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A role of *Excoecaria agallocha L* against streptozotocin induced diabetes and diabetic neuropathy complications in TBARS in Rats

G. Kiran*[1], B.Geethika reddy[2], K.Sushanth sekhar[2], B.Viswanadha reddy

1.Associate professor, Department of pharmacology, A.M Reddy Memorial College of pharmacy, Narasaraopet, Guntur, Andhra Pradesh, India.
2. Department of Pharmacy Practice, Sims college of Pharmacy, Mangaldas nagar, Guntur, Andhra Pradesh, Pin-522509, India.

Corresponding Author: G. Kiran

ABSTRACT

The present study was designed to investigate the effect of different extracts of *Excoecaria agallocha L*. against diabetes mellitus and its related complications. Diabetes was induced by intraperitoneal administration of streptozotocin STZ (60 mg/kg) for the development of diabetic neuropathy. Treatment with both the extracts significantly attenuated the parameters of oxidative stress in sciatic nerve of diabetic neuropathic rats. Also, level of nitrite, TNF-α, TGF-β and IL1β was significantly increased in sciatic nerve of diabetic neuropathy animals, that were ameliorated by treatment with *Excoecaria agallocha L.* extracts. Histopathological changes in sciatic nerve of diabetic rats were also reversed by the treatment. These findings suggested that the *Excoecaria agallocha L*. may be used to manage the diabetes mellitus and its related complications such as diabetic neuropathy.

Keywords: *Excoecaria agallocha*, streptozotocin STZ, etc

INTRODUCTION

Diabetes mellitus is a metabolic disorder characterized by a loss of glucose homeostasis with disturbances of carbohydrate, fat and protein metabolism resulting from defects in insulin secretion, insulin action, or both. Without enough insulin, the cells cannot absorb sufficient glucose from the blood; hence blood glucose levels increase, and result as hyperglycemia. If the blood glucose level remains high over a long period, it can result in long-term damage to organs, such as kidneys, liver, eyes, nerves, heart and blood vessels. Complications in some of these organs can lead to death also. Diabetes mellitus (sometimes called "sugar diabetes") is a condition that occurs when the body can't use glucose (a type of sugar) normally. Glucose is the main source of energy for the body's cells. The levels of glucose in the blood are controlled by a hormone called insulin, which is made by the pancreas.

Excoecaria agallocha L.

*Excoecaria agallocha L. (*Euphorbiaceae) is an ancient mangrove tree also known as Blinding tree; Tillai, Kampetti (Tamil); Tilla, Tella and Chilla (Telugu); Telakiriya, Talia (Singhalese). It is widely distributed in the mangrove region of Pichavaram, Indian coastal regions, Australia from Northern New South Wales, along the northern coast line of Western Australia (1)

Morphological characteristics

Excoecaria agallocha is a deciduous and dioecious tree of about 15 meter height with abundance of latex (2). The latex is toxic in nature and may create blindness and blisters over skin. The stem bark is greenish in colour with lenticellate and fissures arranged vertically. The root part remains conjugated with each other. The leaves are orange-red colored, alternate and ovate elliptic, arranged oppositely with glands. The flowers are yellowish green in color, larger in males than the females, with specific odor. The fruit is encapsulated,

globose with 3lobes, containing black colored numerous seeds. The pollination occurs by the insect ***(Li et al; 2010).***

Traditional Values

The *Excoecaria agallocha* is considered as a sacred plant by the local community of the tamilanadu, and being used for the worship (***3).***Traditionally, the *Excoecaria agallocha* is being used in the various regions of Tamilnadu to treat various ailments like sores, ulcers, as a laxative and purgative. The plant part especially leaves are being used as emetic agent due to its bitter taste. The fumes of bark are also used to treat leprosy ***(4).*** On the other hand *Excoecaria agallocha* leaves are also being used against, snakebites, hemorrhagic fever, rheumatoid etc. The larvicidal, and mosquito repellents property have also documented traditionally ***(5).***

