CHAPTER 1
SUPPRESSION OF OXIDATIVE-NITROSATIVE STRESS 
MEDIATED NEUROINFLAMMATORY CASCADE IN 
EXPERIMENTAL MODEL OF ALCOHOLIC NEUROPATHY

1.1. INTRODUCTION

Chronic alcohol consumption produces a painful peripheral neuropathy for which there is no reliable successful therapy, which is mainly due to lack of understanding of its pathobiology (Monforte et al., 1995; Naik et al., 2006). Alcoholic neuropathy is characterized by spontaneous burning pain, hyperalgesia (an exaggerated pain in response to painful stimuli) and allodynia (a pain evoked by normally innocuous stimuli). Depending on the criteria and patient selection, incidence of peripheral neuropathy ranging from 10% to 50% has been reported (Monforte et al., 1995). Primarily it is an axonal neuropathy characterized by wallerian degeneration of the axons and a reduction in the myelination of neural fibers (Yerdelen et al., 2008). Studies on rat models have indicated that alcohol does have a direct neurotoxic effect on spinal cord and neuronal organelles (Corsetti et al., 1998; Narita et al., 2007). Acetaldehyde, one of the most toxic metabolites of ethanol, has a direct neurotoxic effect (Koike et al., 2001a, 2003).

Oxidative stress is known to play a very important role in the experimental animal models of neuropathic pain. Lee et al. (2007) suggested that reactive oxygen species are importantly involved in the development and maintenance of capsaicin-induced pain, particularly in the process of central sensitization in the rats’ spinal cord. Padi and Kulkarni (2008) demonstrated that chronic administration of minocycline when started early before peripheral nerve injury could attenuate the development of neuropathic pain by inhibiting proinflammatory cytokines release and oxidative and nitrosative stress in mononeuropathic rats. A significant decrease in the activity of antioxidant enzymes (superoxide dismutase and catalase) and an increase in lipid peroxidation were observed in the sciatic nerves of diabetic rats with established neuropathic pain (Sharma and Sayyed, 2006). Dina et al. (2000)
demonstrated that hyperalgesia is present in an established model of chronic alcoholism in the rat and that PKC signaling plays a critical role in the enhanced nociception produced by chronic alcohol.

Although pharmacological pain management offers a significant relief in several pain-related diseases, many patients turn to its supplementation with complementary and alternative medicine. Botanicals used in pain therapy can contribute to restoring the quality of life of a patient and may effect and enhance conventional pain management (Zareba, 2009). Phenolic compounds are widely present in plants and they have recently received considerable attention due to their antioxidant property. Since past decade, the scientific community has shown huge interest in the multiple activities of natural vitamin E i.e. tocotrienols. Tocotrienols possess powerful cardioprotective (Das et al., 2007, 2008), neuroprotective (Khanna et al., 2006; Shichiri et al., 2007), radioprotective (Ghosh et al., 2009), anti-angiogenic (Nakagawa et al., 2004; Shibata et al., 2008), potent natural antioxidant (Schroeder et al., 2006; Maniam et al., 2008), anti-cancer (Nesaretnam et al., 2004), anti-inflammatory (Wu et al., 2008), COX-2 inhibitory (Yam et al., 2009), anti-nociceptive (Kuhad and Chopra, 2009; Tiwari et al., 2009a), insulin sensitizing, hypoglycemic (Chen and Cheng, 2006; Budin et al., 2009) and cholesterol lowering (Chou et al., 2009) properties that often differ from the properties of tocopherols (Serbinova et al., 1991; Serbinova and Packer, 1994; Sen et al., 2007; Budin et al., 2009). The unsaturated side chain of tocotrienol allows for more efficient penetration into tissues that have saturated fatty layers such as the brain and liver (Suzuki et al., 1993; Atkinson et al., 2006). Experimental research examining the antioxidant effects of tocopherol and tocotrienols has revealed that tocotrienols appear superior due to their better distribution in the fatty layers of the cell membrane (Suzuki et al., 1993; Kawakami et al., 2007; Tsuzuki et al., 2007; Maniam et al., 2008).

Green tea polyphenols are natural plant flavonoids found in the leaves and stems of green tea plant. The green tea polyphenols have shown a variety of pharmacological properties such as anti-inflammatory, anticarcinogenic and antioxidant effects (Cavet et al., 2011; Konta et al.,
The green tea polyphenols comprise epigallocatechin gallate (EGCG), epigallocatechin, epicatechin, gallocatechin and catechin. Among these polyphenols, EGCG is a major component responsible for most of biological effects. EGCG has been reported to display a potent antioxidant property because it possesses two triphenolic groups in its structure (Jin et al., 2000). Thus, EGCG is expected to have beneficial effects on free radical-induced cell death including the production of reactive oxygen species (ROS) in nervous tissues and cells. Concerning antioxidant therapy, green tea extract (GTE) appears to be a promising candidate. In addition, green tea extract has been shown to protect the brain (Hong et al., 2000; Suzuki et al., 2004) and heart from ischemia-reperfusion-induced damage (Aneja et al., 2004; Townsend et al., 2004).

Resveratrol, a natural polyphenolic phytoalexin is a potent anti-oxidant and anti-inflammatory agent. It possesses diverse biochemical and physiological actions which includes estrogenic, anti-platelet and anti-inflammatory properties (Wu et al., 2007; De Amicis et al., 2011; Yoon et al., 2011). We have previously reported the beneficial effects of resveratrol in experimental diabetic neuropathy (Sharma et al., 2007). Resveratrol has been found to protect the heart from ischemic–reperfusion injury (Das et al., 1999). In kidney cells, resveratrol was found to exert its protective action through upregulation of NO (Giovannini et al., 2001). The ingestion of trans-resveratrol has been associated with an attenuation of ethanol-induced oxidative damage in neurons and subsequent neuronal cell death (Sun and Sun, 2001; Sun et al., 2002). In vitro studies demonstrated that trans-resveratrol attenuated ethanol-induced increases in ROS levels (Sun et al., 1997). Another in vitro study using monocytes isolated from healthy adult humans demonstrated that resveratrol reduced oxidative stress, increased membrane permeability and reduced activities of apoptosis-related enzymes including Caspase-3, Caspase-8 and phosphatidylserine (Losa, 2003).

The rhizome of turmeric is widely used in indigenous medicine (Nadkarni, 1954). A paste made from powdered rhizome of *Curcuma longa* Linn. mixed with slaked lime applied locally, is an ancient household remedy for sprains, muscular pain and inflamed joints. It is also applied in poultices to
relieve pain and inflammation (Chandra and Gupta, 1972). The volatile oil and curcumin obtained from C. longa exhibit potent anti-inflammatory effect (Chandra and Gupta, 1972). Extensive scientific research on curcumin has demonstrated a wide spectrum of therapeutic effects such as anti-inflammatory (Ammar et al., 2011), antibacterial (Rai et al., 2008), antiviral (Rechtman et al., 2010), antifungal (Sharma et al., 2010), antitumor (Luo et al., 2011), antispasmodic (Itthipanichpong et al., 2003), neuroprotective (Lapchak, 2011; Yadav et al., 2011) and hepatoprotective (Park et al., 2000). The anti-inflammatory effects of curcumin are most likely mediated through its ability to inhibit expression of pro-inflammatory genes such as cyclooxygenase-2 (COX-2), adhesion molecules, chemokines, cytokines, metalloproteinases (MMP), lipooxygenase and iNOS (Huang et al., 1991; Surh et al., 2001; Woo et al., 2005) via down-regulation of transcription factor NF-κB (Aggarwal et al., 2003) and intracellular signaling pathways such as Janus kinase (JAK)-STAT signaling (Kim et al., 2003). Another study has shown that curcumin reduces oxidative damage and amyloid pathology in an Alzheimer transgenic mouse (Lim et al., 2001).

With this background, the present study was undertaken to investigate the effect of tocotrienol, epigallocatechin gallate, resveratrol and curcumin against alcoholic neuropathy and associated neuroinflammatory cascade in rats.

1.2. MATERIALS AND METHODS

1.2.1. Animals

Adult male Wistar rats (150-200 g) bred in Central Animal House facility of Panjab University were used. The animals were housed under standard laboratory conditions, maintained on a natural light-dark cycle and had free access to food (Ashirwad Industries, Mohali, India) and water. Animals were acclimatized to laboratory conditions before the tests. All experiments were carried out between 0900 and 1700 hr. The experimental protocols were approved by the Institutional Animal Ethics Committee of Panjab University and performed in accordance with the guidelines of...
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Committee for the Purpose of Control and Supervision of Experimentation on Animals, Government of India.

1.2.2. Drugs and Reagents

Tocotrienol (mixture of α-, β-, γ-tocotrienol) and epigallocatechin gallate were received as gift sample from Golden-Hope Bioganic, Malaysia Palm Oil Board, Malaysia and DSM Nutritional Products Ltd, Switzerland respectively. Resveratrol and curcumin were purchased from Sigma (St. Louis, MO, USA). Tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β) and transforming growth factor-β1 (TGF-β1) ELISA kits were purchased from R&D Systems, Minneapolis, USA. All other chemicals and reagents used in the present study were of analytical grade.

