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RESEARCH OPINIONS IN ANIMAL & VETERINARY SCIENCES

Effect of ethanolic leaf extract of *Nymphaea odorata* on biochemical and oxidative stress parameters of liver and pancreas in alloxan induced diabetic mice

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Abstract

Nymphaea odorata (*Nymphaeceae)* is a classical medicinal plant, has an *ayurvedic* importance in Indian continent. The study was carried out to find the effect of this plant on the level of DNA, RNA, protein, glycogen and cholesterol, lipid peroxide and activity of antioxidant enzymes, superoxide dismutase (SOD), catalase (CAT), reduced glutathione (GSH), glutathione-s-transeferase (GST) in liver and pancreas of control and alloxan-induced diabetic mice. Male Swiss albino mice weighing about 20-30 g were selected and divided into five groups as control, diabetes induced, glibenclamide treated and with *Nymphaea odorata* leaf extract of 300 and 600 mg/kg b.wt/day. The results indicated that there was a significant decrease in the level of DNA, RNA, protein and glycogen and in the activity of SOD, catalase, GSH, GST and increase in the level of TBARS, cholesterol in the group of alloxan treated mice. However, on treatment with ethananolic leaf extract of *Nymphaea odorata,* the level of DNA, RNA, protein, glycogen and activity of SOD, CAT, GSH and GST, TBARS and cholesterol were recovered significantly in the standard drug and plant extract treated groups. This study suggested that ethanolic leaf extract of *Nymphaea odorata* shows potent antioxidative property.

Keywords: *Nymphaea odorata*; *Diabetes mellitus*; Alloxan monohydrate; Biochemical contents; Oxidative stress

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Introduction

Diabetes mellitus is a metabolic disorder characterized by fasting hyperglycemia and alterations in carbohydrate, fat and protein metabolism, associated with absolute or relative deficiencies in insulin secretion and insulin action (Kameswara et al., 2005). Dehydration and loss of body weight was associated with *diabetes mellitus* (Pupim et al., 2005). The World Health Organization estimates that more than 220 million people worldwide have diabetes, the number will likely become double by 2030 (WHO, 2009). The plant such as *Nymphaea stellata* has antioxidative and antihyperglycemic property. The genus Nymphaea takes its name from the Greek word; numphe which means virgin or water nymphe and is reputed for the anti-aphrodisiac activity of its members.

Nymphaea odorata (water-lily) is known by common names such as fragrant water-lily, American white-lily and Alligator Bonnet amongst many others (Josh and Emily, 2002). It belongs to the family *Nymphaeaceae* which consists of six genera and seventy species (Trease and Evans, 1996) and grows in ponds, marshes and sluggish streams (Odey and Kingdersley, 1993). Compounds such as tannins (tannic acids and gallic acids, anti-microbial), alkaloids (nymphaerine and Nupharine) and glycosides (cardenolide and myrictrin) which are antiseptic, astringent and demulcent have been reportedly isolated from this plant (Trease and Evans, 1996). Although

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Nymphaea odorata plant has been used as a traditional plant treatment in *Ayurveda* medicine in India and North American continent as there are no reports on antioxidant study, it is necessary to carry out detailed scientific investigation on efficiency and mechanism of its action.

The liver plays a pivotal role in preserving glucose homeostasis. A constant glucose level is maintained by the major glucoregulatory hormones: insulin, glucagon, and catecholamines (Stone et al., 1985). Liver cirrhosis is frequently associated with alterations in carbohydrate metabolism, ranging from mild glucose intolerance (in 30 to 75% of patients) to overt diabetes (in about 15% of patients) (Pilkis et al., 1988). Pancreas is dual gland that controls the sugar metabolism. Hence, in the present study alloxan monohydrate is used for the induction of experimental diabetes and further the effect of ethanolic leaf extract of *Nymphaea odorata* was evaluated.

Materials and Methods

Plant material and extraction

The fresh leaves of the *N. odorata* were shade dried for six days and reduced to powder by using dry grinder. This powder was packed into soxhlet apparatus and extracted using absolute ethanol (40-50°C). The extraction was carried out for 38 hrs till the total extraction was achieved. The extract obtained was dried at 45°C in hot air oven till semisolid mass was obtained. The yield obtained was (4.5% w/w) and the extract was stored in a refrigerator at 4°C until used. Alloxan monohydrate was purchased from Sigma-Aldrich, St.Louis, USA. All other reagents used were of analytical grade.

