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SCREENING, *IN VITRO* ANTIOXIDANT AND *IN VIVO* ANTIDIABETIC ACTIVITY OF HYDROALCOHOLIC EXTRACT OF *EUPHORBIA NERIIFOLIA*

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ABSTRACT

Euphorbia neriifolia Linn (E. neriifolia, Euphorbiaceae) is a commonly occurring plant around the dry, rocky, hilly areas of North, Central and South India. The milky latex and leaves of this plant is frequently used by the natives of Central India in case of burn, piles and to treat the deep cracks in soles of legs. This study sought to evaluate the in vitro antioxidant and in vivo antidiabetic potentials of hydroalcoholic extract of leaves of *E. neriifolia* in diabetic rats. Qualitative analysis of various phytochemical constituents and quantitative analysis of total phenolics and flavonoids were determined by the well-known test protocol available in the literature. The oral antidiabetic activity of the extract (100 and 200mg/kg) was screened against alloxan induced diabetes in rats and glibenclamide was used (600 mcg/kg body weight) as standard drug. The investigational drug was administered for 15 consecutive days, and the effect of the extract on blood glucose levels was studied at regular intervals. At the end of the study, the blood samples were collected from all the animals for biochemical estimation. In this study, Body weight, blood glucose, TC, TG and total protein were measured. The effect of hydroalcoholic extract of E. neriifolia leaves elicited significant reduction in blood glucose, lipid parameters (TC, TG, total protein) and significantly increased body weight at the dose of 200 mg/kg when compared with the diabetic-induced control. From the above results, it is concluded that hydroalcoholic extract of *E. neriifolia* leaves possesses significant antidiabetic activity in alloxan induced diabetic rats due to presence of various biomarkers.

Key words: *Euphorbia neriifolia* Linn, Euphorbiaceae, Phytochemical analysis, Antioxidant, Antidiabetic.

INTRODUCTION:

Diabetes mellitus is one of the most common disorders affecting almost 6% of the world population and the dynamics of the diabetes are changing rapidly in low- to middle-income countries (Adeghate et al., 2006). According to International Diabetes Federation's (IDF) estimates, 80% of the world diabetic population will be from low- and middle-income countries in 2030. As per IDF 2011 report, China, India, and the United States of America have a diabetic population of 90.0, 61.3, and 23.7 million, which may be increased up to 129.7, 101.2, and 29.3 million,

respectively in 2030 (Petchi et al., 2013). Globally, diabetes is one of the six major causes of death and also causing various systemic complications. Diabetes mellitus is treated by hormone therapy (insulin) or by administering glucose-lowering agents such as alpha-glucosidase inhibitors, sulfonylureas, biguanides and thiazolidinediones. Development of an adverse event is one of the complications in the treatment of any systemic disorder; hence, many of the research institutes and pharmaceutical companies are involved in drug development to find the molecules with good therapeutic potential and less adverse events (Parasuraman et al., 2010). In the USA, 10-25% of patients experience an adverse drug reaction and these adverse drug reactions are responsible for 3.4-7.0% of hospital admissions (Mandavi et al., 2012). Plants are very useful to mankind. Many of them are used exclusively for medicinal purposes. According to the World Health Organization (WHO), a medicinal plant is a plant which, in one or more of its organs, contains substances that can be used for therapeutic purposes, or which are precursors for chemo-pharmaceutical semi-synthesis. Such plants are in great demand by pharmaceutical companies for their active ingredients (Huai, 2010; Husain et al., 2008). In traditional systems of medicine, many plants have been documented to be useful for the treatment of various systemic disorders. In traditional system leaves *E. neriifolia* (Euphorbiaceae) are used as aphrodisiac, diuretic, in cough and cold, bleeding piles and ano-rectal fistula (Kirtikar and Basu, 1996). The tribal population of Chhattisgarh region uses the milky latex as an ingredient of aphrodisiac mixture. Latex is used to de-root skin warts, ear ache and in arthritis (Pandey, 1992). Plant is bitter, laxative, carminative, improves appetite, useful in abdominal troubles, bronchitis, tumors, leucoderma, piles, inflammation, enlargement of spleen, anemia, ulcers, fever, and in chronic respiratory troubles (Anonymous, 1994; Anonymous, 1952). Topically applied aqueous extract of *E. neriifolia* latex showed wound healing activity in guinea pig by increasing epithelization, angiogenesis, tensile strength and DNA content in wounds (Rashik, 1996). Several triterpenoids like Glut-5-en-3β-ol, Glut-5(10)-en-1-one, taraxerol and β-amyrin has been isolated from powdered plant, stem and leaves of *E. neriifolia* (Chatterjee et al., 1978; Anjaneyulu and Ramachandra, 1965). Antiquorin have been isolated from ethanol extract of fresh root of *E. neriifolia* (Ng, 1990). Neriifolione, a triterpene and a new tetracyclic triterpene named as nerifoliene along with euphol were isolated from the latex of *E. neriifolia* (Ilyas et al., 1998; Mallavadhani et