1.2.3. Induction of Alcoholic Neuropathy and Drug Treatment Schedule

Alcoholic neuropathy was induced by administration of 10 g/kg b.i.d oral gavage of 35% v/v ethanol in double distilled water for 10 weeks. The dose of ethanol was decided on the basis of the existing literature (Dina et al., 2000). After a basal recording of pain related parameters on day 0, rats were randomly selected and divided into nineteen groups of 5-8 animals each. Group 1 comprised of control animals and given double distilled water in place of ethanol by oral gavage. Group 2 animals were administered ethanol (10 g/kg). Group 3, 4, 5 and 6 consisted of α-tocopherol (100 mg/kg, oral gavage) and tocotrienol (50, 100 and 200 mg/kg, oral gavage) treated rats along with administration of ethanol (10 g/kg). Group 7 consisted of control animals which received tocotrienol alone (100 mg/kg, oral gavage). Group 8, 9 and 10 consisted of EGCG (25, 50 and 100 mg/kg; oral gavage) and ethanol (10 g/kg) treated rats. Group 11 consisted of control animals which received EGCG alone (100 mg/kg; oral gavage). Group 12, 13 and 14 consisted of resveratrol (5, 10 and 20 mg/kg; oral gavage) treated rats along with administration of ethanol (10 g/kg). Group 15 consisted of control animals which received resveratrol alone (20 mg/kg; oral gavage). Group 16, 17 and 18 consisted of curcumin (15, 30 and 60 mg/kg; oral gavage) treated rats along with...
administration of ethanol (10g/kg). Group 19 consisted of control animal which received curcumin alone (60 mg/kg; oral gavage).

All the drugs were administered by oral route 1 hr before ethanol dosing daily for ten weeks starting from day 1. Drug solutions were freshly prepared and administered in a constant volume of 5 ml/kg body weight. Tocotrienol was freshly prepared by dissolving in double distilled water after triturating with 5% Tween 80. EGCG was freshly prepared by dissolving in double distilled water. Resveratrol and curcumin were prepared by suspending in 0.5% carboxymethylcellulose. All the behavioral assays were done at 6th, 8th and 10th week. After 10 weeks, blood was collected from the vein of rats and serum was separated. Rats were sacrificed under deep anesthesia and sciatic nerves were rapidly removed and weighed. A 10% (w/v) tissue homogenate was prepared in 0.1 M phosphate buffer (pH 7.4). Homogenates were centrifuged at 200 g for 10 min, at 4 °C and supernatant was used for estimation of lipid peroxidation, nitrite, superoxide dismutase, catalase and reduced glutathione. Serum and nerve cytoplasmic fraction were used for the estimation of TNF-α, IL-1β and TGF-β1. The samples were stored at -80 °C until processed for biochemical estimations.
1.2.4. Behavioral Assessment

1.2.4.1. Assessment of Thermal Hyperalgesia (Tail-immersion Test)

Thermal hyperalgesia was assessed by using tail-immersion test. Rats tail was immersed in a water bath maintained at 42 °C (a temperature that is normally innocuous in normal rats (Courteix et al., 1993; Tiwari et al., 2009a) until tail withdrawal or signs of struggle was observed (cut-off time: 15 sec) As this test involves handling of the animals, one day before the experiment the experimenter handled the rats in the testing environment until they sat quietly in the hand for 15 sec (which corresponds to the cut-off time), 2 or 3 times depending on their capacity to be quiet. On the day of experiment, rats were again handled for 15 sec above the water bath to get the rat used to the condition of the test. No rat should show aversive reaction during handling. Then the tail of the rat was immersed into the water. The reaction time (i.e. the time necessary to observe the withdrawal of the tail from the bath) was measured 2-3 times in order to obtain two consecutive values that differed no more than 10 %, and respecting an interval of at least 15 min between two
measures. The tail of the rat was immediately dried with a soft cellulose paper to avoid tail cooling between two measures. A shortened duration of immersion indicates thermal hyperalgesia.

1.2.4.2 Assessment of Mechanical Hyperalgesia (Randall-Sellito Test)

The nociceptive flexion reflex was quantified using the Randall-Sellito paw pressure device (IITC, Woodland Hills, USA), which applies a linearly increasing mechanical force (in g) to the dorsum of the rat’s hind paw (Taiwo et al., 1989). Nociceptive thresholds, expressed in grams, were applied by increasing pressure to the hind paw until a squeak (vocalization threshold) was elicited. As this test involves animal handling, the experimenter gets the rat used to being handled as following: 3 days before the experiment, rats were handled without escaping from the hand of the experimenter for 20 sec, 2 or 3 times depending on their capacity to be quiet. On the day of the experiment, rats were again handled 2-3 times for 20 sec. No rats should show aversive reaction during handling. Then, the paw of the rat was placed under the tip, and the progressive pressure applied until the rat vocalized. The vocalization threshold was measured 3 or 4 times in order to obtain two consecutive values that differed no more than 10%, and respecting an interval of at least 10 min between two measures.

1.2.4.3. Assessment of Tactile Allodynia (von Frey Hair Test)

Tactile responses were evaluated by quantifying the withdrawal threshold of the hindpaw in response to stimulation with flexible von Frey filaments. Rats were placed individually on an elevated mesh (1 cm² perforations) in a clear plastic cage and adapted to the testing environment for at least 15 min. Von Frey hairs (IITC, Woodland Hills, USA) with calibrated bending forces (in g) of different intensities were used to deliver punctuate mechanical stimuli of varying intensity. Starting with the lowest filament force, von Frey hairs were applied from below the mesh floor to the plantar surface of the hind-paw, with sufficient force to cause slight bending against the paw, and held for 1 sec (Chaplan et al., 1994; Tiwari et al., 2009a). Each stimulation was applied 5 times with an inter-stimulus interval of 4-5 sec. Care was taken to stimulate random locations on the plantar surface. A positive response was noted if the
paw was robustly and immediately withdrawn. Paw withdrawal threshold was defined as the minimum pressure required to elicit a withdrawal reflex of the paw, at least one time on the five trials. Voluntary movement associated with locomotion was not considered as a withdrawal response. Mechanical allodynia was defined as a significant decrease in withdrawal thresholds to von Frey hair application.

1.2.5 Biochemical Assessment

1.2.5.1 Assessment of Oxidative Stress

**Estimation of Lipid Peroxidation**

The malondialdehyde content, a measure of lipid peroxidation, was assayed in the form of thiobarbituric acid-reactive substances by the method of Wills et al. (1965). Briefly, 0.5 ml of post mitochondrial supernatant and 0.5 ml of Tris HCl were incubated at 37°C for 2 h. After incubation, 1 ml of 10% trichloroacetic acid was added and centrifuged at 1000xg for 10 min. To 1 ml of supernatant, 1 ml of 0.67% thiobarbituric acid was added and the tubes were kept in boiling water for 10 min. After cooling 1 ml double distilled water was added and absorbance was measured at 532 nm. Thiobarbituric acid-reactive substances were quantified using an extinction coefficient of 1.56x10^5 M^-1cm^-1 and expressed as nmol of malondialdehyde per mg protein. Tissue protein was estimated using the Biuret method (Lubran, 1978) and the brain malondialdehyde content expressed as nanomoles of malondialdehyde per milligram of protein.

**Estimation of Reduced Glutathione**

Reduced glutathione was assayed by the method of Ellman (1959). Briefly, 1.0 ml of post mitochondrial supernatant (10%) was precipitated with 1.0 ml of sulphosalicylic acid (4%). The samples were kept at 4°C for at least 1 h and then subjected to centrifugation at 1200 g for 15 min at 4°C. The assay mixture contained 0.1 ml supernatant, 2.7 ml phosphate buffer (0.1 M, pH 7.4) and 0.2 ml 5,5-dithiobis (2-nitro benzoic acid) (Ellman’s reagent, 0.1 mM, pH 8.0) in a total volume of 3.0 ml. The yellow color developed was read immediately at 412 nm and the reduced GSH levels were expressed as μmoles/mg protein.
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**Estimation of Superoxide Dismutase**

Cytosolic superoxide dismutase activity was assayed by the method of Kono *et al.* (1978). The assay system consisted of 0.1 mM EDTA, 50 mM sodium carbonate and 96 mM of nitro blue tetrazolium (NBT). In the cuvette, 2 ml of above mixture was taken and to it 0.05 ml of post mitochondrial supernatant and 0.05 ml of hydroxylamine hydrochloride (adjusted to pH 6.0 with NaOH) were added. The auto-oxidation of hydroxylamine was observed by measuring the change in optical density at 560 nm for 2 min at 30/60 s intervals.

**Estimation of Catalase**

Catalase activity was assayed by the method of Claiborne *et al.* (1985). Briefly, the assay mixture consisted of 1.95 ml phosphate buffer (0.05 M, pH 7.0), 1.0 ml hydrogen peroxide (0.019 M) and 0.05 ml post mitochondrial supernatant (10%) in a final volume of 3.0 ml. Changes in absorbance were recorded at 240 nm. Catalase activity was calculated in terms of k minutes⁻¹.

1.2.5.2 Assessment of Nitrosative Stress

**Nitrite Estimation**

Nitrite was estimated in the sciatic nerve homogenate using the Greiss reagent and served as an indicator of nitric oxide production. A measure of 500 μl of Greiss reagent (1:1 solution of 1% sulphamidine in 5% phosphoric acid and 0.1% napthylamine diamine dihydrochloric acid in water) was added to 100 μl of sciatic nerve homogenate and absorbance was measured at 546 nm (Green *et al.*, 1982). Nitrite concentration was calculated using a standard curve for sodium nitrite. Nitrite levels were expressed as μg/ml.

1.2.6. Tumor Necrosis Factor-alpha (TNF-α), Interleukin-1beta (IL-1β) and Transforming Growth Factor beta 1 (TGF-β1) ELISA

1.2.6.1 Estimation of Tumor Necrosis Factor-alpha (TNF-α) levels

Tumor necrosis factor-alpha (TNF-α) levels were estimated using rat TNF-α kit (R&D Systems). It is a solid phase sandwich enzyme linked immunosorbent assay (ELISA), which uses a microtitre plate reader read at
450 nm. Concentrations of TNF-α were calculated from plotted standard curves.

