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In vitro antioxidant activity and effect of ethanolic extract of *Curcuma leucorhhiza* Roxb. on *in vivo* antioxidant status of diabetic rats

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Medicinal plants possess secondary metabolites such as flavonoids, phenolic acids, and tannins, which exhibit significant antioxidant activity by scavenging free radicals. *Curcuma leucorrhiza* Roxb. is a medicinal plant known for its antiseptic, anti-inflammatory and antioxidant properties. This study evaluates the *in vitro* antioxidant potential of its rhizome extracts and assesses the effect of its ethanolic extract on *in vivo* antioxidant status in streptozotocin (STZ) induced diabetic rats. Extracts prepared using chloroform, ethanol, methanol, petroleum ether, aqueous and 50% ethanol were tested via DPPH radical scavenging, FRAP and total phenolic content (TPC) assays. The methanol extract exhibited the highest activity, followed by ethanol. For *in vivo* studies, 30 male Wistar rats were divided into five groups (n=6) *viz*. normal control (Group-I), diabetic untreated (Group-II), metformin treated (5 mg/kg bw, Group-III) and ethanolic extract (absolute) treated (100 and 200 mg/kg bw, Groups IV and V). Treatment with 200 mg/kg ethanolic extract significantly restored antioxidant markers in diabetic rats, comparable to metformin treatment, reducing malondialdehyde (P<0.01) and increasing catalase (P<0.05), superoxide dismutase (P<0.01) and glutathione peroxidase (P<0.05) levels. These findings support the ethnomedicinal use of *Curcuma leucorrhiza* Roxb. and indicate its potential as a natural antioxidant therapy for diabetes associated oxidative stress. Further research is warranted to isolate active compounds, DNA sequence based molecular characterization and evaluate long-term efficacy.

Keywords: Free radicals, Oxidative stress, Phenolic content, Rhizome, Streptozotocin

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The genus *Curcuma* includes several medicinal plants, rich in bioactive compounds with potential therapeutic effects against chronic diseases such as diabetes and cancer[1]. *Curcuma L.* (Zingiberaceae), a genus comprising approximately 80 species, is widely distributed in India and throughout tropical and subtropical regions of Asia, particularly in Thailand, Indonesia and Malaysia[2]. Among these, *Curcuma leucorrhiza* Roxb. is known for its diverse medicinal properties such as antiseptic, anti-inflammatory, antibacterial, antioxidant, antacid, antifungal and carminative effects[2-5]. Curcumin, the major bioactive polyphenol in *Curcuma* species, has been identified as a potent antioxidant, scavenging reactive oxygen and nitrogen species[6]. Its lipophilic nature makes it soluble in ethanol and dimethyl sulfoxide but insoluble in water and ether[7].The rhizomes of *Curcuma leucorrhiza* have long been used in traditional medicine for treating chronic ailments.

Medicinal plants have historically played an important role in traditionally healing practices. Their therapeutic value primarily stems from their secondary metabolites, including alkaloids, terpenoids, phenols, flavonoids, tannins and saponins[8]. These secondary metabolites, produced through plant-specific metabolic pathways, exert significant physiological effects on human health[9]. Among these, phenolic compounds such as flavonoids, phenolic acids and tannins, are recognized for their potential antioxidant activity, free radical scavenging capabilities and metal-chelating, antimutagenic, anticancer and antimicrobial activities[10]. Such bioactive compounds mitigate oxidative stress by balancing reactive oxygen and nitrogen species against endogenous antioxidants within the body[11].

Antioxidants help in neutralizing free radicals generated by environmental pollutants, radiation, chemicals, toxins, spicy and fried foods and physical stress. These reactive species cause damage to DNA, proteins, and membrane lipids while altering gene expression. Antioxidants, therefore, support the body's natural defense mechanism against oxidative stress[12,13]. However, the amounts of these protective antioxidant principles present under the normal physiological conditions are sufficient only to cope with the

physiological rate of free radicals, either from environment or produced within the body. In order to maintain the level of antioxidant in the body for healthy living, external supplementation is necessary[13]. Different studies have investigated the potential of natural antioxidants, particularly polyphenols from medicinal and dietary plants, which may prevent oxidative damage[14,15].

