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Aqueous extract of ginger ameliorated enzymic& non enzymic antioxidant markers in selected brain regions during ethanol withdrawal induced oxidative stress

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ABSTRACT

Alcohol withdrawal (AWD) is characterized by signs of major oxidative stress and the loss of neural cells. The present study was designed to investigate the role of the total aqueous extract from rhizomes of ginger on ethanol withdrawal related oxidative stress and related damage in brain regions of rat. α- Lipoic acid (ALA) (100 mg/kg b.w., i.p.) was used as a standard drug. Silymarin (100 mg/kg b.w., o.p.), a well known antioxidant was used for comparision. The ginger extract improved the level of protective antioxidants such as glutathione peroxidase (GPx), reduced glutathione (GSH), glutathione reductase (GRD), superoxide dismutase (SOD) and catalase (CAT) and inhibited XOD activity in the brain regions under study at a dose of 200 mg/kg, p.o. compared to silymarin or ALA or both combined. Moreover, a decline in the antioxidant enzyme level was observed during chronic ethanol administration too (20% ethanol @ mg/kg, p.o.). Interestingly, significant improvement was recorded with the supplementation of ginger extract by an improvement of the antioxidant enzyme status even in rats with chronic ethanol administration. In addition, a striking difference is observed in the decline in absorbance at 540 nm that reflects mitochondrial PTP opening of rats treated with ginger extract during chronic ethanol administration and ethanol withdrawal compared to rats that are subjected to the identical stress but without extract treatment. The current results indicate the possible utility of ginger rhizome in neuroprotection against neurodegenerative alcohol associated disorders such as ethanol withdrawal.

Keywords: Ginger; Alcohol Withdrawal; Oxidative Stress; Antioxidant System; Neurodegeneration

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INTRODUCTION

Oxidative stress due to an increase of free radical generation or impaired endogenous antioxidant mechanism, is an important factor that has been implicated as one of the reasons in the induced tissue damage in the alcoholics during ethanol withdrawal[1].It is scientifically proved that the augmentation of excitatory neurotransmission due to sudden withdrawal from chronic ethanol may lead to enhanced oxidative stress, which, in concert with reduced inhibitory neurotransmission, may contribute to the symptoms of ethanol withdrawal and associated neurotoxicity [1,2]. It is well established that free radicals are associated with a process that leads to cell degeneration, especially in the brain[3].

Brain metabolically consumes about 20% of the total oxygen consumption by the organism. This causes the accelerated generation of free radicals, especially in the presence of some xenobiotics, such as ethanol. It is suggested that ethanol intoxication could increase cellular redox-active iron, thus contributing to an enhanced steady-state concentration of reactive-free radicals. Catalysis by transition metals, especially iron, is involved in the biosynthesis of free radicals contributing to lipid peroxidation[4]. Ethanol is supposed to facilitate the release of iron ions from storage proteins and in that way it allows the Fenton reaction to occur [5]. This oxidative stress would lead to lipoperoxidative damage and cellular injury. Large amount of unsaturated lipids present in the brain are easy targets for free radicals because they provide readily removable hydrogens[6]. The resulting lipid radicals then rapidly react with molecular oxygen to form peroxyl radicals. This lipid peroxidation (LPO) initiates a series of complex, autocatalytic propagation reactions that generate a variety of thiobarbituric acid reactive substances (TBARs) such as malondialdehyde (MDA), as their end products[7]. Like free radicals, MDA and related carbonyl compounds are highly reactive and could enhance or extend the cellular damage caused by localized radical reactions. On the protein level, they give rise to protein carbonyls[8, 9], further disrupting normal functions of the organism.

Added to this, the content of both exogenous and endogenous antioxidants in the central nervous system (CNS) is very small in comparison with that of other tissues, which in relation to the high level of polyunsaturated fatty acids (PUFAs) makes the CNS exceptionally susceptible to free-radical damage. The antioxidant enzymes superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GSH-Px), and glutathione reductase (GSSG-R) present in the CNS i.e. in the cortex, cerebellum, hypothalamus, striatum, and spinal cord, where they are responsible for the brain's both physical and cognitive functions. Excessive and uncontrolled production of

superoxide and other reactive oxygen species (ROS) can result in cell injury and death[10]. Because mitochondrial respiration generates superoxide anion radical, cells with greater amounts of active mitochondria, such as neurons and myocytes, produce more superoxide than non-excitable cells[10].

However, the consumption of foods rich in antioxidant phytochemicals may help to fight degenerative diseases caused by oxidative stress through an improvement of the body's antioxidant status. The antioxidative capabilities of the CNS also depend on exogenous antioxidants which are provided to the organism during food intake and can be improved by dietary supplementation. A number of medicinal plants are reported to possess ROS scavenging and cytoprotective activity[11-14].Ginger (*Zingiberofficinale*) has been used in the Indian traditional system of medicine for digestive disorders, common cold, and rheumatism[15].Ginger has been shown to posses many pharmacological and physiological activities such as antioxidants, anti-inflammatory, analgesic, anti-carcinogenic, and cardiotonic effects[16]. In this study, we evaluated the effect of a fresh ginger extract in ethanol withdrawal induced neurotoxcity in selected brain regions of male wistar rats. Also, comparisions are made under similar conditions with simultaneous maintenance of groups supplemented with silymarin as well as α-lipoic acid.

