The aim of the present study was to evaluate the possible biological activities of methanolic flower extract of \textit{Nerium oleander} Linn. The evaluated pharmacological activity included antioxidant activity, hepatoprotective activity, anti-Alzheimer activity, ant-inflammatory activity along with neuropsychopharmacological screening. Literature review also suggested the potential activity of the plant in cardiovascular disorders with several cardiotonic phytocompounds isolated. Antibacterial activity was also evaluated for the methanolic extract as a preliminary step in pharmacological screening and was found to be active against several bacteria with lower MIC values. Results of the present research work clearly demonstrated potent pharmacological activity of the extracts evaluated. Among all the fractions, methanolic extract was found to be possessed strong pharmacological activity. The methanolic extract revealed therapeutically considerable hepatoprotective activity against Carbon Tetrachloride (CCL\(_4\)) induced hepatotoxicity, anti-inflammatory activity with COX inhibitory activity, and neuroprotective potential in dementia related to Alzheimer’s diseases. The stronger antioxidant and free radical scavenging activity of the methanolic extract indicated supportive mechanisms in hepatoprotection as well as neuroprotection along with the anti-inflammatory mechanisms of actions. The results were further strengthened by the histopathological photomicrographic observations as well as biochemical estimations.

5.1. Explored The Neuropsychopharmacological Activity

Acute toxicity study (followed OECD guideline-423) indicates that oral administration of 50\% ethanolic extract of \textit{N. oleander} flowers at 2000 mg/kg body weight can be used safely on a mouse. Two dose levels, 100 and 200 mg/kg body wt, have been taken for further experiment. The maximum reduction of locomotor activity was observed after 2 h of drug administration. The result showed that the highly significant reduction in locomotor activity was dose dependent with increased reduction in activity from 100 to 200 mg/kg body wt. Decrease in locomotion implies depression effect on CNS (\textit{Leewanich P et al., 1996}). Also it has been established that an increase in concentration of GABA may lead to CNS depressant effect (\textit{Nagarjun NS et al., 2003}).

The further effect of extract was explored through various activities, which are responsible for increase in GABA concentration, such as potentiation of hexobarbital-induced hypnosis, motor coordination, and anticonvulsant activity. When both dosage levels (200 mg/kg and 100 mg/kg) were compared with the control group, the former’s
potentiation of pentobarbitone induced hypnosis showed better result. The CNS depressants extend barbiturate sleeping time (File SE, et al. 1975). The Sedative-hypnotic drugs induce their effect on the Gabaergic system in the brain (Lundberg D et al. 1975). Inhibition of neuronal output could be facilitated by GABA (inhibitory neurotransmitter) release (Robert E 1974). Loss of righting reflex induced by Phenobarbital is potentiated by GABA agonist (muscimol and THIP) and is inhibited by GABA antagonist (Bicucullin). The activation of GABA receptor partially mediates the sleep response (Mondrup, Pedersoon, 1983: Sivarn et al, 1982; Evans, Hills, 1978).

The findings with extract of *N. oleander* flower are in harmony with the activity shown by other plants. It is plausible that sedative effect in above studies is due to facilitation of GABAergic transmission. This result gives an idea that hydroalcoholic extract of *N. oleander* flowers have effect on GABA receptor. Motor coordination test was conducted on rotarod and it indicated that there is no motor in-coordination at both dose levels. Thus, it implies that extract of *N. oleander* flowers have significant CNS depressant effect through GABA receptor stimulation.

Some other activities evaluate which required GABA augmentation for their treatment like anxiety and epilepsy. HyE of *N. oleander* significantly increased mean number of entries and mean time spent by mice in open arms of elevated plus maze apparatus at the low dose with respect to control, thereby producing anti-anxiety activity.

With respect to these observations, the next study focused on anti-epileptical activity. In this activity we measured the stimulatory effect on GABA receptor. Two different antiepileptic animal models were selected to measure the effect of the two dose levels (100 and 200 mg/kg p.o) in reducing the intensity of seizures. MES and PTZ induced convulsions were the two selected models. *N. oleander* flower extract show anticonvulsant activity in PTZ induced convulsion model. PTZ induced activation of T-type Ca$^{2+}$ channels of thalamic region produced absence seizures or petit mal seizures (O. Carter Snead III). Compounds effective against PTZ induced seizure models, are effective against petitmal type of epilepsy (Vida, 1995). Petitmal seizures can also be prevented by drugs that enhance GABA$_{A}$–BZD receptor mediated neurotransmission such as benzodiazepines and phenobarbitone (Mcdonald and Kelly, 1995).

