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The Pharmacological Evaluation of Epigallocatechin-3-Gallate (EGCG) Against Diabetic Neuropathy in Wistar Rats

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ABSTRACT

The present study was design to evaluate the *Epigallocatechin-3-gallate* (active constituent of Green tea) 200 mg/kg and 400 mg/kg/ p.o dose against Diabetic neuropathy in wistar rats. For the Evaluation of Anti-nociceptive activity of EGCG in diabetic neuropathy, Streptozotocin induced diabetes neuropahty model was used in wistar rats and it was evaluated by Physical parameters: Body weight, Grip strength (Rota-rod), pain-threshold level by Tail Flick Method, Hot plate Method, Formalin Induced Pain test, Biochemical Estimations by Tissue parameters: siatic nerve antioxidants (superoxide dismutase, catalase, lipid peroxidation, glutathione peroxidase) level and Serum parameters: glucose, Total protein and Histopathology of the sciatic nerve was done. After 8 weeks of treatment of *Epigallocatechin-3-gallate* 400 mg/kgdose produced more significant. ***P<0.001, Anti-nociceptive activity in Streptozotocin induced diabetes neuropathy in rat as compare to *Epigallocatechin-3-gallate* 200 mg/kg. Thus the results indicate that the *Epigallocatechin-3-gallate* (EGCG) have dose dependant activity in Diabetic neuropathy in wistar rats.

KEY WORDS: *Epigallocatechin-3-gallate*, Streptozotocin, Diabetic neuropathy.

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INTRODUCTION:

Diabetic neuropathies are a family of nerve disorders caused by diabetes. Up to 50% of all patients with diabetes develop neuropathy and the prevalence of painful neuropathy ranges from 10 to 20% of patients with diabetes People with diabetes can, over time, develop nerve damage throughout the body. Some people with nerve damage have no symptoms. Others may have symptoms such as pain, tingling, or numbness—loss of feeling—in the hands, arms, feet, and legs. Nerve problems can occur in every organ system, including the digestive tract, heart, and sex organs. About 60 to 70 percent of people with diabetes have some form of neuropathy. People with diabetes can develop nerve problems at any time, but risk rises with age and longer duration of diabetes. The highest rates of neuropathy are among people who have had diabetes for at least 25 years. Diabetic neuropathies also appear to be more common in people who have problems controlling their blood glucose, also called blood sugar, as well as those with high levels of blood fat and blood pressure and those who are overweight. Neuropathic pain is generally considered to be one of the most troublesome complications affecting diabetic patients Current therapy for painful neuropathy includes use of antidepressants, ion channel blockers, NMDA receptor antagonists, opioids, topical lidocaine and capsaicin. Pain relief with current therapy is often inadequate and associated with side effects.[1,2]

The hyperglycemia is the backbone of the pathophysiology of diabetes leading to the development of complications like diabetic neuropathy (DN) through many intertwined cellular pathways which have been shown to coalesce into a common fate i.e. oxidative stress. Reactive oxygen species (ROS) are notorious for contributing to cell and tissue dysfunction and damage in DN. Hyperglycemia unleash multiple pathways such as enhanced aldose reductase activity, increased advanced glycation end products and altered protein kinase C activity to induce oxidative stress. Prolonged hyperglycemia, through over production of ROS, is likely to damage dorsal root ganglion mitochondrial DNA, adding to long-term nerve dysfunction. Many studies have supported this hypothesis, including in vivo and in vitro measurement of oxidative stress in sensory neurons and dorsal root ganglion. Exhaustion of natural antioxidant depot in the vascular endothelium and schwann cells of the sciatic nerve may contribute to the neurovascular and metabolic deficits in DN.[3,4]

MATERIALS AND METHODS:

Active compound: The active constituent of the Green tea (*Camellia sinensis*) *Epigallocatechin-3-gallate* (EGCG) was taken as a gift sample from Division of Rheumatology, Department of Medicine, Metro Health Medical centre, Cleveland, Ohio, USA.

Drugs: Biocem, Aliza-80mg (Gliclazide-80 mg) Mefro Pharmaceuticals Pvt Ltd.

Diagnostic kits: Diagnostic kits used for estimation of glucose level and total protein were procured from ANAMOL Laboratories Pvt. Ltd. Maharashtra, India.