In mammals, the enzymatic antioxidant such as superoxide dismutase, catalase, glutathione peroxidase and glutathione reductase[16].Oxidative stress arises when the production of free radicals, such as superoxide, hydroxyl, and peroxyl radicals, surpasses the body′s ability to neutralize them. This imbalance contributes to chronic and degenerative diseases such as cancer, atherosclerosis, Parkinson′s disease, Alzheimer′s disease, diabetes and neurodegenerative disorders[17]. Since, antioxidant enzymes neutralize reactive oxygen species, their upgradation provide greater protection to the organs against degenerative diseases. However, there are limited studies on oxidative stress-induced diseases such as diabetes, which often disrupt the levels of these enzymes.

Several plant extracts have positive effect on the of serum antioxidant enzyme level[18-20]. Mateos *et al*.[21] reported that flavonoids are abundant in plant extracts and effective in reducing lipid peroxidation in hypercholesterolemic rats. The beneficial effect of flavonoids may be mediated through multiple mechanisms such as inhibition of lipid peroxidation, platelet aggregation and enhancement of antioxidant defences[22,21]. Furthermore, the other secondary metabolites (polyphenols) might be responsible for suppressing lipid peroxidation and improving antioxidant capacity in the liver.

Given these considerations, the objective of the present study was to evaluate the *in vitro* antioxidant capacity of the *Curcuma leucorrhiza* Roxb extracts in different solvents and also assess the effect of its ethanolic rhizome extract on the *in vivo* antioxidant status of streptozotocin induced diabetic rats.

Materials and Methods

Plant collection and authentication

The plants of *Curcuma leucorrhiza* Roxb. were collected from Sekmaijin area in Imphal West district of Manipur, India. The plant was identified and authenticated from the accession record of the Institute of Bioresources and Sustainable Development (IBSD), Imphal, as IBSD/Z-21 (Cultiver-Langol, 20-Meitei) under the supervision of Dr L Reena Devi (Assistant professor, Department of Chemistry, Pravabati College, Imphal, Manipur). The collected rhizomes were transported to the postgraduate research laboratory of Department of Veterinary Physiology & Biochemistry, College of Veterinary Sciences & A.H., CAU, Selesih, Aizawl, Mizoram for further processing and experimentation.

Chemicals and reagents

Streptozotocin, Metformin and Chloroform were procured from HiMedia Laboratories Pvt. Ltd., Ethanol, Petroleum ether, Methanol, Ferric Chloride, Sodium Potassium Tartarate, Folin-Ciocalteu reagent were purchased from Merck Limited. Other chemicals, including 1,1-Diphenyl-2-picrylhydrazyl (DPPH), 2,4,6-tri(2-pyridyl)-striazine (TPTZ), 6-hydroxy-2,5,7,8–tetramethyl chromane-2- carboxylic acid (Trolox) and Gallic acid, were obtained from Sigma Chemicals Co. (St. Louis, USA), kits for estimation of *in vivo* antioxidant enzymes such as SOD, Catalase, GPx, MDA were procured from Wuhan Fine Biotech Co. Ltd. All the chemical and reagents used in this investigation were of analytical grade and double glass distilled water was used throughout the investigation.

Animal experiments

The experimental protocol was approved by the Institutional Animal Ethics Committee of the College of Veterinary Sciences and Animal Husbandry, Selesih, Aizawl (CVSC/CAU/IAEC/21-22/P-7, dated 13.11.2022). Adult healthy male Wistar rats (150-200 g) were procured from M/s. Ruata Enterprise, Aizawl. The animals were acclimatized in animal house of C.V.Sc and A.H., CAU, Selesih, Aizawl, Mizoram under standard laboratory condition (12-h light/dark cycle at ambient temperature ranging between 20-25˚C) for seven days. Rats were housed in polypropylene cages in groups of six rats per cage with *ad libitum* access to standard balanced feed and clean drinking water.

