Phytochemical Estimation of Some Active Molecules and Plant Insulin (Glucokinin) From the *Bauhinia variegata* Linn

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Abstract

The study includes the isolation and extraction of some active molecules like (flavonoid and total poly phenolic) and plant insulin (Glucokinin) from leaves and flowers of *B. variegate* L. belonging to the family Leguminosae which cultivated in Iraq. The study aim is to isolation and determined the chemical composition of the active compounds from Bauhinia by using methanol extract (1:7) of leaves plant and the flowers extract using petrol ethyl after that methanol (1:7) for two days under room temperature. The active compounds were separated from methanolic extract used liquid / liquid partition has been employed by silica gel column then the compounds were detected by chemical tests: ferric chloride detector, Potassium dichromate detector and lead acetate detector and sulfuric acid conc. and purity were confirmed by using High Performance Liquid Chromatography (HPLC). The results of the antioxidant activity of leaves (LM1; LM2) and flowers (F) methanolic extracts *in vitro* showed that the extract radical scavenging capacity (EC50) values were (5; 4.9 and 25.5 µg/ml) and possess DPPH radical scavenging activity compared to reference substances BHT and vitamin C (EC50= 4.5 and 4.0µg/ml, respectively). The amount of total phenol in leaves (LM1; LM2) and flowers (F) methanolic extracts for the dose of 5 ; 10 and 25 mg/ ml were (15.07; 18.19 and 32.80µg/g) and (22.78; 45 and 47.92µg/g) and (14.18; 17.64 and 18.65 □g/g) respectively. The results of one- dimensional TLC analyses showed that different flavonoids and phenolic acids were present in the investigated extracts. The greatest number of flavonoids (flavonoid glycosides) and phenolic acidswere determinated in methanolic extract.

Introduction

The antioxidants were divided into primary and secondary. Most of the antioxidants used are primary antioxidants like phenolic compounds for examples phenol acids, flavonoids, anthocyanidins, lignans, tannins, coumarins etc. Secondary antioxidants include metallic complex agents, singlet oxygen and others. Antioxidants help prevent the free radical damage that is associated with cancer and heart disease. Antioxidants can be found in most fruits, vegetables, culinary and medicinal herbs (AL-Jumaily*et al*., 2010;Mierlici, 2009). The preservative effect of medicalplant and herbs suggest the presence of anti oxidative and antimicrobial constituents (Larson, 1997). Phytochemicals are naturally occurring and biologically active plant compounds that have potential disease inhibiting capabilities. The medicinal values of the plants lie in these phytochemicals, which produce definite physiological actions on the human body. (Larson, 1997 ;Hirasa and Takemasa,1998).

The chemical investigation of methanolic extract of the stems of *Bauhinia variegata*Linn.belonging to the family Leguminosae led to the isolation of four bioactive phytoconstituents - Lupeol, β-Sitosterol, Kaempferol and Quercetin (Jash*et al*., 2014).
Another studies shown that the phytochemical screening revealed that *Bauhinia variegata*contained terpenoids, flavonoids, tannins, saponins, reducing sugars, steroids and cardiac glycosides. Pharmacological studies showed that *Bauhinia variegate* exerted anticancer, antioxidant, hypolipidemic, antimicrobial, anti-inflammatory, nephroprotective, hepatoprotective, antiulcer, immunomodulating, molluscicidal and wound healing effects.(Hill, 1952).
*Bauhinia variegata*L. (Fabaceae), commonly known as 'Raktakanchan' is distributed in sub-Himalayan and outer Himalaya of the Punjab and Sikkim. It is also found in Burma and China. Traditionally, various parts of this plant are used as anthelmintic, astringent, anti-leprotic, liver tonic,antibacterial and in the treatment of dysmenorrhea (Al-Santi, 2013). Accordingto different indigenous medicinal systems of India, the plant is also useful for treatment of skin diseases, wounds,edema, dysentery, ulcers, eye disease, piles, hemorrhoids and snake bite (Kirtikar and Basu, 2006). The various biological activities such as antimicrobial, anti-inflammatory, antitumor, cytotoxic andhepatoprotective activities of this plant have also been reported [Azevedo*et al*.,2006; Bodakhe andRam, 2007]. Earlier studies on leaves of *B. variegata*reported the isolation of plant insulin (Glucokinin), an anti-inflammatory triterpenoidsaponin and flavonoids.(Santanu, *et al*., 2011).