MATERIAL AND METHODS

Plant material and extraction:

Aqueous ginger extract was prepared from locally available ginger roots. Ginger rhizomes were purchased fresh from the local market of Tirupati and were authenticated by staff in the Department of Botany at Sri Venkateswara University, Tirupati in India. Whole rhizome of ginger was thoroughly washed, sliced, grated, and ground to a fine paste. A weighed quantity (30gm) of the paste was subjected to continuous hot extraction in a soxhlet apparatus using double distilled water. The extract was evaporated under reduced pressure using a rotary evaporator and then lyophilized until all of the solvent was removed. This aqueous ginger extract (AGE) was stored at 4°C.

Chemicals:

Silyamrinand *alpha*- Lipoic acid were purchased from *Sigma*-Aldrich Co. LLC. All other reagents used were of analytical grade.

Animals

The study involved young (2–3 months old; 200 - 220g) Wistar strain male albino rats purchased from Sri Venkateswara Traders Pvt. Limited, Bangalore and were maintained in the animal house

of the department in polypropylene cages. Standard conditions of humidity (50% relative humidity), room temperature (25 - 28ºC) and 12 h light/dark cycle (6:00 A.M. to 6:00 P.M.) were maintained. A standard rodent diet (M/s Hindustan Lever Ltd., Mumbai), and water was provided *ad libitum*. All experimental procedures were approved by the CPCSEA on Animal Care, Govt. of India, bearing the CPCSEA No. 438 / 01/a / CPCSEA / IAEC / SVU / KSR-1 (dt: 11.09.2008).

Experimental design: The rats were randomly grouped into six (n = 6) and treated as follows:

I. Control received normal saline daily (p.o.),

II. The Ethanol group of rats were treated as following (n=6)

i. Ethanol group-Group that received 20% Ethanol for 6 weeks (orally)

ii. Ethanol + Extract treated group -Group that received 20% Ethanol for 6 weeks and received 200 mg/kg body weight of ginger extract (orally) simultaneously daily

iii. Ethanol + Silymarin treated group-Group that received 20% Ethanol for 6 weeks and received 100 mg/kg body weight of silymarin (orally) simultaneously daily

iv. Ethanol + Lipoic Acid treated group-Group that received 20% Ethanol for 6 weeks and received 100 mg/kg body weight of Lipoc Acid (intra peritoneal (i.p.)) simultaneously daily.

v. Also maintained are ethanol(EtOH) groups that are given combined combinations *viz.*, α-lipoic acid + silymarin; α-lipoic acid+ ginger extract, ginger extract+silymarin at the same doses described above.

III. The Ethanol Withdrawal group of rats were treated as following (n=6)

i. Ethanol Withdrawal group-Group that received 20% Ethanol for 6 weeks and subjected to ethanol withdrawal for 72hrs

ii. Ethanol Withdrawal + Extract treated group -Group that received 20% Ethanol for 6 weeks and subjected to ethanol withdrawal for 72 hrs and received 200 mg/kg body weight of ginger extract (orally) simultaneously daily

iii. Ethanol Withdrawal + Silymarin treated group-Group that received 20% Ethanol for 6 weeks and subjected to ethanol withdrawal for 72 hrs and received 100 mg/kg body weight of silymarin (orally) simultaneously daily

iv. Ethanol Withdrawal + Lipoic Acid treated group-Group that received 20% Ethanol for 6 weeks and subjected to ethanol withdrawal for 72 hrs and received 100 mg/kg body weight of Lipoic Acid (intra peritoneal (i.p.) simultaneously daily.

v. Also maintained are ethanol withdrawal (EW) groups that are given combined combinations of α-lipoic acid + silymarin; lipoic acid+ ginger extract, ginger extract + silymarin at the same doses described above.

Tissue collection

After the period of last dose of ethanol treatment, the animals were sacrificed exactly after 72 hrs by cervical dislocation. Brain tissue was dissected , washed with ice-cold saline, blotted, dry freezed in liquid nitrogen and immediately transferredto the ice chamber at -20°C. Different regions of Brain namely CC, CB, HC and PM were isolated., washed with ice-cold saline, blotted, dry freezed in liquid nitrogen and immediately transferred to the ice chamber at -20°C.

Preparation of rat brain mitochondria

Brain mitochondria were isolated from whole brain homogenized in isolation buffer (in mM: 250 sucrose, 10 Tris·HCl, pH 7.4, and 0.5 K^+ EDTA). The homogenate was centrifuged at 1,000 *g* for 5 min. The supernatant was strained through gauze and recentrifuged at 7,000 *g* for 10 min. The resulting pellet was resuspended in ice-cold isolation buffer, and a new series of centrifugations (1,000 and 7,000 *g*) was performed. The crude mitochondrial pellet was resuspended in a final volume of 10 ml in 3% Ficoll medium (3% Ficoll, 250 mM sucrose, 10 mMTris·HCl, pH 7.4, and 0.5 mM K^+ EDTA). This suspension was carefully layered onto 20 ml of 6% Ficoll medium (6% Ficoll, 250 mM sucrose, 10 mMTris·HCl, pH 7.4, and 0.5 mM K^+ EDTA) and centrifuged for 30 min at 11,500 *g*. The mitochondrial pellet was resuspended in isolation medium and centrifuged for 10 min at 12,500 *g*. The mitochondria were made up to a concentration of ~50 mg protein/ml in the isolation buffer[17].

