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Original Article

Effect of *Artemisia Absinthium* on the Neurochemical Profile of Streptozotocin Induced Diabetic Rat Brain

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ABSTRACT

The study was aimed to investigate the effect of the methanolic leaf extract of *Artemisia absinthium* – MLEAA on the activity of acetylcholinesterase, levels of acetylcholine and lipid profile in the brain tissue of Streptozotocin (STZ) induced diabetic Wistar rats.A total of thirty two adult male rats was divided into four groups, 8 rats in each group; Normal (N), Normal treated (NT) with *Artemisia absinthium*, Diabetic (D) and Diabetic treated (DT) with *Artemisia absinthium*. 55 mg/kg body weight of STZ was used to induce experimental diabetes mellitus in normal adult male Wistar rats by a single dose of intraperitoneal injection (IP).MLEAA treatment for 60 days resulted in marked increase in the levels of total lipids, phospholipids, glycolipids, cholesterol and significant decrease in triglycerides levels in DT group rats when compared to D group rats. Also treatment with *Artemisia absinthium* normalized the levels of Ach by enhancing the activity of AchE in DT group rats.MLEAA treatment of DT group rats for 60 days accentuated the protective effect of *Artemisia absinthium* on brain tissue in STZ induced diabetic Wistar albino rats.

Keywords: Lipid profile, acetylcholinesterase, *Artemisia absinthium*, STZ.

1. INTRODUCTION

Diabetes mellitus is a metabolic derangement associated with abnormalities in major metabolic pathways like carbohydrate, amino acid and lipid. It is featured by hyperglycemia, insulin deficiency, with long-term complications affecting different organs like brain, eyes, heart, kidney and limbs [1].This disorder is divided into insulin dependent diabetes mellitus (IDDM)-Type 1 Diabetes and non-insulin-dependent diabetes mellitus (NIDDM) -Type 2 Diabetes. IDDM is an auto immune condition raised due to damage of insulin producing pancreatic beta cells. NIDDM is

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because of peripheral insulin resistance and impairment of insulin secretion [2, 3].

World Health Organization (WHO) estimates reported that densely populated Asian countries like India and China's expenditure is going to be more than US$1 trillion annually for treating this disease and its complications annually due to huge increase of diabetic cases by 2030 [4,5].

STZ diabetic rat serves as an excellent model to study the molecular, cellular and morphological changes in the brain induced by stress during diabetes [6].In STZ induced diabetic rat models chronic oxidative stress due to the resulting hyperglycemia causes cellular and morphological changes in the brain [7].Through peroxidative mechanism free radicals damage the brain resulting neurochemical and neurophysiological changes that leads to complications like decreased blood flow to the brain, splitting of the blood brain barrier(BBB) and inflammation causing cerebral edema [8]. Finally all these changes subsidize to the everlasting aggravation linked with diabetes mellitus, including kidney failure, blindness, foot amputation, neurological damage, coronary artery disease (CAD) [9-11].

Large requirement of glucose, more oxygen consumption, high lipid content and scanty antioxidant system of brain makes it more susceptible to oxidative stress.STZ induced hyperglycemia in male Wistar rat's causes a decrease in the level of acetylcholinesterase (AChE) due to oxidative stress in the brain tissue of experimental rats [12].Diabetes is allied with mass exodus of deformed neurotransmitter. AchE in brain is chiefly localized in neurons and belongs to a family of hydrolases.Cholinergic function is marked through the hydrolysis of acetylcholine by AchE. It is known to be involved in synaptogenesis and regulates cholinergic nerve and neuromuscular transmission.

If acetylcholinesterase activity is low, the synaptic concentration of acetylcholine will increase than normal. Acetylcholine is the neurotransmitter produced by cholinergic neurons. In the CNS acetylcholine is involved in learning, memory, and mood. Irreversible inhibition or low levels of AChE and accumulation of high levels of Ach in synapse leads to sweating, bronchial constriction, convulsions, paralysis, and death [13].

Deficiency of peptide hormone insulin decreases the lipase activity and increases free fatty acids mobility. The STZ induced diabetic model is thus considered as model of Type-I diabetes mellitus and hyperlipidemia [14]. Diabetes is also responsible for developing premature arteriosclerosis due to increase in TAG (triacylglycerol) and LDL (low density lipoproteins) levels and decrease in HDL (high density lipoprotein) levels [15].Now days along with insulin several oral hypoglycemic drugs are available for the treatment of diabetes but none of the drug offers faultless glycemic control [16]. Throughout the world medicinal plant parts and their extracts are used for treating many diabetic complications. Medicinal plant parts and herbs are included in Ayurveda, Homeopathy, Siddha and Unani in India for the treatment of diabetes mellitus since very long time [17].