Materials and Methods

The leaves of *Bauhiniavariegata* werecollected from Al-Jadiria region-Baghdad city duringNovember 2014and flowers duringApril 2015. Ithas been diagnosed by the College of Science / Baghdad University.The leavesand flowers were washedand dryandthen crushedbyelectricgrindertopowderthen storedinan air tight container until used.
There are many methods using to extracted active compounds and flavonoids from leaves, Frist method: 100 g of the powder was separately extracted with 900 ml of methanol, stirring for 48 hours, and then the solvent was evaporated in vacuum pressure at 40 ^{o}C.

Silicagel (60 mesh) columnchromatography :

A- Silica gel was preparedby weight 40 gram mixed with methanol 99.9% solution left for a time and wash the gel 3 times with same solution , then the slurry was degassing under reduced pressure in order to remove air from the gel. The gel was allowed to equilibrate for 4-5hrs. at room temperature and then poured carefully into a glass column.

B- According to Al-Jumaily*et al*., (2010), the active compounds and flavonoid compound was separated from methanol extracted had been preceded using glass column (4 x 20) cm filled with Silica gel G-60 (A). Five ml of methanol extracted of the leaves was subjected to column and eluted with methanol solution (70%), and the flow rate regulated to be 60 ml/min. The elution had been collected in large tubes for each of mobile phase used and numbered as fractions; all fractions have been tested by ferric chloride solution 1%. Fractions containing flavonoid compound were pooled and concentrated to the required volume. (This extracts called methanolic extracts (LM1).

Second method: the same methanolic extract (1:9) was pass through the silica gel column and after that washed by different solvents (hexane ; petroleum ether ; petroleum ether : chloroform (3:1) ; petroleum ether : chloroform (3:2) and finial washed with methanol 70%. The elution had been collected in large tubes for each of mobile phase used and numbered as fractions; all fractions have been tested by ferric chloride solution 1%. Fractions containing flavonoid compound pooled and concentrated to the required volume.

Third method: the soxhlet extractorby using petroleum ether According to AL- Shahat, (1986) with some modification. 100 gram of the crude powder of leaf was refluxed with 900 ml of petroleum ether (1:9) in soxhlet apparatus for 8 hours. The solution was filtered through a filter paper and evaporated to dryness under vacuum at 40˚C. The dried extract was dissolved by methanol (1:9) and stirring for 48 hrs. and then the solvent was evaporated under vacuum at 40 ^{o}C.The dry extract was dissolved in a small volume 70% methanol and passed through the silica gel column and washed with the same solution. The elution had been collected in large tubes for mobile phase, all fractions had been tested for flavonoid and active compounds. (This extracts called methanolic extracts (LM2).

The flowers extract has been prepared according to the method used by AL- Shahat, (1986)with some modification. 50 gram of the crude powder of seeds was refluxed with 450 ml of petroleum ether (1:9) in soxhlet apparatus for 8 hours to remove oil . The extract has been evaporated to dryness under vacuum at 40 C˚, the dried extract have been weighed and stored at 4 C˚. The dry extract was dissolved in a methanol 70% (1:9) and stirring for 48 hrs. and then the solvent was evaporated under vacuum at 40^{o}C. (This extracts called flowers extracts (F).

Chemical detectionfor tannins and phenolic compounds:

1- **Ferric Chloride Test** :According to Harborne (1984), 1 ml of the methanolic extract was added to 1ml of ferric chloride solution 1%; the appearance of intense green, purple ,blue or black colors indicated the presence of phenolic compound.
2- **Lead acetate Test** : one ml of lead acetate solution was added to 1ml of methanolic extract . The positive result is white jelly residue indicated the presence of tannins.(AL-Tikrity , 1997).
3- **Flavones Test**:Keep 1 mlofextractinatesttubeandadddropsofsulfuricacid Conc. indicatestheemergenceofareddish brown colortopositivedetection (Jaffer *et al*., 1983).
4- The extract is treated with sodium hydroxide; formation of yellow color indicates the presence of flavones .(AL- Shahat, 1986).
5- Detection of Flavonoids: According toJaffer *et al*., (1983) to one ml of methanolic extract added mixed 50% of potassium hydroxide and ethyl alcohol . The positive yellow color indicated the presence of yellow color indicated the presence of flavonoids.

Determination of Protein Concentration

According to the method of Bradford (1976), the protein concentration determined as follow: A 20µl of GTF crude was mixed with 50µl of 1 M NaOH with shaking for 2-3

minutes then 1 ml of Bradford solution was added with shaking. The absorbance was measured at 595 nm by spectrophotometer.

HPLC Method

The methanolic leaf extract and flowers extract were identified to Plant hormones (glucokinin) by (HPLC) according to (Jeandent*et al*., 1994), using ODS- reverse phase column and an elution system under the following conditions as shown in table (1).

