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Jake OO (2002).Pharmaceutical Interactions between Striga hermonthica (Del.) Benth. and fluorescent rhizosphere bacteria Of Zea mays, L. and Sorghum bicolor L. Moench for Striga suicidal germination In Vigna unguiculata . PhD dissertation, Tehran University, Iran.

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African Journal of Pharmacy and Pharmacology

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ARTICLES

Research Articles

Full Length Research Paper

Evaluation of antidiabetic and antioxidant activity of aerial parts of *Hyptis suaveolens* Poit.

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Hyptis suaveolens Poit. (Lamiaceae) commonly called curry leaf and savanna plant which is abundantly found in farmlands. Traditionally, the plant is used in the treatment of diabetes mellitus, fever, eczema, cancers and headache. The present study was aimed to investigate the *in vitro* antioxidant and anti-diabetic potential of aerial parts of *H. suaveolens* Poit at dose levels of 250 and 500 mg/kg, in acute normoglycemic and alloxan induced hyperglycemic rats including oral glucose tolerance and chronic study (11 days), keeping glibenclamide (5 mg/kg) as standard drug. The body weight measurement and selective serum biochemical estimation (blood glucose concentration, ALT, AST, ALP, urea, creatinine, triglyceride, and cholesterol) were undertaken in 11 days treated hyperglycemic rats. The test result revealed that in normoglycemic rats, the decrease in blood glucose level lies between 4 to 15% in extract treated groups, while the hyperglycaemic rats showed a significant ($p<0.05$ to 0.001) fall of blood sugar level in a progressive manner and similar findings also observed in glucose tolerance test to an extent of $p<0.05$. The results of body weight and tested biochemical parameters of blood serum of the 11-days treated animals showed in favor of the anti diabetic activity of the test extracts. In DPPH scavenging anti-oxidant assay, the IC50 value of the test extracts were found as 30.06 and 28.76 µg/ml while the IC50 value of ascorbic acid was 20.92 µg/ml. This study suggests that the aerial part of *H. suaveolens* is endowed with antidiabetic and antioxidant potential.

Key words: Antidiabetic activity, *in vitro* antioxidant, *Hyptis suaveolens,* alloxan.

INTRODUCTION

Diabetes mellitus (DM) is a syndrome which affects more and more people in all countries over the world. It is well known that diabetes mellitus is the commonest endocrine disorder that, according to the World Health Organization (WHO, 2004), affects more than 176 million people worldwide. From an ethnopharmacological perspective, it is important to understand that this disease is one at the interface of conventional biomedical and local (or traditional) treatment. Diabetes mellitus (DM) which not only lead to hyperglycemia also cause many complications, such as hyperlipidemia, hypertension and artherosclerosis

(Bakırel et al., 2008; Sepici et al., 2004; Luo et al., 2004; Abdel-Barry et al., 1997).

Many plant species are known in folk medicine of different cultures to be used for their hypoglycaemic properties and therefore used for treatment of DM (Abdel-Barry et al., 1997; Pushparaj et al., 2000). Despite this, few traditionally used antidiabetic plants have received proper scientific screening.

The plant, *H. suaveolens* (L) Poit commonly known as *W. tulsi* belongs to the family Lamiaceae and is an ethno-botanically important medicinal plant. The plant has been considered as an obnoxious weed, distributed throughout the tropics and subtropics. Almost all parts of this plant are being used in traditional medicine to treat various diseases. The leaves of *H. suaveolens* have been utilized as a stimulant, carminative, sudorific, galactolgogue and

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as a cure for parasitic cutaneous diseases (Anonymous, 1964).

Crude leaf extract is also used as a relief to colic and stomach ache. Leaves and twigs are considered to be antispasmodic and used in antirheumatic and antisudori-fic baths, anti-inflammatory, antifertility agents ((Kirtikar and Basu, 1991) and also applied as an antiseptic in burns, wounds, and various skin complaints. The decoction of the roots is highly valued as appetizer and is reported to contain urosolic acid, a natural HIV-integrase inhibitor (Chatterjee and Pakrashi, 1997).

Fumes of the dried leaves are also used to repel mosquitoes and control insect pests of stored grains. The leaves of the plant have been shown to contain alkaloids, terpenes and volatile oils (Gills, 1992).

The present study has been designed to determine the role of extracts of aerial parts of *H. suaveolens* for *in vitro* antioxidant activity and potential antidiabetic activity if any, against normoglycemic and alloxan induced hyper glycemic rats.

MATERIALS AND METHODS

The plant material used in this study was aerial parts of *H. suaveolens,* collected from road side area from Khargone district Khargone M.P., India, during spring (mid-August to mid-October, 2010) and was authenticated by the Taxonomist Dr. S. K Mahajan, Botany Department, Government P G College Khargone M.P. The plant materials were initially rinsed with distilled water and dried on paper towel in laboratory at (37 ± 1°C) for 24 h and milled into coarse powder by a mechanical grinder.

Preparation of extract

The plant materials (1 kg) were initially defatted with petroleum ether and then extracted with alcohol and water using Soxhlet apparatus. The yield of the plant extracts ethanol (95%) and aqueous measured about 20 g each after evaporating the solvent using water bath. The standard extracts obtained were then stored in a refrigerator at 4°C for further use (Akueshi et al., 2002).

Preparation of the test samples

Glibenclamide (5 mg/kg) was used as the reference control. The test extract was suspended in 25% Tween 20 in distilled water prior to oral administration to the experimental animals. Animals in the control group received only the 25% Tween 20 (2 ml/kg). All the test samples were administered through oral route.

Animals used

Male albino Wistar rats, weighing 150 to 200 g and Swiss albino mice, weighing 20 to 25 g were used. Prior to the experiments, the selected animals were housed in acrylic cages in standard environmental conditions (20 to 25°C), fed with standard rodent diet for 1 week in order to adapt to the laboratory conditions and water was given *ad libitum.* They were fasted overnight (12 h) before experiments, but were allowed free access to water. Six animals were used for each group of study. All the experiments on animals

were conducted in accordance with the internationally accepted principles for laboratory animal use and as per the experimental protocols duly approved by the Institutional Ethical Committee (IAEC No. 1171/C/08/CPCSEA).

Determination of blood glucose levels

Fasting blood glucose concentration was determined using a blood Glucometer (Optimum), based on the glucose oxidase method. Blood samples were collected from the tip of tail at the defined time patterns (Aslan et al., 2007a; b).

Screening for antidiabetic activity

The Screening for antidiabetic activity was followed as per standard procedures (Dash et al., 2001). The test samples were suspended in 25% Tween 20 in distilled water. Glibenclamide (5 mg/kg) was used as reference control during the study. All the test samples were administered through oral route.

Single dose study

Normoglycaemic animals

The animals were fasted for 18 h, but were allowed free access to water before and throughout the duration of experiment. At the end of the fasting period, taken as zero time (0 h), blood was withdrawn (0.1 ml) from the tip of the tail of each rat under mild ether anaesthesia. Plasma was separated following centrifugation and the glucose was estimated by GOD/POD method using Glucose estimation kit from M/s. Sigma Diagnostics (India) Pvt. Ltd., Baroda, India. The normal rats were then divided into six groups of six animals each. Group I served as solvent control and received only vechicle (2 ml/kg) through oral route, Group II received glibenclamide (5 mg/kg) and served as reference control. Groups III to VI received the alcohol and aqueous extract at a dose of 250 and 500 mg/kg, respectively, through oral route. Blood glucose levels were examined after 1, 2, 4, 6, 8 and 10 h of administration of single dose of test and control samples (Table 1).

Alloxan induced diabetic animals

The acclimatized animals were kept fasting for 24 h with water *ad libitum* and injected intraperitoneally a dose of 150 mg/kg of alloxan monohydrate in normal saline. After 1 h, the animals were provided feed *ad libitum.* The blood glucose level was checked before alloxanisation and 24 h after alloxanization as aforementioned. Animals were considered diabetic when the blood glucose level was raised beyond 250 mg/100 ml of blood. This condition was observed at the end of 72 h after alloxanisation. The animals were segregated into six groups of six rats in each. Group I served as normal reference, Group II served as solvent control and received vechicle (2 ml/kg) through oral route. Group III received glibenclamide (5 mg/kg). Groups IV to VII received the test extract at doses of 250 and 500 mg/kg in a similar manner as per the aforementioned experiment. Blood glucose level of each rat was estimated at 1, 2, 4, 6, 8 and 10 h, respectively (Table 2).

Effect on oral glucose tolerance in rats

An oral glucose tolerance test (OGTT) was performed on diabetic

Table 1. Effect of ethanolic and aqueous extracts of *Hyptis suaveolens* on blood glucose level in normoglycemic rats.

Treatment and dose	Blood glucose level (mg/dl)							% decrease at 10 h
	0 h	1 h	2 h	4 h	6 h	8 h	10 h	
Diabetic control (Tween + water)	94.6 ± 1.1	87.2± 4.62	91.43 ±1.86	89.56 ± 0.81	91.58 ±2.23	89.66 ± 0.46	92.67 ±3.22	--
Glibenclamide (5 mg/kg)	91.43 ± 1.31	81.22 ± 2.63	67.53 ±2.34*	58.12 ± 2.61**	54.72±2.44***	73.83 ± 1.42***	71.63±2.81***	21.65
Et. Ext. (250 mg/kg)	89.13 ± 1.2	87.6 ± 1.1	87.2 ±2.65	86.73 ± 1.46	86.33 ±1.43	86.12 ± 0.89*	85.32 ±1.51	4.27
Et. Ext. (500 mg/kg)	88.4 ± 2.43	87.32 ± 2.16	86.49 ±1.87	86.04 ± 1.67	85.6 ±2.69	84.4 ± 1.43**	81.32 ±2.49*	7.94
Aq. Ext. (250 mg/kg)	92.53 ± 1.27	91.46 ± 1.68	89.94 ±1.09	87.11 ± 0.91	86.22 ±2.13	84.11 ± 1.18**	82.11 ±1.89**	11.22
Aq. Ext. (500 mg/kg)	91.18 ± 0.93	87.30 ± 0.78	85.49 ±2.61	85.21 ± 1.37	84.33 ±2.38*	82.66 ± 1.21***	77..42 ±2.73***	15.10

Values are expressed in Mean ± S.E.M of six animals. One Way ANOVA followed by Dunnet's t- test (t-value denotes statistical significance at *$p<0.05$, **$p<0.01$ and ***$p<0.001$ respectively, in comparison to group-I).

Table 2. Effect of ethanolic and aqueous extracts of *Hyptis suaveolens* on blood glucose level in single dose treated alloxan induced hyperglycemic rats.

Treatment	Blood glucose level (mg/dl)							% Age decrease at 10 h
	0 h	1 h	2 h	4 h	6 h	8 h	10 h	
Normal control	93.09±1.38	89.50 ± 0.64	88.50 ±0.64	90.50±0.64	87.59 ±0.64	88.67±2.14	89.76±2.33	-
Diabetic control (Tween + water)	307.5 ± 4.341	297.63±4.93###	301.81±2.89###	291.88± 3.51###	287.89±3.67###	303.77±4.29###	279.61±2.71###	-
Glibencamide (5 mg/kg)	315.7 ± 2.17	258.62±3.21***	198.31±2.89***	139.21±2.63***	126.31±3.28***	118.91±2.96***	92.59±2.87***	70.67
Et. Extract (250 mg/kg)	333.2 ± 3.98	328.38±3.26	307.53±3.24	242.79±3.81***	203.44±2.59***	163.53±4.28***	134.36±3.71***	59.67
Et. Ext.(500 mg/kg)	342.7 ± 4.15	369.68±2.88	319.81±2.36*	212.37±3.77***	158.69±3.91***	131.84±3.28***	126.59±2.93***	62.85
Aq. Ext. (250 mg/kg)	341.09± 5.22	322.23±3.91	282.62±3.11**	236.59±4.12***	157.34±3.86***	148.73±3.57***	131.72±2.98***	61.38
Aq. Ext (500 mg/kg)	358.7 ± 7.25	312.33±	279.36±2.47	186.34±3.26***	153.76±3.41***	137.34±2.15***	119.34±2.38***	66.72

Data represented as Mean ± SEM, (n=6). $p< 0$, 0.05 #significant, ##very significant, ###highly significant as compare to normal control group. $p< 0.05$ *significant, **very significant, ***highly significant as compare to diabetic control group. (One way analysis of variance (ANOVA) followed by Dunnett's t-test).

rats by feeding glucose (5 g/kg) per os. Animals were deprived of food 18 h before and during the experiment but were allowed free access to water. They were divided into 6 groups of 6 rats each. Four groups received the plant extract at the doses as per the aforementioned experiment by os. One group received 5 mg/kg of glibenclamide and the control group received the vehicle. The plant extract, glibenclamide and vehicle were orally administered 1 h before glucose administration. Blood glucose level was determined before drug and glucose administration (−1 and 0 h, respectively) and subsequently at 0.5, 1, 2 and 3 h after (Table 3).

