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Anti-hyperglycaemic and antioxidant effects of *Bidens tripartita* and quantitative analysis on its active principles

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ABSTRACT

Objective(s): *Bidens* species are used for their antidiabetic properties traditionally in many countries. Aim of this study is to evaluate hypoglycaemic and antidiabetic activity of *Bidens tripartita* extract and to identify its active compounds through bioactivity guided isolation technique.

Materials and Methods: Hypoglycaemic effects of *B. tripartita* extract and its sub-extracts were investigated in normal and glucose-hyperglycaemic rats. Streptozotocin induced diabetic rats were used to examine antidiabetic activity of the extract and its sub-extracts after acute and sub-acute administration. Additionally, *in vitro* enzyme inhibitory and antioxidant activities were evaluated. HPLC analyses were carried out to determine the active constituents of the extract and its sub-extracts.

Results: Through *in vivo* bioactivity-guided fractionation process, ethyl acetate and n-buthanol sub-extracts were found to have potent antidiabetic activity. *In vitro* enzyme inhibitory activities of the same sub-extracts were found to be potent. The highest total phenol, flavonoid contents and radical scavenging activity was determined in ethyl acetate sub-extract. According to LC-MS analyses, chlorogenic acid, luteolin and 7-O-glucoside of luteolin (cynaroside) were determined as the main components of the active sub-extracts.

Conclusion: According to our results, *B. tripartita* has potent antidiabetic activity and its active constituents might be beneficial for diabetes and its complications.

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Introduction

Bidens tripartita, commonly known as "three-lobe beggarticks, three-part beggarticks, leafy-bracted beggarticks or trifid bur-marigold, is a flowering plant in the Asteraceae family (1). It is a worldwide weed native to Northern hemisphere growing near the river banks, marshes, ditches and wet sites (2).

Infusion of the aerial parts of *B. tripartita* L. is widely used in the treatment of catarrhal rhinitis, angina, acute respiratory infections, and as an anti-inflammatory in colitis, gout, and infantile rickets in Russia as traditional medicine (3). It is also used as a diaphoretic and a diuretic in nephrolithiasis (4), as an antiseptic and as a bath for children to treat allergy symptoms (5). Decoctions of *Bidens* species are used to treat diabetes in different regions of the world (6-11). Additionally, young leaves of *Bidens* species are added to salads, soups or stews and young shoot tips are used to make tea (12). Hence, they have been used as food and medicine traditionally without noticeable adverse effects for centuries (13).

Many pharmacological studies have been conducted on *B. tripartita* and its anticancer, anti-inflammatory, antimicrobial, antioxidant, antithrombin, antiulcer, hepatoprotective, and hypotensive effects are reported in the literature. The Herba Bidentis Monograph is included both in the World Health Organization Monographs on Medicinal Plants Commonly Used in the Newly Independent States (NIS) in 2010 and in Russian Pharmacopoeia. By revealing more information about *Bidens*, broad studies of this plant can lead to an improved appreciation of the extent of the applications of this herb in medicine (14).

Diabetes is one of the most common and important metabolic disorders in the growing world. As of 2014, an estimated 387 million people have diabetes worldwide. Diabetes can affect many parts of the body and is associated with serious complications, such as heart disease, stroke, blindness, kidney failure, and lower-limb amputation, among other conditions (15). Oxidative stress is known to have the major role in the progress of these conditions. Therefore, it is important to evaluate both the antioxidant potential and the

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hypoglycaemic activity of antidiabetic drugs. On the other hand, one of the therapeutic approaches to treat diabetes is to decrease postprandial hyper-glycaemia and this can be achieved by the inhibition of carbohydrate hydrolyzing enzymes like *α*-amylase and *α*-glucosidase. They are the major enzymes in the digestion of carbohydrates and they are the potential targets in the development of lead compounds for the treatment of diabetes (16). As well as insulin and oral antidiabetics, many traditional plant remedies or herbal formulations exist from ancient times and are still widely used (17).

In the present study, we aimed to; 1. evaluate the potential antidiabetic effect of *B. tripartita* extract and its sub-extracts by *in vivo* models, 2. elucidate the probable antidiabetic mechanism by *in vitro* models, 3. determine the active principles by using bioactivity guided isolation technique, 4. investigate antioxidant capacity and 5. determine the chemical profile of the active extracts

Materials and Methods

Plant materials

Aerial parts of *B. tripartita* L. (Asteraceae) were collected at the end of August in 2011 from the lakeside of Yeniçağa, Bolu (Turkey). The voucher specimen (AEF 25996) is stored in the Herbarium of Ankara University, Faculty of Pharmacy.

Preparation of the extract and sub-extracts

Plant materials were dried under shade and coarsely powdered for extraction. A portion of the material (100 g) was extracted with 80% ethanol (EtOH) (1.5 l) on shaker for 24 hr and filtered. 1 l of 80% ethanol was added to the pulp and extraction was completed on a shaker after 24 hr. Combined ethanol extracts were evaporated to dryness under reduced pressure and then lyophilized (EtOH extract, yield 15.63%).

The EtOH extract (40 g) was dissolved in 500 ml of distillated water and fractionated by successive solvent extraction with chloroform (9 × 500 ml), ethyl acetate (11 × 500 ml) and *n*-butanol saturated with H_2O (7 × 500 ml). Each sub-extract as well as the remaining aqueous sub-extract was evaporated to dryness under reduced pressure to obtain "Chloroform sub-extract" (Yield %:14.60), "Ethyl acetate sub-extract" (Yield %:19.05), "*n*-Butanol sub-extract" (Yield %:15.54) and "Remaining water sub-extract" (Yield %:44.22). All the obtained sub-extracts were used in animal experiments at the doses calculated according to their yields.

In-vivo studies

Animals

Wistar Albino male rats (150-200 g) were used in this study with the approval of Animal Experiments Local Ethical Committee (GUET-06.087). Animals were purchased from the animal breeding laboratories of Experimental Animal Research Center of Gazi University (GUDAM) (Ankara, Turkey) and the experiments were carried on at the same research center. The animals were maintained on standard pellet diet and water *ad libitum* throughout the experiment.