Artemisia absinthium (Wormwood) is an aromatic, perennial shrub that was used as alternative herbal medicine for several health disorders. Even though *Artemisia absinthium* is globally distributed it is naturally distributed in Jammu and Kashmir region in India. Ethno pharmacological evaluation of *Artemisia absinthium* revealed its free-radical scavenging activity, anti-oxidative stress function, antioxidant activity and neurite outgrowth function [18].

These strong principle characteristics of *Artemisia absinthium* provoked us to investigate whether this plant has any protective effect on neurochemical

profiles of brain like acetyl choline, lipid profile and activity of acetyl cholinesterase in STZ induced diabetic rats.

2. MATERIALS AND METHODS

Chemicals

All chemicals of the present investigation were obtained from Sigma Chemical Company (USA) and SISCO Research laboratory Pvt. Ltd, India.

Plant material

Dry powder of MLEAA (methanol leaf extract of *Artemisia absinthium*) was procured from Mahaks Herbal & Aromatic Agro Products, Srinagar, Jammu& Kashmir.Before the utilization of MLEAA extract it was suspended in 5% Tween-80 in distilled water.

Maintenance of lab animals

Adult male Wistar rats of body weight 150–200 g were acclimatized for a week in the animal house and maintained at a standard temperature of 24-28^{o}C with a 12 h light/dark schedule cycle.Rats were fed with a rodent pellet diet and water *ad libitum* under aseptic conditions.The study was conducted in the Post Graduate Department of Pharmacology Laboratory, Sree Siddaganga College of Pharmacy, Tumkur, with due permission from the Institutional Animal Ethics Committee (IAEC) with Regd. no: 123/PO/C/99/CPCSEA.

Experimental Design

Grouping of animals

A total of 32 rats used were divided into four groups of eight in each as: Normal rats (N), Normal rats treated with MLEAA (NT), Diabetic rats (D), Diabetic rats treated with MLEAA (DT).

STZ injection

In D and DT marked groups diabetes was induced after 16 hours of fasting by a single intraperitoneal injection of freshly prepared streptozotocin solution with adosage of 55 mg /kgbody weight [19, 20] in ice cold 0.05 M citrate buffer pH 4.5 at a volume of 0.1 ml per rat. 72 hours after the inoculation of STZ dose, rats with determined plasma glucose levels above 300 mg/dl were considered as diabetic and confirmed for usage in the experimentation.

Artemisia absinthium treatment

The dose of MLEAA (500 mg/kg body weight) in the treatment is based on the previous research reports on the *A. absinthium* extract [21, 22].NT and DT groups were treated daily with MLEAA (500 mg/kg body weight), orally by gastric intubation in 5% Tween-80 in distilled water per rat once a day for two months. N (Normal) and D (diabetic) rats were given distilled water instead of MLEAA. Body weight, fasting plasma glucose and levels of insulin were monitored at 15-day intervals till the end of the study.On the basis of the former experimental results on the dose-dependent antihyperlipidemic effect of *A. absinthium* extract, a dose less than 200 mg/kg b.w. was not expected to be effective in rats [21, 22].

Sacrifice of rats and brain collection

At the end of the experimentation all the rats from different groups were sacrificed by cervical dislocation after 12 hours of starvation and immediately the total brain tissue was dissected out and washed with saline (0.9% NaCl- ice cold) and utilized for the analysis of biological parameters.

Biological Parameters

Methodology

Extraction of lipids from Brain tissue

Folch reagent (2:1 chloroform – methanol mixture) was used to prepare tissue homogenate by centrifuging at 3000 rpm. Then 5 ml of supernatant was mixed with 3 ml of distilled water, centrifuged at 3000 rpm and resulting organic phase was used for the estimation of PL, GL, TG and cholesterol analysis [23].