al., 2004; Parkhe et al., 2018). The present study was undertaken to find out the antidiabetic activity of *E. neriifolia* leaf along with antioxidant activity.

MATERIAL AND METHOD

Plant material

Leaves of *E. neriifolia* were collected from local area of Bhopal in the month of March, 2021.

Chemical reagents

All the chemicals used in this study were obtained from HiMedia Laboratories Pvt. Ltd. (Mumbai, India), Sigma Aldrich Chemical Co. (Milwaukee, WI, USA), SD Fine-Chem. Ltd. (Mumbai, India) and SRL Pvt. Ltd. (Mumbai, India).All the chemicals used in this study were of analytical grade. Alloxan (Central Drug House Pvt.Ltd.,India), Glibenclamide tablets (Daonil; Aventis Pharma. Ltd., India) were procured from the medical store. Biochemical estimation kit (Transasia Bio Medical Limited, Mumbai, India) and other chemicals and solvent obtained from Qualigens, India were used.

Defatting of plant material

Leaves of *E. neriifolia* were shade dried at room temperature. 67.50 gram of shade dried plant material was coarsely powdered and subjected to extraction with petroleum ether by soxhlation method. The extraction was continued till the defatting of the material had taken place.

Extraction

Defatted dried powdered of *E. neriifolia* has been extracted with hydroalcoholic solvent (Ethanol: Water; 75:25) using soxhlation method for 48 hrs, filtered and dried using vacuum evaporator at 40°C. Finally the percentage yields were calculated of the dried extracts.

Qualitative phytochemical screening

Crude extracts were screened to identify the occurrence of primary and secondary metabolites, viz. carbohydrates, alkaloids, glycosides, polyphenols, flavonoids, tannins, saponins, terpenoids, proteins and fixed oils, using standard screening test and phytochemical procedures [18, 19].

Estimation of total phenolic content

The total phenolic content of the extract was determined by the modified Folin-Ciocalteu method (Parkhe and Bharti, 2019) [20]. 10 mg Gallic acid was dissolved in 10 ml methanol, various aliquots of 5- 25µg/ml was prepared in methanol. 10mg of dried extracts of were dissolved in 10 ml methanol and filter. Two ml (1mg/ml) of this solution was used for the estimation of phenol. 2 ml of each extract or standard was mixed with 1 ml of Folin-Ciocalteu reagent (previously diluted with distilled water 1:10 v/v) and 1 ml (7.5g/l) of

sodium carbonate. The mixture was vortexed for 15s and allowed to stand for 15 min for colour development. The absorbance was measured at 765 nm using a spectrophotometer.

Estimation of total flavonoids content

Determination of total flavonoids content was based on aluminium chloride method (Meda *et al.*, 2005) [21]. 10 mg quercetin was dissolved in 10 ml methanol, and various aliquots of 5-25µg/ml were prepared in methanol. 10mg of dried extracts of were dissolved in 10 ml methanol and filter. Three ml (1mg/ml) of this solution was used for the estimation of flavonoid. 1 ml of 2% $AlCl_3$ methanolic solution was added to 3 ml of extract or standard and allowed to stand for 15 min at room temperature; absorbance was measured at 420 nm.