Blood Collection and determination of blood glucose levels

Blood samples were collected from the tip of tail at the defined time patterns and blood glucose concentrations (mg/dl) were determined using an Ascensia-Elite commercial test (Serial No. 9123232, Bayer), based on the glucose oxidase method.

Effect on normoglycaemic animals

Fasting blood glucose level of each animal was determined at initial time, after overnight fasting (12 hr) with free access to water. Tolbutamide (100 mg/kg of body weight [BW]) was used as the reference drug. The extract, sub-extracts and the reference were suspended in 0.5% aqueous carboxymethylcellulose (CMC) suspension in distilled water prior to oral administration to animals (10 ml/kg of BW). Control group was received 0.5% CMC (10 ml/kg BW). Blood samples were collected at 1/2, 1, 2 and 4 hr after the oral administration of test samples.

Effect on glucose-hyperglycaemic animals [OGTT: Oral glucose tolerance test]

After overnight fasting (12 hr), the blood glucose levels of animals were determined and immediately test samples were administered orally. Two g/kg glucose was loaded to the rats orally at the 30th minute and the blood glucose concentrations were determined at the 1st, 2nd and the 4th hour of the experiment.

Effect on diabetic animals

Streptozotocin (STZ, 60 mg/kg, intraperitoneal) dissolved in citrate buffer was used to induce experimental diabetes. Seven days after the injection of STZ, the blood glucose levels were measured and the animals with blood glucose levels higher than 250 mg/dl were considered to be diabetic. Diabetic rats were fasted 8 hr before the experiment.

For determination of acute antidiabetic effect, the blood glucose levels were measured at the beginning of the experiment before the administration of CMC/tolbutamide/extract/sub-extracts. Then, in the blood glucose levels were monitored at the 30th, 60th, 120th, 240th minute of the experiment.

The ethanol extract, its sub-extracts (chloroform, ethyl acetate, *n*-butanol, remaining water), CMC and tolbutamide were administered to the diabetic animals seven days consecutively for determination of antidiabetic effect after sub-acute administration. During the experiment, blood glucose levels were

measured at the 1st, 4th and 8th days at 10.00 a.m. just before the administration of test samples. The effect of all test samples on body weight was also monitored in the same days.

In-vitro studies

Assay for α-amylase inhibitory activity

The *α*-amylase inhibitory activities of *B. tripartita* ethanol extract and its sub-extracts were determined by the method of Ali *et al* (2006) (18). Porcine pancreatic *α*-amylase type VI (EC 3.2.1.1, Sigma) was dissolved in distilled water. As substrate solution, potato starch (0.5%, w/v) in phosphate buffer (pH 6.9) was used. Experiments were carried out with three replicates. Acarbose was used as the positive control. At the end of the experiment absorbances of the mixtures were read at 540 nm. The absorbance (*A*) due to maltose generated was calculated according to following formula:

$A_{\text{Control or Sample}} = A_{\text{Test}} - A_{\text{Blank}}$

The amount of maltose generated was calculated by using the maltose standard calibration curve (0-0.1% w/v) and the obtained net absorbance. Percent of inhibition was calculated as:

$\text{Inhibition } \% = [(\text{Maltose}_{\text{Control}} - \text{Maltose}_{\text{Sample}})/\text{Maltose}_{\text{Control}}] \times 100$

Assay for α-glucosidase inhibitory activity

The method of Lam *et al* (2008) was used to evaluate *α*-glucosidase inhibitory activity (19). *α*-Glucosidase type IV enzyme (Sigma Co, St. Louis, USA) from *Bacillus stearothermophilus* was dissolved in phosphate buffer (0.5 M, pH 6.5). The enzyme solution and extracts dissolved in MeOH-H_2O were preincubated in a 96-well microtiter plate for 15 min at 37 °C. After that, the substrate solution [20 mM *p*-nitrophenyl-*α*-d-glucopyranoside (NPG), Sigma] was added. The mixture was incubated for 35 min at 37 °C. The increase in the absorption at 405 nm due to the hydrolysis of NPG by *α*-glucosidase was measured by an ELISA microtiter plate reader. Acarbose (Bayer Group, Turkey) was used as positive control. The inhibition percentage (%) was calculated by the equation:

$\text{Inhibition } \% = [(A_{\text{Control}} - A_{\text{Sample}})/A_{\text{Control}}] \times 100$

Total antioxidant activity by phosphomolybdenum assay

This assay is based on the reduction of Mo (VI) to Mo (V) by the sample and the subsequent formation of a green phosphate/Mo (V) complex at acidic pH. *B. tripartita* ethanol extract and its sub-extracts were added to test tubes containing molybdate reagent solution (Molybdate reagent:1 ml 0.6 M sulphuric acid, 28 mM sodium phosphate, 4 mM ammonium molybdate). Vortexed tubes were incubated at 90 ◦C for 90 min. Then, tubes were cooled to room temperature and the absorbances of the samples were measured at 695 nm. Results were expressed as mg ascorbic acid equivalent/g extract (20). Trolox was used as positive control.

Radical scavenging activity by DPPH assay

The DPPH (2,2-diphenyl-1-picrylhydrazyl) radical scavenging activities of the extract/sub-extracts were determined according to the method reported by Jung *et al* (2011) (21). Extract/sub-extracts were mixed with DPPH solution and incubated in darkness for 30 min. Then the absorbance was measured at 520 nm utilizing a 96-well ELISA microplate reader (VersaMax, Molecular Devices, USA). Ascorbic acid was used as a positive control at 0.5, 1 and 2 mg/ml concentrations.

Phytochemical analysis

Determination of total phenol content

The method of Zongo *et al* (2010) was used to determine total phenol contents. The extract and sub-extracts were mixed with Folin-Ciocalteu reagent and samples were incubated for 5 min. at room temperature (22). Then, sodium carbonate solution was added. The absorbance of mixture was measured at 735 nm after 30 min. The mean of three readings was used and the total phenol content was expressed in mg of gallic acid equivalents/g extracts. Calibration curve equation was;

y(Abs.)= 5.306x(Conc.)+0.0587 and the coefficient of deter-mination was r^2= 0.9986.