Study of blood glucose level on alloxan induced 11-days treated diabetic animals

The animals were kept fasting for 24 h with water *ad libitum* and injected alloxan monohydrate intraperitoneally at a dose of 150 mg/kg in normal saline. After 1 h, the animals were provided rodent- feed *ad libitum*. The blood glucose level was measured 72 h after administration of alloxan. The animals showing blood glucose level beyond 250 mg/dl were considered for the study. The diabetic animals were segregated into six groups of six rats each. Group II served as solvent control and received only

vehicle (2 ml/kg) through oral route. Group III received glibenclamide (5 mg/kg). Groups IV and V received ethanol extract at doses of 250 and 500 mg/kg. Similarly, Groups VI and VII received aqueous extract at same dose level respectively in a similar manner for 11days. The Group I served as a normal reference. The blood glucose level was measured on 0, 3, 7 and 11th day of treatment (Table 4).

Determination of body weight and serum biochemical of 11- days treated alloxan induced diabetic rats

The body weight of the 11-days treated animals comprised

Table 3. Effect of ethanolic and aqueous extracts of *Hyptis suaveolens* on oral glucose tolerance in normal rats.

Group	Treatments and dose	Blood glucose concentration (mg/dl)					% Reduction at 3 h, w.r.t 5h
		0 min	0.5 h	1 h	2 h	3 h	
I	Normal control	83.75 ± 0.47	86.50 ± 0.64	88.50 ±0.64	83.50 ±0.64	86.50 ±0.64	
II	Solvent control (2 ml/kg)	90.50±0.64	135.52±0.64###	118.83±0.85###	98.50±0.64###	91.50±0.64###	32.48
III	Glibenclamide (5 mg/kg)	89.43 ± 0.40	95.50±1.04***	81.53±0.91***	72.50±0.64***	62.51±0.64***	34.54
IV	Et. Ext. (250 mg/kg)	83.62±0.40	98.25±0.85***	97.61±0.91***	93.50±0.64***	89.13 ± 0.64	9.28
V	Et. Ext. (500 mg/kg)	87.50 ± 0.64	107.31±1.37***	102.32±1.10***	94.50±0.64**	91.50 ± 0.64	14.73
VI	Aq. Ext. (250 mg/kg)	91.50 ± 0.64	116.84±1.10***	109.83±0.85***	102.65 ± 0.91*	92.50 ±0.64**	20.83
VII	Aq. Ext. (500 mg/kg)	84.87 ±0.91	128.36±0.85***	117.36 ±1.10	103.51±0.64***	96.53±0.64***	24.79

Data represented as Mean ± SEM, (n=6). $p<0.05$ #significant, ##very significant, ###highly significant as compared to normal control group. $p<0.05$ *significant, **very significant, ***highly significant as compare to solvent control group. (One way analysis of variance (ANOVA) followed by Dunnett's t-test).

Table 4. Effect of ethanolic and aqueous extract of *Hyptis suaveolens* on blood glucose level in alloxan induced diabetic rats.

Groups	Treatment	Blood glucose concentration (mg/dl)				% Decrease at 11 day
		0 day	3 day	7 day	11 day	
I	Normal control	88.33 ± 2.155	89.33 ± 1.745	89.33 ± 1.820	90.33 1.687	-
II	Diabetic control	407.5 ± 4.341	407.7 ± 6.458###	419.5 ± 5.708###	452.7 ± 10.53###	-
III	Glibenclamide (5 mg/kg)	395.7 ± 27.17	212.3 ± 3.603***	173.5 ± 3.630***	101.8 ± 4.90***	74.27
IV	Et. Ext. (250 mg/kg)	345.2 ± 7.998	297.8 ± 8.080***	143.2 ± 11.57***	164.5 ± 5.97***	52.34
V	Et. Ext.(500 mg/kg)	367.7 ± 15.15	306.7 ± 7.360***	214.8 ± 5.081***	136.3 ± 4.787***	62.93
VI	Aq. Ext.(250 mg/kg)	327.0 ± 21.22	283.8 ± 12.16***	241.3 ± 12.44***	146.3 ± 3.921***	55.25
VII	Aq. Ext.(500 mg/kg)	321.7 ± 18.25	289.3 ± 9.330***	216.2 ± 2.770***	118.7 ± 5.207***	63.10

Data represented as Mean ± SEM, (n=6). p<0, 0.05 #significant, ##very significant, ###highly significant as compared to normal control group. $p<0.05$ *significant, **very significant, ***highly significant as compared to diabetic control group. (One way analysis of variance (ANOVA) followed by Dunnett's t-test).

of similar group distribution as earlier mentioned and was determined by simple weighing, using standard balance on 0, 3, 7 and 11 day of the study. The animals were sacrificed at the end of the study and blood samples were collected by standard method for estimation of serum urea, creatinine, triglycerides, AST, ALT, ALP and cholesterol (Tables 5 and 6).

In vitro antioxidant activity

DPPH radical scavenging activity

Quantitative analysis

The free radical scavenging capacity of the both extract

was determined using DPPH. An ethanolic DPPH solution (0.004%) was mixed with serial dilutions (10 to 100 µg/ml) of crude extracts and after 30 min, the absorbance was read at 515 nm using a spectrophotometer (Schimadzu 1700 UV – visible spectrophotometer). Ascorbic acid was used as reference. The inhibition curve was plotted and IC_{50} values obtained by Probit analysis (Viturro et al.,

Table 5. Effect of ethanolic and aqueous extract of *Hyptis suaveolens* on body weight on treated alloxan induced diabetic rats.

Groups	Treatment	Body weight in (g)			
		0 day	3 day	7 day	11 day
I	Normal Control	182.3 ± 4.2	188.34±3.93	193.22±4.38	199.8 2± 6.8
II	Diabetic control	190.8 ± 3.8	182.56±4.61	173.36±3.28##	164.74 ± 7.2###
III	Glibenclamide (5 mg/kg)	176.5 ± 4.9	182.31±3.72	187.74±2.89**	198.56 ± 6.1**
IV	Et. Extract (250 mg/kg)	182.6 ± 4.6	186.53±3.77	190.87±3.44**	193.53 ± 6.1**
V	Et. Extract (500 mg/kg)	176.2 ± 5.1	187.59±3.46	191.82±4.68**	194. 3 3± 5.8**
VI	Aq. Extract (250 mg/kg)	184.4 ± 4.4	187.37±2.86	191.91±3.73**	192.54 ± 6.0**
VII	Aq. Extract (500 mg/kg)	182.3 ± 4.2	184.73±3.41	188.39±3.66**	196.62 ± 5.1**

Data represented as mean ± SEM, (n=6). p<0.05 # significant, ## very significant, ### highly significant as compare to normal control group. p<0.05 *significant, **very significant, ***highly significant as compare to diabetic control group. (One way analysis of variance (ANOVA) followed by Dunnett's t-test).

1999).

Phytochemical Screening

Phytochemical screening of the prepared extracts was conducted with various qualitative tests to identify the presence of chemical constituents. To perform the tests, the following chemicals and reagents were used: steroids with chloroform and sulphuric acid, tannins with ferric chloride solution, gum with Molish reagents and concentrated sulfuric acid, flavonoids with Mg and HCl and saponins with the capability of producing suds. Alkaloids were tested with Mayer's reagent, Hager's reagent and Dagendorff's reagent. These were identified by characteristic color changes using standard procedures (Ghani, 2003).

Statistical analysis

The data obtained in the animal experiments like body weight, fasting blood sugar and biochemical estimations were subjected to statistical analysis. All values are expressed as Mean ± SEM (Standard Error of Mean). The data were assessed by the analysis of variance (ANOVA). Mean values were considered significantly different if P< 0.05 and 0.001.

RESULTS AND DISCUSSION

Alloxan-induced type 2 diabetes is a chemical model of experimental diabetes mellitus developing a severe hyperglycaemia and widely used in the diabetic studies. This model was used in our investigation to evaluate the effects of aerial parts of *H. suaveolens* ethanolic and aqueous extracts on hyperglycaemia and some metabolic disorders related to diabetic mellitus. The effects of ethanol and aqueous extracts of aerial parts of *H. suaveolens* on fasting blood glucose levels of normal and diabetic rats are presented in Tables 1 and 2 respectively.

The plant extracts induced 4 to 15% fall of fasting blood glucose which is not significant enough to interpret hypoglycemic effect on normal rats (Table 1). Treatment

of normal rats with glibenclamide produced a significant (p<0.01) hypoglycaemic effect from first to 6 h, reaching a 41.15% maximum fall (p<0.01) in the blood glucose, as compared with the normal control group or with time 0. In alloxan induced diabetic rats as shown in Table 2, a dose dependent effect of the plant extract was observed. The test extracts showed a persistent decrease in blood glucose level till the end of 10 h, with maximal decrease noted in aqueous extract at 500 mg/kg dose, reaching 66.72% (p<0.01), while the standard drug glibenclamide showed 70.67% decrease. The test result presented in Table 3, indicates that the test extracts induce reduction in hyperglycaemia during the glucose tolerance test in diabetic rats. The alloxan-induced hyper glycaemia was significantly (p<0.01) corrected by the plant extract at the end of the treatment (11 days) in a sustained dose dependent manner, the result of which is presented in table 4. The maximal reduction 63.10% was observed with aqueous extract at high dose of 500 mg/kg. The potency of the extract in the light of fall of blood sugar level is dose dependent and in the order of aqueous extract followed by ethanol extract. *H. suaveolens* recover the body weight of treated diabetic group in a significant extent (p<0.01) when compared with diabetic control group and approach towards the untreated control normal animal group (Table 5).

The biochemical parameters of plasma urea, creatinine, triglyceride, cholesterol, AST, ALT, ALP values of treated diabetic groups at the end of the treatment (11 days) decreases in a significant extent (p<0.001) when compared with diabetic control group (Table 6). The extent of decrease is in a dose dependent order and the potency rest first with aqueous extract followed by ethanolic extract.

The preliminary phytochemical investigation report indicates that the aqueous extract of *Hyptis suaveolens* found to contain carbohydrates, saponins, tannins, alkaloids, flavonoids, terpenoids, steroids and sterols as

Table 6. Effect of ethanolic and aqueous extract of *Hyptis suaveolens* on some serum biochemical parameters on treated alloxan induced diabetic rats.

Group	Treatment	Urea (mg/dl	Creatinine (mg/dl)	Triglyceride (mg/dl)	Cholesterol (mg/dl)	ALT (U/L)	AST (U/L)	ALP (U/L)
I	Control	42.25±0.47	0.83±0.004	84.25±0.47	63.25±0.75	36.42 ± 0.8	136.14 ± 2.6	118.20 ± 4.8
II	Diabetic control	183±0.64###	1.53±0.008###	145.5±0.64###	172.8±1.10###	64.26 ± 1.2*	170.20 ± 4.8*	134.28 ± 2.2*
III	Glibenclamide (5 mg/kg)	89±0.40***	1.13±0.006***	104.8±0.85***	85±0.91***	42.46 ± 1.4**	141.80 ± 2.6**	128.12 ± 1.9**
IV	Et. Ext.(250 mg/kg)	143.8±0.47***	1.29±0.006***	138.3±0.85***	128.5±1.04***	47.15 ± 1.1***	144.00 ± 4.4***	126.6 ± 3.8***
V	Et. Ext. (500 mg/kg)	109±0.40***	1.27±0.004***	132.5±1.19***	114.5±1.32***	44.26±2.2***	142.00±4.2***	120.26±2.4***
VI	Aq. Ext. (250 mg/kg)	92.50±0.64***	1.17±0.006***	115.0±1.08***	96.75±1.31***	44.26±2.2***	144.22±2.4***	124.24±4.6***
VII	Aq. Ext.(500 mg/kg)	89±0.40***	1.15±0.006***	108.3±0.85***	94.25±1.10***	43.62±2.8***	143.20±4.6***	121.42±1.2***

Data represented as Mean ± SEM, (n=6). $p<0.05$ # significant, ##very significant, ###highly significant as compare to control group. $p<0.05$ *significant, **very significant, ***highly significant as compare to negative control group. (Analysis of variance (ANOVA) followed by Dunnett's test).

Table 7. Effect of ethanolic and aqueous extract of *Hyptis suaveolens* on DPPH scavenging activity.

Test substances	Concentration (µg/ml)				IC_{50} (µg/ml)
	10	20	50	100	
Ascorbic acid	28.8±0.22	62.8 ± 0.70	80.8 ± 1.14	95.17 ± 1.45	20.92
Ethanolic ext.	21.6 ± 0.19***	54.2 ± 0.71***	73.1 ± 1.01**	90.5 ± 1.36*	30.06
Aqueous ext.	22.4 ± 0.23***	57.5±0.33***	72.2.4 ±0.02	93.2 ±1.23	28.76

Data are expressed as Mean ± S.E.M., n = 6; *$p<0.01$ vs. control, ** $p< 0.001$ vs control and ***$p< 0.0001$ vs. control by student's 't' test.

phytoconstituents. DPPH is relatively stable nitrogen and centered free radical that easily accepts an electron or hydrogen radical to become a stable diamagnetic molecule. This activity was increased by increasing the concentration of the sample extract. DPPH antioxidant assay is based on the ability of 1, 1-diphenyl-2-picryl-hydrazyl (DPPH), a stable free radical, to decolorize in the presence of antioxidants. The DPPH radical contains an odd electron, which is responsible for the absorbance at 515 nm and also for a visible deep purple color. When DPPH accepts an electron donated by an antioxidant compound, the DPPH is decolorized, which can be quantitatively measured from the changes in absorbance. The IC50 value of the test extracts was found to be 30.06 and 28.76 µg/ml for ethanolic extracts and aqueous extracts while the IC50 value of ascorbic acid was 20.92 µg/ml (Table 7). From the results, it may be postulated that both the plant extracts have hydrogen donors thus, scavenging the free radical DPPH.

