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FUNCTIONAL ANALYSIS OF FLAVONOIDS IN SOME HIGHER AND LOWER PLANT VEGETABLES EATEN IN EASTERN NIGERIA

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ABSTRACT

Little did this generation know of high plant vegetables eaten in Enugu State of Nigeria and their health benefits? In the present investigation vegetables of high plants (Vitex doniana and Ficus sur) flavonoids are compared with that of lower plants (Genatum africanum, Solanium aethipicium and Achyranthes spondens). The samples were reflux extracted with ethanol respectively. Extensive chromatographic technique like thin layer and column chromatography were used for separation and purification of flavonoids. The absorption and functional characterization were carried out by UV/Visible and Fourier transform infrared spectroscopy (FTIR) respectively. By using the obtained phytochemical, total flavonoids content, UV/Visible and FTIR spectral evidence and referring previous data libraries, the isolated compounds present in ethanol extract of the tested plant leaves were flavonoids.

KEYWORDS: Flavonoids, functional groups, infrared spectra, higher plant vegetables, lower plant vegetables

INTRODUCTION

Long term eating of vegetables offered some protection against chronic diseases such as cancers and heart diseases. Because uncontrolled production of free radicals due to life style was thought to be significantly implicated in the etiology these chronic diseases observations focused attention on the possible role of radical scavenging and radical suppressing nutrients and non-nutrients(Sheetal and Jamuna, 2009) in explaining the apparent benefit of such diets (Liu, 2004). Obesity, hypertension and high blood level of cholesterol are severe threats to the public health and high-fat and high-fructose diet have been implicated to be a cause of these conditions and vegetables can prevent them (Onyeka and Nwambekwe, 2007).

Flavonoids are low molecular weight polyphenol compounds present in all fruits, vegetables, and certain beverages of high/secondary plants that have diverse beneficial biochemical and antioxidant effects which renders them as important class of therapeutically relevant compounds (de Groot and Rauen, 1998), that have anti-viral, anti-cancer, anti-inflammatory, anti-allergic, anti-microbial, anti-diarrhea activities and also shown to inhibit topoisomerase enzymes, reduce DNA mutations in the mixed-lineage leukemia gene (Sak, 2014; Baikar and Malpathak, 2010).

Flavonoids are the most abundant polyphenols in human diet representing about 2/3 of all other phytochemicals. By definition, flavonoid is any of the group of compounds containing a characteristic aromatic nucleus (A and B and a heterocyclic ring C). It is also any group of phytonutrients that are referred to as water-soluble pigments – mostly yellowish in colour, but in

some cases they are red, blue, purple or white. The more colourful a food item is, the richer it will be in flavonoids (Galeotti et al. 2008; Maria et al., 2012).In most cases, Ring B is attached to position 2 of ring C, but it can also bind in position 3 or 4; this with the structural features of the ring B and the patterns of glycosylation and hydroxylation of the three rings, makes the flavonoids one of the larger and more diversified groups of phytochemicals. Their biological activities depend both on the structural characteristics and the pattern of glycosylation (Nicola, 2014).

Previous studies on flavonoids by UV/visible spectroscopy have revealed that most flavonoes and flavonols exhibit two major absorption bands: Band 1 (320 – 385 nm) represents the B ring absorption, while band 11 (250 – 285 nm) corresponds to the A ring absorption. Functional groups attached to the flavonoid skeleton may cause a shift in absorption such as from 367nm in quercetin (3, 5, 7, 3^{I}, 4^{I} - 3, 5, 7,3^{I}, 4^{I}, 5^{I} – hydroxyl group) (Yao et al., 2004).

Flavonoids possess many biochemical properties and the most pronounced of them is their capacity to act as antioxidants. The antioxidant activity depends upon the arrangement of functional groups about the nuclear structure. The mechanisms of antioxidant activity such as radical scavenging and metal ion chelation ability depends on the configuration, substitution and total number of hydroxyl groups (Kelly et al., 2002; Pandey et al, 2012). The B ring hydroxyl configuration is the major determinant of scavenging of ROS, and RNS because it donates hydrogen and an electron to hydroxyl, peroxyl, and peroxynitrite radicals, stabilizing and giving rise to a relatively stable flavonoids radical (Cao et al., 1997).

Chromatography is a broad range of physical methods used to separate and or to analyze complex mixtures. The component to be separated is distributed between two phases: a stationary phase bed and a mobile phase which percolates through the stationary bed. The complex mixtures are flushed through the system at different rates. These differential rates of migration as the mixture moves over adsorptive materials provide separation. Repeated sorption/desorption that take place during the movement of the sample over the stationary bed determine the rate. The smaller the affinity a molecule has for the stationary phase, the shorter the time spent (Lenehan, 2013).

Thin layer chromatography is a special application of adsorption – desorption equilibra between compounds adsorbed on the coated onto a flat surface is utilized. The separation of the components in a mixture is dependent on adsorption – desorption equlibria between compounds adsorbed on the solid stationary phase and in the moving liquid phase. The extent of adsorption of a single component depends upon the polarity of the molecule, the activity of the absorbed, and the polarity of the mobile phase (Freid and Sharma, 1986).

Column chromatography is another common and useful separation technique that involves the same principles as paper/TLC but can be applied to separate larger quantities. It allows the separation and collection of the compounds individually (Still et al., 1978)

This study is aimed at investigating chromatographic separation and functional characterization of flavonoids in higher and lower plant vegetables eaten in Eastern Nigeria.

MATERIALS AND METHODS

SAMPLE COLLECTION AND PREPARATION

The vegetable samples namely *Vitex doniana* (Uchakiri)*, Ficus sur* (Akakaro or Agburu)*, Gentum africanum* (Ukazi), *Achyranuthis spondens (nchanwu),* and *Solanium aethiopicium* (Aghara) leaves were bought from Agbani market, Nkanu West Local Government Area of Enugu State, Nigeria. The samples were soughted and washed with distilled water and left to dried under subdued sun light for 10 days. The samples were ground into fine powdered particles with the aid of manual grinding machine and stored in an air tight nylon bag and labeled for analysis.