Determination of total flavonoid content

Total flavonoid contents of the *B. tripartita* extract and its sub-extracts were measured by the method of Kosalec *et al* (2004) (23). Extract and sub-extracts were dissolved in ethanol. Ethanol, sodium acetate and aluminium chloride solution were added to the samples and the mixture was diluted by distillated water to 5 ml. After 30 min, the absorbance of yellow mixtures was measured at 415 nm. Results were expressed in mg of quercetin equivalents/g extracts. Calibration curve equation was;

y(Abs.)=2.4214x(Conc.)-0.051 and the coefficient of determination was r^2= 0.9998.

LC-MS and HPLC analysis

Solvents and chemicals

HPLC grade methanol and acetonitrile, analytical grade trifluoroacetic acid, and chromatographic grade double-distilled water were used for LC-MS and HPLC analysis. Presence of selected compounds was investigated. Ascorbic acid (57803), caffeic acid (C0625), chicoric acid (06957), chlorogenic acid (C3878), rosmarinic acid (R4033), catechin (43412), hyperoside (00180595), kaempferol (K0133), luteolin (L9283), luteolin-7-O-glucoside (cynaroside)

Table 1. Acute hypoglycaemic and antidiabetic effects of *Bidens tripartita* ethanol extract

Test Model	Group	Dose (mg/kg)	Mean blood glucose concentration (mg/dl) ±SEM (Inhibition %)				
			Initial	½ hr	1 hr	2 hr	4 hr
NG	Control	-	88.0 ± 4.98	110.2 ± 5.57	103.7 ± 5.60	99.8 ± 4.77	109.9 ± 3.56
	Tolbutamide	100	88.0 ± 4.93	81.4 ± 1.69*** (26.1)	68.2 ± 4.06*** (34.2)	57.5 ± 3.48*** (42.4)	60.3 ± 4.33*** (45.1)
	B. tripartita	250	88.7 ± 3.56	95.2 ± 3.51 (13.6)	88.7 ± 1.52* (14.5)	83.2 ± 1.05* (16.6)	97.2 ± 2.18* (11.6)
		500	82.2 ± 1.30	81.8 ± 3.28*** (25.8)	85.5 ± 4.17* (17.3)	79.3 ± 1.98** (20.5)	93.0 ± 4.13* (15.4)
OGTT	Control	-	85.0 ± 4.75	88.0 ± 3.94	134.2 ± 3.11	118.2 ± 5.31	95.6 ± 5.08
	Tolbutamide	100	86.5 ± 4.30	64.6 ± 4.33** (26.6)	80.9 ± 7.51*** (39.7)	52.8 ± 4.76*** (55.3)	44.2 ± 6.90*** (53.8)
	B. tripartita	250	83.2 ± 2.93	90.0 ± 3.90	118.8 ± 5.17* (11.5)	104.2 ± 5.72 (11.8)	81.0 ± 3.31* (15.3)
		500	76.7 ± 1.98	81.5 ± 1.73 (7.4)	117.7 ± 3.57* (12.3)	104.5 ± 2.05 (11.6)	75.5 ± 2.22** (21.0)
Diabetic	Control	-	335.4 ± 19.53	303.4 ± 13.01	294.6 ± 16.60	265.8 ± 18.60	204.4 ± 15.42
	Tolbutamide	100	300.8 ± 17.68	253.7 ± 11.18* (16.4)	235.6 ± 15.40* (20.0)	222.0 ± 7.40* (16.5)	210.0 ± 9.78
	B. tripartita	250	308.8 ± 16.68	260.8 ± 4.64* (14.0)	249.2 ± 13.09* (15.4)	206.0 ± 12.20** (22.5)	158.8 ± 16.21** (22.3)
		500	307.6 ± 16.97	229.4 ± 8.69** (24.3)	214.0 ± 19.40** (27.4)	178.8 ± 12.43*** (32.7)	152.6 ± 8.37* (25.3)

* $P<0.05$, ** $P<0.01$, *** $P<0.001$, SEM: standart error of the mean; NG: normoglycaemic; OGTT: oral glucose tolerance test

(49968), quercetin (Q4951), rutin (R5143) were purchased from Sigma-Aldrich (Germany) and Fluka.

Experiment

Major chemical constituents of active extract and its sub-extracts were determined by using LC-MS. HPLC was used in the quantitative analysis. The analysis was first carried out with an LC-MS system (Agilent Technologies). ACE column (5 µ, C_{18} (150 mm x 4.6) was used for the analysis. Injection volume was 20 µl. Mobile phase was acetonitrile and trifluoroacetic acid (0.03%) in water with a flow rate of 1 ml/min. A mass dedector (G1956) was used to investigate the presence of selected compounds given above.

HPLC analysis were performed by using HP-1200 (Agilent Technologies Inc., California, USA) HPLC with an ACE 5 µ, C18 (150 x 4.6 mm) column. Water (0.03 % trifluoroacetic acid) and acetonitrile were used in gradient elution as the mobile phase with a flow rate 1 ml/min. The composition of gradient was 10:90 (Acetonitrile: TFA Solution) at 0 min and changed to 30:70 (Acetonitrile: TFA Solution) in 30 min. Injection volume was 20 µl. The duration between runs was 10 min. Different wavelengths (210, 254, 280 and 360 nm) was scanned by a G1315D diode array dedector (DAD). Then, DAD was set at 210 nm and peak areas were integrated automatically by computer using Agilent Software. Spectrums of selected compounds, *B. tripartita* ethanol extract and its sub-extracts were compared and their UV spectrums were superimposed. Presence of chlorogenic acid, cynaroside and luteolin were proved. Thus, quantitative analysis of these 3 compounds in *B. tripartita* ethanol extract, ethyl acetate sub-extract and *n*-butanol sub-extract were done.