Preparation of plant extract

The rhizomes were washed under running tap water and after that, followed by successive washes with distilled and deionized water to remove impurities. The cleaned rhizomes were chopped into small pieces and dried in the shade. The dried rhizomes were ground into a fine powder by a

mechanical grinder. The powder was extracted in different solvents like; Chloroform(pure), Ethanol (absolute), Aqueous (double distilled water), Petroleum ether (pure), 50% Ethanol and their antioxidant capacities were estimated. To study the *in vivo* antioxidant enzymes, the rhizome extract was prepared in ethanol. The dried powdered rhizome (700 g) was soaked in 3000 mL of absolute ethanol (1:4.3 w/v) in a conical flask with intermittent stirring for 10 days at room temperature. The mixture was filtered and the filtrate was concentrated under reduced pressure using a rotary vacuum evaporator (MAC, Delhi) as per the method of Zakaria *et al*[23]. The dried extract was stored at -20˚C in air tight containers until further use.

Estimation of i*n-vitro* Antioxidant capacity of the extracts

The *in vitro* antioxidant content of the rhizome extract was estimated by three *in vitro* assays methods such as DPPH free radical scavenging assay, Ferric reducing antioxidant potential assay and Total phenolic content assay.

DPPH free radical scavenging assay

The free radical scavenging activity of the rhizome extracts was measured using the 1,1- Diphenyl-2-picrylhydrazyl (DPPH) method as described by Leong & Shui (2002) with some modifications. 0.1 mM DPPH solution was prepared in methanol and the initial absorbance was measured at 517 nm. A 40 µL aliquot of extract was mixed with 3 mL of DPPH solution and the absorbance was measured at different time intervals until the absorbance remained constant[24]. A standard curve was prepared using Trolox (250 -1250µg/mL) and the free radical scavenging ability of the extracts were expressed as mg Trolox equivalent (TE) per gram of dry rhizome powder.

Ferric reducing antioxidant potential (FRAP) assay

The FRAP assay was conducted as per the method of Benzie & Strain[25]. Briefly, 50µL of extract was added to 3 mL of FRAP reagents (10 parts of 300 mM sodium acetate buffer of pH 3.6, 1 part of TPTZ and 1 part of 20 mM Ferric chloride solution). The reaction mixture was incubated at 37ºC for 30 min and the increase in absorbance was measured at 593 nm using UV/Vis Spectrophotometer[25]. The standard curve was prepared using Trolox (250-1000µg/mL) and the results were expressed as mg Trolox equivalent (TE) per gram of dry rhizome powder.

Total phenolic content (TPC) assay

The total phenolic content of the extracts waw estimated using the Folin-Ciocalteau method[26]. A 100 µL aliquot of extract was added to 1mL of 1:10 Folin-Ciocalteau's reagent and incubated at room temperature for 5 min. Subsequently 900 µl of sodium carbonate (7.5%) solution was added and the mixture was incubated for 1 hour at room temperature. Absorbance was measured at 640 nm using UV/Vis Spectrophotometer[26]. A standard curve was prepared using gallic acid (100 µg/mL) and results were expressed as mg gallic acid equivalent (GAE) per gram of dry rhizome powder.

Diabetes induction

Thirty healthy male Wistar rats (weighing approximately 130±10 g) were randomly assigned to five groups (n=6). The rats were fasted for 24-h before diabetes induction. Streptozotocin (STZ) was administered intraperitoneally at a dose of 40 mg/kg body weight (bw) following the method of Kalaivanan & Pugalendi[27]. Rats with blood glucose levels ≥250 mg/dL after 72-h were considered diabetic[27]. The experimental groups were as follows: control (Group-I), diabetic untreated (Group-II), metformin treated (5 mg/kg bw, Group-III) and ethanolic extract (absolute) treated (100 and 200 mg/kg bw, Groups IV and V).

Assessment of *in vivo* antioxidant status

On day 24 of treatment, Superoxide dismutase (SOD), Catalase, Glutathione peroxidise (GPx) and Malondehyde (MDA), were measured using commercial ELISA kits (Fine Biotech, Wuhan, China) following the manufacturer′s protocol. The Glutathione peroxidise (GPx) and Catalase kits were based on sandwich enzyme-linked immune-sorbent assay technology. The Malondialdehyde (MDA) kit was based on Competitive-ELISA detection method while the Superoxide dismutase (SOD) kit was based colourimetric estimation.