QUALITATIVE TEST FOR FLAVONOIDS

SHINODA TEST: Four pieces of magnesium chips were added to 2mL of the filtrate followed by 10 drops of concentrated hydrochloric acid. A pink, orange, or red to purple colouration indicates the presence of flavonoids (Trease and Evans, 2002).

FERRIC CHLORIDE TEST: To 2mL filtrate, three drops of 10% ferric chloride were added. A green-blue or violet colouration is an indication that phenolic hydroxyl group is present.

DETERMINATION OF TOTAL FLAVONOIDS

The samples (60g) were refluxed with 90% ethanol in the ratio of 1:5 in a round bottom flask for 1hr and allowed to cool to room temperature and filtered. The chlorophyll and impurities were removed with activated charcoal. The dark yellowish filtrate was then concentrating to a constant weight and preserved for analysis.
Aluminum chloride UV/visible spectroscopy method was employed for total flavonoid determination (Ghasemi et al, 2009; Zhishen et al., 1999). Rutin was used as flavonoid standard for the calibration curve. Hundred milligram of rutin was dissolved in 70ml of 60% ethanol (v/v) and the volume made up to 100ml with 30% ethanol (v/v). The standard curve was constructed by diluting 0.0, 0.1, 0.2, 0.3, 0.4, and 0.5ml rutin to 1ml with distilled water to obtain 0, 10, 20, 23, 40, and 50 mg/ml of rutin. To this and 1ml of the samples extracts, 0.5ml of 5% $NaNO_2$ were added and shaken for 5 minutes. Then 0.5ml of 10% $AlCl_3$ was also added and shaken for 5 minutes. Four milliliter of water and 4% NaOH were added and shaken 15 minutes. The absorbance was determined at 510 nm using test tube 0 to zero the UV/Visible spectrophotometer.

CHROMATOGRAPHIC SEPARATION

THIN LAYER CHROMATOGRAPHIC SEPARATION OF FLAVONOIDS

The crude flavonoid was spotted and analyzed using TLC procoated silica gel F_{234} as describe by Hamilton and Hamilton, 1989 method. The chromatogram was developed using ethyl acetate – methanol– formic acid –water (50:2:3:6) solvent system. After the development, the chromatogram was dried and the spots detected with 1% ethanolic solution of aluminium chloride.

COLUMN CHROMATOGRAPHIC FRACTIONATION OF FLAVONIOD

The column chromatography was prepared with chloroform and alumina in a glass column of 60cm length with 1.5cm internal diameter. A 20% solution of the crude flavonoid was gently introduced from the top of the column. The components were fractionated with solvent system chloroform and methanol (8:2). The fractions obtained were monitored by TLC. Identified fractions were combined after correlating their R_F values.

SPECTROSCOPIC ANALYSIS

UV – VISIBLE SPECTROSCOPIC ANALYSIS

The UV-visible analysis was carried out for all the samples at NARICT, Zaria, Nigeria. Solution of the samples fraction (mg/ml), in methanol was used. Peak pick method was employed using UV 250D PC series. Spectroscopic graded methanol without added reagent was used as reference.

INFRARED SPECTROSCOPY

FT-IR spectrum was analyzed to find the most important functional groups of flavonoid.Fractionswere purified with alumina and chloroform andscanned with KBr disk using FT-IR 8400S SHIMADZU (Japan) in the NARICT LAB, Zaria.

RESULTS AND DISCUSSION

In this study, flavonoid extracted using ethanol as a solvent have a deep greenish brown colour with a yield of 4.2 – 9.6gm/ 100g dry material. Table (1) shows the crude yield and total flavonoid content.
The results of the TLC analysis of the extracted flavonoid performed with ethyl acetate – methanol – formic acid – water (50:2:3:6) as mobile phase respectively were presented in Table (2).
The UV-visible spectrum of the isolated flavonoid fractions of the vegetable samples were shown in Table (3).

DISCUSSION

The percent yield of crude flavonoid and total flavonoid (µg/g) of the higher known green leafy vegetables (*F. sur and V.doniana*) are well known and commonly consumed as well as lower green leafy vegetables (*A. spondens, S. aethripicuium and G, africanum*) is presented in Table 1.

Table 1: Percent crude flavonoid and total flavonoid content

Sample	CrudeFlavonoid (%)	Total Flavonoid (mg/g)
Vitex doniana - young	7.4	0.56
Vitex doniana- mature	9.5	0.71
Ficus sur	8.1	0.65
Achyranthes spondens	4.2	0.10
Solanium aethiopicium	4.3	0.12
Genatum africanum	5.7	0.22

Table (2): Results of the TLC analysis and R_F values of extracted flavonoid

Developing Solvent	Samples Flavonoid	No. of Spots	R_F Values
Ethyl acetate methanol formic acid water (50:2:3:6)	Ficus sur (Akakoro/Akpuru)	4	0.63, 0.82, 0.89, 0.90
	Vitex doniana - young	8	0.56, 0.68, 0.79, 0.86, 0.88, 0.89, 0.91, 0.98
	Vitex doniiana - mature	15	0.16, 0.25, 0.26, 0.34, 0.39, 0.44, 0.49, 0.63, 0.68, 0.69, 0.75, 0.76, 0.87, 0.88, 0.89
	Achyranthes spondens (Awa)	2	0.79, 0.95
	Solanium aethiopicium (Anara)	2	0.63, 0.91
	Genatum africanun (Ukazi)	5	0.43, 0.84, 0.88, 0.89, 0.92

The result showed that higher known green leafy vegetables V. doniana (both fresh and old leaves) and F. sur have more percent crude and total flavonoid than lower well-known and commonly consumed vegetable. A. spondens, S. aethipoicuim and G. africanium. V. doniana (mature leaves) have more percent crude and total flavonoid (9.6% and 0.71mg/g) than F. sur (8.1% and 0.65mg/g) followed by the young leaves of V. doniana (7.4% and 0.56mg/g). G. africanium havemore percent crude and total flavonoids (5.7% and 0.22mg/g) among the other lower commonly consumed vegetables investigated (S. aethipoicuim 4.3% and 0.12mg/g and A. spondens 4.2% and 0.10mg/g).

