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Hypoglycaemic Effect of Seed and Fruit Extracts of Laurel Cherry in Different Experimental Models and Chemical Characterization of the Seed Extract

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Abstract: Laurel cherry (*Prunus laurocerasus* L.), an evergreen tree from Rosaceae family, is used as a folk medicine for various medical purposes and against diabetes as well in Northern Anatolia. The present study was performed to evaluate *in-vivo* hypoglycaemic and antidiabetic activities of *P. laurocerasus*. Hypoglycaemic effect of ethanol extracts of the fruit and seeds were tested on normoglycaemic, glucose loaded and streptozotocin induced diabetic rats. The extracts were given orally to rats and blood glucose levels were determined according to the glucose oxidase method. The results indicated that seed extract showed 20% inhibition on blood glucose levels on experimental diabetic animals. Chemical composition of the bioactive seed extract was analyzed by GC-MS. Oleic, linoleic and palmitic acids were detected as the major components of this extract. Therefore, hypoglycaemic activity of the seeds may be arisen from unsaturated fatty acids.

Keywords: Antidiabetic; fatty acids; *Prunus laurocerasus*; Rosaceae; diabetes.

1. Introduction

Prunus laurocerasus L. (synonym: *Cerasus laurocerasus, Laurocerasus officinalis*) known as "laurel cherry" is a plant of the family Rosaceae. It is native to regions bordering the Black Sea in Southwestern Asia and Southeastern Europe, from Albania and Bulgaria east through Turkey to the Caucasus Mountains and Northern Iran. The fruit of the plant is a small cherry 1-2 cm broad, turning black when ripe in early autumn. The seeds contained within the berries are poisonous like the rest of the plant, containing cyanogenic glycosides and amygdalin [1,2].

In Turkey, the fruits of the plant, named as "taflan, karayemis, lazkirazi" are mostly consumed as freshly, dried and also in the form of jam, pulp, marmalade and drinks (fruit juice, tea) in canned or pickled styles [3-5].

Fruits and leaves of the plant have been used as folk medicine from ancient times. Dioscorides mentioned that leaves of the plant helps headaches and burning of the stomach in Materia Medica. Moreover, the juice expels the menstrual flow and urine applied both orally and vaginally [6]. Nowadays, the fruits, leaves and seeds of cherry laurel and its cultivated forms are well-known folk medicinal plants in Turkey and used in the treatment of stomach ulcers, digestive system illnesses,

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bronchitis (seeds), eczemas, haemorrhoids. It is used externally for antipruritic and analgesic effect on local pains [2]. In northern side of Anatolia, the fruits and seeds of the plant are used against diabetes mellitus and its complications widely. Seeds, separated from the fruits after drying in the oven, are grinded and 1-2 dessert spoon of powder are consumed against diabetes in the mornings on an empty stomach [3].

Many studies have been conducted on different parts of the plant to evaluate their biological activities. It has been shown that various parts of the plant possessed antioxidant [7,8], antiinflamatory [9], analgesic [10] and neuroprotective activities [11]. However, any study has not been performed on the antidiabetic activity of the plant so far. Hence, the aim of this study is to evaluate the hypoglycaemic effect of the fruit and the seed extracts of the plant comparatively and to verify the folkloric claim.

2. Materials and Methods

2.1. Plant Material

Fruits of the *Prunus laurocerasus* L were collected from Dogancay Village-Trabzon (Turkey) in August 2009. The plant was identified by M. Aslan and a voucher specimen was deposited in the herbarium of Gazi University Faculty of Pharmacy (GUE 2441).

2.2. Preparation of the Extracts

Fruits were dried in a drying oven until dryness and seeds were pitted. Non-pitted fruits, pitted fruits and seeds were powdered separately and their extracts were prepared. The materials (100 g) were extracted with 1 L ethanol (80%) for 24 hours in a mixer at 37^{o}C. The extracts were filtrated and evaporated under reduced pressure until dryness. Yields of the extracts were calculated for non-pitted fruits: 49.5 %, for pitted fruits: 78.4%, and for seeds: 19.7%.