Antioxidant activity

DPPH method

Total free radical scavenging capacity of the ethanolic extract obtained from *Drypetes roxburghii* was estimated according to the previously reported method with slight modification (Parkhe and Bharti, 2019). Solution of DPPH (6 mg in 100ml methanol) was prepared and stored in dark place. Different concentration of standard and test (10- 100 µg/ml) was prepared. 1.5 ml of DPPH and 1.5 ml of each standard and test was taken in separate test tube; absorbance of this solution was taken immediately at 517nm. 1.5 ml of DPPH and 1.5 ml of the methanol was taken as control absorbance at 517nm. The percentage inhibition of free radical DPPH was calculated from the following equation: % inhibition = [(absorbance of control - absorbance of sample)/absorbance of control] × 100%.

Animals

Wistar rats (150-250 g) were group housed (n= 6) under a standard 12 h light/dark cycle and controlled conditions of temperature and humidity (25±2 °C, 55–65%). Rats received standard rodent chow and water *ad libitum*. Rats were acclimatized to laboratory conditions for 7 days before carrying out the experiments. All the experiments were carried in a noise-free room between 08.00 to 15.00 h. Separate group (n=6) of rats was used for each set of experiments. The animal studies were approved by the Institutional Animal Ethics Committee (IAEC), constituted for the purpose of control and supervision of experimental animals by Ministry of Environment and Forests, Government of India, New Delhi, India.

Acute oral toxicity

Acute toxicity study of the prepared extracts was carried out according to the Organization for Economic Co-Operation and Development

(OECD) Guidelines-423 (Jonsson et al., 2013) the animals were fasted for 4 h, but allowed free access to water throughout. As per the OECD recommendations, the starting dose level should be that which is most likely to produce mortality in some of the dosed animals; and when there is no information available on a substance to be tested in this regard; for animal welfare reasons, The hydroalcoholic extract of *E. neriifolia* (50, 100, 150, 200, 300 mg/kg/day) was administered orally for 4 days of six groups of rats (n=6) and the animals were kept under examination for mortality as well as any behavioral changes. Acute toxicity was determined as per reported method (Shankar et al., 2007).

Induction of experimental diabetes in rats

After fasting, diabetes was induced by a single intraperitoneal injection of 120 mg/kg body weight of 'Alloxan monohydrate' in distilled water. The animals were allowed to drink 5% glucose solution overnight to overcome the drug-induced hypoglycaemia. These animals were tested for diabetes after 15 days and animals with blood glucose (fasting) were selected for experimentation (Adeneye et al., 2007).

Experimental protocol

Five groups of rats were employed in the present study and each group contains six animals, as follows

Group I: Rats served as normal-control and received the vehicle (0.5 ml distilled water/day/rat)

Group II: Rats served as diabetic-control and received the vehicle (0.5 ml distilled water/day/rat)

Group III: Rats (diabetic) were administered of hydroalcoholic extract of *E. neriifolia* (100 mg/kg p.o.) for 15 days

Group IV: Rats (diabetic) were administered of hydroalcoholic extract of *E. neriifolia* (200 mg/kg p.o.) for 15 days

Group V: Rats (diabetic) were administered Glibenclamide (600µg/kg p.o.) for 15 days

Bioassay

On 15^{th} day of treatment, blood samples were collected by retro-orbital plexus puncture method under mild ether anesthesia and serum was separated by centrifugation. Serum glucose, total protein, cholesterol (TC) and total triglyceride (TG) levels were evaluated using a commercial kit (Roy et al., 2005). Body weights of rats were taken before and after treatment (Koleva et al., 2002).

Statistical Analysis

The data were subjected to the analysis of variance (one way ANOVA) to determine the significance of changes, followed by Tukey's post hoc test comparison were made to analyze the significance of difference within the

experimental groups. *P* values of 0.05 or less were taken as significant.