It is generally accepted that alloxan treatment causes permanent destruction of β-cells and impairment of renal function and sulfonyl ureas are known to lower the blood glucose level by stimulating β-cells to release insulin (Pari and Maheswari, 1999). The hypoglycemic effect comparable to glibenclamide suggested that the extract may act by regenerating the β-cells in alloxan-induced diabetes (Ghosh and Suryawanshi, 2001) and the decreased activity in glucose level in OGTT might be due to a decrease in the rate of initial glucose absorption when plant fiber is given orally with glucose (Day et al., 1990). Diabetes mellitus results from the failure to use

glucose for energy, which leads to increased utilization and decrease storage of protein respon-sible for reduction of body weight essentially by depletion of body proteins (Guyton and Hall, 2000). It has been reported that the increase in glycaemia in alloxan or streptozotocin-induced diabetic rats was associated with dislipidoemia charac-terized by elevated serum triglycerides total cholesterol levels (Dhanbal et al., 2006). The improvement of blood glucose level induced by most hypoglycaemic treatment is associated with a reduction of serum triglycerides and total cholesterol. The presence of alkaloids, carbohy-drates, flavonoids, tannins, steroids and/or terpenes in the whole plant of *H. suaveolens* either as single constituents or in combination may be responsible for the observed anti-diabetic activity.

Conclusion

In this study, several animal models and *in vitro* assay were applied to evaluate the antidiabetic activity of ethanolic and aqueous extract of aerial parts of *H. suaveolens* Poit. As a conclusion, it could be speculated that the observed antidiabetic activity of *H. suaveolens* Poit might be related to the presence of tannins, terpenoids, steroids and flavonoid contents and having the potential to impart beneficial therapeutic effect in diabetes.

This study also establishes a correlation between antidiabetic and antioxidant potential and may be of considerable interest in preventing the ill effects of diabetes and oxidative stress *in vivo*. However, the plant extracts should be investigated to find out the chemical compounds responsible for antidiabetic action. Furthermore, *in vitro* antioxidant activity should be evaluated by other antioxidant methods, for example, nitric oxide scavenging activity, reducing power, lipid peroxidation assay.

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Full Length Research Paper

Hepatoprotective effects of *Justicia adhatoda* L. against carbon tetrachloride (CCl$_4$) induced liver injury in Swiss albino mice

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Medicinal plants are believed to be a precious natural reservoir and have been continuously studied for their pharmacological activities against various ailments, liver being no exception. Leaves of *Justicia adhatoda* L. are being extensively used against many human ailments. Hepatoprotective effects of *J. adhatoda* L. leaves and flowers were investigated on carbon tetrachloride (CCl$_4$) induced liver damage in Swiss albino mice. Liver injury was assessed by estimation of biochemical parameters which includes liver function tests and supplemented by histopathological examination of liver. Aqueous, ethanolic and methanolic extracts of different concentrations were given orally and activities of these extracts were compared with standard drug (silymarin). The results showed that *J. adhatoda* leaves and flowers showed unambiguous hepatoprotective activity against CCl$_4$ induced liver toxicity. It was also concluded that different concentrations of leaves and flowers of *J. adhatoda* did not show any sign and symptoms of toxicity and mortality.

Key words: *Justicia adhatoda* L., Swiss albino mice, silymarin, hepatoprotective effects.

INTRODUCTION

Drug-induced liver injury is a major health problem that became a challenge not only for health care professionals, but also for the pharmaceutical industry and drug regulatory agencies which are in search of alternative medicines for hepatoprotection. Drug-induced liver injury accounts for more than 50% of acute and chronic liver failure, which includes about 39% hepatotoxicity caused by overdose of acetaminophen and 13% idiosyncratic liver injury triggered by other drugs (Michael and Cynthia, 2006; Kaplowitz, 2001). Isoniazid and rifampicin are considered as the first line drugs used for tuberculosis therapy and are associated with

hepatotoxicity (Tasduq et al., 2005). Most of the hepatotoxic chemicals damage liver cells mainly by inducing lipid peroxidation and other oxidative damages (Shanmugasundaram and Venkataraman, 2006). Liver is a chemical factory, and i5t is accountable for the regulation, synthesization, storage and secretion of many important proteins, nutrients, chemicals and clears toxin or superfluous substances from the body. Therefore, healthy liver determines the vigor rank of an entity (Gupta et al., 2006). More than 50% of acute and chronic liver failure, which includes approximately 39% hepatotoxicity are caused by overindulgence of acetaminophen and 11% idiosyncratic liver injury triggered by other drugs (Michael and Cynthia, 2006; Kaplowitz, 2001).

Animal cells are equipped with both enzymic and non-enzymic antioxidant defense with varied efficacies that

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protect animals against oxidative stress caused by wide range of toxins including carbon tetrachloride (CCl_4) (Karbownik et al., 2001).

Organism may have an endogenous shielding antioxidant defense system against the damage of free radicals. Superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GSH-Px) are enzymatic antioxidants that catalyze detoxification response of toxic oxygen metabolites. Bilirubin is a dominant lipophilic antioxidant that protects membranes from lipid peroxidation and protects membrane proteins from oxidation (Thomas et al., 2009). It accounts for nearly all of the antioxidant activity of human serum and predominant influence against superoxide and peroxyl radicals (Novotny and Vitek, 2003). Many folk remedies from plant origin are tested for their potential antioxidant and hepatoprotective liver damage in experimental animal model. CCl_4- induced hepatotoxicity model is widely used for the study of hepatoprotective effects of drugs and plant extracts (Qureshi et al., 2010; Yu et al., 2010). Phytochemicals from medicinal plants serve as lead compounds in drug discovery and design (Ncube et al., 2008). *Justicia adhatoda* L. syn. *Adhatoda vasica, Adhaotoda zylenica* is a very popular plant of Himalaya and throughout Northern region of Pakistan. It belongs to family Acanthaceae, subclass Asteridae, and species adhatoda generally known as bakkar and bakkas. *J. adhatoda* is an evergreen, gregarious shrub 3 to 6 m long, large leaves lanceolate 10 to 20 by 4 to 8 cm and flowers are white or purple in short, dense auxiliary pedunculates (Ahmad et al., 2009). Foremost, the uses of this plant extracts are against bronchitis. It is also used as antispasmodic, antioxidant, anti-inflammatory, anti-tussive, antipyretic and antibacterial. Diabetes is also being treated with Justicia by traditional healers in certain areas of Pakistan (Ahmad et al., 2009). The current study was undertaken to evaluate the hepato-protective effects and safety assessment studies of *J. adhatoda* L. against CCl_4-induced hepatic damage in albino mice.

MATERIALS AND METHODS

Plant and chemicals

J. adhatoda leaves and flowers were collected from adjoining areas of Islamabad and Rawalpindi in fine plastic bags duly labeled with numbers and date of gathering of samples. Samples were identified by taxonomist from Department of Botany, Pir Mehar Ali Shah, Arid Agriculture University, Rawalpindi, Pakistan and registered as specimen (voucher specimen numbers 215 and 216). All chemicals used in this experiment were of the analytical status and purchased from Sigma Chemicals USA.

Preparation of plant extracts

Extraction procedure of World Health Organization (WHO, 2011) was modified for this study. A total of 10 g (each) sample of leaves and flowers were weighed and dissolved in ethanol, methanol and water (1:10) and positioned in shaking incubator at 120 rpm, 25°C

for 24 h. Solutions were centrifuged at 14000 rpm for 15 min and were filtered. Finally, solvents were recovered via rotary evaporator and the extracts were dried at 37°C, labeled and stored for further process.

Animals

Swiss albino mice weighing between 30 and 40 g were used in this evaluation and were obtained from National Veterinary Laboratories, Islamabad. They were housed in well ventilated stainless-steel cages at room temperature (24±2°C) in hygienic provision under natural light and dark schedule and were fed on standard laboratory diet. Food and water were given *ad libitum.*

Toxicity studies

Acute toxicity study was conducted according to the Organization for Economic Cooperation and Development (OECD) revised up and down procedure for acute toxicity testing (OECD, 2001). A dose of 400 mg/kg of the aqueous, ethanolic and methanolic extracts of the leaves and flower of *J. adhatoda* were administered to six healthy adult albino mice of either sex in each group.

Experimental induction of hepatic damage

Male Swiss albino mice were selected for experimental assays and were sorted in groups of six mice in each cage. Group I (negative control) mice were administered normal food and water for 15 days. Group II (normal saline control) received normal saline daily for 15 days p.o. Group III (induction control) received intraperitoneally, 25% CCl_4 dissolved in olive oil at dose of 1 ml/kg of body (b.w.) eight twice a week. Group IV (drug or positive control) received CCl_4 intraperitonealy twice a week and standard drug silymarin (100 mg/kg b.w.) on the remaining days of the week for 15 days. Group V to X served as herb treated groups and further divided into two subgroups like groups A and group B. Groups A and B were orally administered extracts of leaves and flower of *J. adhatoda.* Groups VA and VB received CCl_4 intraperitonealy twice a week and methanolic extracts of the leaves and flower, respectively (100 mg/kg b.w.) for the rest of 15 days. Groups VIA and VIB received CCl_4 intraperitonealy twice a week and methanolic extracts of the leaves and flower, correspondingly (200 mg/kg b.w.) up to 15 days. Groups VIIA and VIIB received CCl_4 intraperitonealy twice a week and ethanolic extracts of the leaves and flower, respectively (100 mg/kg b.w.) for 15 days. Groups VIIIA and VIIIB received CCl_4 intraperitonealy twice a week and ethanolic extracts of the leaves and flower, respectively (200 mg/kg b.w.) for 15 days. Groups IXA and IXB received CCl_4 intraperitonealy twice a week and aqueous extracts of leaves and flower, respectively (100 mg/kg b.w.) for 15 days. Groups XA and XB received CCl_4 intraperitonealy twice a week and aqueous extracts of leaves and flower, correspondingly (200 mg/kg b.w.) for 15 days.

Serum separation and liver isolations

At the end of experiment, animals were weighed and were anesthetized with 20% chloroform and blood samples were collected by carotid artery puncture allowed to coagulate at room temperature for half an hour and then centrifuged at 3000 rpm for 10 min. The serum was separated and conserved at -20°C for ensuing analysis. Animals were sacrificed under diethyl ether anesthesia at fasting state, liver tissues were promptly excised and a part of liver homogenized in normal saline (0.9%), centrifuged at 3000 rpm for 10 min and supernatants were kept at -20°C for the
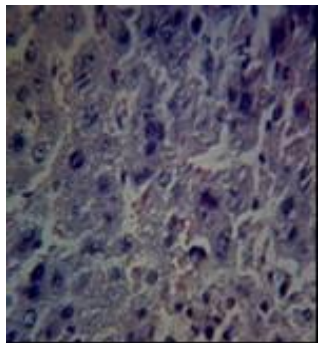
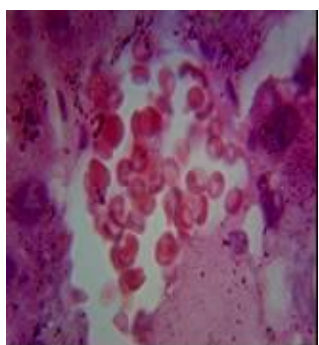**Figure 1.** Microscopic view of liver tissue of normal mice.

Figure 2. Microscopic view of liver obtained from CCl₄ control mice.

assay of biochemical parameters related to oxidative stress.

Determination of body weight increase and biochemical assays

Experimental animals were weighed before and after the onset of toxicity at the end of trial, change in body weight was noted down. Concentrations of aspartate amino transferase (AST), alanine amino transferase (ALT), alkaline phosphatase (ALP), serum total proteins, albumin and plasma total bilirubin were estimated as described by Qureshi et al. (2010) and also using reagent kits purchased from Pioneer Diagnostics Company.

Histopathological examination of liver tissue

Small pieces of liver tissues were transferred to 10% formalin for proper fixation and were processed and embedded in paraffin wax. Sections of 5 to 6 μm in thickness were cut and stained with haematoxylin and eosin. Each liver was microscopically examined to assess the severity of lesions. The degree of liver damage was assessed and lesion grading was done as minimum, moderate and maximum.

Statistical analysis

Statistical analysis of data was performed by using the student's

unpaired t-test and by analysis of variance (ANOVA) under absolutely randomized design (CRD) and the means of the treatment were compared by Duncan's Multiple Range Test (DMRT) (Anitha and Karuppasamy, 2011).