Table (1) Conditions on HPLC of glucokininquantification.

Column	**ODS.**
Column length	25 cm
Flow rate	1ml/min.
Wave length	280nm
Mobile phase	acetonitrile -water (95:5)
Retention time	The time is obtained following the experiment.

DPPH free radical scavenging assay:

Five mL of a freshly prepared 0.004 % DPPH solution 4 mg/100 mL in methanol was mixed with 50 µL of different concentration ofleafmethanolicextract (LM1 , LM2)and flowers extract(F1) 5 , 10 , 15 , 25 , 35 and 50 mg/ml and the absorbance of each dilution, after 30 minutes, was measured at 517 nm. Butylated hydroxyl toluene (BHT) and vitamin C was the antioxidant used as positive control. (Huang *et al,* 2005).All tests were performed in triplicate and the methanol was used as blank solution. The percentage DPPH reduction (or DPPH radical scavenging capacity) was calculated as: % Reduction = (Abs DPPH – Abs Dil.)/Abs DPPH x 100 Where by: Abs DPPH = average absorption of the DPPH solution Abs Dil. = average absorption of the three absorption values of each dilution. With the obtained values, a graphic was made using Microsoft Excel. The EC50 of each extract (concentration of extract or compound at which 50% of DPPH is reduced) was taken from the graphic.

Determination of total phenolic contents The amount of total phenolics in leaf methanolic extract (LM1; LM2 and flower's extract) was determined with the Folin-Ciocalteu reagent. Gallic acid was used as a standard (Figure 1) and the total phenolics were expressed as mg/g gallic acid equivalents (Yau*et al*, 2006) Concentration of 0.005, 0.01, 0.02, 0.03, 0.04 and 0.05 mg/ml of gallic acid were prepared in methanol.Concentration of 10, 25 and 50 mg/ml of pure and partial lignan were also prepared and 0.5 ml of each sample were introduced into test tubes and mixed with 2.5 ml of a 10 fold dilute Folin- Ciocalteu reagent and 2 ml of 7.5 % sodium carbonate. The absorbance was at read at 760 nm spectrometrically. All determination was performed in triplicate.

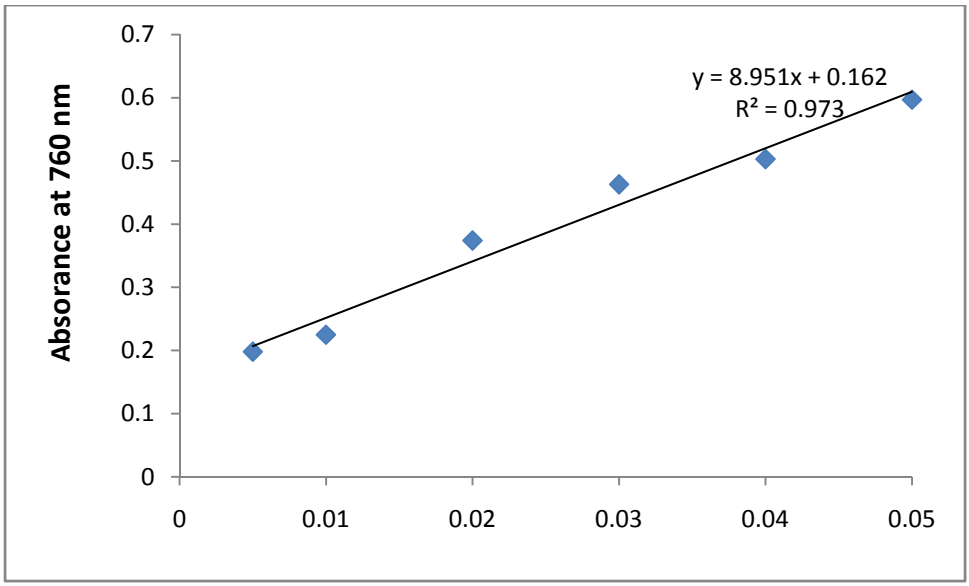


Figure (1): Standard curve of Gallic acid

Chromatography Thin Layer(TLC)

Followed this method mentioned in (Sinisa *et al*.,2001)terms of taking a model of the form and check manner chromatographyth in layeristh in sheets of aluminumcoatedsilica geldimensions20 □ 20cmand thickness0.2 millimeter and processed from a fluka company which contain a fluorescent substance when exposed to a source ofUVmode form the bottom of the plate just 2cmandtheotherspotis a 3 cm of the different extracted (Second method)using a separate system: Petroleum ether: chloroform by (3:1) put this solution in separation bowland letbas in separation tightly closed for half an hour to saturated vaporbasinuser solution and then placed the plates containing models in bas in separation and leavethesolutionuptoaheightof17.1cm **.**