Quantification and validation procedures

Ethanol extract of *B. tripartita* and its *n*-buthanol sub-extract (10 mg) were dissolved in 10 ml of water: methanol (1:1) mixture. Ethyl acetate sub-extract (10 mg) was also dissolved in 10 ml methanol. All solutions were filtered from 0.45 μm filters and directly injected. The stock solutios of the pure compounds were prepared by dissolving 10 mg of chlorogenic acid and luteolin in 10 ml methanol and 10 mg of cynaroside in 10 ml water:methanol (1:1) mixture. For calibration, six concentrations of the standards (0.01-0.5 mg/ml) were prepared by diluting stock solutions. Triplicate 10 µl injections were made for each concentration and the calibration equations were obtained by using peak areas of standard solutions.

Limit of detection (LOD) and limit of quantification (LOQ) were established at a signal to noise ratio (S/N) of 3 and 10, respectively. LOD and LOQ concentrations were experimentally verified by 9 injections of standard compounds. Precision tests were performed by the evaluation of intra-day variations of the same standard solutions of all compounds at the LOQ level. The area values were recorded and RSD% was calculated.

Statistical analysis

Instat-2 software was used to evaluate the data statistically. Values are presented as means±SEM Statistical differences between the treatments and the controls were tested by one-way analysis of variance (ANOVA) followed by the Student-Newman-Keuls test. A difference in the mean values of $P<0.05$ was considered to be statistically significant.

Results

In-vivo activity studies

Hypoglycaemic and antidiabetic effects of B. tripartita after acute administration

Effects of *B. tripartita* ethanol extract on normoglycaemic, glucose loaded and streptozotocin induced diabetic rats are shown in Table 1. Reference drug tolbutamide (100 mg/kg BW) was found to have a potent hypoglycaemic activity (26.1-55.3%) on normoglycaemic and glucose loaded rats. *B. tripartita* extracts reduced blood glucose levels significantly (11.5-25.8%) on normoglycaemic and glucose loaded rats compared to healty control group

Table 2. Sub-acute hypoglycaemic effect of *Bidens* tripartita extract on STZ-induced diabetic rats

Group	Dose	Mean blood glucose conc. (mg/dl) ± SEM (Inh. %)			Mean body weight±SEM (Change % to 1st day)		
	(mg/kg)	1st Day	4th Day	8th Day	1st Day	4th Day	8th Day
Control		335.4±19.53	391.5±17.44	328.7±12.63	180.2±11.64	193.0±11.35 (+7.1)	185.7±13.61 (+3.1)
Tolbutamide	100	300.8±17.68	336.5±15.64* (14.1)	266.3±8.73* (19.0)	194.2±10.09	200.8±9.50 (+3.4)	186.5±10.84 (-4.0)
B. tripartita	250	308.8±16.68	342.4±10.45* (12.5)	282.6±8.25* (14.0)	183.0±11.99	189.8±13.10 (+3.7)	196.2±13.91 (+7.2)
	500	307.6±16.97	252.3±18.44*** (35.6)	195.3±24.93*** (40.6)	169.6±17.76	176.2±30.20 (+3.9)	194.3±30.25 (+14.6)

*P<0.05, **P <0.01, *** P<0.001, SEM: standard error of the mean; STZ: streptozotocin

dose dependently. Additionally, antidiabetic activity of *B. tripartita* ethanol extract was found to be promising (14.0-32.7%) on streptozotocin induced diabetic rats. Antidiabetic effect of the extract at 500 mg/kg (24.3-32.7%) was significant and this effect was higher than the effect of the reference drug tolbutamide (0.0-20.0%) at all measurements.

Antidiabetic effect of B. tripartita after sub-acute administration

Ethanol extract of *B. tripartita* was given to STZ induced-diabetic rats for 8 days at two different doses and the effects on blood glucose levels and body weights were monitored (Table 2). Antidiabetic effect of the extract at 500 mg/kg dose (35.6%, 40.6%) was much higher than the reference drug (14.1%, 19.0%) at 4th and 8th day measurements. Body weights of the animals were increased in all groups (except tolbutamide on the 8th day) compared to the initial body weights.

Hypoglycaemic and antidiabetic effects of Bidens tripartita sub-extracts after acute administration

Chloroform, ethyl acetate, *n*-butanol, and remaining water sub-extracts of *B. tripartita* were obtained by successive solvent extraction of the ethanol extract. Doses of the extracts were calculated according to the percentage of their yields. The sub-extracts were administered at two different doses to rats to determine the hypoglycaemic and antidiabetic effects. None of the sub-extracts reduced blood glucose levels significantly in normoglycaemic animals (Table 3). In oral glucose tolerance test, reference drug tolbutamide lowered blood glucose levels significantly between 28.5-47.7%. Sub-extracts demonstrated significant and promising Hypoglycaemic effect in different ratios (10.3-27.9%). Among all tested extracts, only *n*-butanol sub-extract showed dose dependent effect on glucose loaded animals.

Table 3. Hypoglycaemic effect of *Bidens tripartita* sub-extracts on normoglycaemic and glucose loaded rats