Results and discussions

The crude extracts so obtained after soxhletion extraction process was concentrated on water bath by evaporation the solvents completely to obtain the actual yield of extraction. The yield of hydroalcoholic extracts was found to be 7.9 %. The results of qualitative phytochemical analysis of the crude powder of leaves of *E. neriifolia* were shown in Table 1. Hydroalcoholic extracts of *E. neriifolia* showed the presence of flavonoids, phenols, protein, carbohydrate and saponins. The determination of the total phenolic content, expressed as mg gallic acid equivalents and per 100 mg dry weight of sample. The total flavonoids content of the extracts was expressed as percentage of quercetin equivalent per 100 mg dry weight of sample. TPC and TFC of hydroalcoholic extract of *E. neriifolia* showed the content values of 0.563 and 0.752 respectively Table 2. The DPPH assay has been largely used as a quick, reliable and reproducible parameter to search for the in vitro general antioxidant activity of pure compounds as well as plant extracts (Gonçalves et al., 2005; Dhanabal et al., 2007). The decrease in absorbance by the DPPH radical with increase in concentration of the extract which manifested in the rapid discolouration of the purple DPPH, suggest that the hydro alcoholic extracts of *E. neriifolia* has antioxidant activity due to its proton donating ability. It was found that the extracts exhibited a dose-dependent activity which indicates that DPPH scavenging activity was increased proportionately to the increase in the extracts' concentration. Additionally, the IC_{50} values of scavenging DPPH radicals for the AA and extract were shown in Table 3. Comparing with AA, the IC_{50} value for DPPH radical activity of extract was found to be 85.77. It is well known fact that alloxan monohydrate induces diabetes mellitus in rats by selective necrotic action on the beta cells of pancreas leading to insulin deficiency. Insulin deficiency leads to various metabolic aberrations in animals like increased blood glucose level, increased levels of cholesterol and triglyceride and decreased protein content. As expected in alloxan treated rats, there was significant increase in blood glucose, cholesterol (TC) and triglyceride (TG) levels. The diabetic animals showed significant decrease in blood glucose level after 15 days treatment. Moreover it also decreased the levels of cholesterol (TC) and triglyceride (TG) increased by alloxan

treatment. Alloxan treatment of the rats has showed the loss in body weight as compared to normal rats. However, the hydroalcoholic extract was more effective and results are comparable with that of reference drug, gliblenclamide. Hence, we can say that presence of flavonoid in the ethyl acetate fraction may be responsible for antidiabetic activity. As shown in Table 4 Blood glucose level of animals in all groups was recorded at 0, 8th and 15th day. Progressive decrease in blood glucose level was found in all treatment groups during study. At the end of experiment Glibenclamide 600µg/kg, *E. neriifolia* 100 and 200 mg/kg/p.o. (118.6; 133.6 and 115.1) treated group blood glucose level was decrease significantly ($p<0.01$) at 15thdays, respectively. As shown in Table 5 Glibenclamide 600µg/kg, *E. neriifolia* 100 and 200 mg/kg/p.o. treated group biochemical parameters like TC, TG level was decrease significantly ($p<0.01$) and total protein level was increase as compared to diabetic control group. As represented in Table 6 body weights of animals in all groups were performed at the initial and end of the study. Body weight of animals was significantly ($p<0.05$) maintained in all treatment groups (Glibenclamide 600µg/kg, *E. neriifolia* 100 and 200 mg/kg/p.o. 207.00, 195.00, 204.00) during study as compared to control group (207.10).

Table 1 Phytochemical screening of extract *of E. neriifolia*

S. No.	Constituents	Hydroalcoholic extract	Observation
1.	**Alkaloids**		
	Wagner's Test	-ve	Red coloured
	Mayer's Test	-ve	Green coloured
	Dragendroff's test	-ve	Green coloured
	Hager's test	-ve	Yellow coloured
2.	**Glycosides**		
	Legal's test	-ve	Red coloured
3.	**Flavonoids**		
	Lead acetate	-ve	White coloured
	Alkaline test	+ve	Colourless
4.	**Phenol**		
	Ferric chloride test	+ve	Black coloured
5.	**Proteins**		
	Xanthoproteic test	+ve	Yellow coloured
6.	**Carbohydrates**		
	Fehling's test	+ve	Red precipitated
7.	**Saponins**		
	Foam test	+ve	Foam
	Froth Test	+ve	Foam

8.	**Diterpenes** Copper acetate test	-ve	Sky blue coloured
9.	**Tannins Gelatin Test**	-ve	Green coloured

+ve (Present), -ve (Absent)

Table 2 Estimation of total phenolic and flavonoids content of *E. neriifolia* extract

S. No.	Total phenol content (mg/100mg of dried extract)	Total flavonoids content (mg/ 100 mg of dried extract)
1.	0.563	0.752

Table 3 % Inhibition of ascorbic acid and hydroalcoholic extract of *E. neriifolia* using DPPH method