EXPERIMENTAL ANIMALS:

Male albino rats of wistar strain weighing between 150 and 200 gm were used in the study. The animals were housed under standard environmental conditions containing husk as bedding material (temperature 22±2 ◦C; humidity 60±4%) with a 12 h light/dark cycle at the animal house of The experimental protocol was approved by the institutional animal ethical committee (IAEC) of NIMS institute of pharmacy, NIMS university, Shobha Nagar, Jaipur NU/PH/M/COL/12/66 With CPCSEA Registration no 1302/ac/12 /CPCSEA**.** Animals had free access to water ad libitum. and all procedures were conducted in accordance with the "Guide for the Care and Use of Laboratory Animals.

METHODOLOGY:

Streptozotocin induced diabetes diabetic neuropathy:

Healthy wistar strain albino rats of either sex weighing about 150-200 grams were taken. animals were deprived to food for 16 hours but allowed free access to water after that blood sample was collected from tail of rats and measure blood glucose level by using GOD-POD kit method. Then they were injected with streptozotocin dissolved in 0.1M sodium citrate and citric acid at a dose of 55 mg/kg body weight intraperitoneally. Then animals were kept for 21 days during which food and water was allowed. After 21days of streptozotocin administration blood glucose level, body weight, grip strength and pain sensation measurements were taken. The animals showed fasting blood glucose level above 250 mg/dl considered diabetic rats and after that they were divided into five groups in which each group contained six animals. the blood glucose level, body weight, measurements of each rat were taken at the start and at the end of experiment. After the 4th week of the experiment administered the control, standard and test drug orally daily for 8 weeks.[5,6,7]

Physical parameters :

I. ***Body weight*:** Diabetic animals show reduction in body weight hence body weight of all the animals measured at the completion of study.

II. ***(Grip-strength) Rota-rod test* :** Grip strength used for evaluation of muscle strength during Diabetic Neuropathy. The test was being used to assess muscular strength or neuromuscular function with the help of rota rod apparatus in which the rats were placed

on a horizontal rod rotating at a speed of 25 rpm. The rats which were capable of remaining on the top for 25 sec or more, in three successive trials were selected for the study. The selected animals were divided into five groups (n= 6). The time of stay at the rod was calculated.[8,9]

Measurement of Nociceptive Threshold:

I. ***Tail Flick :*** All groups of animals were experimented for this test. Animals are placed into individual restraining cages leaving the tail hanging out freely. The animals are allowed to adapt to the cages for 30 min before testing. The lower 5 cm portion of the tail is marked and the tail of the rat was immersed in hot water maintained at 55±0.2°C. the basal tail flick latency (withdrawal response of tail) or signs of struggle were observed. The cut off time was 10 sec.

II. ***Hot plate method:*** In this Hot Plate Method animals from the each group were placed on the hot plate which is commercially available, consists of an electrically heated surface. Temperature of this hot plate is maintained at 55-56 ˚C and observation is done up to the time until paw licking or jumping was noted the cut-off time was 10 sec. Then the average basal reaction time was noted after the oral administration of the drugs and test compounds.[10,11,12,13]

Formalin Test :

In this test, each animal was acclimatized to the observation box before any testing began. Then, it was given a subcutaneous injection hind paw using a 25-gauge syringe needle. Each rat was then immediately placed in a Plexiglas box (40 x 40 x 40 cm) positioned over a mirror angled at 45° to allow an unobstructed view of the paws by the observer. Observations to determine nociceptive responses began upon placing the rat into the box and continued for the next 60 min. A nociceptive score was determined for each rat after 15 min in a 5 min block during that period by measuring the amount of time spent in each of the four behavioral categories: 0, the position and posture of the injected hind paw is indistinguishable from the contralateral paw; 1, the injected paw has little or no weight placed on it; 2, the injected paw is elevated and is not in contact with any surface; 3, the injected paw is licked, bitten, or shaken. Then, a weighted nociceptive score, ranging from 0 to 3 was calculated by multiplying the time spent in each category and summing these products and dividing by the total time for each 5 or (300 sec) min block of time.[14,15,16]

Bio-chemical parameters:

I. ***Blood glucose level measurement:*** *The* animals blood was collected from tail of rats for the determination of the blood glucose levels. The plasma was obtained after centrifugation (3000 g for 10 min, 4°C). Blood glucose was estimated by GOD-POD kit method.[17,18]