2.3. Preparation of the Test Samples

The doses of the test samples were calculated according to the yields of the extracts prepared as the traditional usage. A weighted portion of the each extract was suspended in 0.5% aqueous carboxymethylcellulose (CMC) solution in distilled water prior to oral administration to animals (10 mL/kg, b.w.) [b.w.: body weight]. Animals in the control group received only the vehicle (10 mL/kg, b.w.). Tolbutamide (Sigma T0891) (100 mg/kg, b.w.) was used as the reference drug. The test samples (extracts and tolbutamide) were administered orally by using a gastric gavage needle.

2.4. Animals

Male Wistar-albino rats weighing 150–200 g, purchased from the GUDAM (Gazi University Experimental Animal Research Center) Ankara-Turkey, were used in the present study. All rats were kept at room temperature. Throughout the animal experiments were processed following the internationally accepted ethical guidelines for the care of laboratory animals. Prior to the experiments, rats were fed with standard food for 1 week in order to adapt to the laboratory conditions. Sixteen hours before the experiments, they were fasted overnight, but allowed free access to water. Seventy-two rats, included for the study, were divided into 8 groups, each consisting of 6 animals. The body weight and fasting blood glucose levels of all the rats were determined before the start of the experiment. Rats were divided into the following groups:

- Group 1: Control, received only vehicle (0.5% CMC) (5 mL/kg)
- Group 2: Reference, tolbutamide was given at a dose of 100 mg/kg
- Group 3: Extract of non-pitted fruits was given at a dose of 1000 mg/kg
- Group 4: Extract of non-pitted fruits was given at a dose of 2000 mg/kg
- Group 5: Extract of pitted fruits was given at a dose of 1000 mg/kg
- Group 6: Extract of pitted fruits was given at a dose of 2000 mg/kg
- Group 7: Extract of seed was given at a dose of 500 mg/kg

• Group 8: Extract of seed was given at a dose of 1000 mg/kg

2.5. Determination of the Blood Glucose Levels

Blood glucose concentration (mg/dL) was determined using an Ascensia-Elite commercial test (Bayer), based on the glucose oxidase method. Blood samples were collected from the tip of tail at the defined time patterns.

2.6. Effect in Normoglycaemic Rats

Fasting blood sugar level of each rat was determined at the beginning of the experiment, after overnight fasting with free access to water. Blood samples were collected at 30, 60, 120 and 240 min. after the oral administration of test samples.

2.7. Effect in Glucose-Hyperglycaemic Rats (Oral Glucose Tolerance Test, OGTT)

Fasting blood sugar level of each rat was determined at zero time, after overnight fasting with free access to water. Glucose (2 g/kg b.w.) was orally administered 30 min. after the oral administration of the test sample or vehicle (for control). Blood glucose concentrations were measured just before and 30, 60, 120 and 240 min. after the oral administration of the test samples.

2.8. Effect in Diabetic rats (Streptozotocin-Induced Diabetes Model)

Diabetes was induced in rats by the intraperitoneal (i.p.) injection of streptozotocin (STZ) (Sigma S0130), at a dose of 55 mg/kg b.w. dissolved in distilled water (1 mL/kg b.w.). Seven days after the injection, the blood glucose levels were measured. Each animal with a blood glucose concentration level above 250 mg/dL was considered to be diabetic and used in the experiments. Test samples were given orally to the fasted diabetic rats. The blood glucose concentrations of the animals were measured at the beginning of the study and the measurements were repeated 30, 60, 120 and 240 min. after the initial of the experiment.

2.9. Gas Chromatography–Mass Spectrometry (GC-MS) Analysis

2.9.1. Preparation of Sample for GC-MS Analysis:

In order to obtain volatile derivatives, methylation was generated by a modified method described in the European Pharmacopoeia 6.8 part 2.4.22 (Method C). 0.5 g of extract was dissolved in solution of NaOH (20 g/L) in methanol in a 100 mL conical flask and boiled under a reflux condenser for 30 min. 10 mL of borontrifluoride-methanol solution was added to flask then boiled for 30 min. After adding 20 mL of hexane through the condenser, it was boiled for 5 min. Mixture was cooled, 50 mL of saturated NaCl solution was added and shaked for 15 s. A quantity of saturated NaCl solution was added to obtain upper phase in the neck of the flask. 10 mL of upper phase was collected and washed with distillated water three times and dried by anhydrous sodium sulphate.