**Principle**

This assay employs the quantitative sandwich enzyme immunoassay technique. An affinity purified polyclonal antibody specific for mouse TNF-α has been pre-coated onto a microplate. Standards, Controls, and samples are pipetted into the wells and any mouse TNF-α present is bound by the immobilized antibody. After washing away any unbound substances, an enzyme-linked polyclonal antibody specific for mouse TNF-α is added to the wells. Following a wash to remove any unbound antibody-enzyme reagent, a substrate solution is added to the wells. The enzyme reaction yields a blue product that turns yellow when the stop solution is added. The intensity of the color measured is in proportion to the amount of mouse TNF-α bound in the initial step. The sample values are then read off the standard curve.

**Assay Procedure**

1. The reagents and standard dilutions were prepared as suggested by the manufacturer’s instructions.
2. 50 μL of assay diluent was added to each well of the precoated microtitre plate.
3. 50μL of standard or sample was then added per well and were mixed gently. The plate was covered with the adhesive strip and incubated for 2 hours at room temperature.
4. Each well was then aspirated and washed with wash buffer, repeating the process four times for a total of five washes. The plate was then inverted and blotted against a clean paper towel.
5. 100 μL of mouse TNF-Conjugate was added to each well and covered with a new adhesive strip. The plate was again incubated for 2 hours at room temperature.
6. Repeated the aspiration/washes as in step 5.
7. 100 μL of substrate solution was then added to each well and incubated for 30 minutes at room temperature protected from light.
8. 100μL of stop solution was finally added to each well and the absorbance of each well was read in an ELISA reader set to 450 nm.
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Calculations

The average for the duplicate readings was calculated for each standard and sample and the blank values were subtracted. A standard curve was constructed by plotting the mean absorbance for each standard on the y-axis against the concentration on the x-axis. The samples which were diluted were multiplied by the dilution factor to get the exact concentration of the unknown samples.

1.2.6.2 Estimation of Interleukin-1beta (IL-1β) levels

The Quantikine Rat IL-1β Immunoassay is a 4.5 hour solid phase ELISA designed to measure rat IL-1β in cell culture supernates, serum, and EDTA plasma. It contains recombinant rat IL-1β and antibodies raised against recombinant rat IL-1β. This immunoassay has been shown to quantitate the recombinant rat factor accurately.

Principle

This assay employs the quantitative sandwich enzyme immunoassay technique. A polyclonal antibody specific for rat IL-1β has been pre-coated onto a microplate. Standards, Control, and samples are pipetted into the wells and any rat IL-1β present is bound by the immobilized antibody. After washing away any unbound substances, an enzyme-linked polyclonal antibody specific for rat IL-1β is added to the wells. Following a wash to remove any unbound antibody-enzyme reagent, a substrate solution is added to the wells. The enzyme reaction yields a blue product that turns yellow when the Stop Solution is added. The intensity of the color measured is in proportion to the amount of rat IL-1β bound in the initial step. The sample values are then read off the standard curve.

Sample Preparation

Rat serum and rat EDTA plasma samples require a 3-fold dilution into Calibrator Diluent RD5Y. A suggested 3-fold dilution is 50 μL sample + 100 μL Calibrator Diluent RD5Y.

Assay Procedure

1. All reagents and samples were brought to room temperature before use. All samples, standards, and control were assayed in duplicate.
2. Reagents, standard curve dilutions, and samples were prepared.
3. 50 μL of assay diluent RD1-21 was added to each well.
4. 50 μL of standard, control, or sample were added per well and mixed by gently tapping the plate frame for 1 minute and covered with the adhesive strip. Incubated for 2 hours at room temperature.
5. Each well aspirated and washed, repeating the process four times for a total of five washes.
6. 100 μL of Rat IL-1β conjugate was added to each well and incubated for 2 hours at room temperature.
7. The aspiration/wash was repeated as in step 5.
8. 100 μL of substrate solution was added to each well and incubated for 30 minutes at room temperature.
9. 100 μL of stop solution was added to each well and gently tapped the plate to ensure thorough mixing.
10. The optical density of each well was determined within 30 minutes, using a microplate reader set to 450 nm.

Calculations

The average for the duplicate readings was calculated for each standard and sample and the blank values were subtracted. A standard curve was constructed by plotting the mean absorbance for each standard on the y-axis against the concentration on the x-axis. The samples which were diluted were multiplied by the dilution factor to get the exact concentration of the unknown samples.

1.2.6.3 Estimation of Transforming Growth Factor beta 1 (TGF-β1) levels

Transforming Growth Factor beta1 (TGF-β1) was estimated using rat TGF-β1 immunoassay kit (R&D Systems, USA). The assay is based on the quantitative sandwich enzyme immunoassay technique (ELISA) using a microtitre plate reader at 450 nm. Serum samples were diluted 78-fold for ELISA determination of TGF-β1. Concentrations of TGF-β1 were calculated from plotted standard curves.

Principle

This assay employs the quantitative sandwich enzyme immunoassay technique. A monoclonal antibody specific for TGF-β1 has been pre-coated
onto a microplate. Standards, controls and samples are pipetted into the wells and any TGF-β1 present is bound by the immobilized antibody. After washing away any unbound substances, an enzyme-linked polyclonal antibody specific for TGF-β1 is added to the wells to sandwich the TGF-β1 immobilized during the first incubation. Following a wash to remove any unbound antibody-enzyme reagent, a substrate solution is added to the wells and color develops in proportion to the amount of TGF-β1 bound in the initial step. The color development is stopped and the intensity of the color is measured using ELISA reader set at 450 nm.

Activation Reagent Preparation

To activate latent TGF-β1 to the immunoreactive form, the following solutions were prepared for acid activation and neutralization. The solutions were stored in polypropylene bottles at room temperature for up to one month. If any precipitate forms, the solution was gently warmed to 37° C while mixing and cooled to room temperature before use.

Reagents to activate serum/plasma samples

2.5 N Acetic Acid/10 M Urea (250 ml) - 150.2 g of Urea was added to 100 ml of deionized water and was mixed well until dissolved. 35.9 ml of glacial acetic acid was slowly added to the mixture and the final volume was made up to 250 ml with deionized water.

2.7 N NaOH/1 M HEPES (250 ml) - 67.5 ml of 10 N NaOH was added to 140 ml of deionized water. After mixing, 59.5 g of HEPES was added and the final volume was made up to 250 ml with deionized water.

TGF-β1 Sample Activation Procedure

To activate latent TGF-β1 to immunoreactive TGF-β1 detectable by the Quantikine TGF-1 immunoassay, the activation procedure mentioned below was followed:

1. 40μL of 2.5 N acetic acid /10 M urea was added to 40 μL of the serum/plasma and was mixed properly.
2. The solution was incubated for 10 minutes at room temperature.
3. The acidified sample was then neutralized by adding 25 μL of 2.7 N NaOH/1 M HEPES and mixed well.
4. Prior to the assay, the activated samples were diluted 30 times with the calibrator diluents.


**Assay Procedure**

1. All the reagents, standard dilutions, and activated samples were prepared as directed in the kit (MB100; TGF-β1 kit).

2. 50 µL of assay diluent (for serum/plasma samples) was added to each well.

3. 50 µL of standard, control or activated samples were then added to each well and were covered with the adhesive strip. All the wells were incubated for 2 hours at room temperature on a horizontal orbital microplate shaker set at 500 ± 50 rpm.

4. The wells were aspirated and washed for a total of five washes with the wash buffer (400 µL). After the last wash, any remaining wash buffer was decanted by inverting the plate and blotting it against a clean paper towel.

5. 100µL of TGF-β1 conjugate was then added to each well and incubated for 2 hours at room temperature on the shaker.

6. The aspiration/wash steps were repeated as in step 4.

7. 100 µL of substrate solution was then added to each well and incubated for 30 minutes at room temperature on the benchtop protected from light.

8. 100µL of stop solution was finally added to each well and the plate was gently tapped to ensure thorough mixing.

9. The absorbance of each well was read using a microplate reader set to 450 nm within 30 minutes. The concentrations of TGF-β1 were calculated from plotted standard curves.

**Calculations**

The average for the duplicate readings was calculated for each standard and sample and the blank values were subtracted. A standard curve was constructed by plotting the mean absorbance for each standard on the y-axis against the concentration on the x-axis and a best fit curve was drawn through the points on the graph by regression analysis. The samples which were diluted were multiplied by the dilution factor to get the exact concentration of the unknown samples.
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1.2.7. Statistical Analysis

Results were expressed as mean ± SEM. The intergroup variation was measured by one-way analysis of variance (ANOVA) followed by Tukey's test to assess the significance. Two-way ANOVA followed by Tukey's test was employed to discover the inter-group variation in thermal hyperalgesia, mechanical hyperalgesia and mechanical allodynia data by considering day of testing and treatment as two independent variables. Statistical significance was considered at p<0.05. The statistical analysis was done using the SPSS Statistical Software version 16 (SPSS Inc., USA).