Based on the ethnomobotanical and pharmacological reports presented above, we postulate that the HyE of *N. oleander* flowers could have potential activities on the CNS and the
administration of these extracts on animals could produce behavioural modifications. Above observation showed that same important compound present in the extract which has medicinal properties.

For further investigation different solvent fraction of *N. oleander* flowers were obtained by successively extracted in the Soxhlet apparatus. Maximum safe dose of various extracts of *N. oleander* flowers was obtained by acute oral toxicity study followed by OECD guideline 423, such as oral administration of PE, EA, ME and water extract were safe at 2000 mg/kg body weight, however CE was safe at 500 mg/kg body weight. This study explored the putative behavioral effects of the various extract of flowers of *N. oleander* in mice. The EA and CE were proficient to induce motor depressant effects after the oral administration. Thus, single doses of CE (50 and 100 mg/kg) and EA (200 and 400 mg/kg) produced a significant decrease in total motility.

The favourable medicinal property of plant materials evidently results from the combinations of secondary metabolites present in the plant, through additive or synergistic action of several chemical compounds acting at single or multiple target sites associated with a physiological process (Briskin DP 2004). This fact reveals that medicinal activities of plants are distinctive to particular plant species or groups, dependable with the conception that combinations of secondary metabolites in a particular plant are generally taxonomically diverse (Wink M. 1999). Preliminary phytochemical analysis of various extracts of *N. oleander* flowers in this study revealed the presence of alkaloids, tannins, cardiac glycosides, steroids, terpenoids, flavonoids, reducing sugars, and saponins. Going through this case, the anxiolytic and sedative activities observed with CE, EA and ME of flowers was possibly due to the presence of flavonoids, alkaloids, and terpenoids in the plant extract. Some synthetic and natural flavonoids have been bind exclusively and competitively to benzodiazepine receptors and reveal anti-anxiety property in the EPM test in rat and mice (Salgueiro JB et al., 1997), these extracts induce anxiolytic like effect. These findings indicate a remarkable sedative effect of this plant.

In fact, only mice treated with *N. oleander* 25, 50 and 100 mg/kg (CE) and 100, 200 and 400 mg/kg (EA and ME) showed a significant increase of both the percentage of entries and the percentage of time spent in the open arms of the EPM.
Montgomery reported that rodents consistently spend greater time in the closed arms when placed in maze comprising of open and closed arms (Montgomery KC. 1955). Avoidance of the open arm portrays a manifestation of fear and anxiety, based on these assertions EPM test is reliable means of identifying selective anxiolytic effects of drugs. In this context, the effectiveness of *N. oleander* (50 and 100 mg/kg) in relieving anxiety in this model suggests a possible positive modulation of the GABA\(_A\) chloride channel receptor complex. At higher doses of 200 and 400 mg/kg, the anxiolytic effect of *N. oleander* was sustained but diminished in magnitude, being non-significant at the later dose. The EPM model utilized in this research is a suitable animal model as this model has invoked natural stimuli (Dawson GR, Tricklebank MD 1995). A chloroform, Ethyl acetate and methanolic extract were significantly increased the time spent in open arms and the frequency of open arm entries in EPM, thus suggesting an anti-anxiety effect (Fleming T. 2000) In conclusion it was evident that the CE, EA and ME of *N. oleander* flowers showed an anxiolytic effect on mice, and it could serve as a new approach for the treatment of anxiety.