RESULTS

The effects of *J. adhatoda* leaves and flowers on serum enzymes and proteins in CCl₄ induced hepatotoxic mice are summarized in Tables 1 and 2. Administration of 25% CCl₄ (1 ml/kg) drastically reduced body weight (P<0.05) and the total protein and albumin concentration (2.53±0.46, 1.76±0.63). However, it increases serum enzymes, that is, ALT (264.87±5.9), AST (169.36±8.8), ALP (682.6±9.2), and total bilirubin (1.24±0.15) as compared to normal control (P<0.001).

The pretreatment of different extracts of *J. adhatoda* leaves and flowers (200 and 100 mg/kg) in CCl₄ administered mice significantly reduced the toxic changes. Methanolic, ethanolic and aqueous extracts of the leaves and flowers were used in this investigational study. Results in Tables 1 and 2 clearly confirmed that pretreatment of flower extracts of methanol, ethanol and water at higher dose (200 mg/kg) more considerably increased (P<0.001) the body weight (17.16, 12.32 and 16.64%), respectively equivalent to the effect observed with leaves extracts of *J. adhatoda* and silymarin used as standard drug. There is significant reduction (P<0.01) in the level of serum ALT, ALP, AST activities and total bilirubin and increase in the level of total proteins and albumin after pretreatment with *J. adhatoda* leaves and flowers as compared to toxic control. Activity of the plant extracts was similar to silymarin in which flower extracts showed more noteworthy (P<0.05) results as compared to silymarin.

Histopathological studies further confirmed the hepatoprotective effects of *J. adhatoda* leaves and flowers. Liver sections of normal control animals exhibited normal hepatic cells each with well defined cytoplasm, prominent nucleus and nucleolus and well brought out central vein whereas that of toxicant administered group animals showed total loss of hepatic design with centilobular hepatic necrosis, fatty changes vacuolization and blocking of sinusoids, Kepffer cell hyperplasia, crouding of central vein and apoptosis. The administration of *J. adhatoda* extracts reversed the gross disturbances observed in the liver cytoarchitecture (Figures 1 to 9).

DISCUSSION

The CCl₄ is one of the toxic chemicals used to evaluate the effects of herbal and synthetic drugs on liver injury (Yu et al., 2010). Administration of this toxicant results in the increase of liver enzymes and reduction in the activity of antioxidants in the body. When CCl₄ administered in
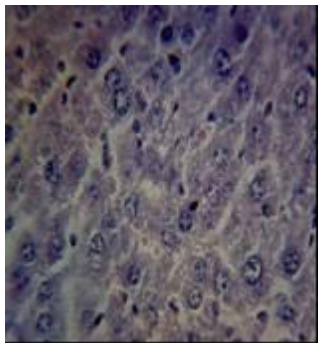
Figure 3. Liver sections from silymarin treated CCl$_4$ injected mice.
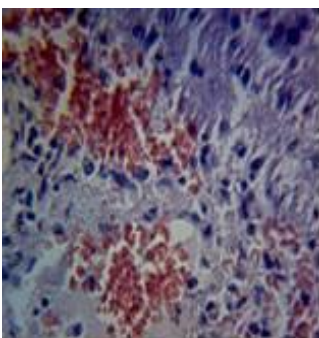
Figure 4. Liver sections from *Justicia* leaves aqueous extract treated CCl$_4$ injected mice.
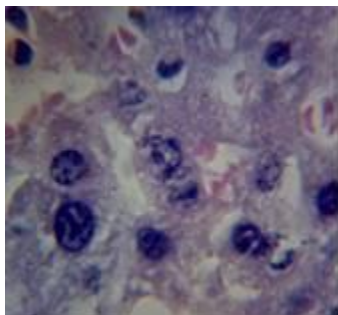
Figure 5. Micros view of liver tissue of mice treated with *Justicia* leaves ethanolic extract.
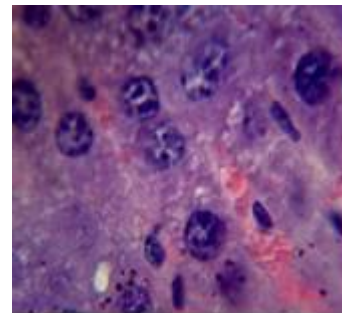
Figure 6. Micros view of liver tissue of mice treated with *Justicia* leaves methanolic extract.
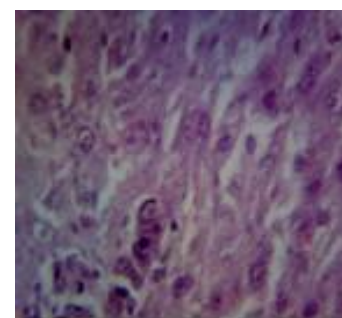
Figure 6. Micros view of liver tissue of mice treated with *Justicia* flowers aqueous extract.
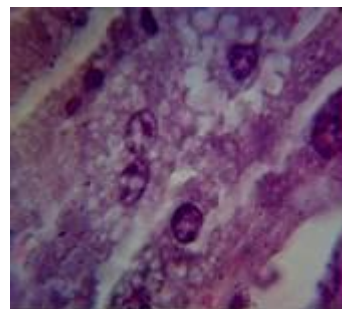
Figure 8. Micros view of liver tissue of mice treated with *Justicia* flowers ethanolic extract.

mice, it causes severe toxicity by interfering with their normal metabolic functions. Inside the cell, CCl$_4$ is biotransformed into trichloromethyl radical (*CCl$_3$) which join together with cellular proteins and membrane lipids and disintegrate them, there by initiating elevated level of lipid peroxidation which is the principle action of CCl$_4$ (Aghel et al., 2007). It is also presumed that herbal drugs inhibit the CYP2E1 enzyme activity in hepatic microsomes *in vivo* hepatocytes which are reflected as their increased levels in serum (Rajesh and Latha, 2004). Pretreatment with *J. adhatoda* leaves restored the liver enzyme parameters showing a dose dependent effect. The reduction of liver enzyme parameter (ALT) was significant and is considered as a specific marker of liver injury due to toxic drugs, alcohol and virus (Sherlock and Dooley, 2002). The protective effect may be the result of stabilization of plasma membrane, thereby preserving the

Table 1. Effects of *J. adhatoda* leaves and flowers on body weight gain in CCl$_4$ administered mice.

Group	Body weight (g)		
	Before experiment	After experiment	Change in weight (%)
Normal control	20.33±0.26	24.43±0.28	18.91±2.69
Solvent control Normal saline (10 ml/kg)	20.14± 0.53	22.72±0.24	12.33±1.83
Induction control (25% CCl$_4$, 1 ml/kg)	20.55±0.23*	18.98±0.50*	-6.61±1.52*
Drug control (Silymarin 100 mg/kg) + CCl$_4$	23.51±0.23[λ]	26.07±0.16[λ]	11.23±1.00[λ]
J.l meth (200 mg/kg) + CCl$_4$	25.29±0.28[α]	28.57±0.31[α]	12.97±0.60[α]
J.l meth (100 mg/kg) + CCl$_4$	20.41±0.22	21.84±0.21	6.99±0.16
J.l eth (200 mg/kg) + CCl$_4$	25.72±0.61[α]	28.80±0.29[α]	11.98±1.59[α]
J.l eth (100 mg/kg) + CCl$_4$	24.99±0.29	26.66±0.41	6.29±0.71
J.l aq (200 mg/kg) + CCl$_4$	26.03±0.65	28.55±0.43	9.68±1.40
J.l aq (100 mg/kg) + CCl$_4$	28.11±0.92	29.89±0.80	6.37±1.51
J.f meth (200 mg/kg) + CCl$_4$	21.89±0.68[βλ]	25.64±0.54[βλ]	17.16±1.63[βλ]
J.f meth (100 mg/kg) + CCl$_4$	28.82±0.86	30.94±1.14	7.35±0.90
J.f eth (200 mg/kg) + CCl$_4$	22.82±1.82[α]	25.74±1.59[α]	12.32±.16[α]
J.f eth (100 mg/kg) + CCl$_4$	25.66±0.56	27.52±0.39	7.26±1.71
J.f aq (200 mg/kg) + CCl$_4$	21.92±0.93[βλ]	25.50±1.09[βλ]	16.64±1.53[βλ]
J.f aq (100 mg/kg) + CCl$_4$	27.00±0.72	28.70±0.49	6.35±2.13

Data are expressed as mean ± standard deviation (SD, n=4). Values significantly different as compared to normal control: *$P<0.05$. Values significantly different as compared to CCl$_4$-administered control: [α]$P<0.05$, [λ]$P<0.01$. Values significantly different as compared to standard drug: [β]$P< 0.001$. J.l meth: *Justicia adhatoda* leaves methanolic extracts, J.l eth: *Justicia adhatoda* leaves ethanolic extracts, J.l aq: *Justicia adhatoda* leaves aqueous extracts, J.f meth: *Justicia adhatoda* flowers methanolic extracts, J.f eth: *Justicia adhatoda* flowers ethanolic extracts, J.f aq: *Justicia adhatoda* flowers aquous extracts.
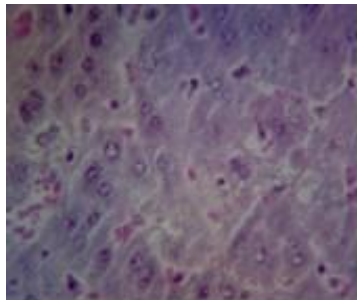
Figure 9. Micros view of liver tissue of mice treated with *Justicia* flowers methanolic extract.

structural integrity of cell as well as the repair of hepatic tissue damage caused by CCL$_4$ (Pari and Murugan, 2004). The lessening in cellular proteins might be due to the disruptive effect on endoplasmic reticulum, of which consequent loss of P450 enzyme will protein synthesis. Less protein merger results in the growth of triglycerides leading to fatty liver (Shenoy et al., 2001).

ALT is more selectively a liver paranchymal enzyme than AST. AST presents two isozymes, one is present in mitochondria, while the other one in cytoplasm (Sapakal et al., 2008). Another key parameter used to assess liver toxicity is bilirubin determination which is a chemical product of hemoglobin which is conjugated with

glucoronic acid in hapatocytes to amplify its water solubility (Sreepriya et al., 2001; Ravi et al., 2005; Rajib et al., 2009). Rise of serum enzymes indicates the harmful effects of CCl$_4$ on liver of animals, because these enzymes are localized in cytoplasm and released after cellular damage (Mohan et al., 2007). The increased level of serum bilirubin might be due to inconsistent production of bilirubin due to excessive breakdown of red blood cells (RBCs) and the incapacity of animal to handle bilirubin due to liver damage which would cause either intra or extra hepatic obstruction (Ahmad et al., 2002). The increase of plasma bilirubin levels by CCl$_4$ further indicates that CCl$_4$ is a toxic agent for liver which is also in agreement with results reported by Vogel (2002), Samudram et al. (2008), Ahsan et al. (2009) and Tsala et al. (2010).

Herbal extracts contain antioxidant phytochemicals like flavonoids and phenolic compounds and triterpenes. Due to the radical scavenging potential of these phytochemicals, toxicity level reduces and body tends back towards normalcy (Laszczyk et al., 2006). Several studies have shown the hepatoprotective effects of *Justicia* leaves (Pandit et al., 2004; Bhattacharya et al., 2005; Krishna et al., 2010), but no work has reported on flowers of *J. adhatoda*. It has been reported that due to its antioxidant potential, *J. adhatoda* ethanolic extracts pretreatment significantly prevents radiation induced chromosomal eccentricity in bone marrow cells and reduces stickiness of chromosomes (Kumar et al., 2007).

Table 2. Effects of *J. adhatoda* leaves and flower extracts on serum enzymes activities and plasma total bilirubin level.