Fig 1: Leaves of the *Excoecaria agallocha L.*

Chemical constituents

A number of primary and secondary metabolites have been well reported in various parts of *Excoecaria agallocha* like, leaves, bark, stem, root and flowers. The plant is abundant in presence of terpenoids (especially, diterpenoids,) along with triterpenoids, phenols, flavones, sterols (phytosterol), tannins, carbohydrates, glycosides and amino acids. ***(6)***. The diterpenoids (specially; 2-Acetoxybeyera-1,15-diene-3,12-dione, Agallocha excoerins D–F, Agallochaone A, Agallochaol A, B, D–F, G–J, K–P, Q , Ribenol, Stachenol, Stachenone and many more) are abundant in various parts of *Excoecaria agallocha* ***(7).***

MATERIALS AND METHODS

Body weight, blood glucose and serum insulin estimation

Body weight of each animal was measured at the start of study and animals with similar weight were grouped together. Body weight of each group was measured periodically till end of study. FBG level was estimated at interval of 15 days by using commercial enzymatic kits procured from Reckon Diagnostics Pvt. Ltd. INDIA throughout the study. Serum insulin was determined by Insulin ELISA kit (DRG, Germany) in blood collected into tubes with anticoagulant.

Biochemical analysis

For biochemical estimations, a 10% (w/v) tissue homogenate (sciatic nerve) was prepared in phosphate buffered saline (pH 7.4) using a Teflon homogenizer. The homogenate was centrifuged at 1000 gm for 10 minutes at 4˚C to remove nuclei and unbroken cells. The pellet was discarded and clear supernatant thus obtained was used to assay thiobarbituric acid reactive substances (TBARS) and level of antioxidant enzymes, *viz.* superoxide dismutase (SOD) and reduced glutathione (GSH).

ANTIOXIDANT ENZYMES

Nitrite estimation

Nitrite was determined in the sciatic nerve using Greiss reagent and it indicated the nitric oxide level. 0.5 ml of Greiss reagent [1% sulphanilamide in 5% phosphoric acid (1:1) and 0.1% napthylamine diamine dihydrochloric acid in water)] was mixed with 0.1 ml tissue homogenate and absorbance was determined at 546 nm ***(8).*** The nitrite concentration was calculated from standard curve of sodium nitrite and expressed as µg/ml.

AGEs estimation in sciatic nerve

Level of AGEs in sciatic nerve was estimated by a method as previously given by (***9).***Sciatic nerve was homogenized using 0.25 M sucrose (2 ml) and then centrifuged at 2000 rpm at 4˚C and collected the supernatant. Pellet was resuspended in 2 ml sucrose solution and again centrifuged to collect the supernatant and mixed it with the previously collected supernatant. Add equal volume of TCA to the supernatant in order to precipitate the protein. Centrifuge the solution at 2000 rpm at 4˚C to obtain a protein pellet which is further mixed with 1 ml methanol to remove lipids. Protein was then dissolved in 1 ml of 1 M NaOH and protein concentration was measured at 280 nm against BSA standard curve. AGEs were then quantified using spectrofluorometer at an emission wavelength 440 nm and excitation at 370 nm, and values were expressed as relative fluorescence units (RFU)/mg protein.

STATISTICAL ANALYSIS

Statistical analysis was performed using Graph Pad Prism 6. Values were expressed as mean ± SEM and one way analysis of variance (ANOVA) was used for statistical analysis. One

way ANOVA followed by Tukey's multiple test.

RESULTS AND DISCUSSION

Effect of Excoecaria agallocha Extracts (AEEA and HAEA) on antioxidant Enzymes and TBARS

Level of antioxidant enzymes (SOD and GSH) reduced significantly in kidney, pancreas and liver of DN rats while level of TBARS increased dramatically in comparison to normal control group.