In the result of the thin layer chromatographic analysis (Table 2) shows that V. doniana mature leaves have 15 spots more than the young leaves with 8 spots followed by *G. africanium* with 5spots, *F. capensis* 4 spots*, S. aethipoicuim* 2 spots and *A. spondens* 2spots respectively.

The UV/Visible spectra's of the sample fractions revealed that they all absorb between 365-416 nm ranges conforming with the characteristic 300-550 nm range (Andersen and Markham, 2006)

Table (3): Result of UV-visible spectroscopic analysis of flavoniod fractions

		Maximum absorption wavelength (max)	
Sample	**Fraction**	**Flavonoid**	**Fraction**
Ficus Pur	Ficus 1	411	Ethyl acetate
(Akakaro or	Ficus 2	415	
Akpuru)	Ficus 3	414	n-Butanol
	Ficus 4	413	
Achyranthes	Awa 1	416	
Spondens (Awa)	Awa 2	411	Ethyl acetate
Solanium aethipicium (Anara)	GE 1	414	
	GE 2	-	Ethyl acetate
Gentaum	A& B	408	
africanum	C	414	Ethyl acetate
(ukazi)	D	365,415	
Vitex doniana	1, 2, 3		
Young (uchakiri)	Y A and Y B	413	-
Vitex domiana	1, 2		
Mature (uchakiri)	M A and M B	365,410	Ethyl acetate

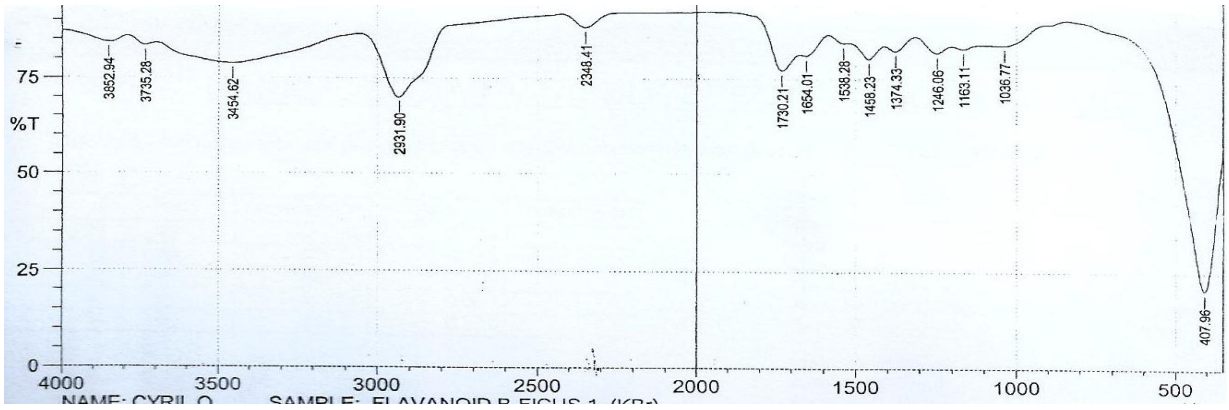

Fig. 6: IR spectra of flavonoids fraction 1 of Ficus surleaves

Table 4: IR spectra data result of *Ficus* leavesfractions of flavonoids

Peak	Intensity	ASSIGNMENT	Peak	Intensity	ASSIGNMENT	Peak	Intensity	ASSIGNMENT	Peak	Intensity	ASSIGNMENT
Ficus 1			**Ficus 2**			**Ficus 3**			**Ficus 4**		
1037	83.74	C—O Str	1065	91.14	C—O Str	1062	50.74	C—O Str	1081	80.40	C—O Str.
1163	82.84	C—O Str									
1246	81.73	C—O Str	1271	91.86	C—O Str	1249	65.85	C—O Str	1255	88.20	C—O Str.
1458	80.23	C—H Bending	1457	89.95	C—H Bending						
1538	84.29	C=C Bending (Aromatic)	1536	87.55	C=C Bending (Aromatic)				1540	76.46	C=C Bending (Aromatic)
1654	81.04	C=O Str (Alkenyl)	1651	84.61	C=O Str (Alkenyl)	1642	40.29	C=O Str (Alkenyl)	1644	59.74	C=O Str (Alkenyl)
1730	77.08	C=O Str (Aldehyde)									
						2107	76.00	C=C Str (Alkenyl)			
			2376	92.26	C=C Acc. Double bond	2379	76.32	C=C Acc. Double bond	2376	87.08	C=C Acc. Double bond
			2927	84.16	C—H Str (Alkyl)	2930	47.16	C—H Str (Alkyl)	2934	72/33	C—H Str (Alkyl)
3454	78.79	O—H Str	3421	78.10	O—H Str	3426	10.37	O—H Str	3437	27.20	O—H Str
3931	69.90	C—H Str (Alkyl)									