The purity of mitochondria was assessed by the assay of specific marker enzyme; succinate dehydrogenase was assayed by the method of Slater and Bonner, 1952[18]. Mitochondrial protein was estimated by the method of Lowry et al.,1951[19].

BIOCHEMICAL ASSAYS

Assessment of mitochondrial membrane swelling

The resulting mitochondrial pellet was used for the assay of mitochondrial swelling and rupture as a marker of PTP opening[20].Mitochondrial membrane swelling was assessed by suspending mitochondria in medium containing phosphate, which induces swelling and rupture more rapidly in vulnerable mitochondrial membranes than healthy membranes[21]. Mitochondria (0.25 mg protein) were suspended in medium containing 250 mM sucrose, 10 mMTris-MOPS, 0.05 mM EGTA, 5 mM pyruvate, 5 mM malate, and 1 mM phosphate (pH 7.4), and absorbance by this

suspension was measured at 540 nm in a Beckman DU 640 spectrophotometer. Intact mitochondria scatter light at 540-nm wavelength; mitochondrial swelling and rupture due to prolonged or excessive mitochondrial PTP opening reduces mitochondrial light scattering and, thus, absorbance[22.]The time required for absorbance to fall to the midpoint between initial and final values was determined to assess how readily PTP open indifferent treatment groups.

Antioxidant profile

Enzymic Antioxidants

Superoxide dismutase (SOD) activity is presented as units per milligram of protein (U mg−1 protein). The activity of superoxide dismutase was assayed spectrophotometrically by the method of Misra and Fridovich[23]. Superoxide dismutase uses the photochemical reduction of riboflavin as oxygen generating system and catalyses the inhibition of Nitrobluetetrazolium (NBT) reduction, the extent of which can be assayed spectrophotometrically at 600nm. Catalase (CAT) activity in the supernatant was measured by recording the rate of decrease in H_2O_2 absorbance at 240 nm[24].The activity of catalase was expressed as µmol H_2O_2/min/mg protein.

Glutathione Peroxidase (GPx) was determined using butylhydroperoxide as a substrate. The optical density was spectrophotometrically recorded at 340 nm. One unit of the enzyme was defined as micromoles (µmol) of reduced nicotinamide adenine dinucleotide phosphate (NADPH) oxidized per minute[25]. The GPx activity was expressed as U/mg protein.

Glutathione Reductase

(GR) was estimated by the method given by Carlberg and Mannervik, 1985 [26]. Then, 2.5 ml buffer, 0.2 ml NADPH, 0.2 ml GSSG, and 0.1 ml supernatant (S) were mixed and allowed to stand for 30s. Absorbance was recorded at 340 nm for 3 min at 30 sec intervals. GR was calculated in terms of nmol/min/mg protein.

Glutathione-S-Transferase

(GST) was estimated as per a method of Habig*et al*., 1974[27]. The reaction mixture consisting of 1.425 ml phosphate buffer (1 M, pH 6.5), 0.2 ml GSH (1.0 mM), 0.25 ml 1-chloro-2,4-dinitrobenzene (CDNB, 1 mM), 20 µl supernatant (S) and 60 µl water was mixed to give a total volume of 3.0 ml. Absorbance was recorded at 340 nm, and the enzyme activity was calculated as nmol CDNB conjugate formed/min/mg protein using a molar extinction coefficient of $9.6 \times 10^3 M^{-1}$ cm^{-1}.

Non enzymic Antioxidants

Vitamin E (α-tocopherol) was determined by the method of Desai, 1984[28]. By addition of 1.6 ml ethanol and 2.0 ml petroleum ether to 0.5 ml sample and centrifuged. The supernatant was

separated and evaporated. To the residue, 0.2 ml of 0.2% 2,2- dipyridyl, 0.2 ml of 0.5% ferric chloride was added and kept in dark for 5 minutes. An intense red coloured layer obtained on addition of 4 ml butanol was read at 520 nm. And is expressed as mg/gm wet wt.

Vitamin C was measured according to Benderitter et al.,1998[29] which is based on the oxidation of ascorbic acid to dehydroascorbic acid by tricholoracetic acid. The dehydroascorbic acid in acidic solution react with 2,4-dinitrophenyl hydrazine forming corresponding hydrazone. The hydrazone when treated with sulfuric acid develop orange-red colour which is measured sphectrophotometrically at 520nm and is expressed as mg/gm wet wt.

Reduced glutathione (GSH) was estimated by the method described by Ellman[30]. Part of the crude homogenate was centrifuged at 9000 rpm for 20 min to obtain the supernatant. 1ml of the sample was mixed with 1 ml of 5% TCA (w/v), the mixture was allowed to stand for 30 min and centrifuged at 2500 rpm for 15 min. 0.5 ml of the supernatant was taken and 2.5 ml of 5'5 '-dithionitrobenzoic acid (DTNB) was added, mixed thoroughly and absorbance was recorded at 412 nm. The results were expressed as µmol/g tissue.

Oxidised glutathione (GSSG) was determined according to Klotzsch and Bergmeyer[31] using 0.1 mM NADPH and 0.4 U/mL GR in 0.2 mM phosphate buffer, pH 7.4. The GSSG content was calculated as nmol per mg protein.

Xanthine Oxidase (XOD) (EC 1.1.3.22) activity was determined by using xanthine as substrate, and by following the rate of reduction of nitrobluetetrazolium at 560 nm[32]. One unit of XOD was defined as the amount of enzyme that produces 1 nmol of uric acid per minute.