II. ***Total protein :*** Total protein measured kinetically at 546 nm according to standard method by using commercially diagnostic kits from anamol laboratories pvt.ltd.[19]

Tissue parameters:

Preparation of heart tissue for estimation of oxidative stress marker:

The heart of each animals was removed after completion of experiment, weighed and the various heart were dissected out and homogenized with 10 times (w/v) ice cold phosphate buffer saline (50mM pH 7.8) in teflon glass homogenizer. The homogenate was centrifuged at 1000 rpm 4 ◦C for 3 min and the supernatant divided into two portions, one of which was used for measurement of lipid peroxidation (LPO) and the remaining supernatant was again centrifuged at 12,000 rpm at 4 ◦C for 15 min and used for the measurement of superoxide dismutase (SOD), catalase (CAT), and glutathione (GSH). was measured by the method of Lowry et al.

Estimation of catalase :

Catalase measurement was carried out by the ability of CAT to oxidize hydrogen peroxide (H_2O_2). 2.25 ml of potassium phosphate buffer (65 mM, pH7.8) and 100_l of the brain homogenate were incubated at 25 ◦C for 30 min. A 650_l H_2O_2 (7.5mM) was added to the brain homogenate to initiate the reaction. The change in absorption was measured at 240 nm for 2–3 min and the results were expressed as CAT mol/min mg of heart. [20,21]

Lipid peroxidation assay :

This assay was used to determine thiobarbituric acid-reactive substances as described by Slater and Sawyer (1971). In 2.0 mL of the tissue homogenate (supernatant) was added 2.0 mL of freshly prepared 10% w/v trichloroacetic acid (TCA) and the mixture was allowed to stand in an ice bath for 15 min, followed by centrifugation at 2500 rpm for another 15 min at 4°C. Two milliliter of clear supernatant solution was mixed with 2.0 mL of freshly prepared 0.67% w/v TBA. The resulting solution was heated in a boiling water bath for 10 min. It was then immediately cooled in an ice bath for 5 min. The absorbance of colour developed was measured by UV/VIS double beam spectrophotometer (systraonic Japan) at 532 nm using 1, 1, 3, 3-tetraethoxypropane as a standard.[22,23]

Estimation of glutathione:

The assay of GSH was determined by method described by Moron *et al.* (1979). One milliliter of tissue homogenate (supernatant) and 1.0 mL of 20% TCA were mixed and centrifuged at 2500 rpm for 15 min at 4°C. In 0.25 mL of supernatant, 2 mL of DTNB (0.6 M) reagent was added. The final volume was made up to 3 mL with phosphate buffer (pH 8.0). The colour developed was read at 412 nm against reagent blank. Different concentrations (10-50 µg) of standard glutathione were processed as mentioned above for constructing standard curve. The amount of reduced glutathione was expressed as µg of GSH /mg of protein. [22,23]

Estimation of superoxide dismutase:

The SOD activity was determined by the method of Misra and Fridovich (1972). 0.5 mL of heart homogenate + 0.5 mL of cold distilled water + 0.25 mL of ice-cold ethanol and 0.15 mL of ice-cold chloroform were mixed well using cyclo mixer for 5 min and centrifuged at 2500 rpm for 15 min at 4°C. To 0.5 mL of supernatant, 1.5 mL of carbonate buffer (pH 10.2) and 0.5 mL of 0.4 M ethylene diamine tetra acetic acid (EDTA) solutions were added. The reaction was initiated by the addition of 0.4 mL of epinephrine bitartarate (3 M) and the change in optic density/minute was measured at 480 nm against reaction blank. SOD activity was expressed as units/mg of protein. Change in optical density per minute at 50% inhibition of epinephrine to adrenochrome transition by the enzyme was taken as the enzyme unit. Calibration curve was prepared by using 10-125 units of SOD.[24,25]

RESULTS:

Physical parameters:

Table 1: Epigallocatechin-3-gallate effect on body weight and grip strength.

Group	Body weight(gm)	Rota-rod (Time in sec)
Normal Control	179.0±0.89	27.00±1.41
Diabetic Control	112.7±1.75$^{\#}$	9.00± 0.89$^{\#}$
Gliclazide10 g/kg	176.8±1.16***	26.00± 1.41***
EGCG 200 mg/kg	134.0±0.89***	15.17±1.16***
EGCG 400 mg/kg	175.2±0.75***	24.83±1.32***

Data were expressed as mean±S.D.(n=6) and analyzed by one way ANOVA followed by, dunnets comparison test.***P<0.001, when compared to Diabetic control group $^{\#}$P<0.001 when compared to Normal control group.