Test Model	Group	Dose (mg/kg)	Mean blood glucose conc. (mg/dl) ± SEM (Inhibition %)				
			Initial	½ hr	1 hr	2 hr	4 hr
NG	Control		70.7±5.24	80.0±1.40	73.5±1.65	63.5±2.56	58.3±4.67
	Tolbutamide	100	68.0±1.81	55.2±4.74** (31.0)	45.2±3.54*** (38.5)	42.7±4.86*** (32.8)	53.0±7.49 (9.09)
	Chloroform SE	78	67.3±0.62	74.7±2.70 (6.6)	69.8±3.10 (5.0)	64.7±2.23	80.7±8.25
		156	73.5±3.77	76.5±2.81 (4.4)	72.3±1.28 (1.6)	69.33±3.06	76.2±3.28
	Ethyl acetate SE	102	71.7±3.11	76.2±2.99 (4.8)	72.5±2.51 (1.4)	68.8±3.67	70.2±2.30
		204	69.3±3.99	73.5±2.88 (8.1)	74.3±3.41	64.0±2.34	70.3±3.58
	n-Butanol SE	83	74.8±1.49	88.2±3.25	81.7±2.69	71.2±1.33	80.5±2.66
		166	66.5±1.98	76.3±2.54 (4.6)	70.0±1.39 (4.8)	63.8±3.28	71.5±5.40
	Remaining water SE	236	72.5±1.84	80.7±1.31	77.0±1.57	69.2±1.78	72.2±3.77
		473	71.3±1.84	87.8±2.48	83.7±2.20	71.2±2.14	77.0±3.37
OGTT	Control		85.4±5.15	93.2±4.14	158.8±2.68	134.3±3.49	105.1±5.09
	Tolbutamide	100	86.0±2.11	66.6±2.93*** (28.5)	83.1±3.23*** (47.7)	78.5±3.58*** (41.6)	59.8±4.77*** (43.1)
	Chloroform SE	78	85.0±1.98	81.7±3.53	142.5±9.96 (10.3)	119.7±2.98** (10.9)	88.3±4.28* (16.0)
		156	85.0±4.01	84.2±2.68	137.3±5.09* (13.5)	119.8±2.99** (10.9)	91.3±4.33* (13.1)
	Ethyl acetate SE	102	90.0±4.57	87.8±4.06	140.2±4.88 (11.7)	103.5±4.16*** (22.9)	82.5±5.25** (21.5)
		204	90.7±4.95	81.8±3.22	117.8±4.37** (25.8)	113.7±4.32** (15.4)	85.5±3.39** (18.6)
	n-Butanol SE	83	84.2±4.93	86.8±5.65	163.2±12.86	124.7±6.18 (7.1)	86.2±3.32** (18.0)
		166	83.2±2.68	85.5±2.91	114.5±5.50** (27.9)	118.7±2.99** (11.6)	84.3±2.72** (19.8)
	Remaining water SE	236	93.8±3.83	88.5±2.98	137.5±7.46* (13.4)	117.2±3.61** (12.7)	87.7±3.77* (16.6)
		473	85.2±2.02	86.3±3.27	154.2±8.45 (2.9)	119.8±2.44** (10.8)	83.2±1.85** (20.8)

NG: normoglycaemic, OGTT: oral glucose tolerance test, SE: sub-extract, * P<0.05, ** P<0.01, *** P<0.001, SEM: standard error of the mean

Table 4. Acute antidiabetic effects of *Bidens tripartita* sub-extracts on STZ-induced diabetic rats

Group	Dose (mg/kg)	Mean blood glucose conc. (mg/dl) ± SEM (Inhibition %)				
		Initial	½ hr	1 hr	2 hr	4 hr
Control		388.0±5.40	377.7±6.75	351.5±2.50	327.0±15.00	300.0±6.50
Tolbutamide	100	381.0±4.25	369.0±14.65 (2.3)	341.0±10.50 (2.9)	331.0±14.00	265.0±13.00* (11.7)
Chloroform SE	78	376.0±8.60	375.5±7.20 (0.58)	363.2±6.40	355.6±11.50	336.8±13.40
	156	391.0±13.90	328.8±13.60 (13.0)	312.6±12.90* (11.1)	294.6±16.70 (9.9)	235.4±11.21** (21.5)
Ethyl acetate SE	102	388.0±3.90	395.0±5.80	363.0±9.50	345.0±10.40	314.3±11.20
	204	371.8±15.59	337.8±16.00 (10.6)	338.6±15.00 (3.7)	272.4±18.00* (16.7)	212.6±9.07*** (29.1)
n-Butanol SE	83	380.0±10.50	412.0±6.80	374.0±6.50	395.0±7.80	268.0±7.60 (10.7)
	166	375.0±15.00	338.4±19.00 (10.4)	293.2±5.00** * (16.6)	255.2±9.40** (22.0)	158.2±5.89*** (47.3)
Remaining water SE	236	380.0±6.70	380.0±7.20	342.0±8.50 (2.7)	334.0±8.90	290.0±10.50 (3.3)
	473	378.0±14.00	415.5±15.70	354.0±18.00	325.5±12.00 (0.5)	273.5±9.50* (8.8)

SE: sub-extract, *P<0.05, **P<0.01, ***P<0.001, SEM: standard error of the mean; STZ: streptozotocin

Results of acute antidiabetic activities of *B. tripartita* sub-extracts are given in Table 4. Except remaining water sub-extract, high doses of all tested sub-extracts showed promising antidiabetic effect on STZ-induced diabetic rats (11.1-47.3%). *n*-butanol sub-extract at 166 mg/kg dose was the most active one, its effect was significant and continuous (16.6-47.3%). Ethyl acetate sub-extract at 204 mg/kg dose lowered blood glucose levels significantly at 2nd and 4th hour measuremets (16.7, 29.1%) and chloroform sub-extract showed a similar antidiabetic effect at 156 mg/kg (9.4-21.5%) after acute administration.

Antidiabetic effects of B. tripartita sub-extracts after sub-acute administration

Antidiabetic activity of high doses of the *B. tripartita* sub-extracts after sub-acute administration was investigated and the results are given in Table 5. According to the 4th and 8th days measurements, chloroform and remaining water sub-extracts were found to be inactive. On the other hand, antidiabetic effect of the ethyl acetate and *n*-butanol sub-extracts were higher than the other sub-extracts and reference drug tolbutamide on severe diabetic animals. Their antidiabetic properties were found to be similar.

Therefore, all other analysis were conducted on these sub-extracts. BW of the diabetic animals were also recorded during the sub-acute administration. However the changes in the body weights of the groups were not significant compared to control group due to short experiment time.