Estimation of total lipids (TL) by gravimetric method

Brain tissue (5g) is homogenized with equal volumes (5ml each) of $CHCl_3$ (chloroform) and CH_3OH

(methanol). Again $CHCl_3$ (5ml) is added to the mixture and homogenized for another 30seconds. Finally water (5ml) is added to this mixture, and the sample is again homogenized for 30 more seconds. Then he mixture is allowed to separate, the lower solvent phase is removed and filtered through a Whatman no. 1 filter paper, the filtrate is collected. This procedure is repeated once again with the addition of chloroform step.At the final step of theprocedure the filtrate is allowed to separate in a graduated cylinder, and the volume of the lower $CHCl_3$layer is recorded. Pre-weighed aluminum pans (3 pans per sample) are used to evaporate 0.5 ml aliquots of the $CHCl_3$ layer overnight in a hood to estimate the total lipid (TL) content of the brain tissue by recording the weights, and then converting to percent lipids [24].

Estimation of triglycerides (TG)

GPO-PAP procedure of Foosati*et al*was applied to estimate Triglycerides (TG) using Liquid Gold Diagnostic kit [25]. Lipid extract of the brain (60µl) in an eppendorff tube, was evaporated using an incubator. Then it is added to 1.0 ml of the TG reagent and incubated at 37 ˚C for 10 minutes. In the same way standard (TG 200 mg %) and blank (H_2O) were also treated. At 505 nm absorbance was recorded and the results are expressed as mg/g tissue after incubation for all the samples.

Estimation of cholesterol (C)

CHOD-PAP enzymatic method was used to estimate total cholesterol content [26]. In an incubator lipid extract (60µl) was evaporated, and then it is added with cholesterol reagent (1ml) and incubated at 37˚C for 10minutes. Blank (H_2O) and Cholesterol standard (200 mg %) were also treated in the same way. At 510 nm, absorbance was recorded and the results are expressed as mg/g tissue after incubation for all the samples.

Estimation of phospholipids (PL)

Connerty*et al* method was implied for the estimation of Phospholipids (PL) [27]. Through Fiske and Subbarow method released inorganic phosphate (Pi) was estimated by digesting PL with H_2SO_4 [28]. About 200 µl of lipid extract was evaporated; it is added to 1 ml of 10 N H_2SO_4 and digested for an hour in a hot water bath. Then H_2O_2 (20µl) was added to it, boiled till colorless liquid is seen and the tubes were cooled. Later phosphorus (Pi) was estimated by Fiske Subbarow method. 1.0 ml of molybdate II, 0.4 ml of ANSA (1-amino-2-napthol-4-sulfonic acid) reagents were added to the digest and the volume was made up to 10 ml with distilled H_2O. At 660 nm developed colour (blue) was recorded after15 minutes of incubation.Phospholipid (PL) content is obtained by multiplication of phosphate (Pi) value by 25.The results are expressed as mg/g tissue.

Estimation of glycolipids (GL)

Roughan&Batt method was used to estimate Glycolipid (GL) content in lipid extract of brain tissue [29]. Tissue homogenate (200 µl) was evaporated, added to 2 ml of 2 N sulphuricacid and digested for two hours in a water bath (boiling). After hydrolytic digestion, it is centrifuged by adding 4 ml of $CHCl_3$ (chloroform). The Supernatant aqueous layer was collected after centrifugation and it is added with 50 µl of 80% C_6H_5OH (phenol) and 4 ml of con.H_2SO_4. The colour (orange) developed was read at 480 nm. Standards of galactose sugar (20-200 µg) were also treated in the same procedure. Multiplication of galactose sugar concentration with 4.45 gives the concentration of glycolipid and the results are expressed as mg/g tissue.

Homogenate Preparationfor the analysis of acetyl choline and acetylcholine esterase levels

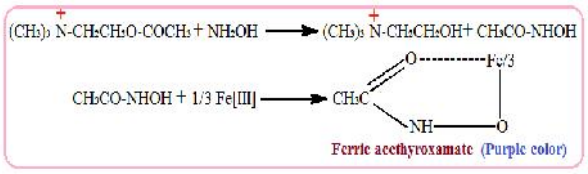
Potassium chloride (KCl -0.15 M) is used to prepare 10% of tissue homogenate using a homogenizer at 0 ˚C. Then the sample was centrifuged at 12,000 rpm for 45 minutes at 4˚C. After centrifugation supernatant was

distributed into tubes (eppendorff), stored at-20 ˚C and used for chemical assays.