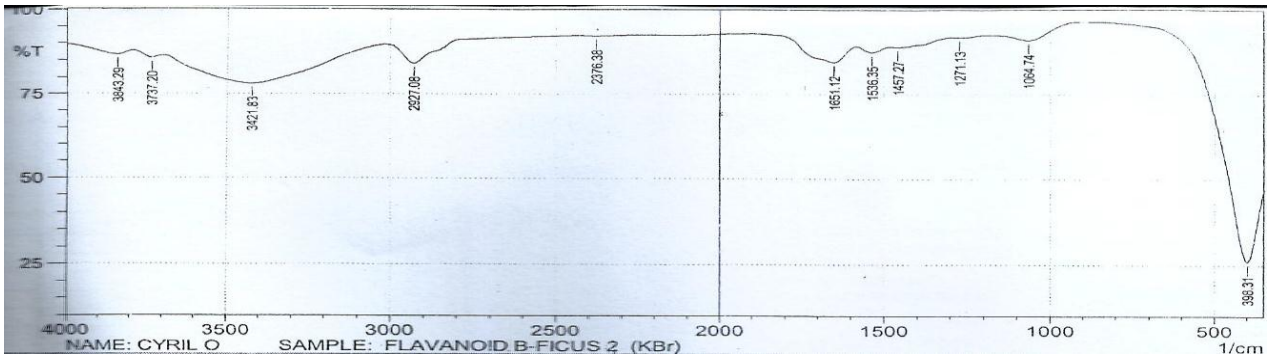

Fig. 7: IR spectra of flavonoids fraction 2 of Ficus surleaves

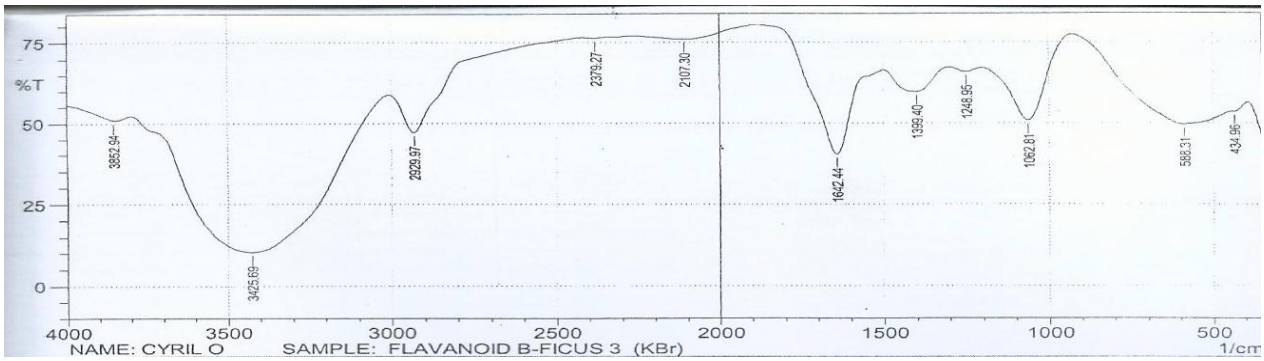

Fig. 8: IR spectra of flavonoids fraction 3 of Ficus surleaves

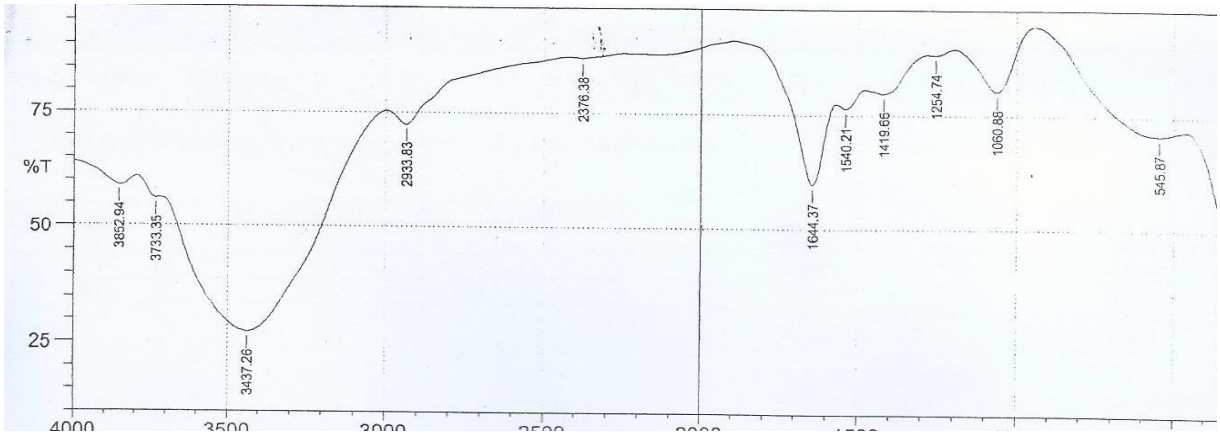

Fig. 9: IR spectra of flavonoids fraction 4 of Ficus surleaves

Table 5: IR spectra data result of *Vitex doniana* mature and young leavesfractions of flavonoids

Peak	Intensity	ASSIGNMENT	Peak	Intensity	ASSIGNMENT	Peak	Intensity	ASSIGNMENT	Peak	Intensity	ASSIGNMENT
Vitex M A			Vitex M B			Vitex Y A			Vitex Y B		
1064	72.21	C—O Str	1062	24.59	C—O Str	1071	63.80	C—O Str	1066	72.29	C—O Str.
						1182	65.47	C—O Str			
1261	72.14	C—O Str	1269	29.59	C—O Str	1255	64.97	C—O Str	1271	70.12	C—O Str.
1458	80.23	C—H Bending				1455	60.55	C—H Bending	1459	63.06	C—H Bending
1535	65.38	**C=C Bending (Aromatic)**	**1523**	**33.82**	**C=C Bending (Aromatic)**	**1534**	**58.39**	**C=C Bending (Aromatic)**	**1532**	**54.37**	**C=C Bending (Aromatic)**
1654	58.96	C=O Str (Alkenyl)	1640	21.41	C=O Str (Alkenyl)	1671	49.60	C=O Str (Alkenyl)	1649	39.80	C=O Str (Alkenyl)
1730	77.08	C=O Str (Aldehyde)									
			213	49.60	C=C Str						

			1		(Alkenyl)						
2353	68.90	C=C Acc. Double bond				2306	62.19	C=C Acc. Double bond	2304	66.20	C=C Acc. Double bond
2929	56.28	C—H Str (Alkyl)	2937	25.62	C—H Str (Alkyl)	2930	40.48	C—H Str (Alkyl)	2938	42.33	C—H Str (Alkyl)
3438	43.96	O—H Str (Alcohol/ phenol)	3417	12.14	O—H Str (Alcohol/ phenol)	3446	30.32	O—H Str	3435	13.19	O—H Str