5.2. Exploring Anti-Convulsant Potential

We have used two different animal model experiments that characteristically described three types of seizures activity. These were demonstrated by the activity against PTZ-induced seizures which correlate with anti-absence activity and activity against electrically induced seizures signifies activity against generalized tonic–clonic and partial seizures (Delgado and Remers, 1998). The *N. oleander* extract had little protection on the animals used in the screening for anticonvulsant activity using PTZ animal model, but it increased seizures latency. However, these effects were not dose-dependent. In MEST-induced convulsion, the *N. oleander* extract significantly protected the animals against seizures, increased the onset and reduced the duration of seizures

The current available antiepileptic drugs (AEDs) that are clinically effective in the management of generalized tonic–clonic and partial seizures such as carbamazepine, phenytoin, primidone, phenobarbital, valproate and lamotrigine all suppress hind limb tonic extension (HLTE) in MEST (Browning, 1992; Rho and Sankar, 1999). Protection against HLTE also indicates the ability of a testing material to inhibit or prevent seizures discharge within the brainstem seizure substrate (Browning, 1992). The ability of the extract to inhibit the HLTE in MEST as compared to phenytoin (100%
protection) in the model suggests anticonvulsant activity for the management of
generalized tonic–clonic and partial seizures. AEDs effective in the therapy of
generalized seizures of (absence or myoclonic) petit mal type such as phenobarbitone,
valproate, ethosuximide and benzodiazepines exhibit dose-dependent suppression of
various seizure pattern induced by PTZ (Loscher et al., 1991). PTZ-induced seizures are
similar to the symptoms observed in the absence seizures and drugs such as valproate
and ethosuximide which are useful in the management of absence seizures inhibit PTZ-
induced seizures (McNamara, 2001).

At cellular level, one of the basic mechanisms of actions of AEDs such as ethosuximide
and valproate is the suppression of T-type calcium currents in thalamic neurons
(Macdonald and Kelly, 1994; Meldrum, 1996; Rho and Sankar, 1999). In PTZ-
induced convulsions, the extract of N. oleander had only increased the latency but not
the incidence of seizures as compared to diazepam. This extract might not be useful in
the management of absence seizures. The observed effects might be in agreement with
the findings of Swinyard et al. (1952) and Swinyard (1969) that not all antiepileptic
drugs have protective value against Sc-PTZ induced convulsions. The biphasic activity
observed in PTZ-induced studies may probably be due to possible interaction between
constituents of the crude extract.

The majority of currently available antiepileptic drugs fall into one of two
pharmacological classes, those that modulate neuronal voltage-gated sodium channels
(e.g. carbamazepine, phenytoin, lamotrigine, and topiramate) and those that modulate
inhibitory GABAergic neurotransmission (e.g. benzodiazepine, vigabatrin and
tiagabine). While, small number of AEDs such as ethosuximide, gabapentin and possibly
levetiracetam, may exert their effects via an interaction with voltage-operated calcium
channels (Wickenden, 2002).

The ability of the extract to exhibit activity against these two types of seizures suggests
that it may act through different mechanisms to elicit its anticonvulsant effects, such as
voltage-gated sodium, calcium, and potassium or GABAergic pathway. The results of
the study have demonstrated that N. oleander possessed anticonvulsant activity on the
animal models investigated and this provides a rationale for its use in medicine for the
management of epilepsy.

5.3. Hepatoprotective Activity
The present study demonstrates the hepatoprotective, prophylactic and antioxidant effects of MENO-F against CCl₄-induced liver injury in rats. CCl₄ is biotransformed in liver by cytochrome P₄₅₀ enzymes to CCl₃ radical which is a very active radical. This active CCl₃ radical reacts with oxygen to produce trichloromethylperoxyl radical (CCl₃O₂·), which is then covalently binds with cellular macromolecules and biomembranes to cause lipid peroxidation of the lipid membranes of the adipose tissue. Peroxide products finally trigger production and leakage of biomarkers like MDA (Malonaldehyde). This whole cascade of biochemical events ultimately causes loss of cellular integrity and hepatic damage (Thabrew et al., 1987). Lipid peroxidation is an important parameter of oxidative stress along with other free radical damage occurred in the biochemical cascade. Therefore, antioxidant efficacy is regarded as one of the utmost important parameter indicative of the possible mechanism of hepatoprotection.