Animals

Laboratory bred 3 to 4 months adult virgin male albino mice weighing about 20 to 30 g were used under standard animal housing condition (temperature controlled 25 ± 2ºC and 12 hrs light/dark cycle) with unlimited access to pellet diet Gold Mohar (Hindustan Lever Ltd., Mumbai) and water *ad libitum.* Animals were randomly divided into control and four treatment groups (distilled water vehicle served as control). Each group consists of 10 mice housed in separate polypropylene cages containing sterile paddy husk as bedding material. Animal studies in the work have been strictly performed as per the Institutional Ethical Committee (IACE) constituted under the guidelines of committee for purpose of control and supervision on experimental Animal (CPCSEA), Ministry of Environments, Government of New Delhi.

Induction of diabetes

Diabetes was induced in male Swiss albino mice by intraperitoneal administration of alloxan monohydrate in concentration of 150 mg/kg body weight dissolved in normal saline. Blood glucose was measured after 72 hour of alloxinisation by glucocard-01 mini glucometer (Accu-check sensor) of ARKAY Factory, Inc. Japan. Since alloxan is capable of producing fetal hypoglycemia as a result of massive pancreatic insulin release, mice were treated with 30 percent glucose solution orally at different time intervals after six hours of alloxan induction, and 5 percent glucose solution was kept in bottles in their cages for next 24 h to prevent hypoglycemia. Mice showing fasting blood glucose levels (>250 mg/dl) were selected for the study.

Treatment

Animals were divided into five groups of ten mice each. Normal untreated mice given only vehicle (distilled water). Diabetic control mice were given a single intraperitoneal dose of 150 mg/kg alloxan monohydrate. Diabetic mice were given a single dose of glibenclamide (500 mg/kg b.wt) by oral administration daily for 45 days. Diabetic mice were given a single dose of ethanolic leaf extract of *Nymphaea odorata* leaves (300 mg/kg b.wt and 600 mg/kg b.wt) by oral administration daily for 45 days. All the experimental animals were sacrificed by cervical dislocation on the 46th day of experiment. The liver and pancreases were dissected out, weighed to the nearest milligram in digital weighing balance (vibra) and were used for the biochemical and oxidative stress parameters such as DNA, RNA, protein, glycogen, cholesterol, and level of superoxide dismutase (SOD), glutathione (GSH), thiobarbituric acid reactive substances (TBARS), Glutathione-s-transferase (GST), catalase (CAT) respectively.

Biochemical Studies

The biochemical contents such as estimation of DNA and RNA was carried out as per the method described by Schneider (1957), protein by Lowry et al. (1951), glycogen by Carrol et al. (1956) and cholesterol by Abell et al. (1952).

Oxidative stress parameters

The liver and pancreas were thawed and homogenized in 10% w/v ice-cold 0.05 M potassium phosphate buffer (pH 7.4). 0.2 ml of the homogenate was used for TBARS estimation and 1.0 ml of the homogenate was mixed with 10% trichloroacetic acid (TCA) and centrifuged for tissue GSH estimation. The remaining homogenate was centrifuged at 40,000 × g for 60 min and the supernatant was used for estimations of superoxide dismutase (SOD) and catalase (CAT).The oxidative stress parameters such as GSH level was measured following the method of Ellman (1959), the product of the reaction between malondialdehyde

(MDA) and TBARS by Okhawa et al. (1979) were measured by a modified method of Esterbauer and Cheesman, (1990). SOD activity was determined by Kakkar et al. (1984), CAT activity was measured by Aebi (1974) and GST activity by Habig and Jakoby (1974).

Statistical analysis

Statistical significance between the control and experimental data were subjected to analysis of variance (ANOVA) together with Dennett's test ($P<0.05$).

Results

Biochemical Studies

The biochemical contents revealed that there was a significant decrease in the level of DNA, RNA, protein and glycogen in the mice treated with alloxan when compared with that of normal control with the exception of cholesterol. However, there was a significant increase in the level of DNA, RNA, protein and glycogen with reduction of cholesterol in the mice treated with alloxan along with glibenclamide and plant extract when compared with that of alloxan treated control (Table 1 & 2). Further, the results also indicated that there was a recovery in the level of DNA, RNA, protein, glycogen and cholesterol in the mice treated with alloxan along with glibenclamide and plant extract when compared with that of alloxan treated control mice.