Effect of Excoecaria agallocha extracts (AEEA and HAEA) on GSH

AEAA increased the level of GSH in kidney, liver and pancreas in a dose dependent manner to 36±1.3, 39±1.7 and 40±1.8 µM/mg protein respectively at 200mg/kg; 54±1.7, 48±1.4 and 55±1.6 µM/mg protein at 400 mg/kg respectively. HAEA increased the level of GSH in kidney, liver and pancreas to 55± 1.5, 59 ± 1.8 and 62± 2.0 µM/mg protein respectively at 200 mg/kg; 61± 1.3, 58± 1.6 and 61±1.7 µM/mg protein respectively at 400 mg/kg in comparison to DN control group (36± 1.3, 39 ± 1.5 and 44 ± 1.7 µM/mg protein respectively). Glimepride increased the level of GSH in kidney, liver and pancreas to 61± 1.3, 57± 1.5 and 64 ± 1.7 µM/mg proteins respectively **(Fig 2)**.

Table 1: Effect of AEEA and HAEA on GSH in diabetic-nephropathy wistar rats

Organs	Normal	Diabetic	AEEA 200	AEEA 400	HAEA 200	HAEA 400	Glimepride 10
Kidney	76± 1.7	36± 1.2#	36± 1.3	54± 1.7*	55±1.5**	61±1.3***	61± 1.3***
Pancreas	63± 1.4	39± 1.5#	39± 1.7	48± 1.4*	59±1.8**	58±1.6***	57± 1.5***
Liver	67± 1.8	44± 1.7#	40± 1.8	55± 1.6*	62±2.0**	61±1.7***	64± 1.7***

*Values are mean ± SD (n=6).Where a represents #P < 0.001 as compared with control and *P < 0.05;**P < 0.01; and ***P < 0.001 in comparison with diabetic control.*