1.3. RESULTS

1.3.1. Effect of tocotrienol, epigallocatechin gallate, resveratrol and curcumin on thermal hyperalgesia in tail immersion test

Before the administration of the ethanol, the mean baseline tail flick latency (13.40 ± 0.19 sec) was not significantly different from that in control group (13.20 ± 0.17 sec). A significant decrease in tail flick latency (i.e., thermal hyperalgesia) was produced in the ethanol administered rats after 6 weeks (2.73 ± 0.19, p<0.05) which was further reduced (1.93 ± 0.19, p<0.05) on 10th week (Fig. 1.1.1, Fig. 1.1.2, Fig. 1.1.3, Fig. 1.1.4). Chronic treatment with tocotrienol (50, 100 and 200 mg/kg), epigallocatechin gallate (25, 50 and 100 mg/kg), resveratrol (5, 10 and 20 mg/kg) and curcumin (15, 30 and 60 mg/kg) significantly reversed thermal hyperalgesia in a dose dependent manner (Fig. 1.1.1, Fig. 1.1.2, Fig. 1.1.3, Fig. 1.1.4). The maximum increase in pain threshold was observed with higher doses of tocotrienol (200 mg/kg), epigallocatechin-3-gallate (100 mg/kg) resveratrol (20 mg/kg) and curcumin (60 mg/kg) (Fig. 1.1.1, Fig. 1.1.2, Fig. 1.1.3, Fig. 1.1.4). Tocotrienol (50 mg/kg) was found to be more effective than α-tocopherol (100 mg/kg) in increasing the tail flick latency in ethanol-treated rats (p < 0.05). There was no significant change in the mean tail flick latency of the control and per se groups over the same time period.
Figure 1.1.1. Effect of tocotrienol (50, 100 and 200 mg/kg) on reaction time in tail-immersion test in control and alcohol administered rats. Values are expressed as mean ± S.E.M. a (p<0.05) different from control group; b (p<0.05) different from ethanol administered group; c (p<0.05) different from rats treated with α-tocopherol or tocotrienol along with ethanol; d (p<0.05) different from basal (0 day). CNTL Control, E Ethanol, α-T(100) α-Tocopherol (100 mg/kg), T(50) Tocotrienol (50 mg/kg), T(100) Tocotrienol (100 mg/kg).

Figure 1.1.2. Effect of epigallocatechin gallate (25, 50 and 100 mg/kg) on reaction time in tail-immersion test in control and alcohol administered rats. Values are expressed as mean ± S.E.M. a (p<0.05) different from control group; b (p<0.05) different from ethanol administered group; c (p<0.05) different from rats treated with epigallocatechin gallate along with ethanol; d (p<0.05) different from basal (0 day). CNTL Control, E Ethanol, EG epigallocatechin gallate (25 mg/kg), EG (50) epigallocatechin gallate (50 mg/kg), EG (100) epigallocatechin gallate (100 mg/kg).
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Figure 1.1.3. Effect of resveratrol (5, 10 and 20 mg/kg) on reaction time in tail-immersion test in control and alcohol administered rats. Values are expressed as mean ± S.E.M. a (p<0.05) different from control group; b (p<0.05) different from ethanol administered group; c (p<0.05) different from rats treated with epigallocatechin gallate along with ethanol; d (p<0.05) different from basal (0 day). CNTL Control, E Ethanol, RVT (5) resveratrol (5 mg/kg), RVT (10) resveratrol (10 mg/kg), RVT (20) resveratrol (20 mg/kg).

Figure 1.1.4. Effect of curcumin (15, 30 and 60 mg/kg) on reaction time in tail immersion test in control and alcohol administered rats. Values are expressed as mean ± S.E.M. a (p<0.05) different from control group; b (p<0.05) different from ethanol administered group; c (p<0.05) different from rats treated with curcumin along with ethanol; d (p<0.05) different from basal (0 day). CNTL Control, E Ethanol, CMN (15) curcumin (15 mg/kg), CMN (30) curcumin (30 mg/kg), CMN (60) curcumin (60 mg/kg).
1.3.2. Effect of tocotrienol, epigallocatechin gallate, resveratrol, curcumin on mechanical hyperalgesia in randall sellito test

The mean baseline paw-withdrawal threshold of ethanol treated (160.33 ± 2.59 gm) was recorded on day 0 before administration of ethanol, which was not significantly different from that in control group rats (160.4 ± 4.31 gm). A significant decrease in mechanical nociceptive threshold (mechanical hyperalgesia) was produced in the ethanol treated rats after 2 weeks (80.48 ± 4.85 gm, p<0.05) and subsequently reduced on 10th week (52.98 ± 3.21 gm, p<0.05) (Fig. 1.2.1, Fig. 1.2.2, Fig. 1.2.3, Fig. 1.2.4). Chronic treatment with tocotrienol (50, 100 and 200 mg/kg), epigallocatechin gallate (25, 50 and 100 mg/kg), resveratrol (5, 10 and 20 mg/kg) and curcumin (15, 30 and 60 mg/kg) significantly increased the pain threshold in ethanol administered rats in a dose dependent manner (Fig. 1.2.1, Fig. 1.2.2, Fig. 1.2.3, Fig. 1.2.4). Tocotrienol (50 mg/kg) was found to be more effective than α-tocopherol (100 mg/kg) in increasing the paw-withdrawal threshold in ethanol-treated rats (p<0.05). In addition, tocotrienol (200 mg/kg), epigallocatechin-3-gallate (100 mg/kg), resveratrol (20 mg/kg) and curcumin (60 mg/kg) per se did not affect mechanical hyperalgesia in control rats.

Figure 1.2.1. Effect of tocotrienol (50, 100 and 200 mg/kg) on mechanical hyperalgesia in randall sellito test in control and alcohol administered rats. Values are expressed as mean ± S.E.M. a (p<0.05) different from control group; b (p<0.05) different from ethanol administered group; c (p<0.05) different from rats treated with α-tocopherol and tocotrienol along with ethanol; d (p<0.05) different from basal (0 day). CNTL Control, E Ethanol, α-T(100) Tocopherol (100 mg/kg), T(50) Tocotrienol (50 mg/kg), T(100) Tocotrienol (100 mg/kg), T(200) Tocotrienol (200 mg/kg).
**Figure 1.2.2.** Effect of epigallocatechin gallate (25, 50 and 100 mg/kg) on mechanical hyperalgesia in Randall-Sellito test in control and alcohol administered rats. Values are expressed as mean ± S.E.M. a (p<0.05) different from control group; b (p<0.05) different from ethanol administered group; c (p<0.05) different from rats treated with epigallocatechin gallate along with ethanol; d (p<0.05) different from basal (0 day). CNTL Control, E Ethanol, EG (25) epigallocatechin gallate (25 mg/kg), EG (50) epigallocatechin gallate (50 mg/kg), EG (100) epigallocatechin gallate (100 mg/kg).

**Figure 1.2.3.** Effect of resveratrol (5, 10 and 20 mg/kg) on mechanical hyperalgesia in Randall-Sellito test in control and alcohol administered rats. Values are expressed as mean ± S.E.M. a (p<0.05) different from control group; b (p<0.05) different from ethanol administered group; c (p<0.05) different from rats treated with resveratrol along with ethanol; d (p<0.05) different from basal (0 day). CNTL Control, E Ethanol, RVT (5) resveratrol (5 mg/kg), RVT (10) resveratrol (10 mg/kg), RVT (20) resveratrol (20 mg/kg).
Figure 1.2.4. Effect of curcumin (15, 30 and 60 mg/kg) on mechanical hyperalgesia in Randall-Sellito test in control and alcohol administered rats. Values are expressed as mean ± S.E.M. a (p<0.05) different from group; b (p<0.05) different from ethanol administered group; c different from rats treated with curcumin along with ethanol; d different from basal (0 day). CNTL Control, E Ethanol, CMN (15) curcumin (15 mg/kg), CMN (30) curcumin (30 mg/kg), CMN (60) curcumin (60 mg/kg).

1.3.3. Effect of tocotrienol, epigallocatechin gallate, resveratrol, and curcumin on tactile allodynia in von Frey hair test

The mean baseline paw-withdrawal threshold (84.60 ± 8.20 g; p<0.05) of ethanol treated rats on day 0 was not significantly different from that in control group (84.73 ± 0.95 g, p<0.05). After 6 weeks of administration, in response to von-Frey hair stimulation, a significant decrease (22.99 ± 2.26 g, p<0.05) in paw-withdrawal threshold (i.e., mechanical allodynia) was produced in the ethanol treated rats which gets further reduced (11.34 ± 0.55 g, p<0.05) up to 10th week (Fig. 1.3.1, Fig. 1.3.2, Fig. 1.3.4). Treatment with tocotrienol (50, 100 and 200 mg/kg), epigallocatechin gallate (25, 50 and 100 mg/kg), resveratrol (5, 10 and 50 mg/kg) and curcumin (15, 30 and 60 mg/kg) significantly and dependently reversed the decreased paw-withdrawal threshold. Tocotrienol was found to be more effective than α-tocopherol in increasing paw-withdrawal threshold in ethanol-treated rats (p < 0.05). There was a significant change in the mean paw-withdrawal threshold of the control group.
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tocotrienol (200 mg/kg), epigallocatechin-3-gallate (100 mg/kg), resveratrol (20 mg/kg) and curcumin (60 mg/kg) per se groups over the same time peri (Fig. 1.3.1, Fig. 1.3.2, Fig. 1.3.3, Fig. 1.3.4).

Figure 1.3.1. Effect of tocotrienol (50, 100 and 200 mg/kg) on mechanical allodynia in von-frey hair test in control and alcohol administered rats. Values are expressed mean ± S.E.M. a (p<0.05) different from control group; b (p<0.05) different from ethanol administered group; c (p<0.05) different from rats treated with α-tocopherol or tocotrienol along with ethanol; d (p<0.05) different from basal (0 day). CNTL Control, E Ethanol, α-T(100) α-Tocopherol (100 mg/kg), T(50) Tocotrienol (50 mg/kg), T(100) Tocotrienol (200 mg/kg).