Statistical analysis

All the experiments were performed in triplicate and data were expressed as mean±standard error (SE). Statistical analysis was conducted using one-way Analysis of variance (ANOVA) in SPSS version 27.0. Differences between groups were considered statistically significant at $P<0.05$[28]. Duncan′s multiple range test was used to determine significant differences between group means.

Table 1 — Antioxidant activity (mean±SE) of the *Curcuma leucorrhiza* Roxb. rhizome extract in different organic solvents and water

Extracts	Concentrations of the solvents	DPPH (mg TE/gm)	FRAP (mg TE/gm)	Total phenolic content (mg GAE/gm)
Chloroform	Pure	2.25±0.30	0.00±0.00	1.23±0.20
Ethanol	Absolute (100%)	2.09±0.10	0.28±0.00	12.02±0.50
Aqueous	Double distilled water	1.06±0.18	0.07±0.01	2.21±0.09
Petroleum Ether	Pure	1.10±0.35	0.00±0.00	1.05±0.17
Methanol	Absolute (100%)	2.70±0.07	0.39±0.01	12.35±0.52
50% Ethanol	50% ethanol in distilled water (v/v)	1.79±0.01	0.16±0.01	5.93±0.21

Results and Discussion

Dried rhizomes of *Curcuma leucorrhiza* Roxb. were extracted in different organic solvents and double distilled water. To assess the antioxidant activities of the extracts, three different *in vitro* assay methods were performed *viz* DPPH free radical scavenging activity, Ferric Reducing Antioxidant Power (FRAP) assay, and Total Phenolic Content assay (TP). The antioxidant activity of the extracts is presented in Table 1.

Among all three assays, the methanol extract showed the highest antioxidant activity. The observed antioxidant activity in DPPH free radical assay were 2.70±0.07 mg TE/gm in methanol extract followed by 2.25±0.30 mg TE/gm in chloroform extract, 2.09±0.10 mg TE/gm in ethanol extract, 1.79±0.01 mg TE/gm in 50% ethanol extract, 1.10±0.35 mg TE in petroleum ether extract and 1.06±0.18 mg TE/gm in aqueous extract. However, in case of FRAP assay the highest activity of 0.39±0.01 mg TE/gm was observed in methanol extract followed by 0.28±0.00 mg TE/gm in ethanol extract, 0.16±0.01 mg TE/gm in 50% ethanol extract and 0.07±0.01 mg TE/gm in aqueous extract. Furthermore, there was no detectable antioxidant activity in the chloroform and petroleum ether extract. In contrast, methanol extract showed the highest total phenolic content similar to DPPH and FRAP assay. The total phenolic content observed were 12.35±0.52 mg GAE/gm in methanol extract followed by 12.02±0.50 mg GAE/gm in ethanol extract, 5.93±0.21 mg GAE/gm in 50% ethanol extract, 2.21±0.09 mg GAE/gm in aqueous extract, 1.23±0.20 mg GAE/gm in chloroform and 1.05±0.17 mg GAE/gm in petroleum ether. As the antioxidant activity of the plant extract in all the three methods are same or little less in ethanol extract compared to that of methanol extract, the plant extract for evaluation of *in vivo* antioxidant status was prepared in absolute (100%) ethanol to avoid use of methanol; the latter being toxic if inhaled by humans during extraction. The antioxidant content of the *Curcuma leucorrhiza* Roxb. rhizome was also reported by other researchers[2,29]. According to Linthoingambi *et al*. the rhizomes of *Curcuma leucorrhiza* Roxb. possess free radical scavenging activity in chloroform, petroleum ether and methanolic extracts and highest activity was observed in chloroform extract[2]. Theanphong *et al*. observed that the essential oils extracted from *Curcuma leucorrhiza* showed strong DPPH radical scavenging activity, hydroxyl radical scavenging activity, hydrogen peroxide scavenging activity and ferric reducing power[29]. Furthermore, several other researchers have reported that the essential oils from Curcuma species are rich in antioxidant activities[1,30,31,32].