Results and Discussion

The leavesof *B. variegata*were collected and dried in shade. The dried leaves were powdered (100gm) and exhaustively extracted by Soxhlet apparatus with methanol for 48h. Then the methanol layer was decanted off. The solvent of the extract was distilled off by using rotary evaporator and the brown syrupy material thus obtained was evaporated to dryness and a brown mass (about 2.45g) was obtained. The preliminary phytochemical studies were performed for testing the different phytoconstituents present in the leavesmethanolic extracts (LM1 & LM2) and flowers extract (F1) (Table 2). The chemical tests revealed the presence of flavonoids,tannins, glycosides, terpenes and steroids.

Table (2): Detection of some active compounds in Solution leaves extracts (M1 and M2) and flowers extract of *Bauhinia vareigata L.*

Phytochemical compound	Methanolic Extract (LM1)	Methanolic Extract (LM2)	Flowers Extracts (F1)
Flavonoids	+	+	+
Tannins	+	+	+
Terpenes	+	+	+

Glycosides	+	+	+
Steroids	+	+	+

(+) Present

Chemical detection of phenolic compounds :

The phytochemical detection of the leaves methanolic extract shows the presence of flavonoids, tannins, phenols as shown in (Table 3).

Table (3): General phenolic compound tests in all leaves methanolic extract (LM1 ,LM2) and flower extract.

Test	Result
1%Ferric chloride solution	Green colour (+)
10%Potassium dichromate solution	Yellow color precipitate
1%Lead acetate solution	White color precipitate
Sulfuric acid Conc.	Reddish brown color (+)

Estimation of total ploy phenol content

The Folin-Ciocalteu method is a rapid and widely-used assay investigating the total ploy phenol content, but it is known that different phenolic compounds gave different responses with this method. The data present in table (4) that showed the total ploy phenol contents of *B. variegata*sample (Gallic acid equivalents, mg/ml) leavesmethanolic extract (LM1 ;LM2) and flower extracts 5 mg/ml were 15.07; 22.78 and 14.18 □g/g respectively, and when using 15 mg/ml of these extracts they gave 32.80; 47.92 and 18.65 □g/g respectively.

The result of the present study showed that the extract of *B. variegata*, methanolic leaves extracts (LM2) which contain highest amount of phenolic compounds which exhibited the greatest antioxidant activity. The high scavenging property of methanolic extracts (LM2) may be due to hydroxyl groups existing in the phenolic compounds. Free radicals are often generated as by products of biological reactions or from exogenous factors. The involvements of free radicals in the pathogenesis of a large number of diseases.(Gyamfi*et al*., 1999).These results agreed with the study by Mishra *et al*.,(2013) about *Bauhinia variegata* Leaf Extracts contained higher total phenolics which different solvent extracted by Soxhlet and maceration different days.

Table (4): Total Phenolic content of leaves (LM1&LM2) and Flowers (F1) MethanolicextractConcentration (mg/ml) Total polyphenol (µg/g).

Concentration (mg/ml)	Total polyphenolic (□g/g)		
	Methanol extract (LM1)	Methanolextract (LM2)	Flower extract (F)
5	**15.07**	**22.78**	**14.18**
10	**18.19**	**45.00**	**17.64**
15	**32.80**	**47.92**	**18.65**

The Protein Concentration Content

The protein concentration in leaves (LM1 &LM2) and flowers (F1) methanolic extracts were determined by Bardford assay. Its appearance that the protein concentration (mg/ml) for these extracts as shows in table (5). From the results we found that the methanolic extract (LM1) gave high protein conc. 0.359 mg/ml follow by the flower extract 0.291 mg/ml and the methanolic extract (LM2) gave 0.283 mg/ml. These results was complemented with TLC results when we running the sample (LM1) on it with mobile phase (petroleum ether : chloroform (3:2) gave the same Rf (0.156) compared with insulin spot.

