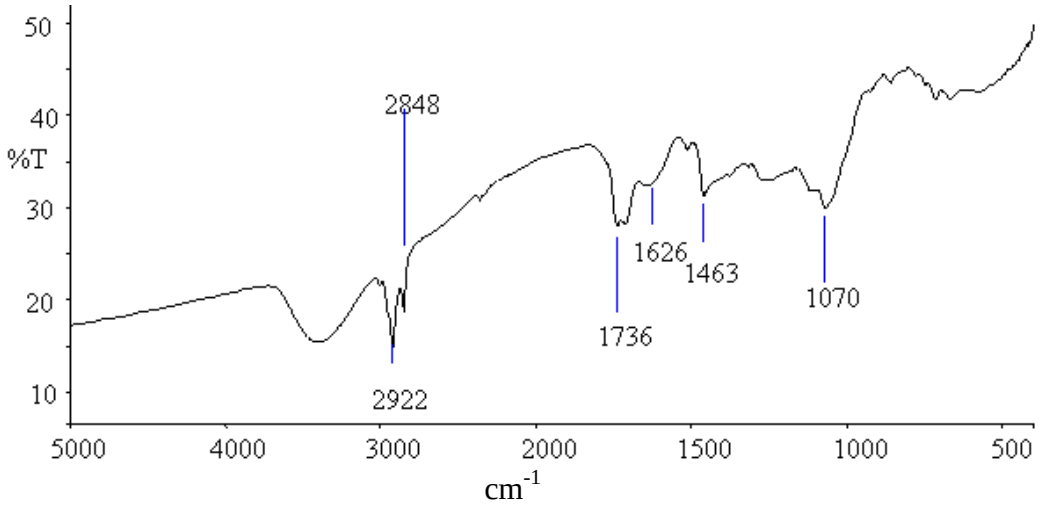
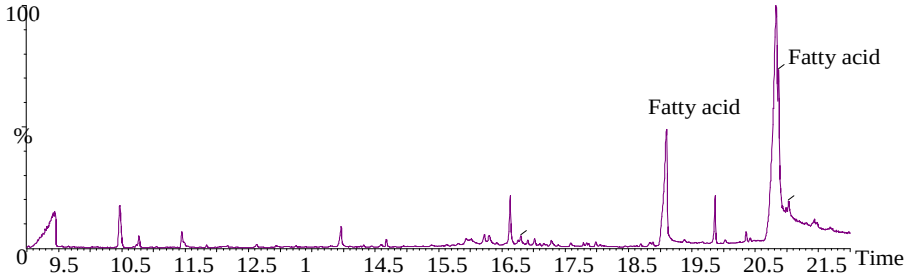


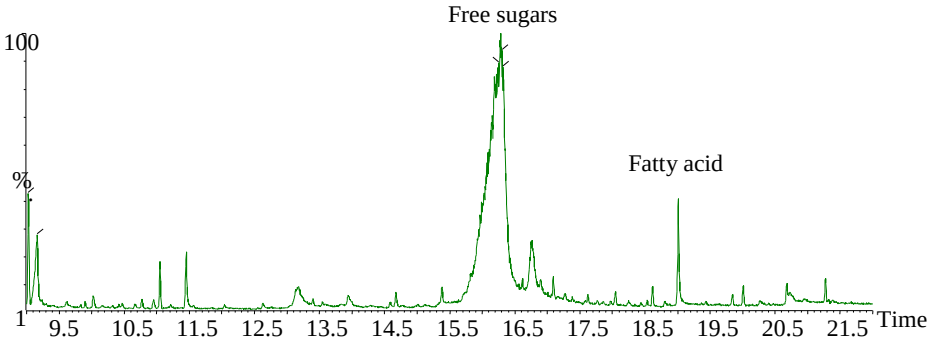
Figure 5. FTIR spectrum for pods dichloromethane extractives

FTIR analysis indicated characteristic hydroxyl group absorption of a sugar unit at 3350 cm^{-1} and C-H vibrations present in aliphatic structure of fatty acids between 2849 and 2922. The C-O absorption of polysaccharides compounds is evident at 1070 and C=O absorption at 1736 confirms a saturated fatty acid. Aromatic C=C skeletal vibrations are weak and difficult to exploit.

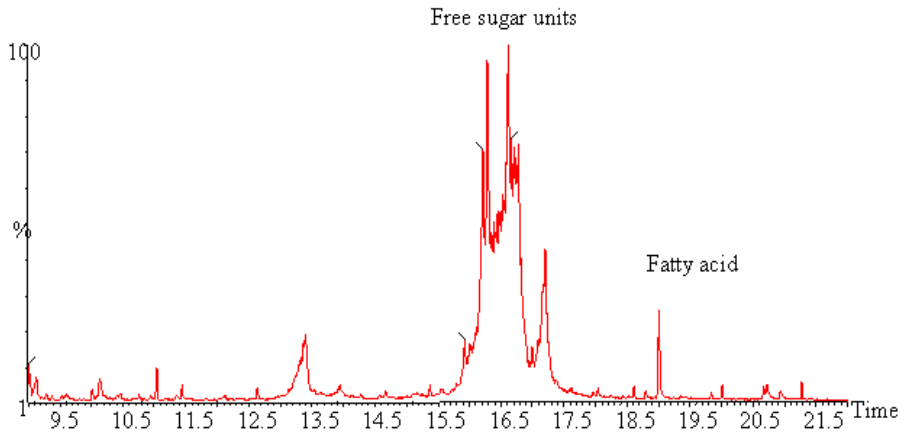
GC-MS analyses of the TMS derivatives of different extractives from *P. Juliflora* whole pods are presented in figure 6.