Following the induction of diabetes, the levels of *in vivo* antioxidants, including Superoxide Dismutase (SOD), Catalase, Glutathione Peroxidase, and Malondialdehyde (MDA), in Wistar rats are altered. Treatment of the diabetic rats with metformin or the ethanol extract of *Curcuma leucorrhiza* Roxb. rhizome was found to improve the *in vivo* antioxidant status. The level of MDA increases (P<0.01) on induction of diabetes where, the level of MDA in normal rats was 140±10.00 ng/mL and the level increased to 410.00±20.81 ng/mL among the diabetic group. Moreover, diabetic rats treated with metformin or the ethanolic extract of *Curcuma leucorrhiza* Roxb. rhizome show an improvement in MDA levels. The level observed among the metformin treated group was 141.67±6.00 ng/mL. Similar to metformin treated group, the 100 mg and 200 mg/kg bw treated groups also show decrease (P<0.01) level of MDA. The observed levels for 100 mg and 200 mg/kg bw treated groups were 130.00±11.54 ng/mL and 140.00±10.00 ng/mL. The level of catalase decreased (P<0.05) upon induction of diabetes, with the observed catalase activity in control rat was 2166.70±0.02U/mL, which decreased to 1300.00±0.01 U/mL in the diabetic group. Treatment of the diabetic rats with metformin or the ethanolic extract of rhizome of *Curcuma leucorrhiza* Roxb. increase the level of catalase. The

level observed among the metformin treated group was 1933.30±0.02U/mL. Similar to metformin treated group, the 100 mg and 200 mg/kg bw extract treated groups also show increasing trend in the level of catalase in a dose dependent manner. The observed levels for 100 mg and 200 mg/kg bw treated groups were 1500.00±57.73U/mL and 1800.66±0.02U/mL respectively.

The GPx activity also decreases on induction of diabetes which was similar to the observation made for catalase activity. The observed GPx activity among the untreated rats was 2050.00±0.01 pg/mL and the level decreased to 1633.30±33.30 pg/mL among the diabetic group. The level observed among the metformin treated group was 2033.30±44.09 pg/mL. Similar to the metformin treated group, the 100 mg and 200 mg/kg bw treated groups also showed an increase trend in the level of catalase however, in a dose dependent manner. The observed levels for 100 mg and 200 mg/kg bw treated groups were 1866.00±44.09 pg/mL and 2050.00±0.01pg/mL respectively. In diabetic rat (Group-II), the level of SOD decreased whereas the plant extract treated animals were showing levels near to normal. The observed activity of SOD among normal rats were 2.12±0.02U/mL and the level decreased to 1.80±0.00 in case of diabetic rats. The metformin treated group was showing better activity than the normal which was 2.57±0.17U/mL and the observed levels in 100 mg/kg and 200 mg/kg *Curcuma leucorrhiza* roxb. treated rats were 2.1±0.05U/mL and 2.06±0.03U/mL respectively which was better than the diabetic group and was near to normal group. The observed levels of *in vivo* antioxidant enzymes among the normal, diabetic and diabetic rats treated with metformin and ethanolic extract of the *Curcuma leucorhiza* Roxb. is presented in Table 2.

In addition, *in vivo* antioxidant status was altered in case of STZ induced diabetic rats which was consistent with the observations made for lipid profile and biochemical changes among the diabetic rats. Hyperglycaemia in diabetes can increase production of free radicals through Amadori rearrangement[33] whereas increased oxidative stress is present in diabetic subjects[34-36] which is due to reduced enzymatic and non-enzymatic antioxidants[37]. Peroxidation of lipids produces highly reactive aldehydes, including MDA, acrolein, 4-hydroxynonenal (HNE), 4-oxononenal (ONE), and isolevuglandins (IsoLGs)[38]. Oxidized lipids are able to produce MDA as a decomposition product and the mechanism is thought to involve formation of prostaglandins, like endoperoxides, from polyunsaturated fatty acid (PUFA) with two or more double bonds[39]. MDA has been documented as a primary biomarker of free radical mediated lipid damage and oxidative stress[40]. Several literature reports that a significant rise in MDA levels in diabetes, indicating greater oxidative damage[41,42]. This increase in MDA is associated to the disruption of cellular integrity through enhanced lipid peroxidation[43,44]. MDA is a marker of lipid peroxidation which reacts with cell membrane phospholipids. As a result, increased level of MDA in diabetics suggests that peroxidative injury may be involved in the development of diabetic complications. It also signifies a reduction in the effectiveness of both enzymatic and nonenzymatic antioxidant defence mechanisms[45].