2.9.2. Gas Chromatography–Mass Spectrometry (GC-MS) Conditions:

The GC/MS analysis was carried out with an Agilent 5975 GC-MSD system (Agilent, USA; SEM Ltd., Istanbul, Turkey). HP-Innowax FSC column (60m × 0.25mm, 0.25µm film thickness, Agilent, USA) was used with a helium carrier gas at 0.8 mL/min. GC oven temperature was kept at 60°C for 10 min. and programmed to 220°C at a rate of 4°C/min., kept constant for 10 min at 220ºC, and then programmed to increase at a rate of 1ºC/min. to 240ºC. The oil was analyzed with a split ratio of 40:1. The injector temperature was 250ºC. Mass spectra were taken at 70 eV and the mass range was from *m*/*z* 35 to 450.

The GC-FID analysis was carried out with capillary GC using an Agilent 6890N GC system (SEM Ltd., Istanbul, Turkey). Flame ionization detector (FID) temperature was set at 300°C in order to obtain the same elution order with GC-MS. Simultaneous injection was performed using the same column and appropriate operational conditions.

2.9.3. Identification and Quantification of Compounds:

Compounds were identified by comparison of the chromatographic peaks retention times with those of authentic compounds analyzed under the same conditions, and by comparison of the retention indices with literature data. Comparisons of MS fragmentation patterns with those of standards and mass spectrum database search were performed using the Wiley GC/MS Library (Wiley, New York, NY, USA), MassFinder software 3.0 (Dr. Hochmuth Scientific Consulting, Hamburg, Adams Library, and NIST Library). Confirmation was also achieved using the in-house "Başer Library of Essential Oil Constituents" database, obtained from chromatographic runs of pure compounds performed with the same equipment and conditions. A C9–C30 *n*-alkane standard solution (Fluka, Buchs, Switzerland) was used to spike the samples for the determination of chromatographic retention indices (RRI). Percent composition was obtained for each constituent on the basis of GC-FID analysis of the oil.

2.10. Statistical Analysis

Values are presented as means ± Standard Error of The Mean (S.E.M.). Statistical differences between the treatments and the controls were tested by one-way analysis of variance (ANOVA) followed by the Student–Newman–Keuls test using the "Instat" statistic computer program. A difference in the mean values of $p<0.05$ was considered to be statistically significant (* $p<0.05$; ** $p<0.01$; *** $p<0.001$).

3. Results and Discussion

As shown in Table 1, tolbutamide, reference drug, possessed potent hypoglycemic activity (31.0-54.3% inhibition) on blood glucose levels of normoglycaemic rats. The seed extract reduced blood glucose levels (14.9-21.5% inhibition) at 1000 mg/kg dose significantly compared to the control group. However, pitted fruit extract showed inhibitory effect on blood glucose level (17.2%) only at 60th min. Rest of the extracts were found ineffective at the doses administered.

Table 1. Effect of *P. laurocerasus* extracts on blood glucose levels of normoglycaemic rats.

Groups	Dose mg/kg	Blood Glucose Concentration (mg/dL) ± S.E.M (Inhibition %)				
		Initial	30th min.	60th min.	120th min.	240th min.
Control	-	90.6±5.8	90.6±4.0	95.6±4.3	82.8±3.3	77.6±4.5
Tolbutamide	100	92.3±5.0	62.5±2.6*** (31.0 %)	44.1±2.9*** (53.8 %)	37.8±3.5*** (54.3 %)	50.4±6.8** (35.0 %)
Non-Pitted Fruit Extract	1000	91.0±4.3	98.5±6.8	84.5±2.4 (11.6 %)	81.6±3.7 (1.4 %)	72.3±5.5 (6.8 %)
	2000	90.6±6.7	113.0±5.1	106.5±7.7	108.5±4.3	95.6±3.1
Pitted Fruit Extract	1000	89.0±4.8	81.9±5.5 (9.6%)	79.2±4.1** (17.2 %)	78.5±4.4 (4.9 %)	68.8±2.0 (11.3 %)
	2000	88.0±6.7	96.5±7.4	93.0±5.8 (2.7 %)	82.5±6.7	76.5±6.1 (1.4 %)
Seed Extract	500	87.8±5.2	82.5±5.0 (8.9 %)	85.2±3.0 (10.9 %)	88.6±3.0	85.0±4.7 (9.5 %)
	1000	90.0±7.8	78.7±3.0 (13.1 %)	75.0±3.0** (21.5 %)	70.5±3.0* (14.9 %)	72.9±3.2 (6.1 %)

n (number of animals in each group)=6, * $p<0.05$, ** $p<0.01$, *** $p<0.001$ results compared to control group