Statistical analysis

All the experiments were performed in triplicate and the values were expressed as percentage change. The statistical analysis was done by one-way ANOVA followed by a tukey post hoc comparison test. In all calculations, a difference at $p<0.01$was regarded as significant.

RESULTS AND DISCUSSION

Mitochondrial membrane swelling.

The effects of ethanol exposure, EW, and ginger extract treatment on mitochondrial membrane swelling, an indication of PTP opening, were assessed by measuring light scattering by mitochondrial membranes. In general, light scattering declined most rapidly in mitochondria from brains of untreated EW rats (Figure. 1). One way-factor ANOVA revealed significant treatment-dependent [F(5, 10)179, P <0.01]and brain region-dependent [F(2, 10)7.8,P <0.01]differences in time to 50% absorbance change. In addition, there was as significant

interaction between treatment and brain region F(10, 75)12, P <0.01], suggesting that different brain regions are not equally vulnerable to PTP opening under specific treatment conditions. Indeed, post hoc Tukey test revealed that cerebellar mitochondria were more vulnerable to swelling and rupture during EW than were cortical and hippocampal mitochondria. In cortical mitochondria, rates of swelling and rupture during ethanol exposure and EW did not differ significantly. Medullary mitochondria were the least affected by ethanol exposure or EW (Figure. 1). Ginger extract produced significant protection only in cerebellar mitochondria during EW (P<0.01); however, there was a tendency to protection in the cortical and hippocampal regions. Collectively, these results indicate that EW increases vulnerability of brain mitochondrial membranes and AGE minimizes this effect of EW.

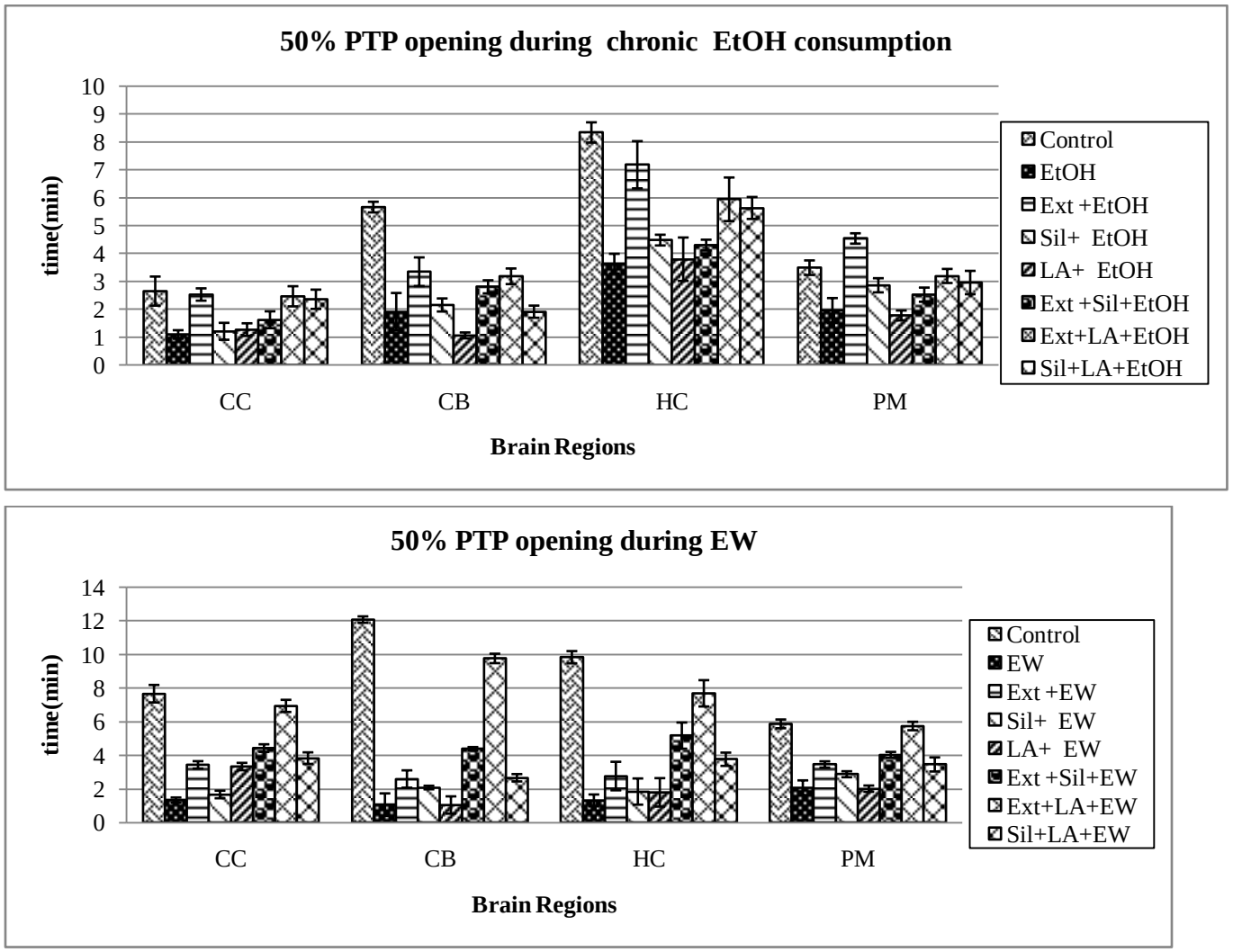


Figure1: Assessment of permeability transition pore (PTP) opening in the Mitochondria of brain regions of rats (n=6), treated with aqueous ginger extract (AGE) and with Silymarin and ALA during chronic ethanol ingestion and ethanol withdrawal induced oxidative stress