AST, ALT and ALP are the serum hepatobillary enzymes present normally in the liver in high concentrations. Upon necrosis or hepatic damage these enzymes will be leaked into the circulation; raising serum concentration of these enzymes (Drotman and Lawhan, 1978). Elevated serum AST, ALT and ALP levels in CCl₄ treated animals indicated cellular breakage and loss of functional integrity of cell membranes in liver (Wolf, 1999, Drotman and Lawhan, 1978). In the present study, increased MDA levels in liver indicated increased lipid peroxidation induced by CCl₄ (Group II animals). This enhanced lipid peroxidation finally triggered hepatic tissue damage. Reduced estimation of SOD in CCl₄ treated animals also suggested failure of antioxidant defense mechanism to block peroxidation damage.

In the present experimental setup, antioxidant activity of the methanolic extract of the flowers of Nerium oleander and the possible mechanism had been investigated by evaluating DPPH and ABTS radical scavenging activity. Reducing power assay, superoxide radical scavenging and hydroxyl radical scavenging were also assessed. Reducing power assay is one of the most investigated significant indicators of antioxidant potential. Mainly capability of bioactive compounds to donate hydrogen and electron reflects reducing power (Jayaprakasha et al., 2000, Ak and Gülcin, 2008). In this assay, the extract showed a good concentration-dependent increase in reducing power. ABTS and DPPH radicals are very popular and well established free radicals used to investigate free radical scavenging power of components in vitro. The present study indicated a strong ABTS and DPPH radicals scavenging activity of the extract.
Degree of lipid peroxidation was also found to be inhibited by the extract as suggested by results of Thiocyanate method. Superoxide anion radical has been known as relatively weak oxidant but the ability to generate more toxic and dangerous singlet oxygen, hydroxyl radical and peroxynitrile radical made superoxide radical a dangerous reactive species (Liu et al., 2005, Halliwell and Chirico, 1993). The results of the present study revealed a good significant hydroxyl radical and superoxide radical scavenging activity of the extract under evaluation.

In view of this, the increased serum level of AST, ALT and ALP enzymes in CCl$_4$ treated animals (Group II) confirmed hepatic damage. As a breakdown product of heme in red blood cells, bilirubin is regarded as a clinical and pathophysiological indicator of necrosis of liver tissues. Pretreatment with Nerium oleander extract in different animal groups (Group IV/V/VI) resulted a significant decrease in serum AST, ALT, ALP and total bilirubin levels as compared to CCl$_4$ treated group (Group II). Prophylactic use of the extract resulted in an inhibition of the degree of hepatic necrosis and concomitantly decreased the leakage of intracellular enzymes by stabilizing hepatic cellular membranes. The results are further confirmed by the histopathological observations. Inhibition of lipid peroxidation to a significant degree is also a predominant mechanism of hepatoprotection as suggested by the significant decrease in MDA levels. Increase in the SOD level was also suggestive of repairment of antioxidant defense system, which plays an important role in hepatoprotection. Based upon the results of this present study, it can be concluded that the MENO-F has proven itself as a significant hepatoprotective as well as a considerable antioxidant.

5.4. Neuroprotective Activity in Dementia Related to Alzheimer Disease.

It is proposed that Aluminum (Al) induces potentiation of the activities of ATP receptors in the brain. Physiologically significant levels of Al could induce neuronal excitotoxicity at normal levels of neurotransmitter. This excitatory mechanism may act together with the disruption of other ATP-mediated signaling pathways (release of acetylcholine) as well as Ca$^{+2}$-mediated excitotoxicity, ultimately leading to the characteristic progress of the AD disease process (Exley C. 1999). The present study investigated the potential synergistic effects of exposure to Cupper (Cu), Al or both metals in promoting inflammatory and oxidative events in rat brain. The design was based on the following observations: Al present in the drinking water enhanced inflammatory markers in the
CNS. Cu is an essential metal and a component of many enzymatic reactions. However, this redox active metal can also mediate the formation of reactive oxygen species (ROS) and can have adverse consequences. Al is a trivalent cation incapable of redox changes and unlike Cu, has no known biological role. Both metals have been associated with neurological impairments. Al has been shown to play a causal role in dialysis encephalopathy and epidemiological studies suggest a possible link between exposure to this metal and a higher prevalence of AD. Various studies suggest that lipid metabolism is altered in the AD. The results obtained from our study have been shown to alter lipid profile to a significant level. Major change in the lipid profile was reflected on LDL and HDL level. Administration of Al and Cu caused a significant rise in the level of LDL and remarkable decrease in the level of HDL. In the present study Vitamin-E caused a highly significant decrease in the level of TG and VLDL while there was a highly significant increase in the serum level of HDL as compared to positive control group along with a significant decrease in the level of LDL as compared with positive control group. At the highest dose level, MENO-F caused highly significant increase in HDL and significant decrease in VLDL, TC and LDL level as compared to positive control group. But at low dose of MENO-F was found to produce any change in other parameter of lipid profile except HDL (Adunsky A et al., 2004; Burgess BL et al., 2006; Florent-Béchard S et al., 2009).