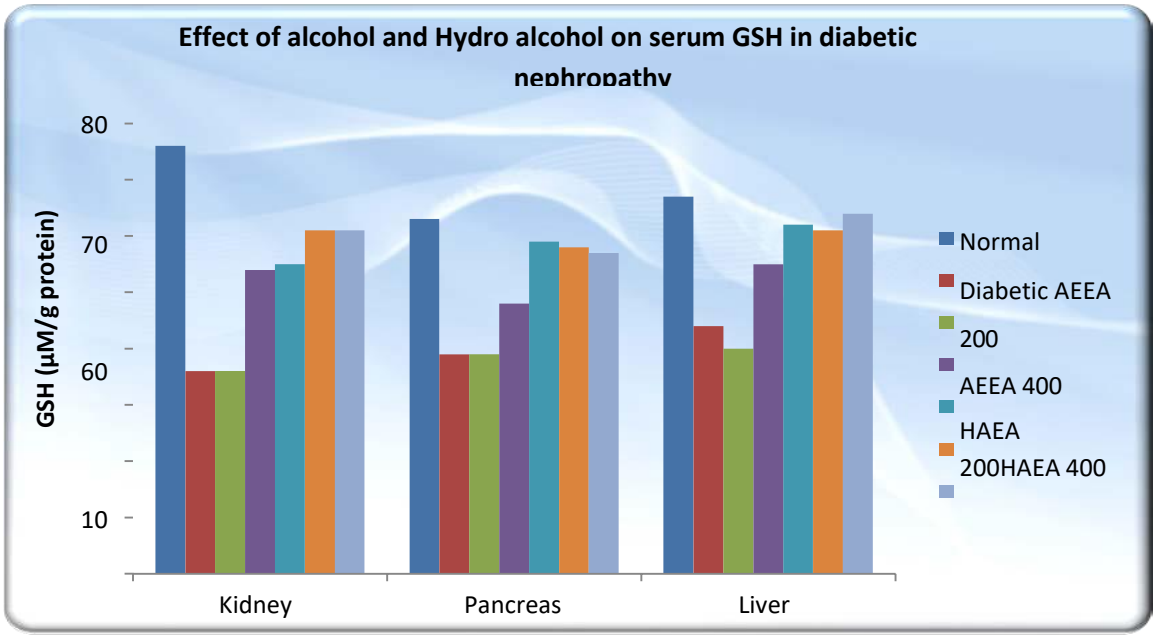


Fig 2: Effect of AEEA and HAEA on GSH in diabetic-nephropathy wistar rats

Effect of Excoecaria agallocha Extracts (AEEA and HAEA) on SOD

Treatment with AEEA and HAEA elevated the level of SOD in kidney, liver and pancreas of DN rats in a dose dependent manner. AEEA increased SOD level in kidney, liver and pancreas to 3.5±0.11, 3.3±0.10 and 3.7±0.09 U/mg protein at 200mg/kg; 2.2±0.07, 2.3±0.05 and 2.6±0.08 U/mg protein at 400 of AEEA Whereas, HAEA increased SOD level in kidney, liver and pancreas to 2.8 ± 0.07, 2.6 ± 0.06 and 2.7 ± 0.04 U/mg protein at 200 mg/kg; 3.6 ± 0.10, 3.8 ± 0.09 and 3.9 ± 0.09 U/mg protein at 400 mg/kg **(Fig 3)**.

Table 2: Effect of AEEA and HEAA on SOD in diabetic-nephropathy wistar rats

Organs	Normal	Diabetic	AEEA 200	AEEA 400	HAEA 200	HAEA 400	Glimepride 10
Kidney	3.5 ± 0.10	1.3 ± 0.09#	3.5 ± 0.11*	2.2 ± 0.07**	2.8 ± 0.07**	3.6 ± 0.10***	3.38 ± 0.04***
Pancreas	3.6 ± 0.12	1.8 ± 0.103	3.3 ± 0.10*	2.3 ± 0.05**	2.6 ± 0.06**	3.8 ± 0.09***	3.46 ± 0.10***
Liver	4.2 ± 0.11	1.9 ± 0.11#	3.7 ± 0.09*	2.6 ± 0.08**	2.7 ± 0.04**	3.9 ± 0.06***	3.54 ± 0.09***