Superoxide dismutase (SOD)

The effect of AGE on SOD content in different brain regions is shown in Figure 2. SOD level in normal group (92.55 units /min /mg protein) was measured to be higher than in ethanol group (45.5 units /min /mg protein). SOD level of AGE 200 mg/kgb.w(o.p.) (52.9 and 62.9 units /min /mg protein, $P < 0.01$) were increased as compare to withdrawal and ethanol groups. SOD level of extract treated groups (59.2 and 71.9 units /min /mg protein, $P < 0.01$) were increased as compare to withdrawal group. Combination of silymarin(100 mg/kg bw, o.p.) and ALA 100 mg/kgb.w. (i.p.)along with ginger extract also restored the SOD level in withdrawal and ethanol groups to the normal level.

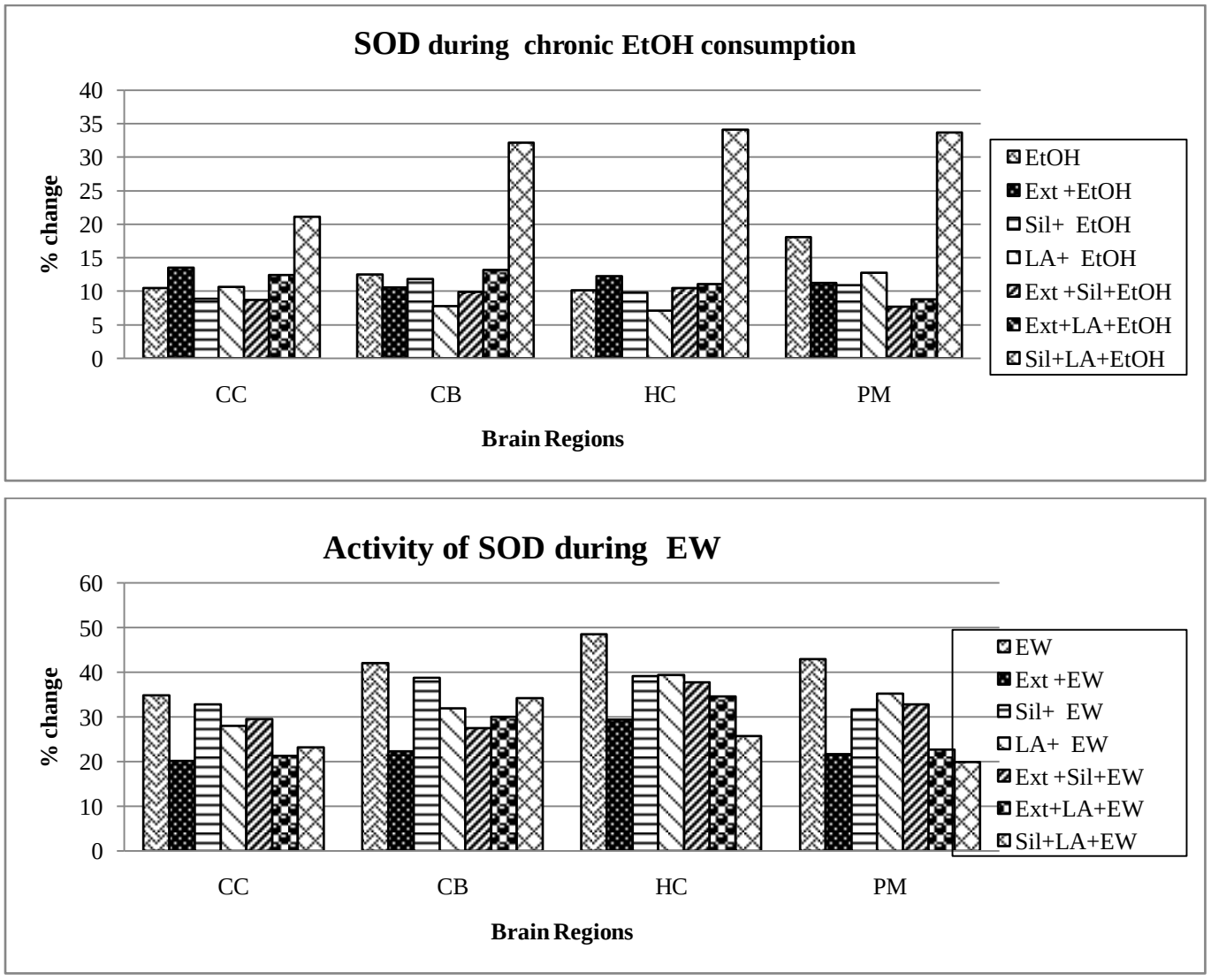


Figure 2: Activity of Superoxide Dismutase (SOD) in the brain regions of rats (n=6), treated with aqueous ginger extract (AGE) and with Silymarin and ALA during chronic ethanol ingestion and ethanol withdrawal induced oxidative stress

Catalase (CAT)

The effect of AGE on catalase activityin different brain regions is shown in Figure 3. CAT level in normal group (185.4 nmol of H_2O_2 decomposed/min/mg protein) was measured to be higher than ethanol group (53.15 nmol of H_2O_2 decomposed/min/mg protein). CAT level of AGE 200 mg/kg bw (o.p.) (70.46 and 100.9 nmol of H_2O_2 decomposed/min/mg protein, $P< 0.01$) were increased as compare to withdrawal and ethanol groups. CAT level of AGE 200 mg/kg (o.p.) groups (87.6 and 120.6 nmol of H_2O_2 decomposed/min/mg protein, $P < 0.01$) were increased as compare to withdrawal group Combination of silymarin100 mg/kg bw, (o.p.) and ALA 100 mg/kgb.w(i.p.)along with ginger extract also restored the CAT activity in withdrawal and ethanol groups to the normal level.