In-vitro activity studies

Inhibition of carbonhydrate digestive enzymes

α-Glucosidase and *α*-amylase inhibitory activities of *B. tripartita* ethanol extract and its sub-extracts were tested at three different concentrations (2, 1, and 0.5 mg/ml) (Table 6). *α*-Glucosidase inhibitory activity of ethanol extract of *B. tripartita* was found to be moderate (49.87-25.02%) at tested

Table 5. Sub-acute hypoglycaemic effect of *Bidens* tripartita sub-extracts on STZ-induced diabetic rats

Group	Dose (mg/kg)	Mean blood glucose conc. (mg/dl)±SEM (Inh. %)			Mean body weight ± SEM (Change % to 1st day)		
		1st Day	4th Day	8th Day	1st Day	4th Day	8th Day
Control		388.5±5.40	424.5±20.00	372.0±10.09	173.5±11.00	182.6±6.00 (+5.2)	190.3±4.20 (+9.5)
Tolbutamide	100	381.0±4.25	456.0±16.00	447.0±19.00	170.0±6.20	179.2±5.40 (+5.4)	186.0±4.40 (+9.4)
Chloroform SE	156	391.0±13.90	385.0±18.00 (9.3)	379.6±15.00	176.0±7.10	181.0±3.60 (+3.12)	185.0±5.20 (+5.1)
Ethyl acetate SE	204	371.8±15.59	396.0±17.50 (6.7)	320.2±17.00* (13.9)	173.0±2.40	176.1±3.40 (+1.8)	176.3±4.60 (+1.8)
n-Butanol SE	166	375.0±15.00	371.5±15.40* (12.5)	333.0±8.50* (10.5)	172.5±3.20	167.0±4.10 (-3.2)	168.1±3.80 (-3.2)
Remaining water SE	473	378.0±14.00	425.0±16.00	392.5±14.90	170.0±2.40	173.0±5.20 (+1.76)	169.2±4.40 (-0.5)

SE: sub-extract, * P<0.05, ** P<0.01, *** P<0.001, SEM: standard error of the mean; STZ: streptozotocin

Table 6. α-Glucosidase and α-amylase inhibitory activity of *Bidens tripartita* ethanol extract and its sub-extracts

Extract	α-glucosidase inhibitory activity (Inhibition % ± SD)			α-Amylase inhibitory activity (Inhibition % ± SD)		
	2 mg/ml	1 mg/ml	0.5 mg/ml	2 mg/ml	1 mg/ml	0.5 mg/ml
B. tripartita EtOH Ext.	49.87 ± 1.16	33.04 ± 1.12	25.02 ± 1.64	-	-	-
Chloroform SE.	35.77 ± 4.50	23.88 ± 3.34	21.05 ± 2.30	6.23 ± 2.88	-	-
Ethyl acetate SE	64.56 ± 0.75	45.24 ± 1.17	27.38 ± 1.76	22.12 ± 3.19	-	-
n-Butanol SE	11.51 ± 1.74	-	-	5.31 ± 0.40	-	-
Remaining water SE.	7.55 ± 1.30	7.48 ± 4.24	7.11 ± 1.40	-	-	-
Reference	1 µg/ml	0.5 µg/ml	0.25 µg/ml	1 mg/ml	0.3 mg/ml	0.1 mg/ml
Acarbose	71.11 ± 2.08	59.76 ± 1.12	47.99 ± 1.76	47.68 ± 2.23	23.75 ± 3.08	5.34 ± 4.67

SE: Sub-Extract

concentrations compared to the reference drug Acarbose (71.11-47.99 at 1-0.25 µg/ml). All sub-extracts showed inhibitory activity on α-glucosidase enzyme at 2 mg/ml. Among these, ethyl acetate sub-extract showed the highest activity (65.56-27.38%). *B. tripartita* ethanol extract was inactive on α-amylase enzyme and its sub-extracts (chloroform, ethyl acetate, and *n*-butanol) have a peddling inhibitory effect only at 2 mg/ml concentration. Reference drug Acarbose showed a dose dependent inhibitory activity against α-amylase enzyme at tested concentrations (47.68% at 1 mg/ml, 23.75 at 0.3 mg/ml and 5.34 at 0.1 mg/ml respectively).

Antioxidant activities

Total antioxidant capacities of extract and sub-extracts were determined by phosphomolibdenum assay. Antioxidant capacity of the ethanol extract was promising (352.51) and close to antioxidant capacity of the reference compound Trolox (382.5). The highest total antioxidant capacity was found in chloroform sub-extract (1532.45). On the contrary, no activity was observed by the same sub-extract on DPPH radical scavenging activity test. All other tested extract and sub-extracts were active in different percentages (62.86-19.35%) at tested concentrations (2-0.5 mg/ml). Ethyl acetate extract was the most active sub-extract (62.86-59.60%) among all and its effect was similar to reference compound ascorbic acid (70.88-65.62%) in DPPH radical scavenging assay.

Phytochemical analysis

Total phenol and flavonoid contents of the ethanol extract and its sub-extracts were measured (Table 7). Total phenol content of the ethanol extract was found to be 120.99 mg gallic acid equivalent in 1 g extract. Total flavonoid content was determined as 127.47 mg quercetin equivalent in 1 g extract. Both total phenol and flavonoid contents were determined very high in ethyl acetate extract (280.93 mg GAE/g extract and 297.62 mg QE/g extract respectively). Total flavonoid content of chloroform sub-extracts (213.79 mg QE/g extract) was higher than the flavonoid content of ethanol extract. Also phenol and flavonoid contents of *n*-butanol sub-extract were found close to the results of ethanol extract as expected.

After determination of phenol and flavonoid contents, active extract and sub-extracts (ethyl acetate and *n*-butanol) were examined for their chemical profile. Among all investigated flavonoids and phenolics; chlorogenic acid, luteolin-7-O-glycoside (cynaroside) and luteolin were found to be the major compounds according to LC-MS and UV analysis. Quantities of these three compounds were measured by using HPLC in ethanol extract of *B. tripartita* and its active sub-extracts. Amount and retention time of the compounds, wavelengths, calibration and validation data are given in Table 8 and 9. These three compounds were found to be in the highest level in ethyl acetate sub-extract (chlorogenic acid; 10.63%, cynaroside 10.87% and luteolin 7.58%). On the other hand, a small amount of chlorogenic acid (4.51%) and cynaroside (0.33%) were determined in the *n*-butanol sub-extract. It was interesting that luteolin is not detected in *n*-butanol sub-extract.