Group	Biochemical parameters					
	ALP (U/L)	AST (U/L)	ALT (U/L)	Plasma total bilirubin (mg/dl)	Total proteins (g/dl)	Total albumin
Normal control	130.29±6.76	39.472 ± 4.24	48.367±6.74	0.146 ± 0.03	7.213±0.80	3.285±0.46
Normal saline (10 ml/kg)	248.177±2.05	41.962± 0.72	47.51±4.29	0.253± 0.01	5.018±0.76	4.010±0.35
Induction control (25% CCl₄, 1 ml/kg)	682.622±9.2*	264.87± 5.91*	169.36±8.8**	1.24± 0.15*	2.53±0.46**	1.764±0.63*
Drug control (Silymarin 100 mg/kg) + CCl₄	283.457±7.5$^{\lambda}$	78.022±1.25$^{\lambda}$	50.665±6.7$^{\alpha}$	0.24± 0.07$^{\alpha}$	5.760±0.64	4.345±0.42
J.l meth (200 mg/kg) + CCl₄	305.46±5.28	125.26 ± 4.58	73.522±8.11	0.372 ± 0.03	6.055±0.90	4.075±0.38
J.l meth (100 mg/kg) + CCl₄	394.535±9.18	179.72 ± 9.90	85.315±3.91	0.543 ± 0.05	3.784±0.35	3.405±0.31
J.l eth (200 mg/kg) + CCl₄	357.107±35.9	81.93 ±7.48$^{\lambda}$	55.022±6.8$^{\alpha}$	0.273 ± 0.03$^{\lambda}$	5.890±0.38	4.367±0.2$^{\alpha\beta}$
J.l. eth (100 mg/kg) + CCl₄	385.142±6.68	137.022±7.23	92.83±2.25	0.542 ± 0.03	4.877±0.25	2.612±0.39
J.l aq (200 mg/kg) + CCl₄	275.42±9.66	71.372±6.23$^{\beta\lambda}$	89.602±5.90	0.194 ± 0.01$^{\alpha}$	6.777±1.83$^{\alpha\beta}$	3.612±0.27
J.l aq (100 mg/kg) + CCl₄	392.567±10.1	155.022 ±9.37	113.225±2.8	0.413 ± 0.03	4.690±0.62	2.345±0.28
J.f meth (200 mg/kg) + CCl₄	246.275±4.2$^{\lambda}$	86.73± 4.10	57.55±6.02$^{\alpha}$	0.152 ± 0.0$^{\alpha\beta}$	6.988±1.31$^{\alpha\beta}$	4.720±0.8$^{\alpha\beta}$
J.f meth (100 mg/kg) + CCl₄	396.257±6.75	166.67 ± 7.4	96.55±5.51	0.676 ± 0.03	4.493±0.32	2.535±0.37
J.f eth (200 mg/kg) + CCl₄	209.49±10.1$^{\alpha}$	65.157±2.30$^{\alpha\beta}$	57.30±3.97$^{\alpha\beta}$	0.418 ± 0.03	6.116±0.48	3.962±0.47
J.f. eth (100 mg/kg) + CCl₄	308.667±8.61	137.187 ±6.92	77.297±3.00	0.696 ± 0.07	3.711±0.29	2.752±0.18
J.f aq (200 mg/kg) + CCl₄	221.71±3.8$^{\alpha\beta}$	90.785± 5.23$^{\lambda}$	60.223±9.0$^{\alpha\beta}$	0.523 ± 0.22	6.212±0.90	3.856±0.68
J.f aq (100 mg/kg) + CCl₄	383.212±9.98	166.26± 9.65	86.377±0.14	0.846 ± 0.07	4.256±0.14	2.610±0.32

Values expressed as means ± standard deviation (SD, n=4). Values significantly varied comparable to normal control: *P<0.001, **P<0.01. Values significantly varied comparable to CCl₄-administered control: $^{\alpha}$P<0.01, $^{\lambda}$P<0.05. Values significantly varied comparable to drug control: $^{\beta}$P<0.05. J.l meth: *Justicia adhatoda* leaves methanolic extracts, J.l eth: *Justicia adhatoda* leaves ethanolic extracts, J.l aq: *Justicia adhatoda* leaves aqueous extracts, J.f meth: *Justicia adhatoda* flowers methanolic extracts, J.f eth: *Justicia adhatoda* flowers ethanolic extracts, J.f aq: *Justicia adhatoda* flowers aquous extracts.

The results indicated that *J. adhatoda* leaves and flowers show explicit hepatoprotective activity against CCl₄ induced liver toxicity. Among these flower extracts of *J. adhatoda* showed more prospective activity at dose of 200 mg/kg b.w.

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Full Length Research Paper

A study on the anti-inflammatory effect of aromatic rhinitis spray

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The aim of this study was to study the anti-inflammatory effect of a compound containing volatile oils of *Flos magnoliae*, *Centipeda minima* and *Mentha haplocalyx*. The anti-inflammatory effect of the compound was investigated using xylene-, egg white- and cotton pellet-induced inflammation method. The medium- and high-doses of the compound (0.04 and 0.08 ml/kg) significantly inhibited the xylene-induced ear swelling in mice; low-, medium- and high-doses of the compound (0.02, 0.04, and 0.08 ml/kg) all had significant inhibitory effects on degree of paw swelling 30 min after the induction of inflammation; high-dose of the compound (0.08 ml/kg) significantly lowered the weight of cotton pellet granuloma in rats. The compound has a preferable therapeutic effect on both the acute and chronic inflammations, which can be developed as an anti-inflammatory traditional Chinese medicine.

Key words: Anti-inflammation, *Flos magnoliae*, *Centipeda minima*, *Mentha haplocalyx*, volatile oil.

INTRODUCTION

The formula of the aromatic rhinitis spray consists of three traditional Chinese herbs, namely *Flos magnoliae*, *Centipeda minima* and *Mentha haplocalyx*. The nasal spray is mainly used in the treatment of acute and chronic rhinitis. *F. magnoliae* is the dry bud of the Magnoliaceae plants such as *Magnolia biondii* Pamp., *Magnolia denudate* Desr., or *Magnolia sprengeri* Pamp. (The State Pharmacopoeia Committee of China, 2010). Through the analysis and identification, it was found out from the study of Qin et al. (2001) that the main composition of volatile oil from *F. magnoliae* were aromatic alcohol compounds and unsaturated ester compounds. Modern pharmacological studies have shown that *F. magnoliae* has an astringent effect, which can improve local blood circulation and promote the absorption of secretions, thereby diminishing inflammation, clearing nasal passage, and relieving or eliminating symptoms (Tao, 1996); it also has an anti-allergic effect, which can effectively fight against the nasal itching, sneezing, runny nose, and other symptoms caused by allergic rhinitis (Wen-bin et al., 2002). *C. minima* is the whole dry plant of the *C. minima* (L.) A. Br. et Aschers., which belongs to the Compositae plant family, it has the effects of dispersing wind-cold, clearing nasal passage, and relieving cough. Its volatile oil has an anti-inflammatory effect (Ren-an et al., 2001, 2006; Wen-kui et al., 2000). *M. haplocalyx* is the dry aerial part of *M. haplocalyx* Briq., which belongs to the Lamiaceae plant family. Its main composition is volatile oil, and the main active ingredient in the volatile oil is menthol. In this study, the anti-inflammatory effect of a compound containing these three herbs was studied, thus providing the basis for the development of the compound.

MATERIALS AND METHODS

Reagents and apparatus

F. magnoliae (Anhui Yiyuan Bio-Engineering Co., Ltd); *C. minima* (Anhui Shuzhong Pharmaceutical Co., Ltd.); *M. haplocalyx* (Anhui Shuzhong Pharmaceutical Co., Ltd); prednisone (Hubei Xianhe

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Pharmaceutical Co., Ltd);other reagents were all of analytical grade; DHG-101-3A electro thermostatic blast oven (Gongyi Yuhua Instrument CO., Ltd); volatile oil extractor (Beijing Midwest Technology Co., Ltd); electronic balance (Beijing Sartorius Instrument System Co., Ltd).

Animals

National Institutes of Health (NIH) strain mice (50) of either sex, weighing 18 to 22 g were provided by the Laboratory Animal Center of the China Medical University. Sprague Dawley (SD) rats (80), half male and half female, weighing 130 to 150 g were provided by the Laboratory Animal Center of the China Medical University.

Extraction of volatile oil

F. magnoliae, *C. minima* and *M. haplocalyx* herbs were crushed, and a total of 200 g of three kinds of powders were weighed at a certain proportion (2:1:1). Volatile oil was extracted using the determination method A as prescribed in the appendix XD of the Volume I of the "Chinese Pharmacopoeia" 2010 edition. After continuous reflux extraction for 8 h, 2.6 ml of oily matter was obtained, which was prepared in certain concentrations later when administering (Wei et al., 2010).

Xylene-induced ear inflammation experiment in mice

Healthy male mice (50) were randomly divided into blank control, positive control (prednisone, 10 mg/kg), compound low-dose, compound medium-dose, and compound high-dose groups. Animals in each group were continuously administered the compound once daily for 5 days. 0.5 h after the last administration on the fifth day, 30 µl of (15 µl for each the outer and inner ears) xylene was applied to the right ear of mice to induce inflammation; xylene was not applied in the left ears, which served as the controls. 1 h after the induction of inflammation, mice were sacrificed, ear pieces at the same site of the left and right ears were removed with a 8 mm-diameter puncher, and the swelling inhibition rate was calculated taking the weight difference between the left and right ear pieces as the degree of swelling (Liu-ying et al., 2005; Maria et al., 2010).

Egg white-induced paw swelling experiment in rats

healthy SD rats (40) weighing 130 to 150 g were randomly divided into 5 groups, namely blank control, positive control (prednisone), compound low-dose, compound medium-dose, and compound high-dose groups according to their body weights. Animals in each group were intragastrically administered the compound twice per day for 5 consecutive days. Before the experiment, paw volume of each rat was determined three times using the paw volume determination method, and the average value was taken as the normal paw volume of every rat before administration. 30 min after the last administration, right rear paw of each rat was subcutaneously injected with 0.1 ml of 10% fresh egg white saline solution to induce inflammation.

5, 30, 60, 120, and 180 min after the induction of inflammation, the volume of right rear paw of each rat was determined, respectively using the same method, change in value of the right rear paw volume of each rat before and after the induction of inflammation was calculated, and the anti-inflammatory effect of the drug was estimated by the degree of swelling (Shu-yun et al., 2002;

Shen et al., 2008).

Cotton pellet granuloma experiment in rats

Healthy SD rats (40) were randomly divided into 5 groups according to their body weights. On the first day, rats in each group after administration of ether anesthesia and routine disinfection were anesthetized, under sterile conditions, the left and right armpits of these rats were separately implanted with a sterilized cotton pellet (weight 50±1 mg, high-pressure sterilized, each added with 1 mg/0.1 ml of ampicillin, and dried in the 50°C oven). The compound was administered once daily for 7 consecutive days, on the 8th day, the rats were sacrificed by cervical dislocation, and the cotton pellets were removed, and placed in a 60°C oven for 12 h, and were weighed by subtracting the weights of the original cotton pellets, and the net weights of granulomas were obtained (Shu-yun et al., 2002; Alfreda and Takayuki, 2010; Ji-Yan et al., 2012).

Statistical methods

Experimental results were analyzed using Statistical Package for Social Sciences (SPSS) software version 13.0. T-test was used for the mean comparison between the two groups. Multiple pairwise comparisons were done using analysis of variance.

RESULTS AND DISCUSSION

Results of xylene-induced ear inflammation experiment in mice

The results of the xylene-induced ear inflammation experiment in mice are shown in Table 1. The results revealed that the compound medium- and high-dose groups significantly inhibited xylene-induced ear swelling in mice ($P<0.05$).

Results of egg white-induced paw swelling experiment in rats

The results of the egg white-induced paw swelling experiment are shown in Table 2. The results revealed that: compared with the blank control group, compound low-, medium- and high-dose groups significantly inhibited paw swelling 30 min after the induction of inflammation ($P<0.01$); compound medium- and high-dose groups had inhibitory effects on degree of paw swelling 60 min after the induction of inflammation.

Results of cotton pellet granuloma experiment in rats

The results of the cotton pellet granuloma experiment are shown in Table 3. The experimental results revealed that: the granuloma weights were significantly lowered in the high-dose compound group ($P<0.01$), granuloma weights were also markedly lowered in the medium-dose compound group ($P<0.05$).

Table 1. Effect of the compound on xylene-induced ear swelling in mice ($\overline{x}\pm S$).

Group	Dose (ml/kg)	Number of animals	Degree of ear swelling (mg)	Inhibition rate (%)
Blank control	-	10	21.4±3.87	-
Prednisone	10mg/kg	10	8.9±3.73**	58.14
Low-dose	0.02	10	20.8±4.13	2.80
Medium-dose	0.04	10	18.2±3.96*	14.92
High-dose	0.08	10	16.3±4.51*	23.82

Comparison with the blank control group, *P<0.05; **P<0.01

Table 2. Effect of the Compound on Egg White-Induced Paw Swelling in Rats ($\overline{x}\pm S$)

Group	Normal paw volume (ml)	Degree of swelling at different time periods (ml)				
		5 min	30 min	60 min	120 min	180 min
Blank control	1.78±0.12	0.25±0.12	0.72±0.16	0.41±0.13	0.19±0.12	0.11±0.09
Prednisone	1.87±0.21	0.14±0.11	0.27±0.13**	0.21±0.15*	0.12±0.15	0.03±0.03
low-dose	1.85±0.13	0.17±0.23	0.49±0.13**	0.31±0.16	0.17±0.17	0.07±0.05
medium-dose	1.79±0.15	0.15±0.24	0.41±0.20**	0.27±0.16*	0.15±0.06	0.05±0.04
high-dose	1.82±0.17	0.14±0.16	0.32±0.24**	0.23±0.14*	0.14±0.08	0.05±0.05

Comparison with the blank control group, *P<0.05; **P<0.01

Table 3. Effect of the Compound on Cotton Pellet Granuloma in Rats ($\overline{x}\pm S$).