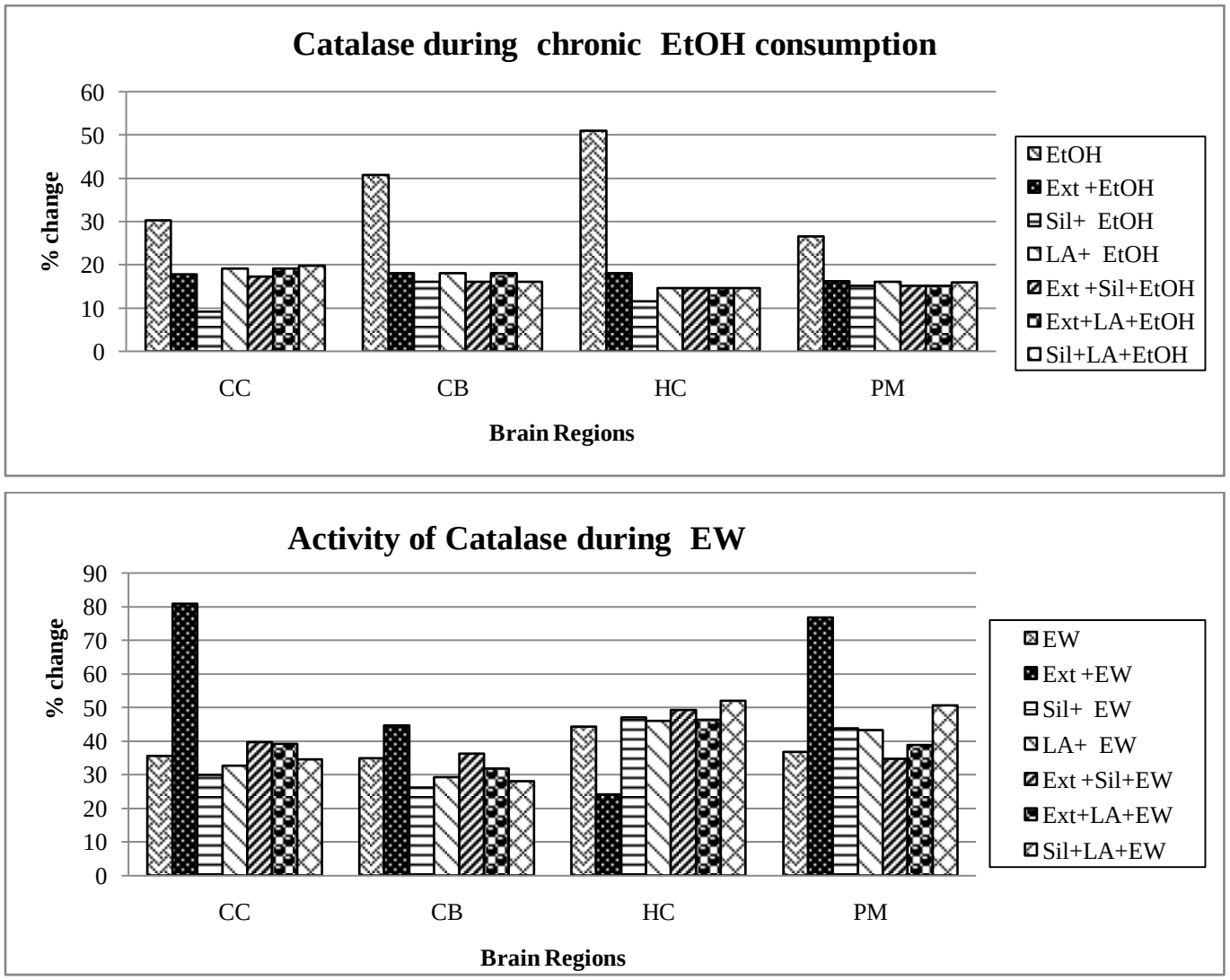


Figure 3: Activity of Catalase (CAT) in the brain regions of rats (n=6), treated with aqueous ginger extract (AGE) and with Silymarin and ALA during chronic ethanol ingestion and ethanol withdrawal induced oxidative stress

Glutathione peroxidase (GP_X)

The effect of AGE on GP_X content in different brain regions is shown in Figure 4. GP_Xlevel in normal group (297.18 nmol of GSH oxidized/min/mg protein) was measured to be higher than in withdrawal group (180.1 nmol of GSH oxidized/min/mg protein). GP_X level of AGE 200 mg/kg (o.p.) withdrawal and ethanol groups (218.47 and 236.35 nmol of GSH oxidized/min/mg protein, $P < 0.01$) were increased as compare to respective extract untreated groups. GP_X level of extract treated groups (227.02 and 252.42 n mol of GSH oxidized/min/mg protein, $P < 0.01$) were increased as compare to withdrawal and ethanol groups. Combination of silymarin100 mg/kg bw, (o.p.) and ALA 100 mg/kgb.w. (i.p.)along with ginger extract also restored the GP_X level in withdrawal and ethanol groups to the normal level.