Figure 1.3.2. Effect of epigallocatechin gallate (25, 50 and 100 mg/kg) on mechanical allodynia in von-frey hair test in control and alcohol administered rats. Values are expressed as mean ± S.E.M. a (p<0.05) different from control group; b (p<0.05) different from ethanol administered group; c (p<0.05) different from rats treated with epigallocatechin gallate along with ethanol; d (p<0.05) different from basal (0 day). CNTL Control, E Ethanol, EG (25) Epigallocatechin gallate (25 mg/kg), EG (50) epigallocatechin gallate (50 mg/kg), EG (100) epigallocatechin gallate (100 mg/kg).
Figure 1.3.3. Effect of resveratrol (5, 10 and 20 mg/kg) on mechanical allodynia in von-frey hair test in control and alcohol administered rats. Values are expressed as mean ± S.E.M. a (p<0.05) different from control group; b (p<0.05) different from ethanol administered group; c (p<0.05) different from rats treated with resveratrol along with ethanol; d (p<0.05) different from rats treated with ethanol alone (0 day). CNTL Control, E Ethanol, RVT (5) resveratrol (5 mg/kg), RVT (10) resveratrol (10 mg/kg), RVT (20) resveratrol (20 mg/kg).

Figure 1.3.4. Effect of curcumin (15, 30 and 60 mg/kg) on mechanical allodynia in von-frey hair test in control and alcohol administered rats. Values are expressed as mean ± S.E.M. a (p<0.05) different from control group; b (p<0.05) different from ethanol administered group; c (p<0.05) different from rats treated with curcumin along with ethanol; d (p<0.05) different from rats treated with curcumin alone (0 day). CNTL Control, E Ethanol, CMN (15) curcumin (15 mg/kg), CMN (30) curcumin (30 mg/kg), CMN (60) curcumin (60 mg/kg).
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1.3.4. Effect of tocotrienol, epigallocatechin gallate, resveratrol and curcumin on ethanol-induced changes in lipid peroxidation

Malonaldehyde (MDA) levels were increased significantly in the sciatic nerve of ethanol treated rats (3.58 ± 0.16 nmoles/mg pr, p<0.05); as compared to control group (0.70 ± 0.05 nmoles/mg pr). Chronic treatment with tocotrienol (50, 100 and 200 mg/kg), epigallocatechin gallate (25, 50 and 100 mg/kg), resveratrol (5, 10 and 20 mg/kg) and curcumin (15, 30 and 60 mg/kg) significantly produced a significant (p < 0.05) and dose-dependent reduction in MDA levels in sciatic nerve of ethanol treated rats (Table 1.1). More potent reduction in MDA levels were found in tocotrienol treated rats as compared to α-tocopherol group (p < 0.05). In addition, tocotrienol (200 mg/kg), epigallocatechin-3-gallate (100 mg/kg), resveratrol (20 mg/kg) and curcumin (60 mg/kg) per se did not affect lipid peroxidation in the sciatic nerves of control rats.

1.3.5. Effect of tocotrienol, epigallocatechin gallate, resveratrol and curcumin on ethanol-induced changes in the antioxidant profile

The reduced glutathione levels and enzyme activity of superoxide dismutase and catalase were significantly decreased in the sciatic nerve of ethanol treated rats as compared to control group. This reduction was significantly reversed by treatment with tocotrienol (50, 100 and 200 mg/kg), epigallocatechin gallate (25, 50 and 100 mg/kg), resveratrol (5, 10 and 20 mg/kg) and curcumin (15, 30 and 60 mg/kg) in sciatic nerve of ethanol treated rats (Table 1.1). Reduction in endogenous antioxidant enzyme level was reversed more potently with tocotrienol than α-tocopherol (p < 0.05). In addition, tocotrienol (200 mg/kg), epigallocatechin-3-gallate (100 mg/kg), resveratrol (20 mg/kg) and curcumin (60 mg/kg) per se did not alter endogenous antioxidant levels in the sciatic nerves of control rats.

1.3.6. Effect of tocotrienol, epigallocatechin gallate, resveratrol and curcumin on ethanol-induced nitrosative stress

Nitrite levels were significantly elevated in sciatic nerve of ethanol treated rats (305.83 ± 4.80 μg/ml, p<0.05); as compared to control group (93.50 ± 6.25 μg/ml, p<0.05). Chronic treatment with tocotrienol (50, 100 and
200 mg/kg), epigallocatechin gallate (25, 50 and 100 mg/kg), resveratrol (5, 10 and 20 mg/kg) and curcumin (15, 30 and 60 mg/kg) significantly and dose dependently inhibited this increase in nitrite levels in sciatic nerve of ethanol treated rats (Table 1.1). In addition, tocotrienol (200 mg/kg), epigallocatechin-3-gallate (100 mg/kg), resveratrol (20 mg/kg) and curcumin (60 mg/kg) per se did not affect nitrite levels in the sciatic nerves of control rats.

1.3.7. Effect of tocotrienol, epigallocatechin gallate, resveratrol and curcumin on serum and nerve TNF-α levels

TNF-α levels were markedly increased both in serum (150.75 ± 1.57 pg/ml; Fig. 1.4) and sciatic nerve (103.45 ± 3.96 pg/ml; Fig. 1.5) of ethanol treated rats as compared to control group (28.25 ± 6.5 pg/ml; 13.92 ± 1.52 pg/ml; p<0.01). Chronic treatment with tocotrienol (50, 100 and 200 mg/kg), epigallocatechin gallate (25, 50 and 100 mg/kg), resveratrol (5, 10 and 20 mg/kg) and curcumin (15, 30 and 60 mg/kg) significantly decreased these elevated cytokine levels both in serum (Fig. 1.4) and sciatic nerve (Fig. 1.5) of ethanol treated rats in a dose dependent manner (p<0.05). a-Tocopherol (100 mg/kg) produced 14% reduction whereas tocotrienol (50, 100 and 200 mg/kg) produced 23%, 38% and 56% reduction of serum TNF-α levels, respectively. However, tocotrienol (200 mg/kg), epigallocatechin-3-gallate (100 mg/kg), resveratrol (20 mg/kg) and curcumin (60 mg/kg) per se did not affect serum and neural TNF-α levels of control rats.
Table 1.1. Effect of tocotrienol, epigallocatechin gallate, resveratrol and curcumin on lipid peroxide, reduced glutathione, superoxide dismutase (SOD), catalase and nitrite levels (mean ± SEM) in sciatic nerve of ethanol administered rats

<table>
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<th>Treatment</th>
<th>LPO (nmoles/mg protein)</th>
<th>GSH (umoles/mg protein)</th>
<th>SOD (units/mg protein)</th>
<th>Catalase (k/min)</th>
<th>Nitrite (µg/ml)</th>
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<td>CNTL</td>
<td>0.75 ± 0.08</td>
<td>0.138 ± 0.009</td>
<td>3.983 ± 0.117</td>
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<td>E</td>
<td>3.77 ± 0.17&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.019 ± 0.001&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.305 ± 0.011&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.10 ± 0.114&lt;sup&gt;a&lt;/sup&gt;</td>
<td>298.60 ± 7.46&lt;sup&gt;a&lt;/sup&gt;</td>
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<td>E + α-T (100)</td>
<td>3.09 ± 0.03&lt;sup&gt;b,c&lt;/sup&gt;</td>
<td>0.032 ± 0.001&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.507 ± 0.031</td>
<td>1.05 ± 0.29</td>
<td>246.50 ± 5.02&lt;sup&gt;b,c&lt;/sup&gt;</td>
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<td>E + T (50)</td>
<td>2.64 ± 0.11&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.041 ± 0.002&lt;sup&gt;b,c&lt;/sup&gt;</td>
<td>0.705 ± 0.016&lt;sup&gt;b,c&lt;/sup&gt;</td>
<td>1.17 ± 0.166</td>
<td>215.20 ± 2.23&lt;sup&gt;b,c&lt;/sup&gt;</td>
</tr>
<tr>
<td>E + T (100)</td>
<td>2.35 ± 0.03&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.061 ± 0.002&lt;sup&gt;b,c&lt;/sup&gt;</td>
<td>1.049 ± 0.028&lt;sup&gt;b,c&lt;/sup&gt;</td>
<td>1.75 ± 0.570</td>
<td>185.00 ± 4.68&lt;sup&gt;b,c&lt;/sup&gt;</td>
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<td>E + T (200)</td>
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<td>0.085 ± 0.002&lt;sup&gt;b,c&lt;/sup&gt;</td>
<td>1.505 ± 0.084&lt;sup&gt;b,c&lt;/sup&gt;</td>
<td>1.70 ± 0.317</td>
<td>143.21 ± 5.31&lt;sup&gt;b,c&lt;/sup&gt;</td>
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<td>T (200)</td>
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<td>E + EG (25)</td>
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<td>0.046 ± 0.004&lt;sup&gt;b,d&lt;/sup&gt;</td>
<td>0.970 ± 0.040&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1.86 ± 0.080&lt;sup&gt;b&lt;/sup&gt;</td>
<td>265.50 ± 5.02&lt;sup&gt;b,d&lt;/sup&gt;</td>
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<td>E + EG (50)</td>
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<td>0.063 ± 0.003&lt;sup&gt;b,d&lt;/sup&gt;</td>
<td>1.220 ± 0.080&lt;sup&gt;b&lt;/sup&gt;</td>
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a (p<0.05) different from control group; b (p<0.05) different from ethanol administered group; c (p<0.05) different from rats treated with a-tocopherol or tocotrienol along with ethanol; d (p<0.05) different from rats treated with EGCG along with ethanol; e (p<0.05) different from rats treated with resveratrol along with ethanol; f (p<0.05) different from rats treated with curcumin along with ethanol. CNTL Control, E Ethanol, α-T(100) α-Tocopherol (100mg/kg), T(50) Tocotrienol (50 mg/kg), T(100) Tocotrienol (100 mg/kg), T(200) Tocotrienol (200 mg/kg), EG (25) epigallocatechin gallate (25 mg/kg).
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1.3.8. Effect of tocotrienol, epigallocatechin gallate, resveratrol and curcumin on serum and nerve IL-1β levels