Results given in Table 2 show that *P. laurocerasus* fruit extracts showed a weak inhibitory effect (12.6-14.1 %) at the tested doses, while tolbutamide had potent hypoglycemic activity (32.0-49.0 %) on blood glucose levels of glucose-loaded rats. However, the seed extract showed a remarkable lowering effect (11.8-23.3 %) on blood glucose level of glucose-loaded hyperglycaemic rats.

Table 2. Effect of *P. laurocerasus* extracts on blood glucose levels of glucose-loaded rats (OGTT).

Groups	Dose mg/kg	Blood Glucose Concentration (mg/dL) ± S.E.M (Inhibition %)				
		Initial		30th min.	120th min.	240th min.
Control	-	101.8±4.6	105.8±5.0	146.2±5.5	110.7±5.2	93.8±4.9
Tolbutamide	100	101.8±3.3	62.5±4.7*** (40.9 %)	91.3±6.4*** (37.5 %)	75.2±5.6*** (32.0 %)	47.6±3.4*** (49.0 %)
Non-Pitted Fruit Extract	1000	101.8±7.0	126.4±7.8	143.4±14.5	126.0±10.2	100.8±5.2
	2000	100.0±2.9	122.5±7.5	125.6±5.6* (14.1 %)	114.5±7.3	113.8±4.0
Pitted Fruit Extract	1000	100.8±6.1	118.9±6.3	127.8±6.8* (12.6 %)	103.5±4.6 (6.5 %)	98.0±4.8
	2000	101.0±4.0	128.5±3.3	138.6±4.8 (5.19 %)	120.3±4.9	101.8±4.9
Seed Extract	500	102.6±4.2	104.1±6.7	112.0±2.6** (23.3 %)	97.0±4.3* (11.8 %)	89.8±4.0 (4.3 %)
	1000	99.6±7.2	109.7±5.9	117.7±8.6** (19.5 %)	110.4±6.1	105.8±4.8

n (number of animals in each group)=6, * p<0.05, ** p<0.01, *** p<0.001 results compared to control group

Acute antidiabetic effect of the extracts of *P. laurocerasus* in streptozotocin-induced diabetic rats is presented in Table 3. The seed extract showed an outstanding effect on the measurement of 120th and 240th minutes at the dose of 500 and 1000 mg/kg. This effect was more potent than the effect of reference drug tolbutamide. Tolbutamide possessed a moderate effect on 240th min. measurement. Extracts prepared from pitted and non-pitted fruits were found ineffective in experimental diabetic rats.

As mentioned above, many different pharmacological activities of laurel cherry have been examined by researchers [7-9,12]. In a previous study, *P. laurocerasus* leaf and fruit extracts have found to be neuroprotective and antioxidant [8,11]. This is the first study on the hypoglycaemic properties of fruits and seeds of laurel cherry based on folkloric usage.

Previous phytochemical studies have revealed that seeds of plant are rich in saturated and polyunsaturated fatty acids such as palmitic, stearic, oleic and linoleic acid [12]. Additionally, phytosterols occur in the seeds of cherry laurel [13]. Ripe fruits contain carbohydrates (xylose, arabinose, fructose, glucose, sorbitol, saccharose), phenolic acids (vanillic acid, caffeic acid, protocathecuic acid, *p*-coumaric acid, *p*-hydroxybenzoic acid, ferulic acid) and anthocyanins [14,15].

In present study, GC-MS was used to determine phytochemical composition of ethanol extract of seeds that showed potent antidiabetic activity. Methyl oleate (41.1%), methyl linoleate (33.0%) and methyl palmitate (15.2%) were found to be major compounds in the methylated extract.

According to the literature surveys, many studies have been found evaluating the effect of lipids; free, unsaturated, monounsaturated and polyunsaturated fatty acids on progression of diabetes, blood glucose concentrations, insulin levels and antioxidant parameters in tissues of diabetics [16-18].