Oxidative stress parameters

The oxidative stress parameters revealed that there was a significant decrease in the level of GSH, CAT, SOD, and GST and increase in the level of TBARS in the mice treated with alloxan when compared with that of normal control. However, there was a significant increase in the level of GSH, CAT, SOD, and GST in the mice treated with alloxan along with glibenclamide and plant extract when compared with that of alloxan treated control (Table 3 & 4). Further, the results indicate that there was a recovery in the level of GSH, CAT, SOD, and GST and decrease in the TBARS level in the mice treated with alloxan along with glibenclamide and plant extract when compared with that of alloxan treated control mice.

Table 1: Effect of ethanolic leaf extract of *Nymphaea odorata* on biochemical contents of liver in alloxan induced diabetic albino mice

Groups	Treatment (mg/kg/d)	Biochemical contents (µg/mg wet weight of tissue)				
		DNA	RNA	Protein	Glycogen	Cholesterol
I	Normal Control	1.96±0.06^{a}	3.68±0.02^{a}	224.30±2.42^{a}	6.78±0.35^{a}	10.30±0.30^{c}
II	Alloxan Control (150)	0.88±0.01^{d}	2.20±0.01^{d}	168.21±2.32^{d}	5.32±0.22^{d}	14.42±0.31^{a}
III	Alloxan+Glibenclamide (150)	1.68±0.03^{b}	3.38±0.03^{b}	198.34±1.32^{b}	6.61±0.10^{b}	9.89±0.28^{d}
IV	Alloxan+*N. odorata* (300)	1.20±0.03^{c}	2.34±0.03^{c}	176.32±4.42^{c}	6.11±0.13^{c}	12.13±0.42^{b}
V	Alloxan+ *N. odorata* (600)	1.22±0.01^{c}	2.48±0.04^{c}	182.33±3.37^{c}	6.48±0.22^{b}	12.88±0.38^{b}

Values are mean ± SEM of 10 animals; $^{a-d}$Different superscripts in a column differ significantly ($P<0.05$)

Table 2: Effect of ethanolic leaf extract of Nymphaea *odorata* on biochemical contents of pancreas in alloxan induced diabetic albino mice

Groups	Treatment (mg/kg/d)	Biochemical contents (µg/mg wet weight of tissue)				
		DNA	RNA	Protein	Glycogen	Cholesterol
I	Normal Control	1.88±0.03^{a}	3.42±0.03^{a}	212.20±4.02^{a}	6.52±0.33^{a}	10.20±0.30^{d}
II	Alloxan Control (150)	1.70±0.02^{c}	3.01±0.01^{d}	180.20±3.02^{d}	5.48±0.22^{d}	13.20±0.32^{a}
III	Alloxan+Glibenclamide (500)	1.82±0.05^{a}	3.38±0.02^{a}	205.10±1.06^{a}	6.48±0.22^{a}	9.88±0.20^{d}
IV	Alloxan+*N. odorata* (300)	1.62±0.01^{d}	3.22±0.02^{c}	188.22±2.12^{c}	6.14±0.30^{c}	12.11±0.42^{b}
V	Alloxan+*N. odorata* (600)	1.77±0.04^{b}	3.28±0.05^{c}	196.30±4.03^{b}	6.32±0.33^{b}	10.57±0.32^{c}

Values are mean ± SEM of 10 animals; $^{a-d}$Different superscripts in a column differ significantly ($P<0.05$)

Table 3: Effect of ethanolic leaf extract of *Nymphaea odorata* on oxidative stress parameters of liver in alloxan induced diabetic albino mice

Groups	Treatment (mg/kg/d)	Antioxidant	Oxidative stress by-products	Oxidative stress enzymes		
		GSH[1]	TBARS[2]	Catalase[3]	SOD[4]	GST[5]
I	Normal Control	10.68±0.10^{a}	0.22±0.02^{c}	168.21±3.21^{a}	46.51±1.32^{a}	4.82±0.08^{a}
II	Alloxan Control (150)	9.22±0.20^{d}	0.32±0.03^{a}	134.32±3.38^{d}	34.42±1.42^{d}	3.23±0.09^{d}
III	Alloxan+Glibenclamide (500)	10.22±0.10^{b}	0.20±0.02^{c}	152.28±2.11^{b}	38.88±2.31^{b}	4.24±0.07^{b}
IV	Alloxan+*N. odorata* (300)	9.68±0.12^{c}	0.28±0.03^{b}	138.31±2.28^{d}	36.77±3.10^{c}	3.41±0.08^{d}
V	Alloxan+ *N. odorata* (600)	9.88±0.13^{c}	0.26±0.03^{b}	142.11±3.22^{c}	38.61±2.66^{b}	3.82±0.06^{c}