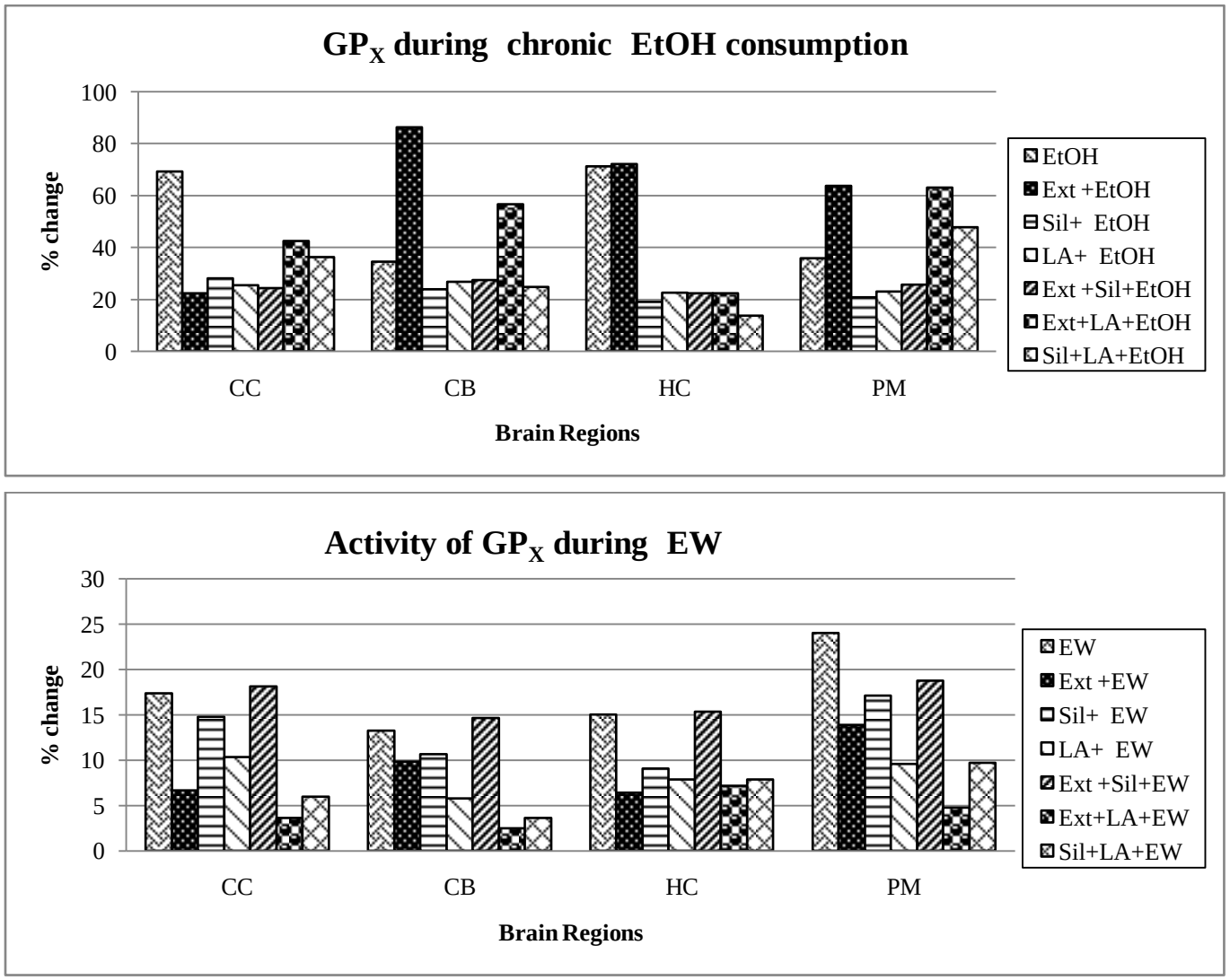


Figure 4: Activity of Glutathione peroxidase (GP_X) in the brain regions of rats (n=6), treated with aqueous ginger extract (AGE) and with Silymarin and ALA during chronic ethanol ingestion and ethanol withdrawal induced oxidative stress

Glutathione reductase (GR)

The effect of AGE on GR content in different brain regions is shown in Figure 5. GR level in normal group (27.2 nmol of GSSG utilized /min/mg protein) was measured to be higher than in ethanol group (10.3 nmol of GSSG utilized /min/mg protein). GR level of AGE 200 mg/kg bw (o.p.) treated withdrawal and ethanol groups (16.21 and 18.08 nmol of GSSG utilized /min/mg protein, $P < 0.01$) were increased as compare to withdrawal group. GR level of extract treated groups (17.15 and 20.5 nmol of GSSG utilized /min/mg protein, $P < 0.01$) were increased as withdrawal and ethanol groups. Combination of silymarin100 mg/kg bw, (o.p.) and ALA 100 mg/kgb.w(i.p.)along with ginger extract also restored the GR level in withdrawal and ethanol groups to the normal level.