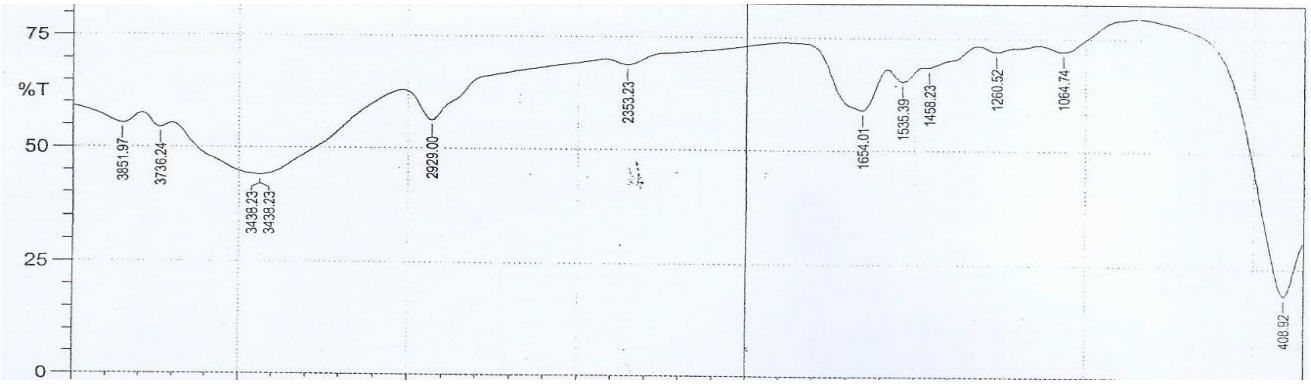

Fig. 10: IR spectra of flavonoids fraction 1 of Vitex doniana matureleaves

Table 6: IR spectra data result of *Genatum africanum* leavesfractions of flavonoids

Peak	Intensity	ASSIGNMENT	Peak	Intensity	ASSIGNMENT	Peak	Intensity	ASSIGNMENT	Peak	Intensity	ASSIGNMENT
Genat-umA			Genat-umB			Genat-umC			Genat-umD		
1066	44.96	C—O Str	1066	93.80	C—O Str	1057	42.60	C—O Str	1062	66.25	C—O Str.
			1271	93.41	C—O Str	1251	50.05	C—O Str	1254	77.60	C—O Str.
			1457	86.42	C—H Bending	1437	51.00	C—H Bending	1436	72.09	C—H Bending
1536	38.78	C=C Bending (Aromatic)	1532	84.55	C=C Bending (Aromatic)	1508	55.37	C=C Bending (Aromatic)	1525	69.53	C=C Bending (Aromatic)
1649	34.68	C=O Str (Alkenyl)	1649	84.28	C=O Str (Alkenyl)	1647	43.78	C=O Str (Alkenyl)	1649	62.69	C=O Str (Alkenyl)
			2131	49.60	C=C Str (Alkenyl)						
24	40.81	C=C	230	66.20	C=C Acc.	234	82.67	C=C Acc.	230	89.45	C=C Acc.

02		**Acc. Double bond**	**4**		**Double bond**	**8**		**Double bond**	**5**		**Double bond**
2935	**56.04**	**C—H Str (Alkyl)**	**2938**	**42.33**	**C—H Str (Alkyl)**	**2934**	**47.66**	**C—H Str (Alkyl)**	**2935**	**67.77**	**C—H Str (Alkyl)**
3454	**23.70**	**O—H Str (Alcohol/phenol)**	**3435**	**13.19**	**O—H Str (Alcohol/phenol)**	**3400**	**33.48**	**O—H Str**	**3404**	**39.44**	**O—H Str**