Table 7. Total phenol, total flavonoid contents and antioxidant activities of *Bidens tripartita* ethanol extract and its sub-extracts

Extract	Total phenol ontent (mg GAE/g ± SD)	Total flavonoid content (mg QE/g ± SD)	Total antioxidant capacity (AAE ± SD)	DPPH radical scavenging activity (Inhibition % ± SD)		
				2 mg/ml	1 mg/ml	0.5 mg/ml
B. tripartita EtOH Ext.	120.99 ± 6.02	127.47 ± 4.70	352.51 ± 67.59	28.65 ± .18	25.57 ± 1.38	21.35 ± 3.00
Chloroform sub-ext.	58.04 ± 3.93	213.79 ± 8.27	1532.45 ± 39.02	-	-	-
Ethyl acetate sub-ext.	280.93 ± 11.05	297.62 ± 20.84	136.18 ± 30.70	62.86 ± .80	59.91 ± 0.46	59.60 ± 1.16
n-Butanol sub-ext.	104.34 ± 5.95	95.12 ± 4.01	91.94 ± 22.53	27.96 ± .75	25.19 ± 5.63	19.35 ± 0.80
Remaining water sub-ext.	26.44 ± 0.94	25.88 ± 0.63	23.11 ± 17.03	46.70 ± .62	40.40 ± 3.46	30.58 ± 14.31
References			3 mg/ml	2 mg/ml	1 mg/ml	0.5 mg/ml
Ascorbic acid	NT	NT		70.88 ± .47	68.27 ± 1.36	65.62 ± 4.67
Trolox	NT	NT	382.50 ± 17.03	NT	NT	NT

Total flavonoid contents of the extracts are expressed as mg quercetin equivalent (QE)/g extract and total phenol contents are expressed as mg gallic acid equivalent (GAE)/g extract.

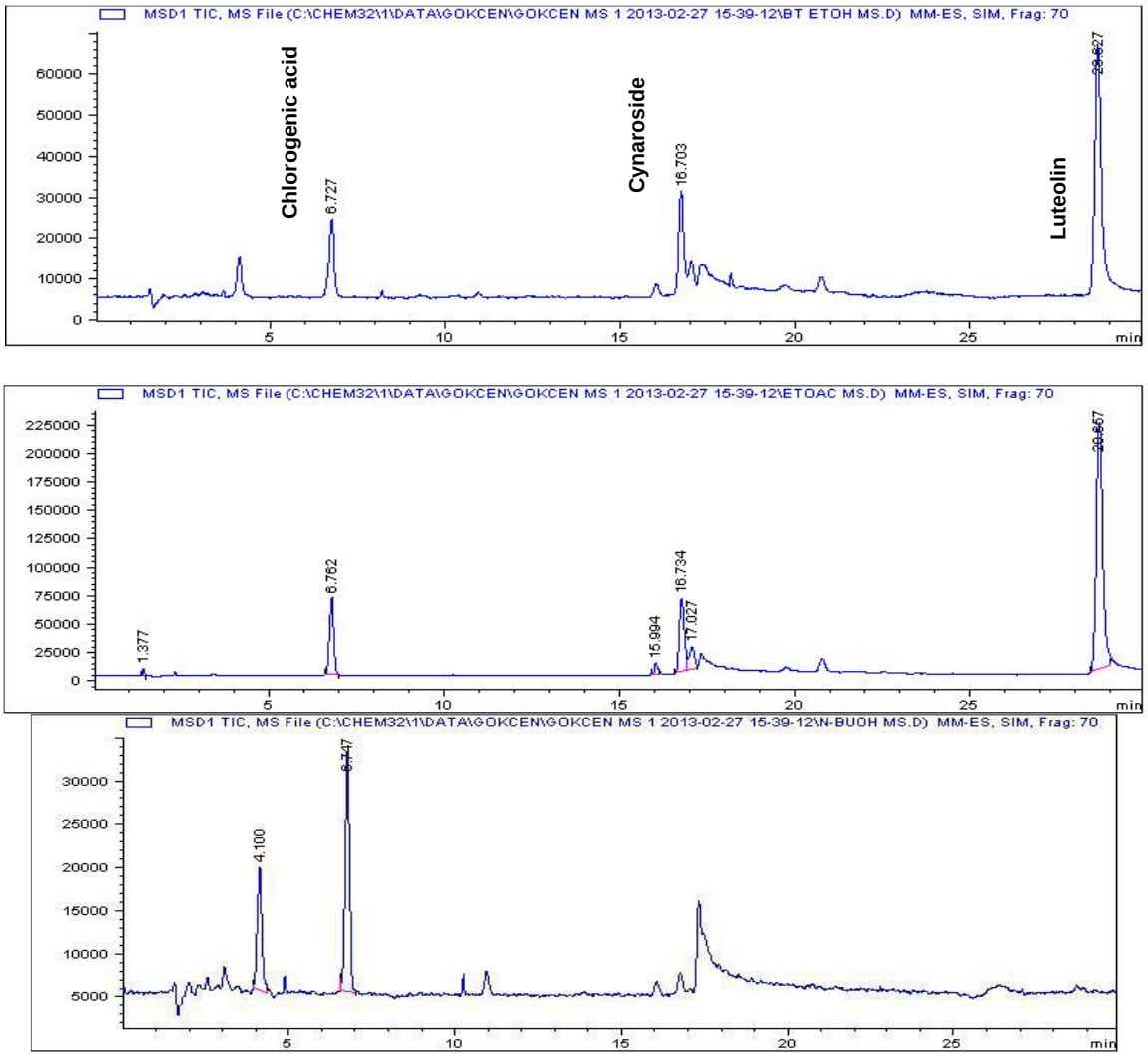

Figure 1. LC-MS chromatograms of *Bidens tripartita* extract, ethyl acetate and *n*-butanol subextracts

Table 8. Chlorogenic acid, cynaroside and luteolin contents of *Bidens tripartita* plant, its ethanol extract and its active sub-extracts

Extract	Chlorogenic acid	Cynaroside	Luteolin
B. tripartita	0.8066	0.6363	0.3267
B. tripartita EtOH ext.	5.160	4.07	2.09
Ethyl acetate sub-ext.	10.63	10.87	7.58
n-Butanol sub-ext.	4.51	0.33	-

Results are given as w/w %

Discussion

For centuries, plants are used to cure many ailments and several species of medicinal plants are used in the treatment of diabetes mellitus. Among them, *Bidens* species have attracted the attention of many scientists for a long time. Many studies have been conducted on the antidiabetic activity and antidiabetic constituents of *Bidens pilosa* extracts so far. According to the results of these elaborate animal experiments, it has been proved that extracts prepared from leaves and aerial parts of *B. pilosa* have antidiabetic effect. These studies have also revealed that *B. pilosa* extracts stimulate insulin secretion from pancreatic islets and increase insulin levels, decrease HbA1c and blood glucose levels and improve insulin sensitivity. Additionally, they can prevent autoimmune diabetes by modulating the differentiation of helper T cells. (24-28).