Group	Number of animals	Dose (ml/kg)	Weight of granuloma (mg)
Blank control	8	-	67.8±6.40
Prednisone	8	10mg/kg	35.6±3.50**
low-dose	8	0.02	51.3±5.20
medium-dose	8	0.04	44.7±2.50*
high-dose	8	0.08	40.3±3.10**

Comparison with the blank control group, *P<0.05; **P<0.01

DISCUSSION

At present, volatile oils of single herb medicines have been widely studied (Liu-ying et al., 2005; Maria et al., 2010; Shu-yun et al., 2002; Tian-qin et., 2006; Zhi-gang et., 2005; Guang-liang et., 2001), but studies on the compound of the three herbs are scanty. In this paper, the volatile oil from the three herbs was studied in general. *F. magnoliae* is pungent in flavor, warm in nature, and acts on the lung and stomach channels, which has a favorable nasal passage clearing effect, a good therapeutic effect on nasal diseases, and can help stop headaches. *C. minima* is pungent in flavor, and warm in nature, it acts on the lung channel, clears the nasal passage, facilitates nine orifices, and expels wind phlegm. Supplemented by the *M. haplocalyx* aroma, it can also relieve stuffiness. Volatile oil of the three traditional Chinese medicines was extracted to make the nasal spray, the spray is easy to use, with smaller droplets and large functioning area, can evenly dispersed in the nasal cavity, difficult to drain, and highly bioavailable. The method used in this study is simple and practical, which includes acute and chronic inflammatory animal models, experimental results also showed the inhibitory effect of the compound on both the acute and chronic inflammations, and can significantly reduce mouse xylene-induced ear swelling and rat carrageenan induced paw swelling and a significant reduction in granuloma weight in rats. This is consistent with the effect of single herb medicine reported in other literature, thus providing the reference for further study of the compound. However, its mechanism to play anti-inflammatory effects remains to be further studied.

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Full Length Research Paper

The effect of honey gel on cesarean incision pain: A triple blind clinical trial

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Honey is considered as a remedial in the Qur'an. Today, honey is used in wound healing and pain relief. This study was designed to assess the effects of honey gel on postoperative pain and analgesic need after cesarean section. This triple blind randomized clinical trial was conducted on women who underwent cesarean section in Imam Ali Hospital, Iran. Patients were categorized into three groups: drug (37 patients), placebo (38 patients), and control (54 patients) by random allocation. Patients in the drug group received 25% local honey gel, while placebo group received similar gel without honey twice a day for 14 days. No intervention was given in control group. Pain was measured using visual analogue scale on the 7 and 14th day after surgery. Data were analyzed using Chi-square and analysis of variance (ANOVA). The mean pain intensity did not differ significantly between groups on the 1st day, but on the 7th day it was 14.44, 26.41 and 25.73 (P=0.01), and on the 14th day it was 0.27, 5.84 and 4.07 (P=0.02) in the drug, placebo and control groups, respectively. The need for analgesics in the first 10 days was 11.5% in drug group, 62.6% in placebo group and 45.9% in control group which was significantly different (P=0.02). Until the 14th day, no patient needed analgesics in the drug group while 40 and 60% of patients in placebo and control groups required analgesic, which did not show a significant different (P=0.09). Honey gel was effective in cesarean wound pain and in reducing the need for analgesics.

Key words: Cesarean section, pain, wound healing, wound infection, honey.

INTRODUCTION

Pain is one of the most important problems in postoperative cares which has received a lot of attention in recent years. Today, pain is considered as the fifth vital sign (Khorgami et al., 2009). Postpartum period is associated with several problems due to the physical and mental stresses of which one of it is pain. Pain may affect the positive sense of being a mother and infant care, and its consistency may lead to a phobia of sexual contact and loss of relaxation and prepare the ground for depression

(Yerbi, 2000). One of the most important causes of pain after delivery is cesarean wound pain which is an important concern for most women (Pillitteri, 2003). Cesarean is one of the most important gynecology surgeries, and the most common surgery today (Scott et al., 2002). Women who undergo cesarean section should be able to take care of their infant besides tolerating cesarean wound pain and the postoperative recovering time. Thus, they required intensive cares (Pillitteri, 2003; Samadi et al., 2010).

The most prevalent treatments for analgesia are narcotics (Fredman et al., 1999; Vanessa et al., 2002), oral analgesics like nonsteroidal anti-inflammatory drugs (NSAIDs), codeine-containing products and acetaminophen

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(Noori and Al-Wailli, 2001). Narcotics cause sedation in mother and disturb the relationship between the mother and her infant (Samadi et al., 2010). Moreover, they slow down intestinal motility and lead to constipation, and are also secreted in the breast milk (Fredman et al., 1999; Vanessa et al., 2002). NSAIDs are effective in pain relief, but they have considerable side effects, particularly on the digestive tract (Pillitteri, 2003; Cambitizi et al., 2000). In order to reduce the side effect, methods of local analgesics, including local anesthesia (Fredman et al., 1999; Ke et al., 1998) and herbal ointment (Samadi et al., 2010) have been considered for pain relief of cesarean wound.

Today, the use of non-classic or traditional medicine treatments is raising and Egyptians have mentioned the medical uses of honey in their 2000 BC documents (Namias, 2003). In the "Holy Qur'an" also, the remedial effects of honey have been noted (Qur'an, 2003). In recent years, studies on animals (Molan, 2001a, b; Sakhavar and Khadem, 2008) have discussed its beneficial effects on skin wound healing, in some clinical trials (Vardi et al., 1998) treating burning (Subrahmanyam, 1996) and wound infection after cesarean (Al-Waili and Saloom, 1998; Chang and Cuellar, 2009). Honey has antimicrobial effect (Yilmaz et al., 2009; Sazegar et al., 2011; Oluwatosin et al., 2000) and affects wound healing and pain relief (Subrahmanyam, 1996; Molan and Betts, 2004; Mphande et al., 2007). The reasons may be its high osmolality, acidity, presence of inhibitors like hydrogen peroxide, flavonoids and phenolic acids (Nagai et al., 2001). Honey reduces inflammation, edema and wound exudation (Molan, 2001b; Tonks et al., 2001). It improves wound healing through stimulation of angiogenesis, epithelialization and granulation, and clean the wound from bacteria so as to prepare an appropriate condition for wound healing (Ndayisaba et al., 1993).

Previous studies have shown that local application of honey has remarkable effects on wound healing, treatment of wound infection (Al-Waili et al., 1999; Phuapradit et al., 1992), pain relief and their irritations (Al-Waili and Saloom, 1999; Mphande et al., 2007; Ozlugedik et al., 2006).

Considering the importance of pain relief and complications of cesarean wound in postpartum period and improving mothers' health, and the fact that honey is an easily accessible and cheap material, this study was designed to assess the effects of honey on postoperative pain in cesarean section. Based on previous reports, it seems that there has been no study about the effects of honey on pain of cesarean wound measured by visual analogue scale (VAS).

MATERIALS AND METHODS

After the written approval of the Ethical Committee of Mazandaran University, this triple blind clinical trial (for intervention and placebo group so that, all participants, investigators and statistical analyzers were not aware of participants' groups and only the head of research

research was aware of groups) was conducted on 132 women who fitted the inclusion criteria in Imam Ali Hospital (in the North of Iran) between 2010 and 2011. The inclusion criteria included education level at least fifth grade class of primary school, gestational age 37 to 42 weeks based on the last menstrual period (LMP) or sonography, Pfannenstiel skin incision and had informed consent to enroll in the study, the maximum time of rupture of membrane was 12 h and health of infants. The exclusion criteria included record of medical and obstetric history, need for transfusion during surgery, and cesarean duration more than 60 min, smoking and diseases or drugs which may affect wound healing.

Based on the study by Mphande et al. (2007), the sample size was calculated considering the confidence level (z_1) of 95%, study power (z_2) of 95%, improvement rates of 85% in patient and 50% in control group, and also considering loss to follow up to 10%; at least 40 patients were considered for each group. In order to gather required sample size, first, fitted persons were targetedly selected and after studying the information form, giving informed consent were randomly allocated to the three groups of drug, placebo or control using permuted block randomization.

The main drug (honey gel) and placebo were prepared by pharmacist. The types of honey were assessed and compared for components including reducing carbohydrates, sacarose, fructose and glucose and their ration, defining invert sugar, humidity percentage, microscopic evaluation for pollen and finally the most qualified honey (from coriander and Goat's- thorn flowers) was selected. Honey gel was prepared on the basis of a formulation to do this. The combination of honey, glycerin, crabapple, methylparaben, triethanolamine, propyl paraben, and distilled water were considered as gel formation. First, water was deionized and autoclaved, crabapple was added and was held for 24 h in a condition like laminar flow, then they were mixed (400 min^{-1}) and other materials were added. Meanwhile, the amount of inverted sugar was measured for standardization. The amount of honey in this gel was 25% based on the defined concentration in previous studies (Mphande et al., 2008). In order to prepare placebo with similar form, consolidation colour and odour, all mentioned materials except honey were used. The final products of honey gel and placebo were submitted to the laboratory to confirm their disinfection.

Data were collected using case selection form (inclusion and exclusion criteria), personal and current pregnancy information form, postoperative form, daily drug consumption form (gel, analgesics and antibiotics), physical activities and complications in cesarean wound site (burn, pruritus, redness and warming in wound). Pain intensity was measured using VAS. VAS is a standard and valid tool (Huskisson, 1974) and its reliability is also confirmed by r=0.90. The degree of mothers satisfaction was measured by a question (to what extent have you been satisfied with using the ointment) arranged on a Likert scale (very satisfied-satisfied-no different-dissatisfied-very dissatisfied).

Patients received a supposition of diclofenac 50 mg in the first 24 h and if pain still existed, they received 25 to 50 mg pethidine intramuscularly. Based on the pain, NSAIDs were prescribed in the following days.

All forms were filled by a researcher in the first 24 h after the cesarean section in the women ward. The required training regarding care of cesarean wound and sutures, personal hygiene and nutrition were given face to face. Moreover, pain intensity was measured using VAS in the supine position.

Then, in both the drug and placebo groups, a packet of coded gel was given to the mothers and its instruction was explained. The mothers had to first wash their hand and suture site, then rub the gel over the sutures twice a day (12 ± 2 h) for 14 days, and leave it open for 15 min. They were instructed to complete the check list of the instructions every day. The control group did not receive any drugs and only underwent control visits. Pain intensity was measured on the 7 and 14th days using VAS in all participants.

Moreover, the completed check list by the mothers including physical activities, complication in wound site (burn, pruritus, redness and warming in wound) and drug consumption (gel, analgesics and antibiotics) were assessed.

All participants were operated by a gynecologist with 20 years of experiences. The cesarean duration was less than 25 min and the incision length was less than 12 cm in all patients; all the three groups were similar regarding these two variables.

After data collection, they were coded and analyzed using Statistical package for Social Sciences (SPSS) software version 16. The cases under the study were described using descriptive statistics. Qualitative variables in three groups were compared using Chi-square test. Quantitative variables in three groups were compared using one-way analysis of variance (ANOVA). The significance level was considered as $P < 0.05$.

RESULTS

One hundred and forty-six (146) women were included in the study. After excluding some cases in each group due to intervening factors, finally 37 cases in the drug group, 38 cases in placebo group and 54 cases in control group were studied (Figure 1). The age of patients in the three groups did not differ significantly (27.77 ± 4.97, 26.57 ± 4.88 and 26.14 ± 6.06 years, respectively; $P > 0.05$). Moreover, there was no significant difference between studied groups regarding the education level of patients, the mother's job, gravidity and type of cesarean (emergent, elective) (Table 1).

During follow up period, pain intensity decreased significantly in all the three groups from first stage (day 1) to third stage (day 14) of the study (Table 2).

The mean pain intensity in cesarean wound site did not differ significantly on the 1st day between groups, but on the 7th day it was 14.44, 26.41 and 25.73 ($P = 0.01$), and on the 14th day it was 0.27, 5.84 and 4.07 ($P = 0.02$) in groups drug, placebo and control, respectively (Table 2).

All participants in the three groups have consumed milk, meat, fruit, vegetables and cereals each day. The need for analgesics in the first 10 days in the drug, placebo and control groups were 11.5, 62.6 and 45.9% which was significantly different ($P = 0.02$). Until the 14th day, none of the patients in the drug group consumed analgesics, while 40% of patients in placebo group and 60% of cases in control group needed analgesics. These differences were not statistically significant ($P = 0.09$), (Figure 2). The satisfaction rate in the drug group (61%) was significantly more than placebo (17.01%) and control (22%) groups ($P < 0.001$).

Regarding wound complications, there was less wound burn and pruritis in the honey group as compared to the other groups, but this difference was not significant ($P = 0.13$). Redness ($P = 0.001$) and warming ($P = 0.003$) were significantly less prevalent in the honey group than those in the other two groups.

DISCUSSION

In this study, the pain intensity was measured using VAS which showed that on the 7 and 14th days after cesarean, it was significantly lower in the honey group as compared to placebo and control groups.