Table (5): The protein concentration in different extraction solution form *Bauhinia Variegata* L. Leaf and flowersmethanolic extracts.

sample	Absorbance at 595nm	Protein conc. mg/ml
Flowers (F)	0.193	0.291
LM1	0.237	0.359
LM2	0.187	0.283

Evaluation of antioxidant activity DPPH radical scavenging assay

Exogenous chemical and endogenous metabolic processes in the human body or in the digestive system might produce highly reactive free radicals, especially oxygen derived radicals, which arecapable of oxidizing biomolecules, resulting in cell death and tissue damage (Mau *et al*., 2001). Figure (2) illustrates the concentration of DPPH radical due to the scavenging ability of the methanolic extract (LM1 &LM2) and flowers extract and standard BHA and vit C were used as references. The radical scavenging capacity (EC50) were found to be 5 ; 4. 90 and 25.5 µg/ml respectively in methanolic extract (LM1 ; LM2 and F) which are the concentration that decreases the initial DPPH radical concentration by 50%. On the other hand the (EC50) of vit C and BHT were 4.5 and 4µg/ml respectively. When the experimental doing with minimum concentration between (2-10 □g/ml) (Figure 3), the radical scavenging capacity of the methanolic extract (LM1 &LM2) and flowers extract were 2.8 ; 4 and 10 □g/ml respectively. The antioxidant activity of plant extracts containing polyphenol components is due to their capacity to be donors of hydrogen atoms or electrons and to capture the free radicals.

The results of the DPPH· free radical scavenging assay suggest that components within the extracts are capable of scavenging free radicals via electron or hydrogen donating mechanisms and thus should be potent enough to prevent the initiation of deleterious free radical mediated chain reactions, presumably because of its phytochemical constituents. Lower absorbance of the reaction mixture indicates higher free radical scavenging activity. All the absorbance values in both the assays were compared to the control system (without extract) used.

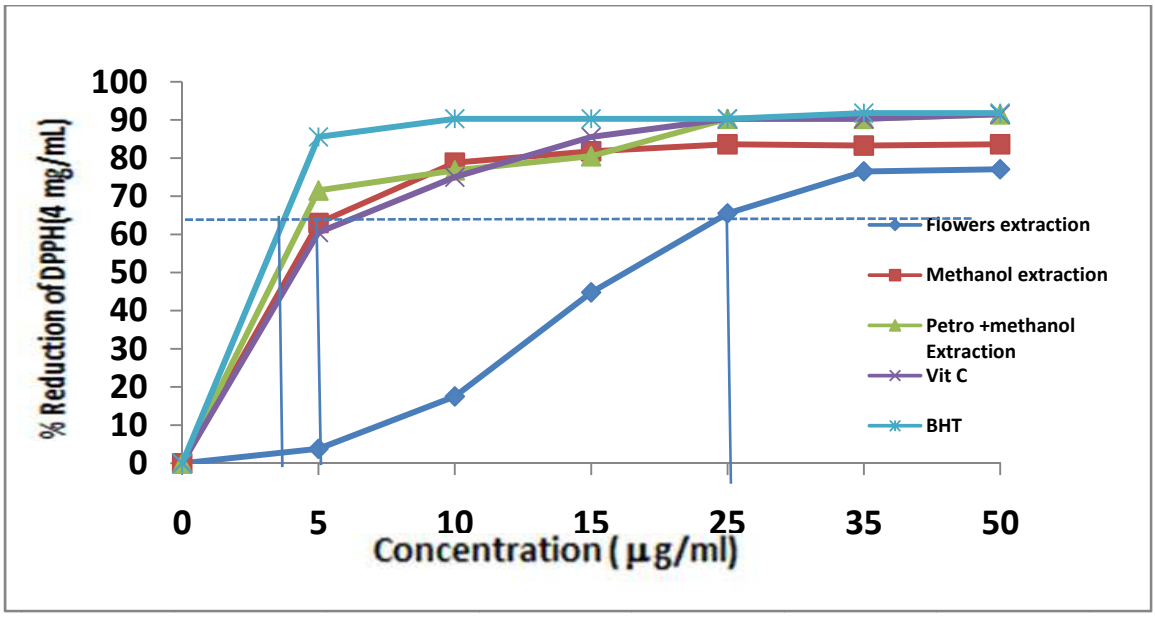


Figure (2): Percentage of DPPH reduction using different extraction(leaves and flowers)
With concentration (5-50 □g/ml) and appropriate controls after 30 min of exposure. The corresponding EC50 are alsooutlined.