Both serum (142.34 ± 7.8 pg/ml; Fig. 1.6) and neural (38.56 ± 1.63 pg/ml; Fig. 1.7) IL-1β levels of ethanol treated rats were markedly increased as compared to control group (32.34 ± 1.45 pg/ml; 5.67 ± 0.33 pg/ml; p<0.01). Chronic treatment with tocotrienol (50, 100 and 200 mg/kg), epigallocatechin gallate (25, 50 and 100 mg/kg), resveratrol (5, 10 and 20 mg/kg) and curcumin (15, 30 and 60 mg/kg) significantly decreased IL-1β levels both in serum (Fig. 1.6) and sciatic nerve (Fig. 1.7) of ethanol treated rats in a dose dependent manner (p<0.05). Tocotrienol was found to be more effective than α-tocopherol in decreasing both the serum and nerve IL-1β levels in ethanol-treated rats (p<0.05). However, tocotrienol (200 mg/kg), epigallocatechin-3-gallate (100 mg/kg), resveratrol (20 mg/kg) and curcumin (60 mg/kg) per se did not affect serum and neural IL-1β levels of control rats.

1.3.9. Effect of tocotrienol, epigallocatechin gallate, resveratrol and curcumin on serum and nerve TGF-β1 levels

TGF-β1 levels were significantly increased both in serum (205.6 ± 5.4 pg/ml; Fig. 1.8) and sciatic nerve (155.6 ± 2.2 pg/ml; Fig. 1.9) of ethanol treated rats as compared to control group (50.5 ± 4.5 pg/ml; 60.3 ± 3.01 pg/ml; p<0.01). Chronic treatment with tocotrienol (50, 100 and 200 mg/kg), epigallocatechin gallate (25, 50 and 100 mg/kg), resveratrol (5, 10 and 20 mg/kg) and curcumin (15, 30 and 60 mg/kg) significantly decreased TGF-β1 levels both in serum (Fig. 1.8) and sciatic nerve (Fig. 1.9) of ethanol treated rats in a dose dependent manner (p<0.05). Tocotrienol treatment produced more significant decrease in both serum and nerve TGF-β1 in ethanol administered rats (p < 0.05). However, tocotrienol (200 mg/kg), epigallocatechin-3-gallate (100 mg/kg), resveratrol (20 mg/kg) and curcumin (60 mg/kg) per se did not affect serum and neural TGF-β1 levels of control rats.
Figure 1.4 Effect of tocotrienol (50, 100 and 200 mg/kg), epigallocatechin gallate (25, 50 and 100 mg/kg), resveratrol (5, 10 and 20 mg/kg) and curcumin (15, 30 and 60 mg/kg) on serum TNF-α levels in ethanol administered rats. Data are expressed as mean ± S.E.M. a (p<0.05) different from control group; b (p<0.05) different from ethanol administered group; c (p<0.05) different from rats treated with α-tocopherol or tocotrienol along with ethanol; d (p<0.05) different from rats treated with EGCG along with ethanol; e (p<0.05) different from rats treated with resveratrol along with ethanol; f (p<0.05) different from rats treated with curcumin along with ethanol. C = control, E = ethanol, D = α-tocopherol, D1, D2 and D3 refer to three different doses of tocotrienol, epigallocatechin gallate, resveratrol and curcumin.

<table>
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<tr>
<th>Groups</th>
<th>Tocotrienol</th>
<th>α-tocopherol</th>
<th>Epigallocatechin gallate</th>
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<th>Curcumin</th>
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<td>E+D2</td>
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Figure 1.5 Effect of tocotrienol (50, 100 and 200 mg/kg), epigallocatechin gallate (25, 50 and 100 mg/kg), resveratrol (5, 10 and 20 mg/kg) and curcumin (15, 30 and 60 mg/kg) on TNF-α levels in sciatic nerve of ethanol administered rats. Data are expressed as mean ± S.E.M. a (p<0.05) different from control group; b (p<0.05) different from ethanol administered group; c (p<0.05) different from rats treated with α-tocopherol or tocotrienol along with ethanol; d (p<0.05) different from rats treated with EGCG along with ethanol; e (p<0.05) different from rats treated with resveratrol along with ethanol; f (p<0.05) different from rats treated with curcumin along with ethanol. C = control, E = ethanol, D = α-tocopherol, D1, D2 and D3 refer to three different doses of tocotrienol, epigallocatechin gallate, resveratrol and curcumin.

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Figure 1.6 Effect of tocotrienol (50, 100 and 200 mg/kg), epigallocatechin gallate (25, 50 and 100 mg/kg), resveratrol (5, 10 and 20 mg/kg) and curcumin (15, 30 and 60 mg/kg) on serum IL-1β levels in ethanol administered rats. Data are expressed as mean ± S.E.M. a (p<0.05) different from control group; b (p<0.05) different from ethanol administered group; c (p<0.05) different from rats treated with α-tocopherol or tocotrienol along with ethanol; d (p<0.05) different from rats treated with EGCG along with ethanol; e (p<0.05) different from rats treated with resveratrol along with ethanol; f (p<0.05) different from rats treated with curcumin along with ethanol. C = control, E = ethanol, D = α-tocopherol, D1, D2 and D3 refer to three different doses of tocotrienol, epigallocatechin gallate, resveratrol and curcumin.
Figure 1.7 Effect of tocotrienol (50, 100 and 200 mg/kg), epigallocatechin gallate (25, 50 and 100 mg/kg), resveratrol (5, 10 and 20 mg/kg) and curcumin (15, 30 and 60 mg/kg) on IL-1β levels in sciatic nerve of ethanol administered rats. Data are expressed as mean ± S.E.M. a (p<0.05) different from control group; b (p<0.05) different from ethanol administered group; c (p<0.05) different from rats treated with α-tocopherol or tocotrienol along with ethanol; d (p<0.05) different from rats treated with EGCG along with ethanol; e (p<0.05) different from rats treated with resveratrol along with ethanol; f (p<0.05) different from rats treated with curcumin along with ethanol. C = control, E = ethanol, D = α-tocopherol, D1, D2 and D3 refer to three different doses of tocotrienol, epigallocatechin gallate, resveratrol and curcumin.

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Figure 1.8 Effect of tocotrienol (50, 100 and 200 mg/kg), epigallocatechin gallate (25, 50 and 100 mg/kg), resveratrol (5, 10 and 20 mg/kg) and curcumin (15, 30 and 60 mg/kg) on serum TGF-β1 levels in ethanol administered rats. Data are expressed as mean ± S.E.M. a (p<0.05) different from control group; b (p<0.05) different from ethanol administered group; c (p<0.05) different from rats treated with α-tocopherol or tocotrienol along with ethanol; d (p<0.05) different from rats treated with EGCG along with ethanol; e (p<0.05) different from rats treated with resveratrol along with ethanol; f (p<0.05) different from rats treated with curcumin along with ethanol. C = control, E = ethanol, D = α-tocopherol, D1, D2 and D3 refer to three different doses of tocotrienol, epigallocatechin gallate, resveratrol and curcumin.
Figure 1.9 Effect of tocotrienol (50, 100 and 200 mg/kg), epigallocatechin gallate (25, 50 and 100 mg/kg), resveratrol (5, 10 and 20 mg/kg) and curcumin (15, 30 and 60 mg/kg) on TGF-β1 levels in sciatic nerve of ethanol administered rats. Data are expressed as mean ± S.E.M. a (p<0.05) different from control group; b (p<0.05) different from ethanol administered group; c (p<0.05) different from rats treated with α-tocopherol or tocotrienol along with ethanol; d (p<0.05) different from rats treated with EGCG along with ethanol; e (p<0.05) different from rats treated with resveratrol along with ethanol; f (p<0.05) different from rats treated with curcumin along with ethanol. C = control, E = ethanol, D = α-tocopherol, D1, D2 and D3 refer to three different doses of tocotrienol, epigallocatechin gallate, resveratrol and curcumin.

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1.4. DISCUSSION

Rats chronically fed ethanol exhibited mechanical and thermal hyperalgesia and tactile allodynia all of which are symptoms frequently occurring in patients with painful peripheral neuropathy (Scadding, 1982). Ethanol-induced hyperalgesia became evident at 6 weeks and progressively increasing till the end of the study i.e. 10 weeks. These results are consistent with the previous reports demonstrating neuropathic-pain state in the rats following chronic alcohol consumption (Dina et al., Miyoshi et al., 2007; Narita et al., 2007). Studies have suggested that chronic ethanol increases oxidative damage to proteins, lipids and DNA (Mansoor et al., 2001; McDonough, 2003). Oxidative injury has been implicated in the pathophysiology of neural injury. Neural tissues are poor in antioxidant defense enzymes such as catalase and superoxide dismutase. Endogenous oxidative stress leads to nerve dysfunction in rats with chronic constipation (Kim et al., 2004). Reactive oxygen species trigger second messenger molecules involved in central sensitization of dorsal horn cells (Ali and Salter, 2003). They activate spinal glial cells which in turn play an important role in chronic pain (Raghavendra et al., 2003). Reduced glutathione is a major intracellular scavenger of free radicals in cytoplasm. Depletion of glutathione increases the susceptibility of neurons to oxidative stress-induced hyperalgesia (Wullner et al., 1999).