In this study, we aimed to evaluate the antidiabetic activity potential of *B. tripartita* ethanol extract. Results of our experiments revealed that the extract has potent hypoglycaemic effect on normoglycaemic and glucose loaded rats and also promising antidiabetic effect on streptozotocin induced diabetic rats by acute and sub-acute administration. Sub-extracts lowered blood glucose levels in oral glucose tolerance test remarkably. Additionally, ethyl acetate and *n*-butanol sub-extracts have shown outstanding antidiabetic activity after acute and sub-acute administration to diabetic animals.

Table 9. Linear regression data, precision of the method at the LOQ level (n=9) and other method validation data

Parameters of analysis and validation	Chlorogenic acid	Cynaroside	Luteolin
Wavelength (nm)	210	210	210
Retention Time (min)	6.770	16.777	29.586
Calibration Curve, r^2	y=6892.2x-31.614, r^2=0.9991	y=20236.62x-15.2951 r^2=0.9999	y=28020x-46.072, r^2=0.9998
Slope RSD %	0.358	0.057	0.218
Intersection RSD %	2.822	1.249	6.177
Peak Area (Mean), RSD %	35.02857, 3.421	40.69286, 0.585	39.73714, 0.850
LOD (µg/ml), RSD %	1.0386, 3.047	0.924, 3.003	1.024, 1.858
LOQ (µg/ml), RSD%	3.1158, 3.421	2.7725, 0.585	3.072, 0.849

RSD % = (SD / Mean) X 100, SD: Standart Deviation

In enzyme inhibition assays, ethyl acetate was the most effective sub-extract on *α*-glucosidase and *α*-amylase enzymes. Also its antioxidant activity was found to be remarkable by phospomolibdenum and DPPH radical scavenging assays. It is estimated that high phenol and flavonoid contents may be responsible for the antioxidant activity of this sub-extract. According to LC-MS and HPLC analysis, major compounds in the ethyl acetate and *n*-butanol sub-extracts were determined as cynaroside and chlorogenic acid, also luteolin was detected in the ethyl acetate sub-extract (Figure 1).

It is well-known that the genus *Bidens* is rich in flavonoid and polyacetylene derivatives. Thus, these compounds are commonly considered as chemotaxonomic markers for the species in this genus. Lv and Zhang have studied the chemical constituents of *B. tripartita* and they have isolated 14 flavonoids and 2 polyacetylenes from the aerial parts of the samples collected from China (29). Additionally, phytochemical studies on *B. tripartita* herb have shown the presence of flavones, flavanones, chalcones, aurones, coumarins, carotenoids and volatile compounds (30).

In a recent study (30), luteolin, cynaroside and flavanomarein have been detected and quantified in *B. tripartita* herb and flower samples collected from Poland. The amount of flavanomarein, cynaroside and luteolin is 0.229, 0.116 and 0.047 (in % of dry weight) respectively in flower extract and 0.157, 0.179 and 0.031 (in % of dry weight) respectively in herb extract. Cynaroside and luteolin content of Turkish *B. tripartita* samples (Table 8) have been found higher than Poland samples. Cynaroside amount in the ethanol extract is 4.07% and luteolin amount is 2.09% in our study. Moreover, DPPH radical scavenging activity of ethyl acetate extracts of *B. tripartita* flower and herbs have been investigated in the same study. Ethyl acetate extracts have exhibited a significant radical scavenging activity (flower extract: 98%, herb extract: 89%). These results are also compatible with our antioxidant activity results.

In recent years, many *in-vivo* and *in-vitro* studies have been conducted on the antidiabetic activities of phenolic acids and flavonoids. Chlorogenic acid is a major polyphenolic compound found in many plants and is an ester of caffeic acid and quinic acid. It has been isolated as one of the main active compounds in *Cecropia obtusifolia.* Its antidiabetic effect has been investigated on STZ induced diabetic rats at 10 and 15 mg/kg doses. Blood glucose levels of diabetic animals in chlorogenic acid administered groups have been decreased significantly compared to diabetic control (31). In *in-vitro* studies, *α*-glucosidase and *α*-amylase inhibitory activity of chlorogenic acid has been found to be significant and dose dependent (32, 33).

On the other hand, luteolin has shown a high inhibitory activity against yeast *α*-glucosidase (92% at 200µM) and against porcine pancreatic *α*-amylase (61% at 500 µM) (34). It has also inhibited rabbit muscle glycogen phosphorylase (IC_{50}=31.7 µM), aldose reductase enzymes (IC_{50}=5.1 µM) and inhibited sorbitol accumulation on erythrocytes (79.2% at 200 µM) (35). In addition, luteolin and cynaroside have been found to have strong inhibitory activity on yeast *α*-glucosidase and porcine pancreatic *α*-amylase (36). Rauter *et al* have evaluated antihyperglycaemic and protective effects of many flavonoids on STZ induced diabetic rats (37). Blood glucose contentrations of diabetic animals after 7 day administrations and on glucose tolerance test have been significantly reduced by cynaroside (4 mg/kg BW).

Conclusion

In the light of literature survey and according to our results, it can be concluded that *Bidens tripartita* extracts containing chlorogenic acid, cynaroside and luteolin have antidiabetic acitivity and its active components reveal this activity via many pathways. Further studies are necessary to obtain toxicological data and to observe long-term effects of the extracts and their active components.

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