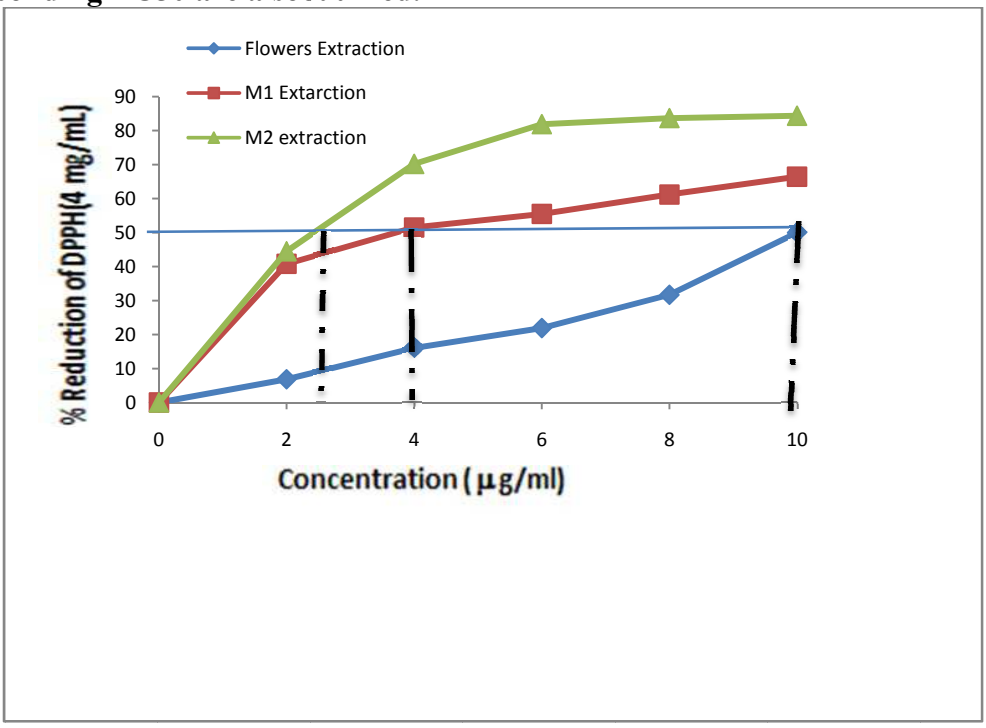


Figure (3): Percentage of DPPH reduction using different extraction(leaves and flowers)
With concentration (2-10 □g/ml) and appropriate controls after 30 min of exposure. The corresponding EC50 are alsooutlined.

Thin Layer Chromatography (TLC)

Figure (4) shown the TLC chromatogram of the methanolic leaves extract which passive on silica gel 60G column and eluted with different solvent.The detection in this method gave the spots were characterized by Rf- values and color under UV light. Same result characterized by appearing of a dark spot on the silica gel thin layer according to Harbone (1973), where the Rf. value of flavonoids was 0.847 (violet/yellow) and 0.935 (blue/light blue); glycoflavones with Rf 0.321 (dark violet) and Anthocyanins with Rf 0.146 (dark red) (Figure 1),this result agree with Al-Jumaily*et al*., (2012) and Ćetkovićet al.,(2003).

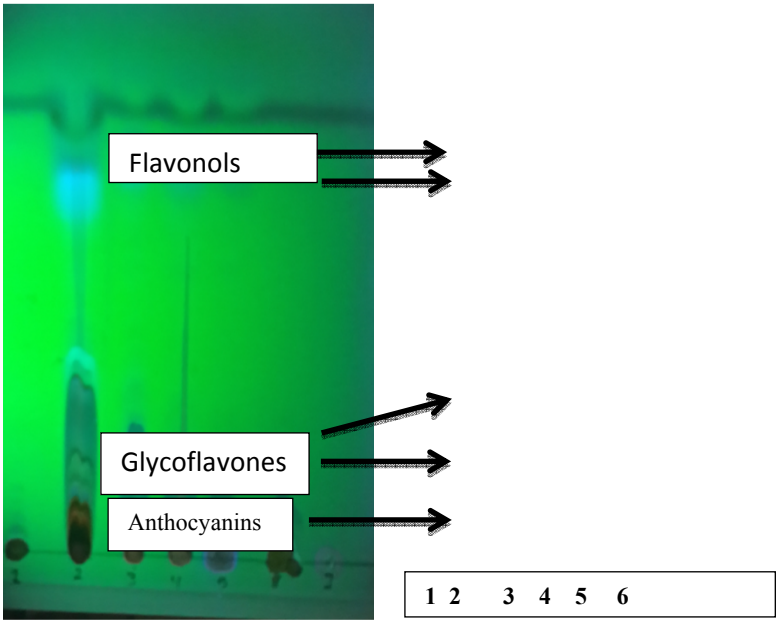
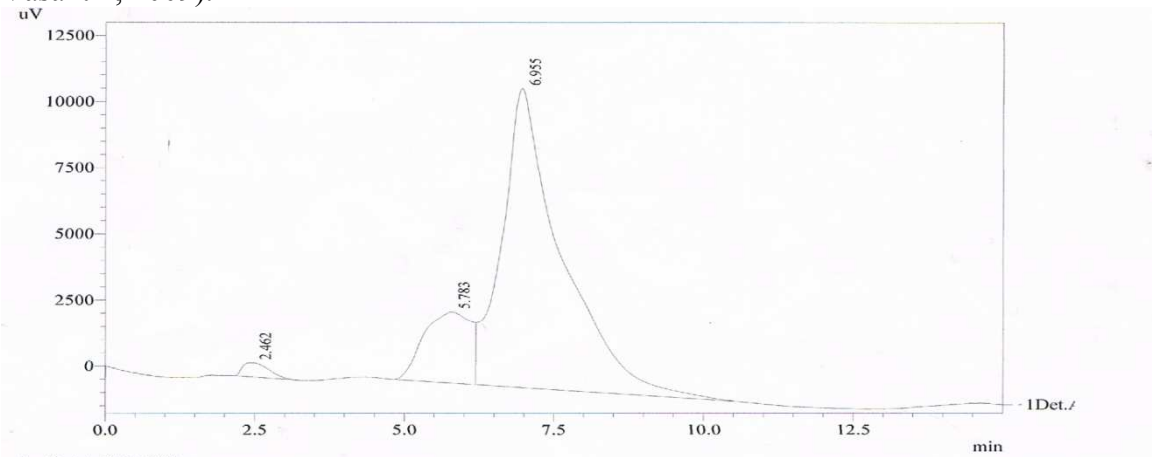