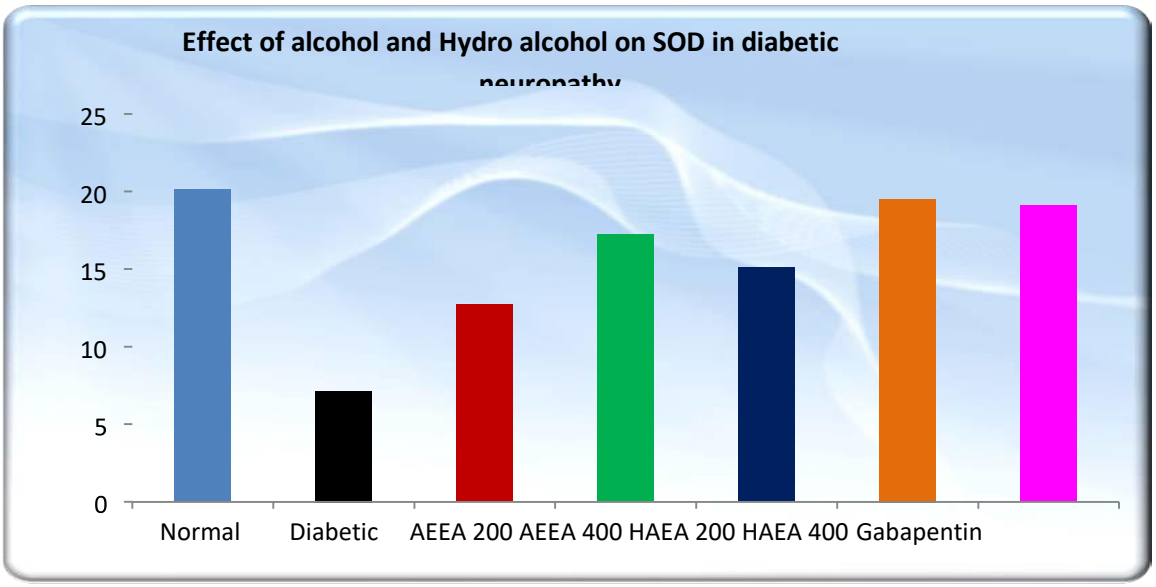


Fig 3: Effect of AEEA and HAEA on SOD level in diabetic neuropathy wistar rats

Effect of Excoecaria agallocha extracts (AEEA and HAEA) on TBARS

AEEA at 200 and 400 mg/kg markedly alleviated the level of TBARS in sciatic nerve to 7.17 ± 0.10, and 4.31 ± 0.23 nmol/mg protein respectively in comparison to DPN control rats (8.78 ± 0.09 nmol/mg proteins). Moreover, HAEA (200 and 400 mg/kg) reduced TBARS level to 6.45 ± 0.42 and 3.25 ± 0.12 nmol/mg protein respectively. Gabapentin 30 mg/kg also reduces TBARS level to 1.67 ± 0.11 nmol/mg protein **(Fig 4)**.

Table 3: Effect of AEEA and HAEA on TBARS level in diabetic-neuropathy wistar rats

Treatment Group	TBARS (nmol/mg protein)
Normal	1.78 ± 0.19
Diabetic	8.78 ± 0.09#
AEEA 200	7.17 ± 0.10*
AEEA 400	4.31 ± 0.23**
HAEA 200	6.45 ± 0.42*
HAEA 400	3.25 ± 0.12***
Gabapentin 30	1.67 ± 0.11***

*Values are mean ± SD (n=6).Where a represents P < 0.001 as compared with control and *P < 0.05; **P < 0.01; and ***P < 0.001 in comparison with diabetic control.*