Mphande et al. (2007) in a study compared the effects of honey and sugar ointment on surgical wound healing, and concluded that in the group with honey ointment the surgical wounds were healed in a shorter period of time. After 3 weeks, 86% of patients treated with honey ointment versus 72% with sugar felt no pain in the wound site (Mphande et al., 2007). Moreover, a study by Subrahmanyam (1996) showed that honey ointment decreases the required time for wound healing and pain intensity as compared to silver sulfadiazine. These researchers reported that topical application of honey has antioxidant effects and reduce the lipid peroxide serum levels, and leads to better wound healing (Subrahmanyam, 1996). In a study by Omidvari et al. (2011), topical use of honey as compared to daily washing did not have any specific effect on the process of healing in dermatitis related to the breast cancer radiotherapy, nor did it reduce pain intensity in the first 2 weeks of intervention (Omidvari et al., 2011). Meanwhile, a limited number of studies have shown that the use of topical honey leads to pain in the first 12 to 20 min (Subrahmanyam, 1996) which is in conflict with our study. The observed difference may be due to the types of wound. These researchers used honey for infected and inflammatory wounds which may have led to irritation and sensitization of nociceptors and caused pain. But in our study, honey gel was used for uninfected and clean wounds, which led to decrease in pain. Moreover, the honey type and quality of honey may also be effective.

In tissue damage and its healing process, reactive oxygen species (ROS) is produced which is an agent of inflammation and tissue damage. On the other hand, the inflammation itself produces prostaglandins and causes pain, edema and exudation in wound site. It is interesting that by increasing the production of phagocytes, honey restricts the production of ROS in tissue which subsequently results in decrease in pain, edema and exudation (Tonks et al., 2001). Honey through increasing the number of lymphocytes B, T, phagocytes and stimulation of monocytes leads to production of cytokines, interleukins 1 and 6 and tumor necrosing factor. These agents have antimicrobial effects and activate the immune system of the body (Tonks et al., 2001). Meanwhile, the existing antioxidant in honey inhibits concentration of free radicals, which are responsible for its anti-inflammatory effects (Henriquins et al., 2006). Its anti-inflammatory properties through reducing edema, exudation and improving the blood flow may reduce pain in wound site (Molan, 2001b). Results of the present study showed that wound complications like burning, pruritus and redness were lower in the honey group than the other two groups, which imply better wound healing and lower irritating symptoms and less pain. The results of a study by Ozlugedik et al. (2006) showed that pain intensity measured by VAS after tonsillectomy
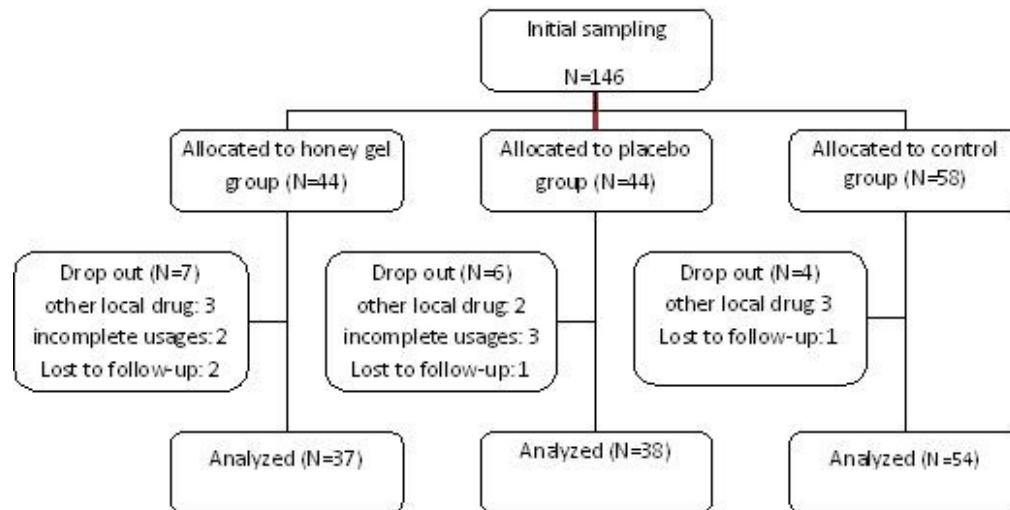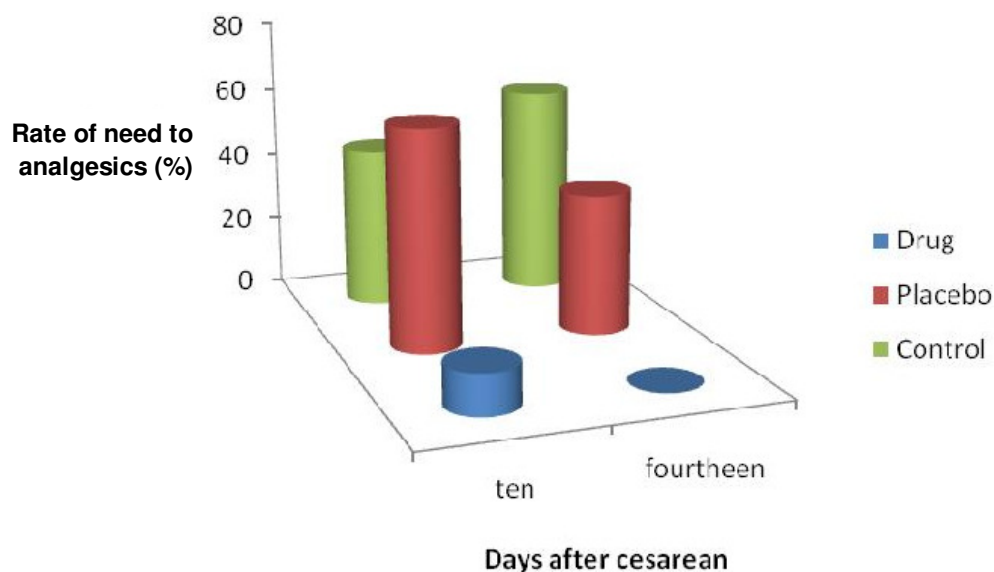
Figure 1. Flow diagram of study.

Figure 2. Frequency of analgesic use for 10 and 14 days after surgery in three studied groups.

and the need for analgesics were significantly lower during 14 days after surgery in the honey plus acetaminophen group as compared to acetaminophen alone. Oral honey has an important role in decreasing acute pain and inflammation of wounds through decreasing prostaglandin E2, F2α and thromboxane B2 (Ozlugedik et al., 2006). In this study, besides reducing pain intensity in wound site, the need for analgesic was significantly lower in honey group in comparison with those in the other groups. None of the cases took analgesic up to the end of the 2nd week, but in the other groups the need for analgesics continued, which may be an evidence for decreasing wound pain in honey group. Several studies have illustrated that honey like other

topical antibacterial agents is more effective in healing infected (Al-Waili and saloom, 1999; Ingle et al., 2007) and uninfected wounds and in decreasing pain intensity (Subrahmanyam, 1996; Mphande et al., 2007).

Honey can inhibit the growth of 60 types of bacteria like airborne, non-airborne, negative and positive gram stain (Molan et al., 1992) so that no living organism is able to live in honey. Its anti bacterial effects and the presence of substance called glunas in honey accelerates healing of tissue lesions (Ashtiyani et al., 2011).

The satisfaction rate also was greater in honey group as compared to that in the other two groups. This may be due to the decrease in pain, inflammation, irritating symptoms of wound healing and rapid relief. Researchers

Table1. Frequency of some variables in three groups.

Variable	Drug group n (%)	Placebo n (%)	Control n (%)	P-value
Mother`s education				
Primary school	11 (29.7)	18 (21.1)	18 (42)	
Guide school	15 (40.5)	20 (52.6)	28 (52.8)	0.25
University	11 (29.7)	10 (26.3)	7 (13.2)	
Mother`s job				
Housekeeping	28 (75.5)	30 (78.9)	50 (92.6)	
Occupied	9 (24.3)	8 (21.1)	4 (7.4)	0.06
Father`s education				
Primary school	3 (8.1)	5 (13.2)	11 (20.4)	
Guide school	23 (52.1)	22 (60.6)	35 (64.8)	0.2
	11 (29.7)	10 (26.3)	8(14.8)	
Cesarean type				
Elective	10(27.02)	13(34.21)	21(38.88)	
Emergent	27(72.97)	25(65.78)	33(61.11)	0.14
Antibiotic consumption				
Regular	32 (88.9)	34 (94.4)	48 (88.9)	
Irregular	1 (11.0)	2 (5.6)	6 (11.1)	0.1
Numbers of pregnancy				
1	15 (40.5)	24 (63.2)	33 (61.1)	
2	22 (59.5)	14 (36.8)	21 (38.9)	0.22

Table 2. Comparison of pain intensity in cesarean wound in first 7th and 14th days after surgery in three studied groups.

Group Variable	Drug n (mean ± SD)	Placebo n (mean ± SD)	Control n (mean ± SD)	p-value ANOVA
Pain intensity 24 h after cesarean	37 (78.91 ± 17.44)	38 (78.55 ± 20.95)	54 (82.7 ± 17.68)	0.6
Pain intensity 7th day after cesarean	37 (14.44 ± 15.20	38 (25.73 ± 18.70)	54 (26.41 ± 21.31)	0.01
Pain intensity 14th day after cesarean	37 (0.27 ± 1.66)	38 (4.07 ± 8.99)	54 (5.8 ± 12.47)	0.02

also found similar findings in previous studies (Ingel et al., 2007). Limitations of this study were: (1) clinical criteria were used, (2) there was no pathologic examination and (3) various concentrations of honey were not compared.

Conclusion

The current study has evaluated the usefulness of honey in postoperative pain relief and wound healing in patients undergoing caesarean section. The strength of the study lies in blinding and objective assessment of pain scores. This paper is interesting, because of the fact that honey is practically devoid of any side effects.

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Full Length Research Paper

Efficiency of intrathecal glial cell line-derived neurotrophic factor on nitric oxide and nitric oxide synthase activity in rat with neuropathic pain

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The aim of this study was to investigate the efficiency of intrathecal glial cell line-derived neurotrophic factor (GDNF) on nitric oxide (NO) and nitric oxide synthase (NOS) activity following a spinal nerve ligation (SNL) of male Sprague Dawley rats. The rats were randomly divided into four groups: normal (control), sham-operated, SNL (SNL followed by a physiological saline injection into the subarachnoid space), and GDNF (SNL followed by a GDNF injection into the subarachnoid space). Each group was divided into three subgroups (n = 10). The rats in each subgroup were euthanised 3, 7, and 14 days after the operation. Rat behaviour was evaluated before euthanising, and the ipsilateral spinal cords were harvested after euthanising to determine the NO content and NOS activity. Compared with the control and sham-operated groups, the NO content and NOS activity in the SNL group increased significantly 3 days after the operation; this increase was maintained until 14 days after the operation ($P < 0.01$ or 0.05). A significant decrease was observed in the NO content and NOS activity in the GDNF group compared with the SNL group. The decrease continued until 14 days after the operation ($P < 0.01$ or 0.05). The results indicated that the NO and NOS activity in the rat spinal cord are associated with SNL-induced neuropathic pain. The decreased neuropathic pain from the intrathecal GDNF is correlated with the decrease in NO content and NOS activity in the spinal cord.

Key words: Glial cell line-derived neurotrophic factor, neuralgia, nitric oxide, nitric oxide synthase.

INTRODUCTION

Glial cell line-derived neurotrophic factor (GDNF) is a small protein that was isolated and purified from the mouse glial cell line B49 in 1993 (Lin et al., 1993). GNDF has been proven to be closely associated with neuropathic pain owing to its nutritional and improved effect on the primary afferent neuron regeneration, as well as its role in the damage sensory formation of the spinal dorsal horn (Boucher et al., 2000; Wieseler et al., 2004; Airaksinen et al., 2006; Ricart et al., 2006). A number of studies demonstrate that in the nociceptive information delivery process, nitric oxide (NO), as a messenger, is involved in the pain regulation of the

peripheral and central nervous system at different levels, particularly in the pain regulation of the spinal cord.

Increasing evidence shows that nitric oxide synthase (NOS) inhibitors have a significant anti-nociceptive effect, and that spinal cord plasticity based on the NO synthesis system plays an important role in the maintenance and occurrence of pain following a nerve injury (Meller et al., 1992, 1994; Yaksh, 1999). Previous study findings show that a subarachnoid injection of GDNF can significantly reduce the neuropathic pain, mechanical hyperalgesia, and cold-induced persistent pain in rats (Jia et al., 2009). The mechanism of NO and NOS effects in the spinal cord is unclear. The aim of this study was to induce a rat neuropathic pain model through spinal nerve ligation (SNL) and to observe the changes in NO content and NOS activity to investigate the GDNF mechanism on

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neuropathic pain.