Figure (4): Partition separation of the methanolicleaves extract, eluted with differents solvent. 1. Methanolicextract 70% 2. Haxane 3. Petroleum ether 4.Hexane and petroleum ether (3:1) 5.Hexane and petroleum ether (3:2) 6. Wash with all perversely solvent and eluted with methanol 70% .

High performance liquid chromatography

All Plant insulin (glucokinni) contain at least one aromatic ring for amino acids (tryptophan) and, consequently, efficiently absorb UV light it is evident that absorb well in the UV range and UV detection is therefore a convenient method to localize a plant insulin in the effluent of a column (Koona*et al*., 2010). However, no single wavelength is sufficient for their simultaneous monitoring in various natural plant extracts. Detection at 280 nm is most generally used for the simultaneous separation of mixtures of plant insulin, although for dual monitoring 250 and 280 nm, can beideal wavelengths used liquid chromatography technology, high efficiency to determine the purity of this compound, the results showed the time of detention in Figures (5, 6 and 7) for each of the Human insulin and flower methanolic extract (F) and leaves methanolic extract (LM1) which manifestation in (6.955; 7.031 and 7.083 minutes) respectively.(Table 6).From this table also could shows that the Plant insulin (glucokinni) concentrationwere 77.79% ; 24.40% and 44.88% respectively.

The observated from the isolation of plant insulin (glucokinni) from *B. variegate*leaves suggested that insulin signaling pathwaywas conserved through evolution and the high concentration suggesting its involvement in carbohydrate metabolism in facilitating glucose transport across membranes similar to its role in animals.(Sangeetha and Vasanthi, 2009).

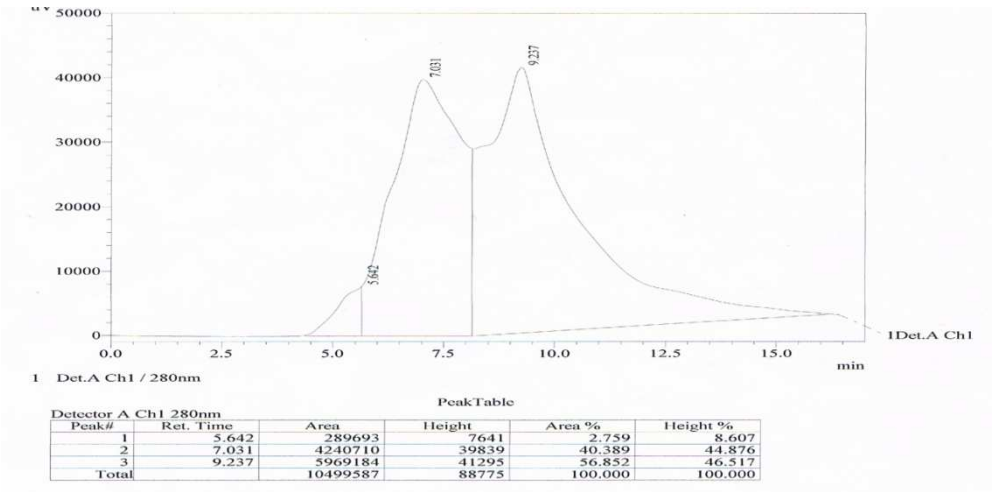


PeakTable

Detector A Ch1 280nm

Peak#	Ret. Time	Area	Height	Area %	Height %
1	2.462	16407	536	1.727	3.683
2	5.783	146929	2697	15.469	18.526
3	6.955	786497	11323	82.804	77.790
Total		949833	14555	100.000	100.000

Figure (5): HPLC quantification of insulin standard, retention time (6.955 min) and the absorbance of Insulin.