Estimation of acetyl choline

Procedure described by Metcalf and Augustinson (methods of biochemical analysis) was opted for the estimation of acetyl choline (Ach) content in the brain tissue [30, 31]. Principle of this procedure is - added alkaline hydroxylamine (NH_2OH) reacts with acetyl group of acetyl choline (Ach) content of the brain tissue to form acetyl hydroxamate, in acidic medium it reacts with ferric chloride ($FeCl_3$) to form colouredcomplex (purple) then its absorbance is measured at 540 nm spectrophotometrically against the blank. Finally acetyl choline (Ach) content is expressed as µmoles of Ach/g of tissue [32].

Principle



Estimation of Acetylcholinesterase activity

Ellman*et al* method of colorimetric determination of acetylcholinesterase activitywas used for the estimation of Acetycholinesterase (AchE) in the brain homogenate [33]. Acetyl thio-choline upon enzymatic hydrolysis (AchE), produces acetate and thiocholine. Later thiocholine reactswith dithiobisnitro-benzoate (DTNB) and develops yellow color. The absorbance of the developed yellow color is read at 412 nm by spectrophotometer. The AchE activity was measured and expressed as µmoles of Ach hydrolysed/min/mg protein by using molar extinction coefficient (ε - epsilon) of DTNB (14.3x103) [34, 35].

Principle

Statistical data analysis

The results were expressed as mean ± S.E.M. Research data was analyzed for significant difference using Duncan's Multiple Range (DMR) test ($P < 0.05$) (Duncan, 1955) [36].

3. RESULTS

Effect of MLEAA on AchE and Ach

Table 1and figures 1&2 represents the activity of AchE and the content of Ach in the tissue extract of brain of experimental rats. In D rat's 23% eloquent decrease of AchE activity and 57 % increase in concentration of Ach were reported when compared with N rats. Treatment with the extract of *A.absinthium* for 60 days in DT group rats resulted in the 56% indicative rise in the AchE activity and 46% of prognostic decline in the Ach content in comparison to D group rats. At the end of the 60 days study, NT group rats have shown 38% higher activity of AchE and 23% of minor decrease in the content of Ach when compared to N group rats. Current study of MLEAA treatment of STZ induced diabetic rats has maintained the normal levels of Ach by regaining the AchE activity within two months.

Table 1: Effect of *Artemisia absinthium* methanol extract (MLEAA) on ACh content and AChE activity in the brain of STZ induced diabetic rats

PARAMETERS	N GROUP	NT GROUP	D GROUP	DT GROUP
Acetylcholine esterase (µmoles of Achhydrolysed /min/mg protein)	0.312±0.013^{a}	0.432±0.021^{d}	0.241±0.006^{a}	0.376±0.024^{b}
Acetylcholine (µmoles of Ach/g tissue)	3.27±0.17^{b}	2.52±0.13^{a}	5.13±0.14^{c}	2.98±0.18^{a}

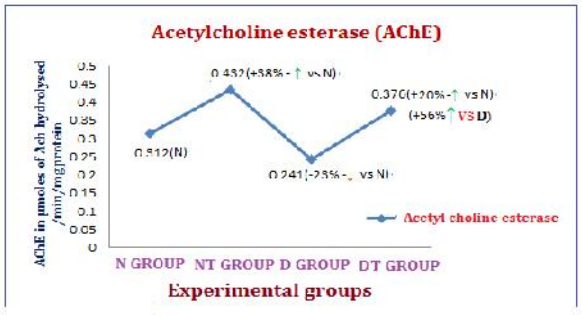


Fig 1: Effect of *MLEAA* on AChE activity in the STZ induced diabetic rat brain.

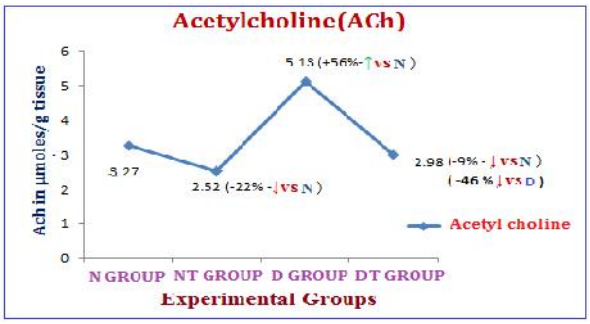


Fig 2: Effect of *MLEAA* on ACh content in the STZ induced diabetic rat brain.