Cholesterol excess is also found to be linked with the amyloid excess and which ultimately cause AD (Florent-Béchard S et al., 2009; Fonseca ACRG et al., 2010; Raygani AV et al., 2006). This hypothesis is further confirmed by the results of this study. Experimental studies suggest that high cholesterol accelerates the production of Aβ in AD, by shifting apolipoprotein (APP) metabolism from α to β cleavage products (Raygani AV et al., 2006). Normal level of Cholesterol support elements of neural integrity as a precursor of steroid hormones estrogens, androgens, and vitamin D), provides structural integrity and modulates fluidity of cell membranes, and is essential for basic synaptic integrity and neurotransmission. A high level of serum lipid especially TC, TG and LDL leads to production of free oxygen species, Aβ and resulting in adverse events like neuronal loss, dementia and ultimately AD (Serra JA et al., 2004; Takechi R et al., 2008).

The high rate of oxygen consumption per unit mass of tissue renders the brain especially vulnerable to the deleterious effects of oxidative stress, which can arise from the
overproduction of ROS and/or from a deficiency of the antioxidant defense systems. Oxidative stress is an important factor that may be involved in pathogenesis of neurodegenerative diseases. There are considerable evidences that oxidative stress occurs in neurodegenerative diseases (Serra JA et al., 2004). Metal-catalyzed hydroxyl radicals are potent mediators of cellular injury, affecting every category of macromolecule, and are central to the oxidative injury hypothesis of AD pathogenesis. It is evident from various studies that the overproduction of ROS by Al and Cu leads to oxidative stress. Oxidative stress represents a significant pathway that leads to the destruction of both neuronal and vascular cells. Modulation of level of SOD, TBARS, GSH and GSSG as markers of the brain antioxidant defense system in our study clearly demonstrated neuroprotection. In addition, assessment of the level of MPO and S. Nitrite in the brain tissues of positive control animals as a measure of inflammation indicated Al and Cu induced inflammatory cascade in the brain along with the production of ROS.

At all the studied dose levels MENO-F were found to reduce the level of MPO, S. Nitrite and TBARS as compared to PC group in a highly significant manner. While the level of SOD, GSH and GSSG were found to be significantly raised. Results from our study furthermore confirmed the evident neuroprotection by the significant decrease in the level of MPO, S. Nitrite and AChE when compared to PC group. The level of SOD was also found to be significantly raised further establishing the neuroprotection provided by MENO-F in terms of buildup of antioxidant defense enzyme system against the oxidative stress caused by Al and Cu.

Histopathology report confirmed the hallmark signs of AD along with the neuroprotective potential of Vitamin-E. The histopathological observations confirmed the inflammation and neuronal damage in the hippocampus and cerebral cortex regions of the brain. These two structures of the brain play an important role in the memory and cognition. In our study these two structures of the rat brain were found to be severely affected and signs of neuronal integrity damage and inflammation were clear from the histopathological observations, clearly strengthen the findings of Radial eight arm maze task. Microscopic examination of the stained tissue of brain reveals the significant changes in the cerebral cortex and hippocampus. Animals of the positive control group were severely affected due to oxidative stress and inflammation resulting in cerebral and hippocampus changes leading to neurodegeneration, ultimately resulting in the dementia and AD. MENO-F at 200 mg/kg dose level clearly demonstrated a moderate
neuroprotection in terms of inflammation. At the dose level of 400 mg/kg, MENO-F demonstrated maximum neuroprotection as evident by the absence of signs of astrogliosis and pyknosis with no active inflammatory cells along with normal neurovascular integrity of the brain.
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Discussion


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