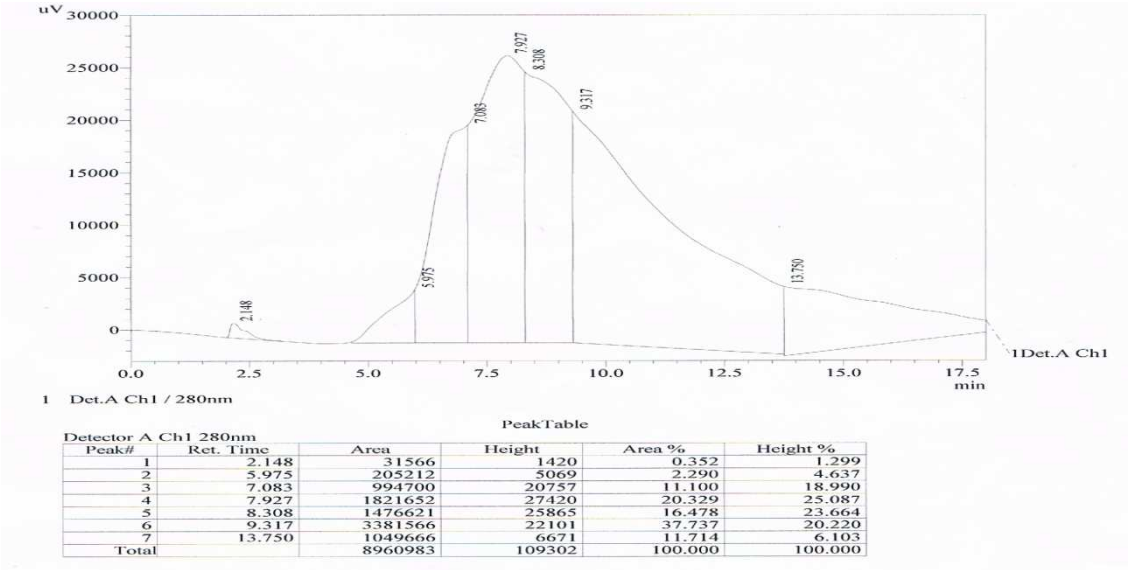


PeakTable

Detector A Ch1 280nm

Peak#	Ret. Time	Area	Height	Area %	Height %
1	5.642	289693	7641	2.759	8.607
2	7.031	4240710	39839	40.389	44.876
3	9.237	5969184	41295	56.852	46.517
Total		10499587	88775	100.000	100.000

Figure (6): HPLC quantification of flowers methanolic extract, retention time (7.031 min) and the absorbance of Insulin.



Peak Table

Detector A Ch1 280nm

Peak#	Ret. Time	Area	Height	Area %	Height %
1	2.148	31566	1420	0.352	1.299
2	5.975	205212	5069	2.290	4.637
3	7.083	994700	20757	11.100	18.990
4	7.927	1821652	27420	20.329	25.087
5	8.308	1476621	25865	16.478	23.664
6	9.317	3381566	22101	37.737	20.220
7	13.750	1049666	6671	11.714	6.103
Total		8960983	109302	100.000	100.000

Figure (7): HPLC quantification of leaves methanolic extract, retention time (7.083 min) and the absorbance of Insulin.

Table (6): The retention time and plant insulin (glucokinni) concentration in Leaves extracts (LM2) and Flowers extract (F).

Retention time (minute)	Human Insulin control	Leaf extract (LM2)	Flowers extract
6.955	77.79	----	--
7.031	---	24.40	---
7.083	---	--	44.88

CONCLUSION

The *Bauhinia variegata*leaves and flowers methanolic extract gave higher phenolic and hydroxyl groups or theirsynergistic properties to using as safe and potent hydroglycemic and hydrolipidemic agent in future study. And we concluded that the Plant insulin (Glucokinin) may be responsible for some of the actions at plant extracts for their antidiabetic properties.

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