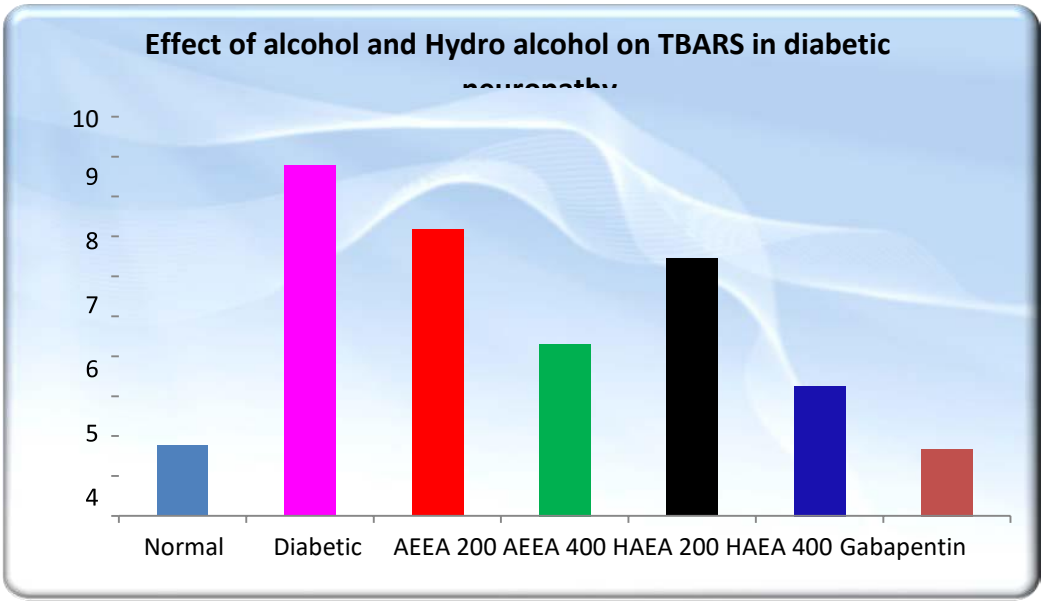


Fig 4: Effect of AEEA and HAEA on TBARS in diabetic neuropathy wistar rats

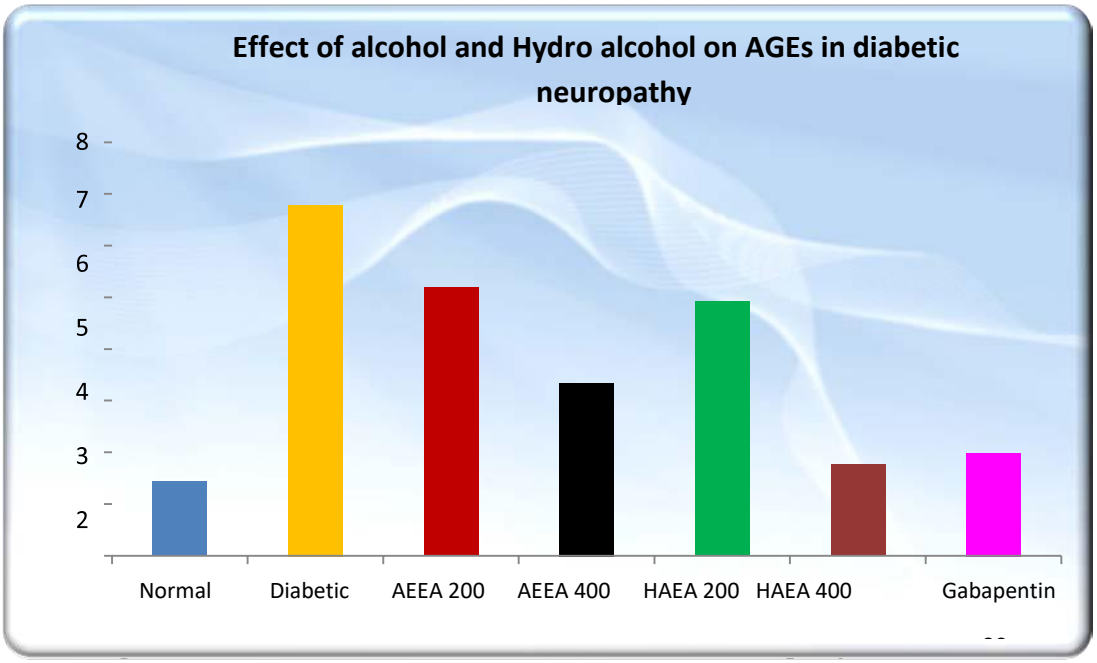
Effect of Excoecaria agallocha extracts (AEEA and HEAA) on AGEs in sciatic nerve

Administration of AEEA and HAEA significantly ($p < 0.001$) ameliorated AGEs level in sciatic nerve as compared to DPN control rats (6.78 ± 0.13 RFU/mg protein). AEEA at 200 and 400 mg/kg reduced AGEs to 5.18 ± 0.10, and 3.34 ± 0.09 RFU/mg protein respectively whereas, HAEA (200 and 400 mg/kg) reduced AGEs level to 4.91 ± 0.07, and 1.76 ± 0.05 RFU/mg protein respectively. Gabapentin 30 mg/kg also reduces AGEs level to 1.99 ± 0.08 RFU/mg proteins **(Fig 5)**.

Table 4: Effect of AEEA and HAEA on AGEs in diabetic-neuropathy wistar rats

Treatment Group	RFU/mg protein
Normal	1.45 ± 0.11
Diabetic	6.78 ± 0.13#
AEEA 200	5.18 ± 0.10*
AEEA 400	3.34 ± 0.09**
HAEA 200	4.91 ± 0.07**
HAEA 400	1.76 ± 0.05***
Gabapentin 30	1.99 ± 0.08***

*Values are mean ± SD (n=6).Where a represents #P < 0.001 as compared with control and *P < 0.05; **P < 0.01; and ***P < 0.001 in comparison with diabetic control.*