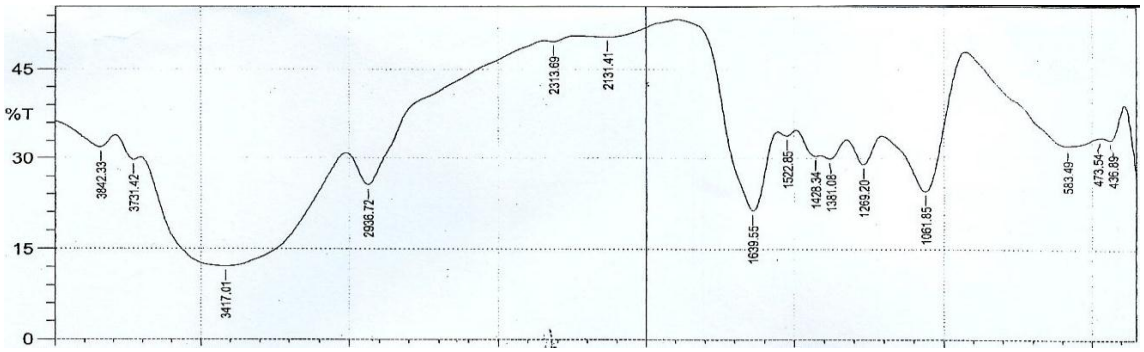

Fig. 11: IR spectra of flavonoids fraction 2 of Vitex doniana matureleaves

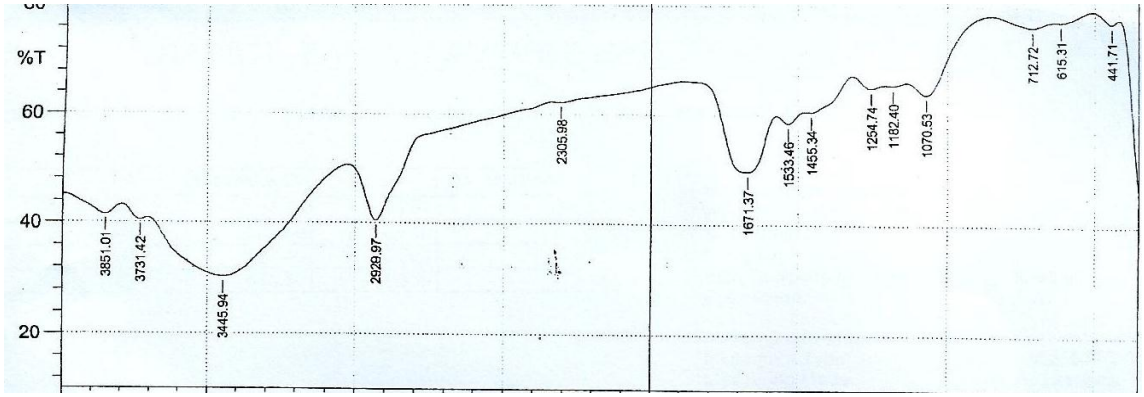

Fig. 12: IR spectra of flavonoids fraction 1 of Vitex doniana youngleaves

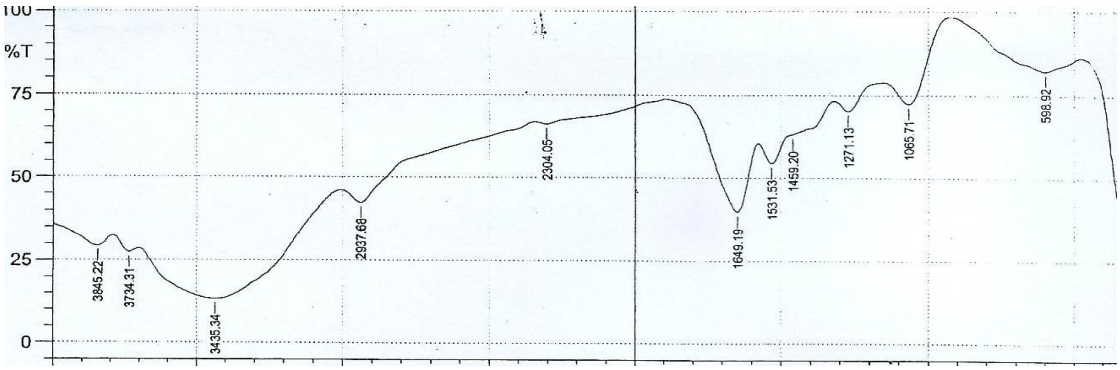

Fig. 13: IR spectra of flavonoids fraction 2 of Vitex doniana youngleaves

The appearance of broad band at 1300 – 1000 cm-1 represents presence of stretching vibration of alcoholic and phenolic group's containing C-O vibration of aromatic compounds. Bands at 1500-

1300 cm-1 represents methyl groups, the bands in this region provide information on the bending vibration of C-H bonds of aromatic ring (Yong-Cheng, 2011).