Measurement of muscle grip strength (Rota-rod) was used to diagnose the diabetic neuropathy at the start and at the end of experiment. The muscle grip strength reduced significantly in all STZ treated groups that showed the induction of diabetic neuropathy. In the Diabetic control group the muscle grip strength was significantly *(P<0.001)*lower as compared with the normal control group. The grip strength of Gliclazide and all test group animals showed grip strength significantly *(P<0.001)*increased when compared to the Diabetic control group. The grip strength of Gliclazide drug (10 mg/kg) and EGCG-400mg/kg treated animals were found to be comparable with normal control group.

The body weight increased normally in normal control rats, while STZ induced diabetic rats (Diabetic control) showed a significantly *(P<0.001)* decreased in body weight, The body weight was significantly increased *(P<0.001)*in treated groups as compared to the diabetic control group (Table.1).

Measurement of nociceptive threshold :

Table 2: Epigallocatechin-3-gallate effect on the reaction time in tail flick, hot plate and (Test score) in formalin test.

Group	Tail flick (Reaction time in sec)	Hot plate (Reaction time in sec)	Formalin (Testscore)
Normal Control	9.00±0.89	8.66± 0.51	1.233±0.10
Diabetic Control	3.16±0.75$^{\#}$	2.66± 0.51$^{\#}$	2.81±0.07$^{\#}$
Gliclazide10 mg/kg	8.16±0.75***	7.66± 0.51***	1.33±0.10***
EGCG 200 mg/kg	3.66±0.51^{ns}	4.33± 0.51***	2.15±0.05***
EGCG 400 mg/kg	7.00±0.89***	7.00± 0.63***	1.40±0.14***

Data were expressed as mean±S.D. (n=6) and analyzed by one way ANOVA followed by dunnets comparison test. ***P<0.001, ns=not significant, when compared to Diabetic control group, $^{\#}$P<0.001 when compared to Normal control group.

In this investigation we observed hyperglycia in diabetic control group and a significant improvement *(P<0.001)* in hot plate and tail flick response that is pain-threshold of diabetic animals with the treatment of Gliclazide 10 mg/kg. The response with dose of EGCG-200, EGCG-400mg/kg mg/kg was found to be significantly *(P<0.001)*. Increase in pain threshold and analgesic action of Gliclazide 10 mg/kg, EGCG-400 mg/kg was found to be near normal control group. There is no significant difference was found in EGCG-200mg/kg, when compared with the diabetic control group in the tail flick responses and significant decreased *(P<0.001)* in pain threshold was found in tail flick and hot plate response in diabetic control group when compared with normal control group (Table.2).

In formalin test the results demonstrated that there is an intensified nociceptive response and exaggerated hyper-algesic behavior in response to noxious stimuli in diabetic control group rats. The administration of Gliclazide10mg/kg, EGCG-200mg/kg, EGCG-400mg/k, produced a significant *(P<0.001)* antinociceptive effect when compared with the diabetic control group rats (Table.2).

Bio-chemical parameters:

Table 3: Effect of EGCG on the glucose and total protein level in rats blood serum.

Group	Serum glucose mg/dl	Total protein g/dl
Normal Control	81±0.89	6.15±0.10
Diabetic Control	267.2±1.47$^{\#}$	4.78±0.17$^{\#}$
Gliclazide10 mg/kg	82.00±0.89***	5.53±0.17***
EGCG 200 mg/kg	132.80±1.72***	4.91±0.17^{ns}
EGCG 400 mg/kg	83.00±0.63***	5.36±0.13***

Data were expressed as mean±S.D. (n=6) and analyzed by one way ANOVA followed by dunnets comparison test. ***P<0.001 ns=not significant, when compared to Diabetic control group, $^{\#}$P<0.001 when compared to Normal control group.