Effect of Excoecaria agallocha extracts (AEEA and HAEA) on Nitrite level

Nitrite level in sciatic nerve was significantly elevated in the diabetic rat as compared to normal control rats which was significantly reduced in a dose dependent manner on treatment with *Excoecaria agallocha* extracts (AEEA and HAEA).AEEA at 200 and 400 mg/kg produced significant decrease in Nitrite level in sciatic nerve to 428.10 ± 10.67, and 231.05 ± 10.78 µg/ml respectively in comparison to DPN control rats (551.09 ± 10.54 µg/ml). Moreover, HEAA at 200 and 400 mg/kg reduced Nitrite level to 376.02 ± 10.88, and 162.09 ± 10.34 µg/ml respectively. Gabapentin 30 mg/kg also reduces nitrite level to 129.11 ± 10.11 µg/ml **(Figure 6)**.

Table 5: Effect of AEEA and HAEA on Nitrite level in diabetic-neuropathy wistar rats

Treatment Group	Nitrite Level (µg/ml)
Normal	111.67 ± 10.34
Diabetic	551.09 ± 10.54#
AEEA 200	428.10 ± 10.67*
AEEA 400	231.05 ± 10.78**
HAEA 200	376.02 ± 10.88**
HAEA 400	162.09 ± 10.34***
Gabapentin 30	129.11 ± 10.11***

*Values are mean ± SD (n=6).Where a represents #P < 0.001 as compared with control and *P < 0.05; **P < 0.01; and ***P < 0.001 in comparison with diabetic control.*

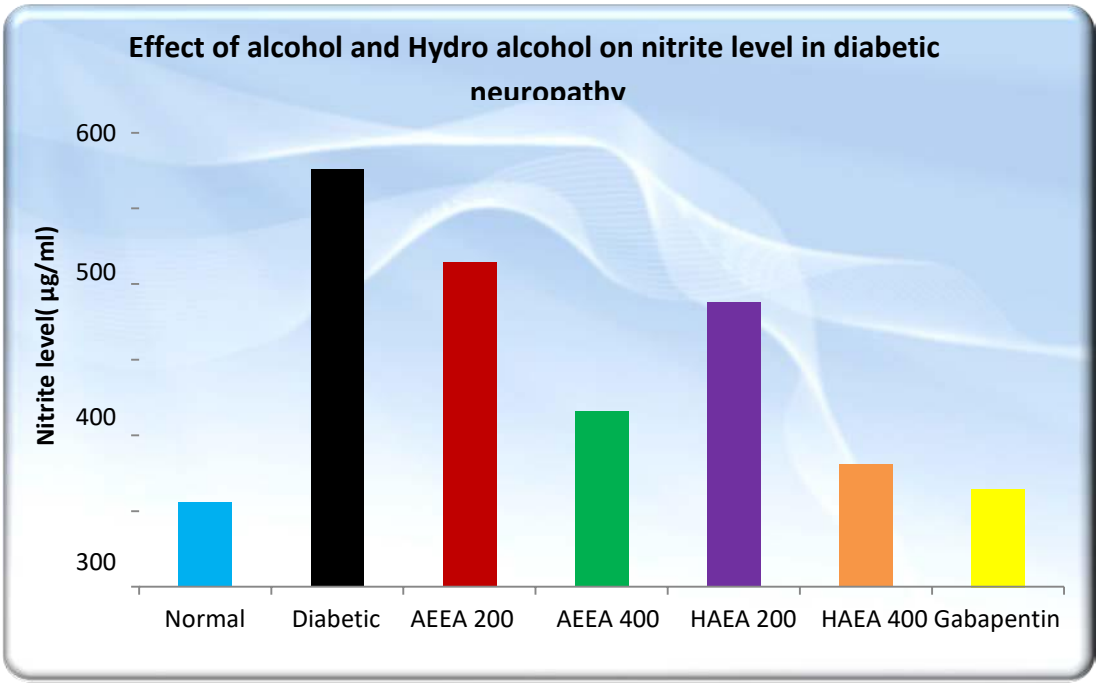


Fig 6: Effect of AEEA and HAEA on Nitrite level in diabetic neuropathy wistar rats