GPx catalyses the reduction of lipid peroxides and inactivation of H_2O_2 resulting in an increased consumption of GSH[36]. The depletion of GSH impairs the activity of antioxidant enzymes as well as that of chain breaking aqueous and lipid phase antioxidants[46,47].

In diabetic condition, the reduction in serum SOD activity levels could be due to excessive consumption in the autoxidation process and increased excretion from the inflammatory kidney in nephropathy[48,49]. About 50% of SOD in erythrocytes of diabetic patients is glycated, resulting in low activity of SOD[50]. Catalase catalyses the degradation of H_2O_2 in

Table 2 — *In vivo* antioxidant activity (mean±SE) of normal, diabetic, diabetic rats treated with metformin and ethanolic extract (100 mg/bw and 200 mg/bw)

Group	MDA (ng/mL)	Catalase U/mL)	GPx (pg/mL)	SOD (U/mL)
Group-I	140.00±10.00^{a}	2166.70±0.02^{c}	2050.00±0.01^{b}	2.12±0.02^{b}
Group-II	410.00±20.81^{b}	1300.00±0.01^{a}	1633.30±33.30^{a}	1.80±0.00^{a}
Group-III	141.67±6.00^{a}	1933.30±0.02^{b}	2033.30±44.09^{b}	2.57±0.17^{c}
Group-IV	130.00±11.54^{a}	1500.00±57.73^{ab}	1866.00±44.09^{a}	2.10±0.05^{b}
Group-V	140.00±10.00^{a}	1800.66±0.02^{abc}	2050.00±0.01^{b}	2.06±0.03^{b}
P value	0.00**	0.03*	0.04*	0.00**

[Different superscripts (a, b, c) within the same column indicate statistically significant differences between groups ($P< 0.05$). Values sharing the same superscript do not differ significantly. **Significant $P<0.01$, *Significant $P<0.05$]

the cells in physiologic condition. The serum level of catalase is decreased in diabetic state[51,52].

Several plant extracts have shown significant positive effect on the level of serum antioxidant enzymes of diabetic rats[18-20]. Flavonoids, which are abundantly present in plant extracts, are very effective in reducing lipid peroxidation in hypercholesterolemic rats[21].The beneficial effect of flavonoids may be mediated by one or more mechanisms such as inhibiting lipid peroxidation, platelet aggregation and enhancing of antioxidant defense[22,21]. Furthermore, the other secondary metabolites i.e., polyphenols might be responsible for suppressing the extent of lipid peroxidation and enhancing the antioxidant capacity in liver. In the present investigation, the improvement in serum antioxidant status of the diabetic rats on treatment with *Curcuma leucorrhiza* Roxb. may be due to high content of secondary metabolites like curcumins, alkaloids, flavonoids, terpenoides, phenols, tannins, saponins etc.

Conclusion

This study found that treatment with *Curcuma leucorrhiza* extract improves the *in vivo* antioxidant status in STZ induced diabetic Wistar rat. The ethanolic extract (absolute) emerged as the most therapeutically relevant *in vivo*, significantly restoring antioxidant defenses in streptozotocin-induced diabetic rats. The level of SOD, Catalase, GPx decreased while the level of MDA was increased on induction of diabetes. However, the study′s reliance on morphological authentication of the plant material indicated the need for future molecular characterization to ensure species consistency.

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Conflict of interest

The authors declare that they have no conflict of interest.

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