Values are mean± SEM of 10 animals; $^{a-d}$Different superscripts in a column differ significantly ($P<0.05$); [1]µmole of glutathione (GSH)/mg protein; [2]nmoles thiobarbaturic acid (TBARS)/gm protein; [3]µmole of H_2O_2 protein; [4]SOD unit/mg; [5]Glutathione-s-transferase (GST) µmole/min/mg/protein

Table 4: Effect of ethanolic leaf extract of *Nymphaea odorata* on oxidative stress parameters of pancreas in alloxan induced diabetic albino mice

Groups	Treatment (mg/kg/d)	Antioxidant	Oxidative stress By-products	Oxidative stress enzymes		
		GSH[1]	TBARS[2]	Catalase[3]	SOD[4]	GST[5]
I	Normal Control	14.61±0.49[a]	0.32±0.03[b]	182.31±2.10[a]	42.31±2.21[a]	4.88±0.08[a]
II	Alloxan Control (150)	10.42±0.36[d]	0.36±0.02[a]	166.28±3.23[d]	38.23±3.45[d]	3.41±0.09[d]
III	Alloxan+Glibenclamide (500)	13.23±0.55[b]	0.28±0.02[c]	178.11±3.11[b]	40.22±.3.38[b]	4.33±0.07[b]
IV	Alloxan+*N. odorata* (300)	12.21±0.68[c]	0.31±0.03[b]	168.38±4.24[d]	38.81±3.22[d]	3.63±0.08[d]
V	Alloxan+ *N. odorata* (600)	12.82±0.46[c]	0.25±0.01[d]	172.32±2.88[c]	39.22±2.88[c]	3.98±0.09[c]

Values are mean± SEM of 10 animals; [a-d]Different superscripts in a column differ significantly (P<0.05); [1]µmole of glutathione (GSH)/mg protein; [2]nmoles thiobarbaturic acid (TBARS)/gm protein; [3]µmole of H_2O_2 protein; [4]SOD unit/mg; [5]Glutathione-s-transferase (GST) µmole/min/mg/protein

Discussion

The present study evaluates the effects of *Nymphaea odorata* on the biochemical and oxidative stress parameters in alloxan induced oxidative damage. The present results indicate that alloxan induced oxidative damage alters the biochemical and oxidative stress parameters damaging the insulin secreting β-cells of the pancreas resulting in decreased endogenous insulin release. Alloxan, a β-cytotoxin, induces *diabetes mellitus* in mice become hyperglycemic in a short period of time, followed by hepatic glucose overproduction (Milagro et al., 2000). Intraperitoneal administration of alloxan (150 mg/kg) effectively induced *diabetes mellitus* in normal mice.

Interestingly *Nymphaea odorata* leaf extract supplementation to mice was able to considerably reduce the alloxan induced oxidative damage showing its antioxidative potential. The antioxidative effect of *N. odorata* extract could be linked to more than one mechanism. The possible mechanism includes stimulation of oxidative stress enzyme activity and subsequent damage of reactive oxygen species (ROS) released after alloxinisation. In this context, a number of other plants such as *N. stellata* have also been reported to have antihyperglycaemic and insulin release stimulatory effect (Prince et al., 1998; Bhandarkar et al., 2004; Kaleem et al., 2006).

In the present study, there was a significant decrease in the levels of DNA and RNA in the liver and pancreas of mice treated with alloxan. The reduced level of DNA and RNA may be due to hyperglycemia induced activation of protein kinase- C (PK-C) (Koya et al., 1998) increased formation of glucose-derived advanced glycation end products (Brownlee et al., 1995). Alloxan induced DNA fragmentation in pancreatic islets and cell damage have been attributed to the production of toxic free radicals (Takasu et al., 1991).