MATERIALS AND METHODS

Animal selection and grouping

Healthy male Sprague Dawley rats aged six weeks and weighing 180 to 200 g were provided by the Vital River Laboratory Animal Technology [Beijing, China; No. SCXK (Jing) 2007 to 2008]. All procedures were approved by the Animal care committee (Ningbo University, Ningbo, China) and were in accordance with the Chinese law on animal experiment. The rats were housed in sawdust-lined plastic boxes, with five rats per box, natural illumination, and free access to food and water. The rats were randomly assigned to four groups: normal control, sham-operated, SNL (SNL followed by a physiological saline injection into the subarachnoid space), and GDNF (PeproTech, Inc., Rocky Hill, NJ, USA) (SNL followed by a GDNF injection into the subarachnoid space). Each group was further divided to three subgroups (n = 10) according to the time at which the rats were euthanised: 3, 7, and 14 days post-surgery. After euthanising, the spinal cord tissue from the affected side of the rats was harvested to measure the NO content and NOS activity.

Preparation of the neuropathic pain rat models

The SNL rat models were established according to the methods reported by Kim and Chung (1992). Following an intraperitoneal injection of 1% sodium pentobarbital (40 mg/kg), the rats were placed in a ventral position such that the Ping iliac spine (L6) was in a horizontal position. A 1.5 cm incision in the skin was made above and below the midline of the back. The left paravertebral muscles were bluntly dissected between L4 and S2 to expose the L6 transverse process and sacral cornu. Part of the L6 transverse process was removed to expose the L4 to 5 spinal nerve. The L5 spinal nerve was isolated and tightly ligated using a 6 to 0 silk suture and the L6 spinal nerve was dissociated from the sacral cornu and ligated by performing a hemostasis and incision suture. The rats from the sham-operated group underwent a spinal nerve exposure without ligation. The same person performed all of the experimental procedures to maintain consistency.

Subarachnoid catheter

All the operations were carried out under sterile conditions according to previously reported methods (Storkson et al., 1996). Following an intraperitoneal injection of 1% sodium pentobarbital (40 mg/kg), the rats were placed in a prone position and a longitudinal incision was made from L5 to 6. A self-made guided needle was inserted vertically, positioned to the subarachnoid space with a clear sense of breakthrough and movement in the rat's tail, and the needle was inclined to the rat's head. A polyethylene catheter (PE-10) was introduced into the subarachnoid space through the self-made guided needle, and the catheter tip was positioned 3 to 3.5 cm near the lumbar enlargement of the spinal cord.

The distal end of the catheter was tunnelled subcutaneously to emerge at the neck. After implanting the intrathecal catheter, the rats displaying evidence of motor dysfunction were sacrificed. The location of the catheter tip was confirmed by pumping an intrathecal infusion of 20 µL 2% lidocaine to induce motor paralysis of the hind limbs within 30 min. The eligible rats were housed in sawdust-lined plastic boxes, with five rats per box, natural illumination, and free access to food and water.

Determination of the NO content in the spinal cord tissues

After the sacrifice, the vertebral lamina was removed and a 0.2 g spinal cord tissue from the lumbar intumescent segment was rinsed with cold physiological saline (0 to -4°C) and then placed into a tube containing cold physiological saline to prepare a 10% spinal cord tissue homogenate (pH 7.5, 0.025 mol/L sucrose, 0.05 mol/L Tris-HCl, 0.1 mmol/L ethylenediaminetetraacetic acid). The homogenate was centrifuged for 10 min at 6,000 r/min, and 500 µL supernatant was harvested. All procedures were performed at 0 to 4°C. All kits used in these procedures were supplied by the Nanjing Jiancheng Bioengineering Institute (Nanjing, China). Considering its active chemical nature, NO was converted into NO_3^- and NO_2^- in vivo. We determined the NO content by measuring the amount of NO_3^- and NO_2^-, expressed as µmol/g protein. The Lowry protein assay was used.

Determination of total NOS (TNOS) and inducible NOS (iNOS) activities

NO is biosynthesised endogenously from L-arginine and oxygen by NOS enzymes, and NO reacts with nucleophilic substances to produce coloured compounds. Total NOS activity was determined based on this principle. The constitutive NOS and iNOS activities were measured based on their different sensitivities to calcium. The NOS activity (U/mg protein) was calculated using the following equation: [(Absorbance$_{550}$ test - Absorbance$_{550}$ blank) / Molar absorption coefficient × Total volume of reaction solution / sample volume (µL) × 1 / (Coloured optical path × Reaction time)] / Protein content (mg/L).

Statistical analysis

All statistical analyses were processed using the SPSS 13.0 software (SPSS Inc., Chicago, IL, USA) and expressed as Mean ± standard deviation (SD). The normality test was conducted using the Kolmogorov–Smirnov test, one-way analysis of variance was employed for an intergroup comparison, and paired t test was used for intragroup comparisons. A P-value less than 0.05 was considered statistically significant.

RESULTS

Assessment of the NO content

Compared with the control and sham-operated groups, NO content was significantly increased in the SNL group on day 3 and remained so until 14 days after the operation ($P < 0.01$ or 0.05). In the GDNF group, NO content was significantly lower than in the SNL group, staying at this level until 14 days after the operation ($P < 0.01$ or 0.05). No difference was observed in the NO content between the sham-operated and GDNF groups (Table 1).

Assessment of the TNOS activity

Compared with the control and sham-operated groups, TNOS activity in the SNL group was significantly increased on day 3 and was maintained until 14 days after the

operation ($P < 0.01$). The TNOS activity was significantly lower in the GDNF group compared with the SNL group; this behaviour was also maintained until 14 days after the operation ($P < 0.01$ or 0.05). A significant difference in TNOS activity was observed between the sham-operated and GDNF groups ($P < 0.01$ or 0.05) (Table 2).

Assessment of the iNOS activity

Compared with the control and sham-operated groups, iNOS activity in the SNL group was dramatically increased on day 3 and continued to increase until 14 days after the operation ($P < 0.01$). Seven days after the operation, iNOS activity was significantly lower in the GDNF group compared with the SNL group, remaining at this level until 14 days after the operation ($P < 0.01$ or 0.05). No significant difference was observed in iNOS activities of the sham-operated and GDNF groups until 14 days after the operation ($P < 0.01$) (Table 3).

DISCUSSION

The present study demonstrated that NO content and NOS activity significantly increased in the SNL group on post-operation days, and that the intrathecal administration of GDNF significantly reduced the SNL-induced NO production and NOS activity in the spinal cord. GDNF, a member of the transforming growth factor beta superfamily, is the most active growth factor for motor neurons (Watabe et al., 2001; Malcangio, 2003; Paratcha et al., 2003; Dong et al., 2006; Honq et al., 2008). Jia et al. (2009) found that SNL rats experienced a 50% reduction in paw withdrawal threshold on the operated side and an increased number of paw lifts on a 5°C cold plate one day after the operation. These effects were still evident until 14 days after the operation. This behaviour indicated that SNL rats exhibit cold hyperalgesia. In our previous study, a subarachnoid injection of GDNF reduced the mechanical and cold hyperalgesia expression in the SNL rats at 3 and 5 days after the operation, respectively (Jia et al., 2009).

NO is a new type of non-classical neurotransmitter and messenger molecule that participates in the pain regulation of the peripheral and central nervous system in various ways. Results showed that the NO content increased significantly in the rats with SNL-induced hyperalgesia until 14 days after the surgery. These results are in accordance with the results of Mabuehi et al. (2003). These findings further prove that the NO produced in the spinal cord plays an important role in pain information transmission and hyperalgesia occurrence (Wang et al., 2001), although the mechanism through which this occurs is complex.

When the peripheral nerve impulse generated by a noxious stimulation is inserted into the spinal cord through the Aδ and C fibres, excitatory amino acids from the spinal cord dorsal horn neurons are precipitated to bind with N-Methyl-D-Aspartate (NMDA) and non-NMDA receptors to realise a continued polarisation of the spinal cord dorsal horn neurons. After the excitatory post-synaptic potential (EPSP) is formed, the Ca^{2+} channels are opened and the Ca^{2+} influx binds with calmodulin to activate the NOS. L-arginine reacts with Nicotinamide adenine dinucleotide phosphate (NADPH) and O_2 to produce NO+NADP+NADPH in the NOS presence. The NO produced rapidly diffuses in and out of the cells to activate soluble guanylate cyclase and generate monophosphate (cGMP), activating the protein kinase to increase the release of neurotransmitters, accumulate prostaglandin, and enhance the response of the NMDA receptors in postsynaptic neurons. Consequently, the spinal cord dorsal horn nociceptive neurons are excited, which, in turn induces pain (Li and Clark, 2001; Tao and Johns, 2002; Yoon et al., 2005). A large amount of NO harvested from persistent noxious stimuli can activate guanylate cyclase to produce more cGMP. The generated cGMP can penetrate into the deep dorsal horn to enhance the response of mass dynamic neurons to external stimuli (Lin et al., 1997).

This study confirmed that the intrathecal administration of GDNF significantly reduces the SNL-induced NO production in the spinal cord, thereby controlling the formation and development of the central nervous system sensitisation.

NOS is an important factor in limiting NO synthesis, and the *in vivo* biological role of NO mainly depends on NOS activity. Thus, measuring NOS is a key link in studying the biological effects of NO. The iNOS system has been described as calcium-insensitive, and is mainly found in macrophages and astrocytes. Under pain stimulation, the astrocytes in the spinal dorsal horn are vitalised by the iNOS, and the NO generation is significantly promoted. In this study, we found that the iNOS activity in the spinal cord was enhanced in the SNL-induced neurotrophic pain rats, accounting for 20% of the TNOS. Considering this calcium-insensitive characteristic, no difference was observed in the *in vitro* and *in vivo* measurement of iNOS activity. iNOS is expressed in activated microglia, and massive amounts of NO can activate astrocytes to produce prostaglandin, enhancing the excitability of the pain transmission neurons. As a result, an increased iNOS activity effectively promotes pain occurrence (Lui and Lee, 2004; Naik et al., 2006).

In this study, the neuronal presynaptic membrane was depolarised after the peripheral nerve injury; therefore, glutamate or NMDA was released into the synaptic cleft to bind with the NMDA receptor or other excitatory amino acid receptors. As the receptor channel opened, the Ca^{2+} influx coupled with calmodulin protein activated the NOS with the assistance of the NADPH. The L-arginine then reacted with NADPH and O_2 to produce NO, activating

Table 1. Effects of GDNF on nitric oxide contents in the spinal cord of SNL rats (μmol/g protein, Mean ± SD).

Group	3 days	7 days	14 days
Control (n=6)	3.26±0.14	3.22±0.12	3.30±0.17
Sham-operated (n=10)	3.11±0.38	3.37±0.38	3.32±0.19
SNL (n=10)	3.63±0.34**	3.71±0.30*	3.80±0.22**
GDNF (n=10)	3.28±0.22[#]	3.36±0.25[#]	3.48±0.28[##]

GDNF: glial cell line-derived neurotrophic factor; SNL: spinal nerve ligation; *$P < 0.05$, **$P < 0.01$ vs. sham-operated group; *$P < 0.05$, **$P < 0.01$ versus sham-operated group; [#]$P < 0.05$, [##]$P < 0.01$ versus SNL group.

Table 2. Effects of GDNF on total nitric oxide synthase activity in the spinal cord of SNL rats (U/mg protein, Mean ± SD).

Group	3 days	7 days	14 days
Control (n=6)	11.77±2.22	11.73±2.12	11.74±2.05
Sham-operated (n=10)	11.60±1.61	11.64±1.95	11.79±1.99
SNL (n=10)	15.95±1.85**	17.60±2.02**	17.90±1.79**
GDNF (n=10)	14.08±1.81**[#]	13.61±2.33*[##]	13.79±2.03**[#]

GDNF: Glial cell line-derived neurotrophic factor; SNL: spinal nerve ligation; *$P < 0.05$, **$P < 0.01$ versus sham-operated group; [#]$P < 0.05$, [##]$P < 0.01$ versus SNL group.

Table 3. Effects of GDNF on inducible nitric oxide synthase activity in the spinal cord of SNL rats (U/mg protein, Mean ± SD).

Group	3 days	7 days	14 days
Control (n=6)	2.25±0.53	2.21±0.54	2.15±0.59
Sham-operated (n=10)	2.52±0.60	2.52±0.66	2.41±0.44
SNL (n=10)	3.79±0.57**	3.84±0.69**	3.63±0.53**
GDNF(n=10)	3.16±0.96	2.84±0.55[##]	2.83±0.48**[##]

GDNF: glial cell line-derived neurotrophic factor; SNL: spinal nerve ligation; *$P < 0.05$, **$P < 0.01$ versus sham-operated group; [#]$P < 0.05$, [##]$P < 0.01$ versus SNL group.

the soluble guanylate cyclase to generate cGMP. The generated cGMP, as a secondary messenger, was involved in the protein phosphorylation, induced the c-Fos expression and switched on the related genes to participate in the pain or nociceptive transmission. The intrathecal administration of GDNF can reduce the SNL-induced NOS activity, thereby reducing the NO synthesis to alleviate the sensitisation of the spinal cord neurons and reduce the nociceptive information transmission to the central nervous system (Myung, 2006; Naik et al., 2006).

Conclusion

The intrathecal administration of GDNF can effectively relieve SNL-induced neuropathic pain. The mechanisms of this effect correlate to the decrease of NO content and NOS activity in the spinal cord.

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