Effect of MLEAA on lipid profile

Total lipids (TL) comprises phospholipids (PL), glycolipids (GL), cholesterol and triglycerides (TG).Brain tissue lipid profile of normal (N), normal treated (NT), diabetic (D) and diabetic treated (DT) rat groups are displayed in table 2& figures3-7.Diabetic rats (D) exhibited significant decrease in TL (62%) content when compared with normal group (N). *Artemisia absinthium* treatment for 2 months improved TL (103%) content greatly in DT rats when compared to D group. But this marked hike in TL content in DT did not reach the normal values. Thus DT rats showed somewhat lesser TL (24%) content when compared to N group. NT rats displayed a minor (9%) but not marked reduction in TL content when compared to N rats.

D rats displayed quite downturn in the levels of PL, GL, and cholesterol (35 %, 72 % and 25 %) but very much boost in TG (54 %) levels when compared to N rats at the end of experimentation. DT rats after systematic medication with *Artemisia absinthium* extract for 60 days exposed growth in the levels of PL, GL, and cholesterol (26 %, 140 % and 21%) and much decline in TG (34%) levels when compared to D rats. PL, GL, and cholesterol levels of DT rats did not reach the normal values of N rats though TG levels were normalized. NT rats show cased a slight rise in GL (29 %) and reduction in cholesterol and PL (4.6% and 7.8 %) and slight growth (2.6%) in TG levels when compared to N rats.

Table 2: Effect of *Artemisia absinthium* methanol extract (MLEAA) on lipid profile (TL, PL, TG, GL and cholesterol) of the brain tissue in STZ induced diabetic rats.

Parameters	N group	NT group	D group	DT group
Total lipids (mg/g tissue)	340.36±13.42 ^{d}	307.12±5.32 ^{c}	127.15±16.32 ^{a}	258.27±4.56 ^{b}
Phospholipids (mg/g tissue)	67.42±1.45^{c}	62.11±1.32^{c}	43.32±2.12^{a}	54.72±1.15^{b}
Triglycerides (mg/g tissue)	7.84±0.12^{a}	7.63±0.22^{b}	12.14±0.31^{d}	7.96±0.54^{c}
Cholesterol (mg/g tissue)	30.21±0.85^{d}	28.82±0.23^{c}	22.51±1.24^{a}	27.43±0.51^{c}
Glycolipids (mg/g tissue)	3.72±0.16^{c}	4.82±0.23^{d}	1.01±0.11^{a}	2.43±0.21^{b}

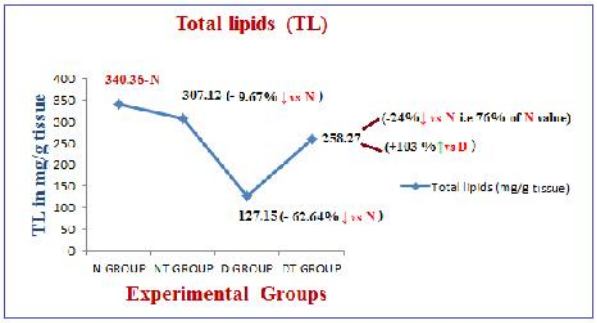


Fig 3: Effect of *MLEAA* on Total lipid content in STZ induced diabetic rat brain

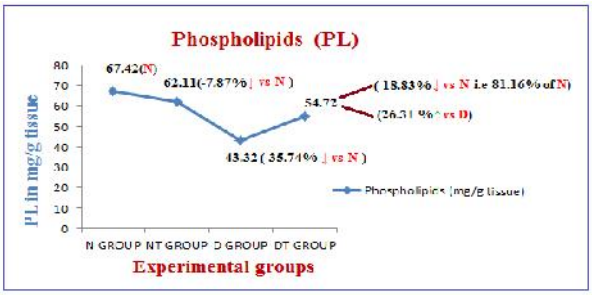


Fig 4: Effect of *MLEAA* on Phospholipid (PL) content in STZ induced diabetic rat brain.