Dichloromethane extractives



Acetone extractives



Toluene/ethanol extractives

Figure 6. GC-MS chromatograms of the different compounds in pods extractives

GC chromatograms are difficult to exploit due to the presence of numerous products. Toluene / ethanol and acetone extractives are very similar showing sugars and fatty acids. Chromatogram of dichloromethane indicates mainly the presence of fatty acids.

Based on GC-MS, FTIR, ^{1}H NMR analysis, NIST library and literature information it can be concluded that the pods contain fatty acids such as hexadecanoic acid, octadecanoic acid, palmitic acid, dehydroabietic acid, oxyhydroabietic acid, free sugars such as sucrose and glucose, mannose, galactomanans and traces of aromatic compounds as shown in figure 7.

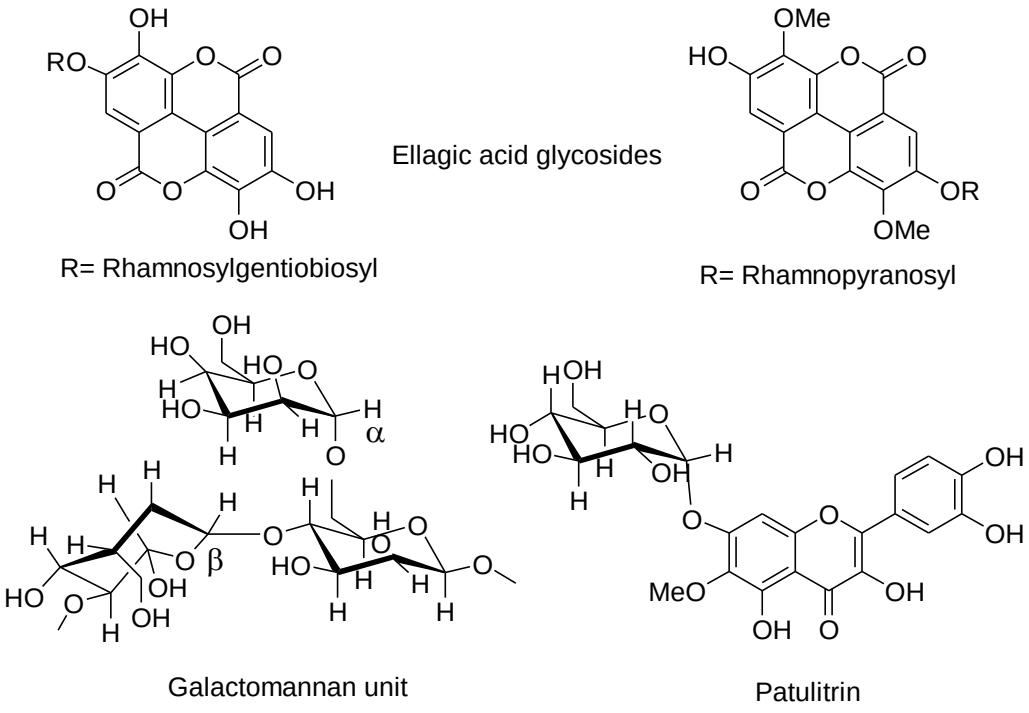


Figure 7. Some components contained in pods extractives of *P. juliflora*

Presence of such products in the pods of *P. juliflora* has been previously reported corroborating our results (Liu *et al.*, 2008; Lopez *et al.*, 2006; Silva *et al.*, 2002; Sawal *et al.*, 2004; Choge *et al.*, 2007). Different bioactive compounds in plants when consumed together

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are modified by synergistic interactions among themselves leading to toxicity (Wang *et al.*, 2011; Haida *et al.*, 2017).

CONCLUSION

Prosopis juliflora leaves and pods contain 8.5% and 6.3% extracts respectively. Pod extracts contain galactommanans, mannose, saturated and unsaturated fatty acids and free sugar described in the literature as food supplement and medicine in animals and humans. Leaves of *P. juliflora* contain alkaloids such as tryptamine, piperidine, phenethylamine and juliprosopine described in literature as having antifungal and plant growth inhibiting properties as well as capable of inducing neuronal damages in animals. GC-MS analysis further indicates the presence of an important quantity of fatty acids such as hexadecanoic and octadecanoic acids, glucopyranose, hydroquinone, glucopyranosides and galactose sugar in leaves extractives. Such diverse chemical products in pods and leaves of *P. Juliflora* when consumed together are modified by synergistic interactions among themselves to induce toxicity in ruminants and livestock in Kenya.

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