S. No.	Concentration (µg/ml)	% Inhibition	
		Ascorbic acid	*E. neriifolia* extract
1	10	46.45	10.47
2	20	49.25	26.58
3	40	60.53	31.52
4	60	72.58	42.86
5	80	86.35	49.68
6	100	91.74	51.47
IC_{50} (µg/ml)		**18.70**	**85.77**

Table 4 Effect of hydroalcoholic extract of *E. neriifolia* treatment on blood glucose (mg/dl) in normal and diabetic rats

Group	Treatment	Blood glucose (mg/dl)		
		Days 0	Days 8	Days 15
I	Normal	94.57 ± 4.6	105.2 ± 4.12	108.3 ± 4.1
II	Diabetic Control	271.4 ± 6.3	277.1 ± 7.2#	281 ± 6.4#
III	Diabetic + hydroalcoholic extract of *E. neriifolia* (100 mg/kg)	257.8 ± 5.1	154.3 ± 3.8***	133.6 ± 3.8***
IV	Diabetic + hydroalcoholic extract of *E. neriifolia* (200 mg/kg)	253.1 ± 4.7	141.2 ± 3.1***	115.1 ± 3.5***
V	Diabetic + Glibenclamide (600µg/kg)	251.6 ± 5.3	135.4 ± 2.8***	118.6 ± 3.3***

Values are expressed as mean±S.E.M (n = 6).Values are statistically significant at # p<0.001 vs. normal group; *P < 0.001, **P < 0.01vs. diabetic control group (Two-way ANOVA test).

Table 5 Effect of hydroalcoholic extract of *E. neriifolia* treatment on biochemical parameters in normal and diabetic rats

Group	Treatment	TC (mg/dl)	TG (mg/dl)	Total protein(g/dl)
I	Normal	91 ± 2.8	82.3 ± 3.6	9.1 ± 0.65
II	Diabetic Control	190.2 ± 4.4	125.5 ± 4.5	5.2 ± 0.41
III	Diabetic + *E. neriifolia* (100 mg/kg)	110.5 ± 5.1**	85.5 ± 3.2*	7.9 ± 0.43**
IV	Diabetic + *E. neriifolia* (200 mg/kg)	102.2 ± 4.5**	80.8 ± 3.4*	8.1 ± 0.45**
V	Diabetic + Glibenclamide (600µg/kg)	98.8 ± 4.5**	78.4 ± 2.8*	9.2 ± 0.51**

Values are expressed as mean±S.E.M (n = 6).Values are statistically significant at $^{\#}p<0.001$ vs. normal group; *P < 0.001, **P < 0.01vs. diabetic control group (One-way ANOVA followed by Tukey's post hoc test).

Table 6 Effects of hydroalcoholic extract of *E. neriifolia* on body weight

Group	Treatment	Initial weight (g)	Final weight (g)
I	Normal	165.00 ± 8.90	207.10 ± 9.06
II	Diabetic Control	175.00 ± 8.40	155.00 ± 8.78
III	Diabetic + hydroalcoholic extract of *E. neriifolia* (100 mg/kg)	165.00 ± 10.00	195.00 ± 7.32
IV	Diabetic + hydroalcoholic extract of *E. neriifolia* (200 mg/kg)	169.00 ± 9.20	204.00 ± 8.42
V	Diabetic + Glibenclamide (600µg/kg)	171.00 ± 7.80	207.00 ± 8.16

Values are expressed as mean ± SD of six samples from each group. (Two-way ANOVA test).

CONCLUSION

Despite ongoing scientific research on this species, this study constitutes the first attempt to determined the phytochemical compositions as well as the antioxidant, antidiabetic activities of *E. neriifolia* leaves hydroalcoholic extracts that could be found despite the throughout literature survey so far as we know. The knowledge of phytochemical constituents of the plant is the basic approach to identify novel secondary metabolites as unmodified form, semi-synthetic or drug templates. This study delineates that hydroalcoholic extracts could be potentials in free-radical scavenging and antidiabetic activity. So, it can be assumed that different active secondary metabolites were present in these extracts. Furthermore, the activity of this plant

constituent can help to elucidate the justification for the ethno medicinal use of this plant species scientifically. Based on our findings, further studies are necessary to elucidate the mechanism lying with these effects of the plant extracts and could be open a new window in the search for new bioactive drug lead components of this plant extracts.

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