Vassiliou et al. have demonstrated that a high oleic acid diet has decreased blood glucose level of Type II diabetic mice compared to a high fat diet with no oleic acid [18]. Oleic acid rich diet was also found to be beneficial for diabetic patients clinically by Madigan et al. [16].

It has been also reported that intracerebroventricular administration of oleic acid which is a long chain fatty acid markedly inhibits glucose production and food intake in the literature [17]. The anorectic effect of oleic acid is independent of leptin and accompany by decrease in the hypothalamic expression of neuropeptide Y. Furthermore, oleic acid provides a signal of “nutrition abundance” to discrete areas within the central nervous system. This signal in turn activates a chain of neuronal events apparently designed to promote switch in fuel sources from carbohydrates to lipids and to limit the further entry of exogenous and endogenous nutrients in the circulation. This restrain may be required for the maintenance of energy and metabolic homeostasis, and its failure could contribute to

weight gain and glucose intolerance. The unveiling of the nutrient-activated neuronal circuitry may lead to innovative approaches to the treatment of obesity and Type II diabetes.

Table 3. Effect of *P. laurocerasus* extracts on blood glucose levels of streptozotocin-induced diabetic rats.

Groups	Dose mg/kg	Blood Glucose Concentration (mg/dL) ± S.E.M (Inhibition %)				
		Initial	30th min.	60th min.	120th min.	240th min.
Control	-	546.7±18.9	544.3±19.7	524.9±24.7	528.9±11.9	535.9±15.1
Tolbutamide	100	546.6±39.9	565.6±32.5	588.7±29.1	495.0±53.0 (6.4 %)	425.2±17.9*** (20.7 %)
Non-Pitted Fruit Extract	1000	532.7±21.5	572.8±9.6	541.7±22.9	564.6±14.6	536.1±20.7
	2000	546.0±31.8	576.8±26.4	566.7±30.7	539.7±28.6	505.3±38.2 (5.7 %)
Pitted Fruit Extract	1000	511.67±25.3	578.8±12.9	566.77±12.5	544.4±21.0	507.9±23.4 (5.2 %)
	2000	546.9±21.4	588.9±3.8	563.9±34.6	540.1±30.5	533.3±36.7
Seed Extract	500	534.5±42.1	561.7±23.4	512.7±22.3 (2.3 %)	481.9±21.0 (8.8 %)	419.7±10.8*** (21.7 %)
	1000	540.0±12.3	519.5±25.5 (4.6 %)	500.3±17.3 (4.7 %)	454.2±14.4* (14.0 %)	417.0±12.4*** (22.2 %)

n (number of animals in each group)=6, * p<0.05, *** p<0.001 results compared to control group

Table 4. Results of gas chromatography analysis of the methylated seed ethanol extract *of P. laurocerasus.*

No	RRI	Compound	%
1	1000	Decane	0.1
2	1100	Undecane	0.2
3	1203	Limonene	0.2
4	1641	Methyl benzoate	0.2
5	2020	Methyl myristate	0.2
6	2226	Methyl palmitate	15.2
7	2237	Methyl madelate	0.2
8	2251	(*Z*)-7-Hexadecanoic acid methyl ester	0.3
9	2260	Methyl palmitoleate	1.5
10	2331	Heptadecanoic acid methyl ester (=*Methyl margarate*)	0.1
11	2431	Methyl stearate	3.2
12	2456	Methyl oleate	41.1
13	2467	Methyl elaidate	2.2
14	2509	Methyl linoleate	33.0
15	2583	Methyl linolenate	0.5
16	2624	Methyl arachidate	0.7
17	2644	11-Eicosenoic acid methyl ester	0.2
		TOTAL	**99.1**

RRI: Relative retention indices calculated against n-alkanes

4. Conclusion

As mentioned above, methyl oleate (41.1%) was found to be the major compound in the methylated seed extract. Investigations revealed that oleic acid and diet rich in oleic acid decreased blood glucose levels, inhibited glucose production and food intake in diabetics. In the light of these findings, it might be hypothesized that oleic acid is the main compound responsible for the antidiabetic activity of *P. laurocerasus* seed extract rich in fatty acids [16-18].

Additionally, the seeds of the plant are eaten as folk remedy in the north side of Anatolia to lower blood glucose levels for at least two weeks. Thus, further studies are needed to determine the long term antidiabetic effects and toxicities of the seed extract and oleic acid.

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