Blood glucose level of all experimental groups, except normal control group was increased significantly *(P<0.001)* after the STZ injection. In the diabetic group (Diabetic control) the blood glucose levels increased to the maximum measurable value of 267.2±1.47 mg/dl at the end day of the experiment and found to be significant increased *(P<0.001)* compared to the value of normal control group which was 81.0 ± 0.89 mg/dl. On the last day the animals treated with Gliclazide drug (10 mg/kg), EGCG-200 mg/kg and EGCG-400 mg/kg, were observed with significant decreased,*(P<0.001)* in their blood glucose as compared to Diabetic control group of animals. The blood glucose level of Gliclazide (10 mg/kg), treated group and EGCG-400 mg/kg on the day 42 was found to be comparable with control group. The total protein value decreased significantly*(P<0.001)* in diabetic control group in compared with the Gliclazide drug (10 mg/kg), EGCG-400mg/kg and normal control but not significantly decreased as compared to EGCG-200 mg/kg. (Table.3)

ENZYMATIC ESTIMATION:

Antioxidant:

1. *Superoxide dismutase (SOD):*
2. *Glutathione peroxidase (GSH):*
3. *Catalase (CAT):*
4. *Lipid peroxidation (LPO) :*

Table 4: Effect of EGCG on the GSH, LPO, SOD and CAT level in the rats sciatic nerve

GROUP	SOD (unit/mg protein)	LPO (nmoles /mg protein)	GSH (nmole/mg protein)	CAT (K/min)
Normal Control	12.91±0.10	1.498±0.023	0.031±0.0018	2.84±0.05
Diabetic Control	4.40±0.037$^{\#}$	3.657±0.025$^{\#}$	0.019±0.0011$^{\#}$	0.55±0.10$^{\#}$
Gliclazide 10 mg/kg	12.64±0.11***	1.55±0.01***	0.029±0.0018***	2.77±0.04***
EGCG 200 mg/kg	10.59±0.46***	1.76±0.04***	0.024±0.0007***	0.88±0.07***
EGCG 400 mg/kg	11.98±0.18***	1.58±0.01***	0.028±0.0014***	2.70±0.04***

Data were expressed as mean±S.D. (n=6) and analyzed by one way ANOVA followed by, dunnets comparison test.

***P<0.001, when compared to Diabetic control group. $^{\#}$P<0.001 when compared to Normal control group.

In this study lipid peroxidation level was significantly reduced *(P<0.001)* when compared to diabetic control group by when treated with EGCG 200 mg/kg and 400 mg/kg doses. SOD, CAT, GSH activity was significantly improved *(P<0.001)* following treatment with EGCG 200 mg/kg and 400 mg/kg, Gliclazide drug (10 mg/kg) doses when compared to diabetic control group the EGCG was significantly effective in lowering the free radical or oxidative stress level in both low and high doses. The diabetic control group showed significant increased in LPO level*(P<0.001)* and significant decreased *(P<0.001)* level of SOD,CAT,GSH in the sciatic nerve tissue as compared to normal control group with the EGCG treatment (Table.4).

Statistical analysis:

Result were expresses as mean ±SEM. The statistical significance of any difference in each parameter among the group was evaluated by ane-way –ANOVA using dunnets multiple comparison test.

HISTOPATHOLOGY OF THE SIATIC NERVE:

Morphological changes were examined in the sciatic nerve of all the groups of the experiment. In the peripheral nerves of the diabetic rat, there was reduction in the number of myelinated fibers and there appeared to be more small fibers in the sciatic nerve in Diabetic control group when compared to normal control and treated groups rats. EGCG 400 mg/kg treated group was found to be more effective than EGCG 200 mg/kg group.