DISCUSSION

The diabetic rats had increased levels of creatinine and urea which are considered as significant markers of renal function and this is in agreement with the present result. Oxidation stress in diabetes mellitus has been shown to coexist with impairment in the endogenous antioxidant status. A marked increase in the concentration of TBARS in STZ induced diabetic rats indicated enhanced lipid peroxidation leading to tissue injury and failure of the endogenous antioxidant defense mechanisms to prevent over production of free radicals. Lipid peroxidation is usually measured in terms of TBARS as a biomarker of oxidative stress. In the present study, it was observed that there was a significant ($p<0.01$) decrease in the TBARS levels of the *Excoecaria agallocha* extracts treated rats in comparison with diabetic control rats (***10).***

Glutathione plays an important role in the endogenous non-enzymatic antioxidant system. It primarily acts as a reducing agent and detoxifies hydrogen peroxide in the presence of the enzyme glutathione peroxidase. The depleted reduced glutathione (GSH) may be due to reduction in GSH synthesis or degradation of GSH by oxidative stress in STZ induced hyperglycemic rats. *Excoecaria agallocha* extracts treatment significantly ($p<0.01$) elevated the serum reduced glutathione levels towards normal in diabetic rats. The results showed that the antihyperglycemic activity of *Excoecaria agallocha* extracts was accompanied by enhancement in non- enzymatic antioxidant protection.

The destruction of superoxide radical or $H_2O_2$by SOD would ameliorate STZ toxicity, as would substances able to scavenge of hydroxyl radical. The altered balance of antioxidant enzymes caused by decrease in SOD activities may responsible for the inadequacy of antioxidant defense in combating ROS mediated damage. The decreased activities of SOD may response to increased production of $H_2O_2$and $O_2$by the auto oxidation of glucose and non-enzymatic glycation. A reduced activity of SOD in serum has been observed during diabetes and this may result in a number of deleterious effects due to the accumulation of superoxide radicals and hydrogen peroxides. *Excoecaria agallocha* extracts treated rats showed decreased lipid peroxidation, which is associated with increased activity of SOD. This means that the extract can reduce reactive oxygen free radicals and improve the activities of the serum antioxidant enzymes ***(11).***

Moreover, increased formation of AGEs activates nuclear factor- κB (NF- κB) that further activates pro-inflammatory gene expression. These changes results in cytokines and growth factors expression (*viz*., IL-1β, IGF-1, TNF-α and TGF-β). Activation of this pro-inflammatory cascade is a major cause of neuronal damage.

Tissue damage, inflammation or injury to the nervous system is cardinal cause of chronic neuropathic pain. Chronic treatment, *Excoecaria agallocha L* reduced the formation of AGEs as well as produced significant; dose dependent inhibition of generation and release of cytokines (IL-1 β, TNF- α, TGF-β). It may be postulated that these plant extracts can directly attenuates the production of inflammatory cytokines and also reduced the formation of AGEs, thereby reducing neuronal apoptosis. Numerous studies indicated that natural antioxidants administration in hyperglycemic situation and also toxicity produces significant neuroprotective effects (***12).***

CONCLUSION

The Results were obtained in the present study suggested that *Excoecaria agallocha L*. have higher amount of phenols, which are known to scavenge free radicals. Also these plant extracts inhibited the formation of AGE's in kidney, increased the level of antioxidant enzymes like SOD, GSH and decreased the level of TBARS (marker of Lipid peroxidation). Thus, these plants modulated diabetic neuropathic pain via reducing the formation of AGEs and amelioration of oxidative/nitrosative stress in peripheral nerves. Finally, it can

be concluded that *Excoecaria agallocha L*. ameliorated diabetes and its complications (diabetic neuropathy).

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