Nitric oxide is also implicated in neuropathic pain (Levy and Zochodne, 2004). It sensitizes spinal neurons and also contributes to sensitization of central neurons by disinhibition (Lin et al., 1999). Moreover, uncontrolled production of nitric oxide coupled with deficient superoxide dismutase to production of notorious peroxynitrite, which is several times more electrophile than its parent substances, hydrogen peroxide and OH· like species (Dicker and Cederlind, 1992). A significant increase in MDA and nitrite levels and marked decrease in the activity of superoxide dismutase, catalase and depletion of reduced glutathione in the sciatic nerve of ethanol treated rats was observed.

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suggesting the involvement of oxidative and nitrosative stress in the development of alcoholic neuropathy in rats (Tiwari et al., 2009a, 2011).

Various clinical studies suggest the beneficial effects of anti-oxidant therapy on signs and symptoms of neuropathic pain conditions. Ranieri et al. (2009) found that 6 week oral treatment with alpha-lipoic acid and gamma-linolenic acid, the potent natural anti-oxidants, in synergy with rehabilitation therapy improved neuropathic symptoms and deficits in patients with radicular neuropathy. Recently, Pace et al. (2010) also found that treatment with vitamin E (alpha-tocopherol 400 mg/day) for 3 months protects against cisplatin-induced peripheral neuropathy in phase III trials. Thus, treatment of neuropathic pain conditions with natural anti-oxidants seems to hold a promising approach. Moreover, as pathogenesis of alcoholic neuropathy involves generation of free radicals with subsequent activation of downstream signaling pathways, we found it worthwhile to investigate the potential of natural polyphenolic antioxidants i.e. vitamin E isoforms (α-tocopherol vs tocotrienol), epigallocatechin gallate, resveratrol and curcumin in attenuating symptoms associated with alcoholic neuropathy along with amelioration of biochemical deficits in rats chronically fed ethanol for 10 weeks.

Chronic treatment with both the isoforms of vitamin E (α-tocopherol vs tocotrienol), epigallocatechin gallate, resveratrol and curcumin significantly prevented thermal and mechanical hyperalgesia and tactile allodynia along with attenuation of MDA and nitrite levels in the sciatic nerve of rats chronically fed ethanol in a dose-dependent manner. Apart from this, decreased neural levels of endogeneous antioxidants (reduced glutathione, superoxide dismutase and catalase) were also significantly restored on treatment with α-tocopherol, tocotrienol, epigallocatechin gallate, resveratrol and curcumin. Moreover, tocotrienol produced more potent effects as compared to tocotrienol both at behavioral and biochemical level.

Vitamin E is a natural, highly tolerable and cost effective molecule. Most of the vitamin E-sensitive neurological disorders are associated with elevated levels of oxidative damage markers. This has led to a popular hypothesis stating that the neuroprotective effects of vitamin E are wholly mediated by its antioxidant property (Vatassery, 1998). Beyond the
nonspecific antioxidant effect specific effects of Vitamin E, which includes gene regulation, have been revealing and non-antioxidant properties of tocopherols are current topics of interest (Azzi and Stocker; 2000). Sharma and Sayyed (2006) found that two weeks treatment with trolox (water-soluble derivative of vitamin E) in the dose of 10 and 30 mg/kg initiated at 6th week of STZ injection significantly improved motor nerve conduction velocity (MNCV), nerve blood flow (NBF) and inhibited thermal hyperalgesia. Trolox treatment also improved the activity of antioxidant enzymes and inhibited lipid peroxidation in sciatic nerves of diabetic rats suggesting its potential in diabetic neuropathy. Tocotrienol has been reported to inhibit oxidative stress in plethora of disease conditions like ischemia-reperfusion injury, alcoholic complications, depression, diabetic complications (Kuhad et al., 2009; Kuhad and Chopra, 2009; Tiwari et al., 2009a, 2009b, 2009c; Das et al., 2012). The experimental research examining the antioxidant, free radical scavenging effects of tocopherol and tocotrienols has revealed that tocotrienols appear superior to α-tocopherol due to their better distribution in the fatty layers of the cell membrane (Suzuki et al., 1993). At nM concentrations, α-tocotrienol, in contrast with α-tocopherol, protects against glutamate-induced neuronal death in mice by suppressing inducible pp60 c-src kinase activation (Sen et al., 2000) as well as by suppressing inducible 12-lipoxygenase activation (Khanna et al., 2003). Findings from our laboratory suggested that tocotrienol can prevent the diabetic neuropathy and associated cognitive deficits by decreasing the oxidative-nitrosative stress and inflammatory cascade (Kuhad et al., 2009).

EGCG was found to prevent ethanol-induced hepatotoxicity and inhibited development of a fatty liver (Yun et al., 2007). Kim et al. (2009) found that EGCG treatment inhibited both tetrodotoxin-sensitive and tetrodotoxin-resistant Na (+) currents in rat dorsal root ganglion neurons in a concentration-dependent manner and suggested its potential to develop as an analgesic agent. He et al. (2009) also suggested that chronic treatment with EGCG (2 mg/kg or 6 mg/kg) for 4 weeks significantly improved the cognitive deficits induced by 2 week administration of D-galactose in mice as measured in water maze. EGCG showed neuroprotective effect in a wide variety of
animal models by inhibition of enhanced oxidative-nitrosative stress and augmentation of antioxidant defense capacity (Kim et al., 2009; Wang et al., 2009). The EGCG pre-treatment abolished ethanol-induced lipid peroxidation of the cell membrane, loss of total superoxide dismutase (SOD) activity and suppressed ethanol-induced gene expressions of Mn- and Cu/Zn-SOD (Asaumi et al., 2006). EGCG administration led to a dose-dependent decrease in the degree of liver injury and expression of iNOS and nitrotyrosine in the carbon tetrachloride-treated mice (Chen et al., 2004). EGCG treatment elevated total superoxide dismutase and glutathione peroxidase activities, decreased malondialdehyde contents and reduced the cell apoptosis index and expression of cleaved caspase-3 in the hippocampus of aging mice induced by D-galactose indicating the anti-oxidative and anti-apoptotic potential of EGCG (He et al., 2009).

Resveratrol, a naturally occurring phytoalexin, is reported to have many beneficial effects on human health against atherosclerosis and cardiovascular diseases, inhibition of proliferation of a number of cell lines and neuroprotective activity (Kaneko et al., 2011; Karalis et al., 2011). Recently, Kumar and Sharma (2010) demonstrated NF-κB inhibitory action of resveratrol as probable mode of neuroprotective action in experimental model of diabetic neuropathy. Resveratrol has also been found to exert a strong inhibitory effect on superoxide anion and hydrogen peroxide production by macrophages stimulated by lipopolysaccharide or phorbol esters. It has hydroxyl-radical scavenging activity and has recently also been found to possess glutathione-sparing activity (Bowers et al., 2000). Moreover, resveratrol was found to strongly inhibit NO generation in activated macrophages, as measured by the amount of nitrite released into the culture medium and resveratrol strongly reduced the amount of cytosolic iNOS protein and steady-state mRNA levels, thereby reducing the formation of peroxynitrite, a potent oxidant. As an antioxidant, resveratrol also reduced oxidative stress in blood platelets (Olas and Wachowicz, 2002). It has also been reported that resveratrol inhibited PKC activity, and therefore has an effect on associated signaling networks which may, in part, underlie the mechanism(s) by which this agent exerts neuroprotective properties (Bastianetto et al., 2009).
Curcumin has many pharmacological activities including powerful antioxidant activity, analgesic (Sharma et al., 2006), anti-inflammatory (Jain et al., 2009) and neuroprotective properties (Thiyagarajan and Sharma, 2004; Kuhad and Chopra, 2007; Zhao et al., 2008; Ataie et al., 2010). Studies have shown that curcumin is a powerful scavenger of the superoxide anion, the hydroxyl radical and nitrogen dioxide (Unnikrishnan and Rao, 1995). Curcumin is also known to exhibit anti-hyperalgesic (Sharma et al., 2006) and anti-inflammatory effects in wide variety of animal models (Xu et al., 2005). Curcumin increased the pain threshold in rats fed with ethanol in all the behavioural paradigms of pain which is in line with evidence from previous studies done in our laboratory where curcumin attenuated the pain in diabetic (Sharma et al., 2006) and reserpinized rats (Arora et al., 2011).

Proinflammatory cytokines are known to play a very important role both in initiation and maintenance of pain in various neurological disorders. TNF-α, IL-1β and IL-6 and in the dorsal horn are increased after nerve lesion and have been implicated in contributing to nerve-injury pain, presumably by altering synaptic transmission in the CNS, including the spinal cord (Inoue, 2006). TNF-α has been demonstrated as an important mediator of neuropathic pain (Ignatowski et al., 1999). Prophylactic treatment with TNF-α inhibitors efficiently reduces hyperalgesia suggesting that TNF-α seems to contribute to the initiation of neuropathic pain (George et al., 1999). Uceyler et al. (2007) had also reported that the patients with the complex regional pain have increased mRNA and protein levels for TNF-α. Like TNF-α, IL-1β is also known to contribute significantly in various models of neuropathic pain. Nerve-injury induced release of IL-1β might contribute to the central sensitization associated with chronic neuropathic pain (Sabrina et al., 2008). Wolff et al. (2006) also found that genetic impairment of interleukin-1 signaling attenuates neuropathic pain, autotomy, and spontaneous ectopic neuronal activity, following nerve injury in mice. Interleukin-1 has been implicated in the modulation of pain perception under various inflammatory conditions.