However, there was significant recovery in the levels of nucleic acids with the possible treatment with plant extract. It may be due to action of plant secondary metabolite tannins and gallic acid (Trease and Evans, 1996) that reduces the ROS produced during alloxinisation. The level of protein and glycogen was decreased in the mice treated with alloxan. This may be due to the oxidative damage that might have increased oxidizable substrate (carbohydrate or lipid), and increase rate of autoxidation of substrate, decline in the antioxidant defence, or combination of all these processes (Halliwell et al., 1999).

The increased glycogen level in the treated diabetic mice may be due to increased level of insulin which has increased glycogenesis and decreased glycogenolysis or gluconeogenesis. Plants like *Momordica charantia* (Reyes et al., 2006), *Syzygium cordatum* (Musabayane et al., 2005) and *Brassica juncea* (Khan et al., 1995) increase the concentration of glycogen which is in accordance with our investigation. The increased insulin levels in diabetics will normalize the protein levels by increasing protein synthesis or decreasing protein glycosylation and protein degradation. The same was observed in this study with the treatment of ethanolic leaf extract of *Nymphaea odorata*. The protein level in liver and pancreas of diabetic mice which were lower than normal were increased after treatment with plant extract. This may be due to faulty glucose utilization causing hyperglycemia and mobilization of fatty acids from adipose tissue for energy purpose (Shih et al., 1997). The lipid changes associated with *diabetes mellitus* are attributed to increased flux of free fatty acids into the liver secondary to insulin deficiency or resistance (Solono et al., 2005; Chahil et al., 2006).This results in excess fatty acid accumulation in the liver, which is converted to triglycerides (Mooradian et al., 2009; Shih et al., 1997).

In the present study, there was a decrease in the GSH level. Under *in vivo* condition, GSH acts as an antioxidant and has been reported to be decreased in *diabetes mellitus* (Baynes et al., 1997). The decrease in GSH levels represents increased utilization of GSH due to oxidative stress (Anuradha et al., 1993). The normal GSH level was recovered upon the administration of *Nymphaea odorata* leaf extract due to action of plant secondary metabolites such as tannic acid and gallic

acid as reported by Trease and Evans (1996). Our study showed a significant increase in tissue TBARS in mice. The increased TBARS content of diabetic mice suggests the peroxidative injury may be involved in the development of diabetic complications. TBARS level in liver and pancreas were significantly lower in the plant extract treated group compared to the diabetic control rats. The above result suggests that the plant extract may exert antioxidant activities and protect the tissues from lipid peroxidation due to secondary metabolites such as alkaloids, tannins, flavonoids and cardiac glycosides present in the plant (Trease and Evans, 1996). In a similar report, Kamalakkannan and Stanely (2004) investigated on the antidiabetic and antioxidant activity of *Aegle marmelos* in streptozotocin-induced diabetic rats showed a significant increase in TBARS and hydroperoxides in the liver.

In the present study, the level of SOD, CAT and GSH were decreased in the liver and pancreas of mice treated with alloxan. It may be due to the damage brought about by oxidative stress as expected to be exacerbated if the antioxidant enzymes themselves are damaged and inactivated by oxidative stress, ultimately result in the perturbation of cellular redox status (Shin et al., 2006). The observed decrease in SOD activity may be due to inactivation by H_2O_2 or by glycation of enzymes (Sozmen et al., 2001). Oral treatment of leaf extract of the plant caused a significant increase in SOD activities in diabetic mice. This is due to scavenging action of alkaloids such as nymphaerine and nupharine (Trease and Evans, 1996). The decrease in CAT activity could result from inactivation by glycation of enzyme (Yan et al., 1997). CAT reduces hydrogen peroxide produced by dismutation reaction. It also prevents the generation of hydroxyl radicals, thereby protecting the cellular constituents from oxidative damage in liver and pancreas. The reduced activity of CAT in alloxan treated mice results in the accumulation of H_2O_2, which produces deleterious effects. In the present study, it was observed that the ethanolic leaf extract caused a significant increase in the activity of CAT in diabetic mice. This action may be attributed to the presence of plants secondary metabolites such as alkaloids, tannins, glycosides those may act as potent antioxidants

Conclusion

In conclusion, the present investigation showed that ethanolic leaf extract of *Nymphaea odorata* possesses an antioxidant potential and also protects lipid peroxidation and thus prevents oxidative damage.

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