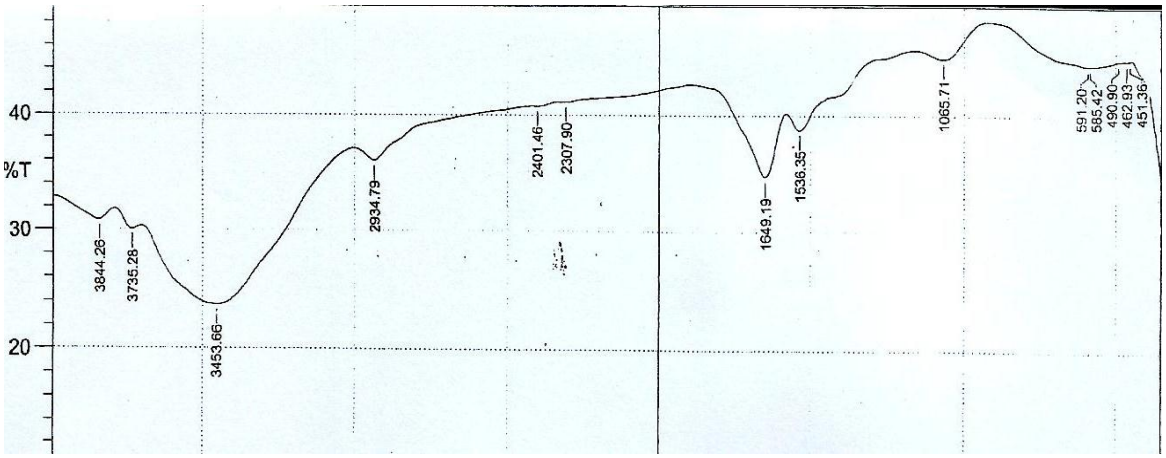

Fig. 14: IR spectra of flavonoids fraction A of Genatum africanum leaves

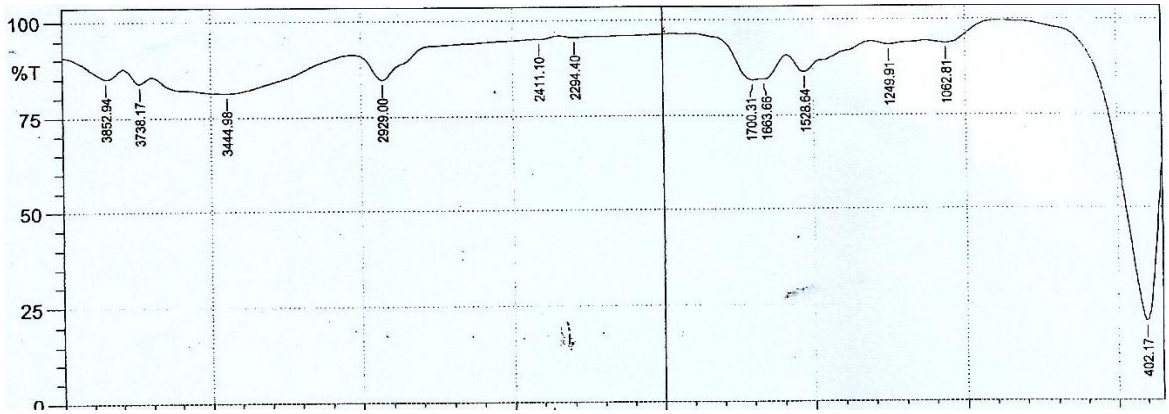

Fig. 15: IR spectra of flavonoids fraction B of Genatum africanum leaves

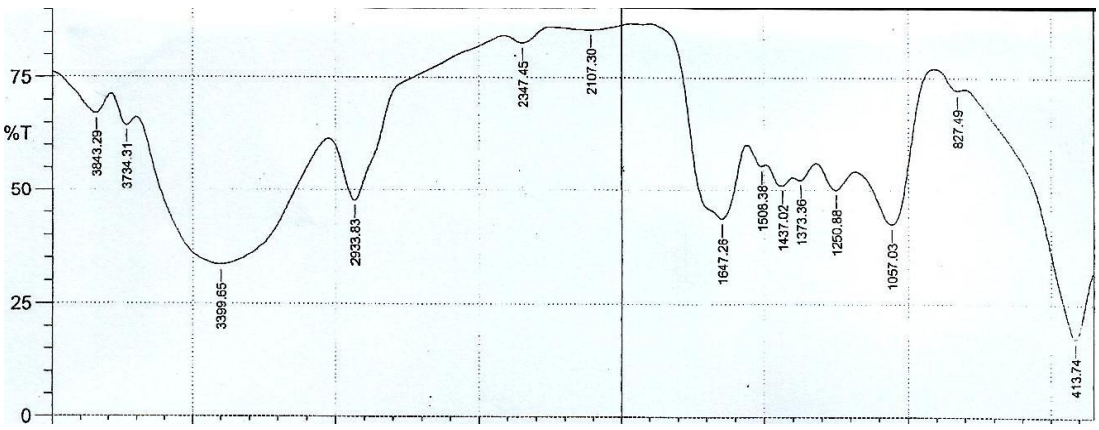

Fig. 16: IR spectra of flavonoids fraction C of Genatum africanum leaves

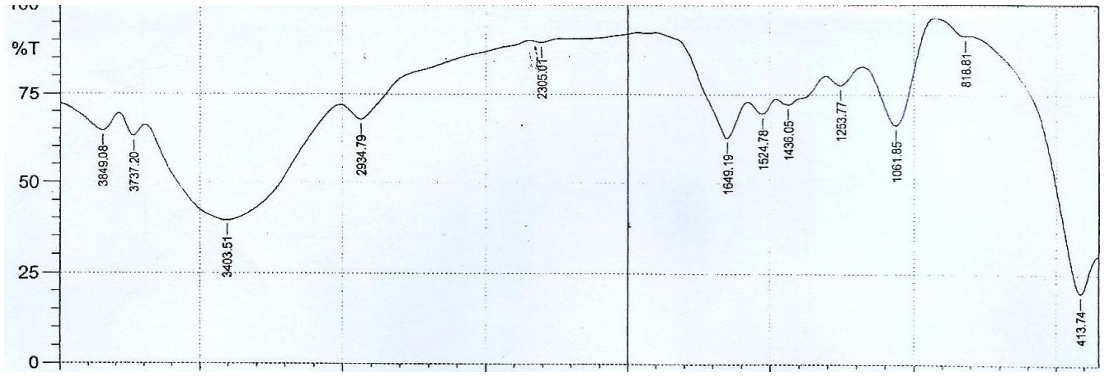

Fig. 17: IR spectra of flavonoids fraction D of Genatum africanum leaves

Table 7: IR spectra data result of *Solanium aethiopicium* and *Achyranthes spondens* leaves fractions of flavonoids

Peak	Intensity	ASSIGNMENT	Peak	Intensity	ASSIGNMENT	Peak	Intensity	ASSIGNMENT	Peak	Intensity	ASSIGNMENT
Solani-um GE 1			Solani-um GE 2			Achyr-anthes 1			Achyr-anthes 2		
1060	86.77	C—O Str	1058	41.29	C—O Str	1065	88.51	C—O Str	1066	73.46	C—O Str.
1247	89.88	C—O Str	1253	51.67	C—O Str	1250	92.85	C—O Str	1247	81.12	C—O Str.
1450	87.86	C—H Bending	1457	86.42	C—H Bending	1447	89.74	C—H Bending	1394	75.21	C—H Bending
1537	87.64	C=C Bending (Aromatic)	1545	48.89	C=C Bending (Aromatic)	1539	91.13	C=C Bending (Aromatic)			
			1648	29.52	C=O Str (Alkenyl)	1653	85.32	C=O Str (Alkenyl)	1643	35.28	C=O Str (Alkenyl)
1711	79.04	C—C Str.				1715	85.90	C—O Str.			
			2103	63.17	C=C Str (Alkenyl)						
2293	95.52	C=C Acc. Double bond	2321	63.54	C=C Acc. Double bond	2348	82.67	C=C Acc. Double bond	2400	95.25	C=C Acc. Double bond
2928	76.21	C—H Str (Alkyl)	2932	35.83	C—H Str (Alkyl)	2929	76.85	C—H Str (Alkyl)	2934	67.22	C—H Str (Alkyl)
3416	77.08	O—H Str (Alcohol/ phenol)	3424	10.32	O—H Str (Alcohol/phenol)	3422	72.18	O—H Str	3444	10.63	O—H Str