Transforming growth factors have also been implicated in the generation of pathological pain states at both peripheral and CNS sites (Lewin and Mendell, 1993) and in the development and progression of diabetic
neuropathy (Anjaneyulu et al., 2008). In diabetic DRG and nerve, TGF-β isoforms applied directly in-vitro reduce neurite outgrowth, and this effect is partially reversed by TGF-β neutralizing antibody. These findings implicate upregulation of TGF-β in experimental diabetic peripheral neuropathy and indicate a novel mechanism of cellular injury related to elevated glucose levels (Anjaneyulu et al., 2008). Ethanol treatment is also known to cause an increase in expression of TGF-β1 and CYP2E1 in the centrilobular area of the liver and involves increased production of intracellular ROS and depletion of GSH which results in mitochondrial membrane damage and loss of membrane potential, followed by apoptosis (Zhuge and Cederbaum, 2006). Acetaldehyde, a toxic metabolite of ethanol, activated TGF-β signaling by stimulating the secretion and activation of latent TGF-β1, as well as by inducing the expression of the TGF-β -RII gene in cultured hepatic stellate cells (Chen, 2002). These findings correlate with the observation that cytokines (TNF-α, IL-1β and TGF-β1) are implicated in the initiation of neuropathic pain which further activates downstream signaling pathway including activation of p38 mitogen-activated protein kinase (MAPK), protein kinase C (PKC), c-Jun N-terminal kinase (JNK), and NF-κβ (Masamune et al., 2006). Dina et al. (2000) suggested the involvement of PKC in neuropathic pain induced by chronic alcohol consumption which was acutely attenuated by intradermal injection of nonselective PKC or selective PKC inhibitors injected at the site of nociceptive testing. Western immunoblot analysis indicated a higher level of PKC in dorsal root ganglia from alcohol-fed rats, supporting a role for enhanced PKC second messenger signaling in nociceptors contributing to alcohol-induced hyperalgesia. Miyoshi et al. (2007) reported that selective inhibitors of the mGlu5 receptor and PKC effectively reversed the induction of the neuropathic pain-like state by chronic ethanol consumption. PKC activity also contributes to neuropathic pain induced by cancer chemotherapy (Aley and Levine, 2002).

In the present study, a marked rise in the proinflammatory cytokines (TNF-α, IL-1β and TGF-β1) release was observed in ethanol fed rats. Chronic treatment with α-tocopherol, tocotrienol, epigallocatechin gallate, resveratrol and curcumin significantly and dose dependently inhibited production and
release of these cytokines both in serum and sciatic nerve of ethanol fed rats. These results are in line with the observation that ghrelin, a potent anti-inflammatory agent by inhibiting proinflammatory cytokines such as TNF-α, IL-1β and IL-6 ameliorated neuropathic pain (Guneli et al., 2007). Tocotrienols have been shown to inhibit inflammatory cytokines such as C-reactive protein, and IL-8 levels (Singh and Devaraj, 2007), TNF-α, and cytokine-induced neutrophil chemoattractant-1 mRNA levels (Hybertson et al., 2005). Previous findings from our laboratory also suggested inhibitory action of tocotrienol on proinflammatory cytokines (TNF-α and IL-1β) in diabetic neuropathy (Kuhad and Chopra, 2009a) and associated cognitive deficits (Kuhad et al., 2009) in rats. Very recently, tocotrienol rich fraction of palm oil was found to reduce extracellular matrix production by inhibiting transforming growth factor-β1 in human intestinal fibroblasts (Luna et al., 2011). Thus, it might be possible that both the isoforms of vitamin E exert the anti-nociceptive action by attenuating the increased oxidative stress and cytokine (TNF-α, IL-1β and TGF-β1) levels which may result in further inhibition of PKC, an important mediator of neuropathic pain. This assumption gets further strengthened by report from Sen et al. (2006) suggesting that at the posttranslational level, α-tocopherol inhibits PKC, 5-lipoxygenase and phospholipase A2. The more potent effects (behavioral and biochemical) of tocotrienol in the present study are in agreement with the previous findings from other research groups (Serbinova et al., 1991; Suzuki et al., 1993; Sen et al., 2000; Khanna et al., 2003). Tocotrienols are similar to tocopherols except that they have an isoprenoid tail with three unsaturation points instead of a saturated phytol tail. The unsaturated side chain of tocotrienol allows for more efficient penetration into tissues that have saturated fatty layers such as the brain and liver and may be attributed to more potent effects of tocotrienol (Suzuki et al., 1993).

Treatment with EGCG significantly reduced the cytokines (TNF-α, IL-1β and TGF-β1) both in serum and sciatic nerve of ethanol-administered rats and these findings are in accord with results from Yuan et al. (2006), who also found that 6-wk treatment with EGCG prevented alcohol-induced liver injury in rats by blunting the elevated expressions of TNF-α, COX-2 and iNOS. Administration of EGCG prevented diabetic nephropathy in rats by inhibiting
increased oxidative stress and protein expressions of COX-2, NF-κβ and TGF-β1 in the kidneys of diabetic animals (Yamabe et al., 2006). Chen et al. (2002) also reported that EGCG treatment markedly suppressed the activation of cultured hepatic stellate cell (HSC) by blocking transforming growth factor-beta signal transduction and by inhibiting the expression of alpha 1 collagen, fibronectin and alpha-smooth muscle actin genes. EGCG also suppressed ethanol-induced p38 mitogen-activated protein (MAP) kinase phosphorylation, alpha-smooth muscle actin production and activated transforming growth factor-beta1 (TGF-β1) secretion into the medium induced by acetaldehyde, the most toxic metabolite of ethanol (Asaumi et al., 2006).

There are several reports suggesting the inhibition of TGF-β by resveratrol as probable mode of protective action in various animal models. Chavez et al. (2008) found that treatment with resveratrol prevents fibrosis, NF-κβ activation and TGF-β increase induced by chronic carbon tetrachloride treatment in rats. In addition, resveratrol not only reduced mRNA expression of fibrosis-related genes such as TGF-β1, collagen type I and alpha-smooth muscle actin but also decrease hydroxyproline in rats with dimethylnitrosamine-induced liver fibrosis (Hong et al., 2010). In another study, by Bureau et al. (2008), treatment with resveratrol not only decreased TNF-α and IL-1β but also attenuated caspase-3 levels against lipopolysaccharide induced neuroinflammation in glial cells. It has also been reported that resveratrol inhibited PKC activity and therefore has an effect on associated signaling networks which may, in part, underlie the mechanism(s) by which this agent exerts neuroprotective properties (Bastianetto et al., 2009). Previous study from our laboratory also suggested that resveratrol treatment not only attenuated production of proinflammatory cytokines IL-1β, IL-6 and TNF-α but also augmented the secretion of anti-inflammatory cytokine IL-10 significantly (Sharma et al., 2007).

Curcumin exerts anti-inflammatory and growth inhibitory effects in TNF-α treated cells through inhibition of NF-κβ and mitogen activated protein kinase pathways (Cho et al., 2007). The findings are supported by previous studies conducted in our laboratory in which curcumin attenuated enhanced
proinflammatory cytokines (TNF-α, IL-1β) in various pathophysiological conditions (Bishnoi et al., 2008; Kuhad et al., 2009). A recent study by Awad and El-Sharif (2011) suggested that curcumin protects the kidneys and other vital organs against ischemia/reperfusion injury in rats by significantly inhibiting TGF-β and caspase-3 in kidney and lung tissues. Apart from this, curcumin also markedly inhibited the induction of nuclear factor-kappa β, transforming growth factor β-1 and interleukin-8 and produced gastroprotective effects against pylorus-ligation-induced gastric ulcer in rats (Mei et al., 2009). Rungseesantivanon et al. (2010) found that curcumin supplementation improved diabetes-induced endothelial dysfunction significantly by decreasing superoxide production and inhibiting PKC activity. Recent study from our laboratory suggested protective potential of curcumin in attenuating pain-depression dyad in reserpinized rats by inhibiting enhanced levels of TNF-α, IL-1β, NF-κβ and caspase-3 (Arora et al., 2011).

Thus, it is clear from the above discussion that chronic alcohol administration for 10 weeks in rats resulted in significantly decreased nociceptive threshold along with increased oxido-nitrosative stress and pro-inflammatory cytokines (TNF-α, IL-1β, TGF-β1) both in serum and sciatic nerve of rats and treatment with tocotrienol, epigallocatechin gallate, resveratrol and curcumin prevented all the behavioral, biochemical and molecular deficits in a dose-dependent manner.

Antioxidant property of tocotrienol, epigallocatechin gallate, resveratrol and curcumin may be responsible for protecting against the oxidative stress-mediated activation of PKC signaling, an important mediator of neuropathic pain, possibly by increasing the endogenous defense capacity to combat oxidative stress induced by chronic alcohol administration. In addition to potent antioxidant activity, the suppression of nitrosative stress and elevated cytokine (TNF-α, IL-1β, TGF-β1) levels both in serum and in sciatic nerve also contribute significantly in preventing alcoholic neuropathy in rats.

Therefore, the major finding of the study is that chronic treatment with tocotrienol, epigallocatechin gallate, resveratrol and curcumin significantly prevented thermal and mechanical hyperalgesia and tactile allodynia in rats.
chronically fed ethanol by attenuating oxidative-nitrosative stress and modulating neuroinflammatory signaling cascade and thus these interventions may find a place in therapeutic armamentarium for the treatment of patients suffering from alcoholic neuropathy.