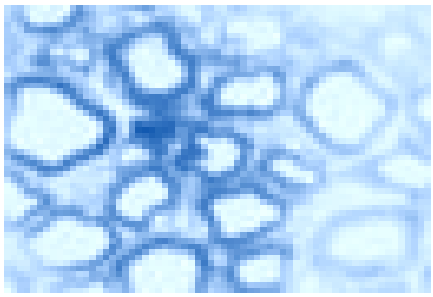

Fig-1. Normal Control

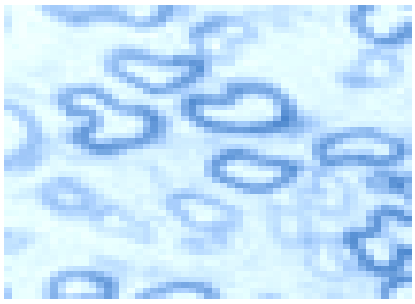

Fig-2. Diabetic Control

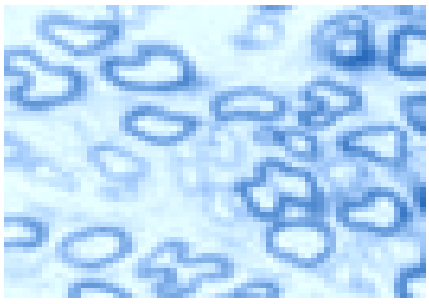

Fig-3. EGCG 200 mg/kg

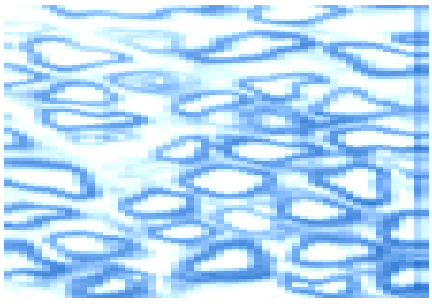

Fig-4. EGCG 400 mg/kg

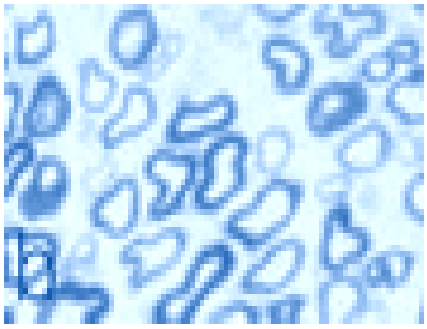

Fig-5.Gliclazide10 mg/kg

DISCUSSION:

The objective of the present study was to evaluate the *Epigallocatechin 3-gallate* (active constituent of Green tea)200mg/kg and 400 mg/kg p.o dose against Diabetic neuropathy and pyrexia in experimental animals. The STZ induced diabetic rat is the most commonly employed animal model of painful diabetic neuropathy. Acute hyperglycemia induced by STZ elicits activation of the polyol pathway with sorbitol accumulation, myo-inositol depletion and reduction of Na+,K+-ATPase activity. The decrease in myo-inositol causes depletion of phosphoinositides, followed by poor calcium mobilization and impaired protein kinase C activity, eventually resulting in impaired Na+, K+-ATPase activity. Thus, nerve Na+/K+ pump activity is reduced; fibers become Na+ loaded, Na+ channels become inactivated and MNCV decreases Hyperglycemia in diabetic neuropathy accompanied by weight loss were seen in adult rats treated with STZ in diabetic control group. The body weight increased normally in normal control rats, while STZ induced diabetic rats (Diabetic control) showed a significantly *(P<0.001)* decreased in body weight. EGCG treated groups exhibited increased body weight as comparative to diabetic groups. which is may be possible due to restored muscle wasting.

Diabetic neuropathy is a long-term complication of diabetes It is known that diabetic neuropathy is a nerve degenerative disease characterized by axonal degeneration, nerve fiber demyelination, and a reduction in the number of medium to large diameter nerve fibers, particularly in peripheral nerves. Decreased Na+ ,K+ -ATPase activity and motor nerve conduction velocity(MNCV) have been reported to be restored by aldose reductase inhibitors. dietary myo- inositol supplementation. gangliosides, prostaglandin E1, anti- oxidants or essential fatty Acid. In diabetic rats cAMP is one of the intrinsic intracellular modulators which regulates Na+ ,K+-ATPse activity or the Na+/K+ pump in various tissues. The significant improvement in the Grip strength and pain threshold level in rota-rod, tail flick, hot plate and formalin test in EGCG 400 mg/kg treated group may be a consequence to improved in the antioxidant defense system improvement because due to its highly anti-oxidant property as well as due to controlling the sciatic nerve oxidative damage from the ROS that's why increased in SOD, CAT and GSH activity.

CONCLUSION:

The Present study results have been obtained in carefully controlled experiments with laboratory animals where psychological factors can presumably be ruled out. the observation indicates that hyperglycemia and oxidative stress is responsible for the development of diabetic neuropathy Protective effect of EGCG against Diabetic neuropathy could be due to controlling hyperglycemia and reducing oxidative stress.This could provide a rationale for the use of *Epigallocatechin-3-gallate* in

fever folk medicine. Thus present study therefore also supports the traditional claims of herbal medicines.

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