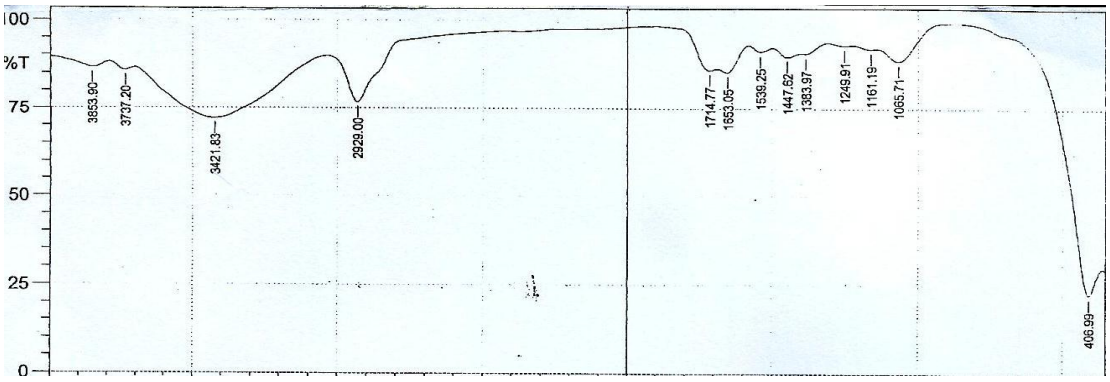

Fig. 18: IR spectra of flavonoids fraction 1of Achyranthes spondensleaves

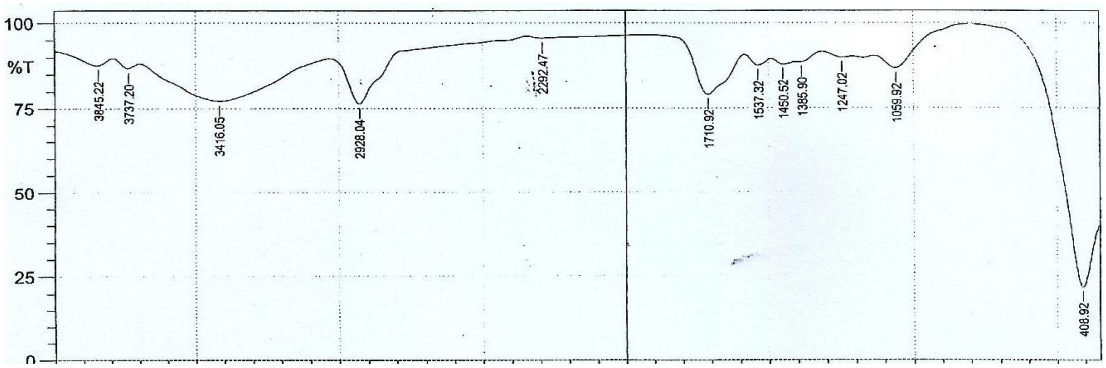

Fig. 19: IR spectra of flavonoids fraction 2of Achyranthes spondensleaves

This is region (2000-1500 cm-1) of double bonds of which carbonyl group is the most important that give the very strong intensity in their IR spectra. Weak IR absorption bands in this region reveal the existence of carbonyl group as impurities. Most bands in this study, within this region reveal strong absorption bands and represents presence of carbonyl group.

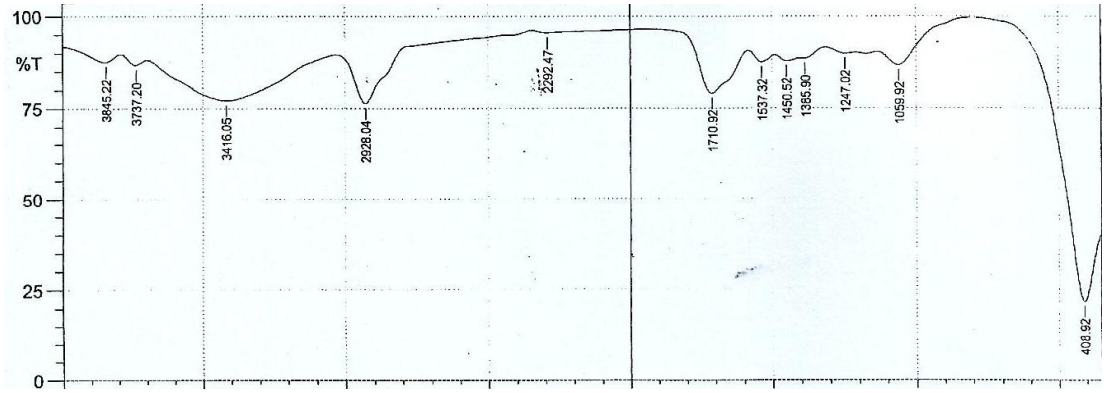

Fig. 20: IR spectra of flavonoids fraction 1of Solanium aethipiciumleaves

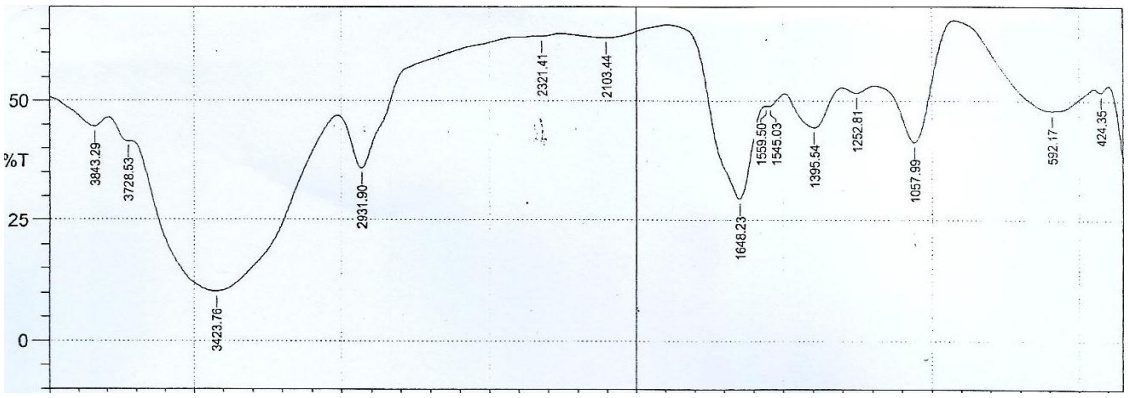

Fig. 21: IR spectra of flavonoids fraction 2of Solanium aethipiciumleaves

The region of triple bonds and accumulated double are within IR absorption band of 2500-2000 cm-1 (Yong-Cheng, 2011).. In this study they represents accumulated C=C double bonds with medium or low intensity.

Absorption bands between 2940-2920 cm-1represents CH_2 while 2960 and 2870 cm-1 represents CH_3 groups respectively. I f the intensities of the peaks at2940 and 2920 cm-1 are considerably stronger than those at 2960 and 2870 cm-1 it reveals that the sample contains many CH_2 groups with few CH_3 groups (Yong-Cheng, 2011). In this study there are more CH_3 groups in the lower plant vegetables while CH_2 groups dominate in higher plant vegetables.

The absorption bands of hydroxyl groups are situated in the 3550-3200 cm-1 region. For the fact there is an absorption at1300-1000 cm-1 that represents the presence of stretching vibration of alcoholic and phenolic group's containing C-O vibration of aromatic compounds (Yong-Cheng, 2011) confirming that phenols in the aromatic ring of these compounds are flavonoids.

CONCLUSION

The total flavonoid content, absorption and functional groups of the higher and lower plants vegetables were compared. Higher known green leafy vegetables have more percent crude and total flavonoid than lower well-known and commonly consumed vegetable. The number of flavonoids, absorption and functional groups varies among the leafy vegetables investigated. The consumption of higher leafy vegetables should be introduced to this generation because of the nutritional and therapeutic benefits of the high content of flavonoids.

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