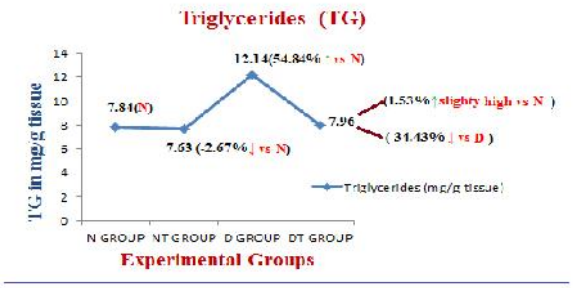


Fig 5: Effect of *MLEAA* on Triglyceride (TL) content in STZ induced diabetic rat brain

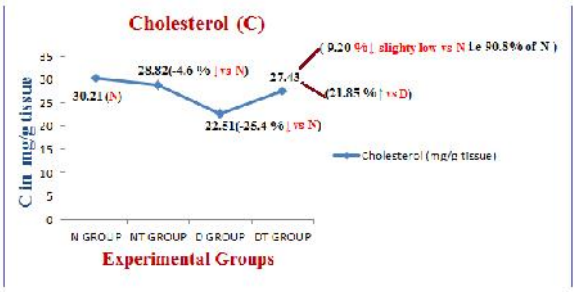


Fig 6.Effect of *MLEAA* on Cholesterol (C) content in STZ induced diabetic rat brain

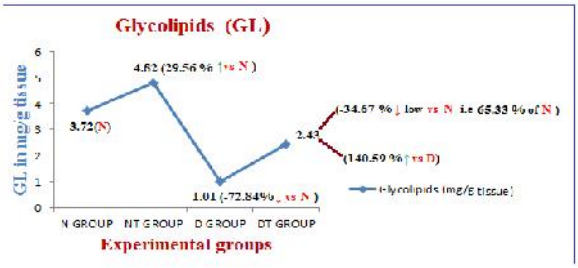


Fig 7: Effect of *MLEAA* on Glycolipids (GL) content in STZ induced diabetic rat brain

4. DISCUSSION

AchE is one of the hydrolase enzyme confined to neurons of the brain. Its cholinergic neural affair is due to hydrolysis of neurotransmitter acetylcholine (Ach).AchE is located on the post-synaptic membrane through covalent linkage to phosphatidylinositol-glycolipid [37].Many published studies have reported decline in the activity of AChE in brain and RBC membrane in diabetic condition [38].According to the reports of Olney et al., 1986 shrinking in the levels of AChE leads to accumulation of acetylcholine, hyperactivity of cholinergic neurons, convulsion and epilepsy [39]. Decrease in the levels of acetyl cholinesterase acts as an indicator for diabetic neuropathy, although its contribution is direct or not. Hyperglycemic condition in the STZ-induced diabetic rats, activity of AChE in muscle end-plate is reduced. Decline in the activity of AChE subsidize pathology and consecutive muscle weakness [40].

In STZ induced diabetic rats debilitated ACh metabolism causes muscle weakness due to decreased number of acetylcholine (ACh) containing vesicles as well as degeneration of mitochondria within motor nerve endings [41]. Acetylcholine is released when electrical inclination of the axon reaches a motor endplate. It diffuses across the synaptic cleft and binds to nicotinic receptors on the muscle fibers, causing them to contract [42, 43].Hike in the AChE activity may be due to regaining of normal insulin and blood glucose levels.Thus MLEAA treatment for 60days has restored plasma insulin, which has led to normalization of AchE activity and Ach levels in brain of DT rats. Our earlier published reports declared the antidiabetic activity of *Artemisia absinthium* [21, 44].The decrease in membrane fluidity of diabetic brain could be due to free radical lipid peroxidation of membrane phospholipids, which is generated by perpetual hyperglycemia.

The decreased PL, GL and cholesterol and increased TG content of D rats compared to N rats indicate the presence of lipid damage or oxidation. Changes in the STZ induced diabetic rat brain may be due to nerve decadence. Our reports of decreased PL, GL and cholesterol content in diabetic rats are in agreement with earlier studies [45]. Diabetes has been linked with an expanded peril of developing early on atherosclerosis due to increase in TG and LDL levels and decrease in HDL levels. Thus dyslipidemia is a well-known characteristic feature of diabetes due to altered lipid metabolism in many tissues.MLEAA treatment of diabetic rats has acknowledged the normolipemic, anti-hypertriglyceridemic properties of *Artemisia absinthium*.

5. CONCLUSION

It is concluded that constructive chattel of MLEAA *(Artemisia absinthium*) is anticipating in counteracting against hypertriglyceridemia and is helpful in maintaining normal levels of Ach, AChE and lipids (PL, GL and cholesterol) in STZ induced diabetic rats. This study has undeniably furnished proof for the safe use of the leaves of *Artemisia absinthium* by conventional therapists in the prescription of diabetes mellitus. However, further investigation is required for the recognition of complete anti-diabetic actions of the *Artemisia absinthium*.

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