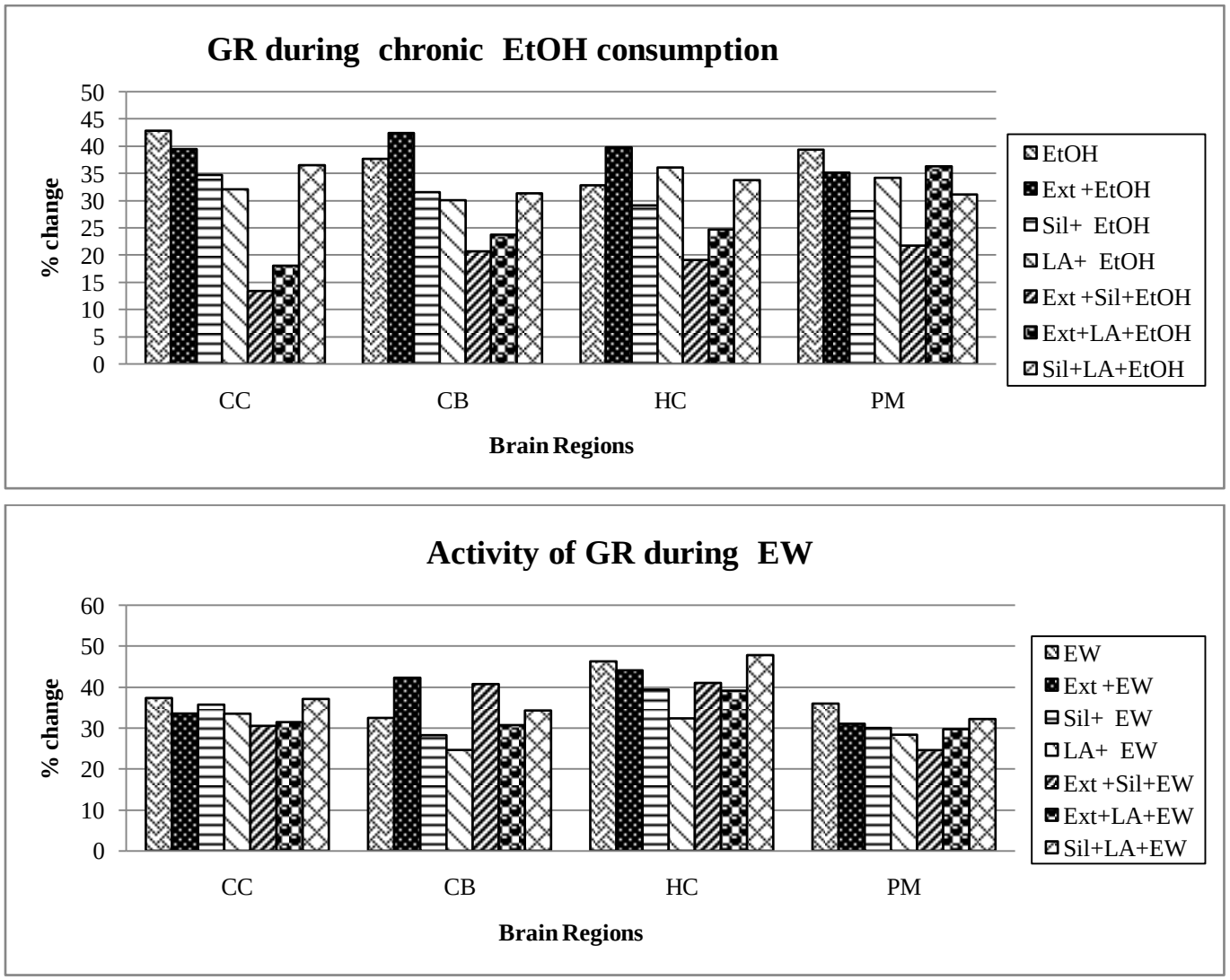


Figure 5: Activity of Glutathione Reductase(GR) in the brain regions of rats (n=6), treated with aqueous ginger extract (AGE) and with Silymarin and ALA during chronic ethanol ingestion and ethanol withdrawal induced oxidative stress

Glutathione transferase (GST)

The effect of AGE on GST content in different brain regions is shown in Figure 6. GST level in normal group (280.06 nmol of CDNB conjugate formed/min/mg protein) was measured to be higher than in withdrawal group (154.01 nmol of CDNB conjugate formed/min/mg protein). GST level of AGE 200 mg/kg bw (o.p.) withdrawal and ethanol groups (165.6 and 207.1 nmol of CDNB conjugate formed/min/mg protein, $P < 0.01$) were increased as compare to withdrawal group. GST level of extract treated groups (180.13 and 238.06 nmol of CDNB conjugate formed/min/mg protein, $P < 0.01$) were increased by as compare to withdrawal and ethanol groups. Combination of silymarin100 mg/kg bw, (o.p.) and ALA 100 mg/kgb.w. (i.p.)along with ginger extract also restored the GST level in withdrawal and ethanol groups to the normal level.The levels of non enzymic antioxidants namely Vit E, Vit C, GSH, GSSG and XOD compared to control in extract untreated ethanol and withdrawal rats as well as ginger extract, silymarin, ALA treated rats are expressed as percent changes over control group(Figure 7,8,9, 10 and11). Also combination of the treatments are also verified and found to be statistically insignificant compared to control at $P < 0.01$.

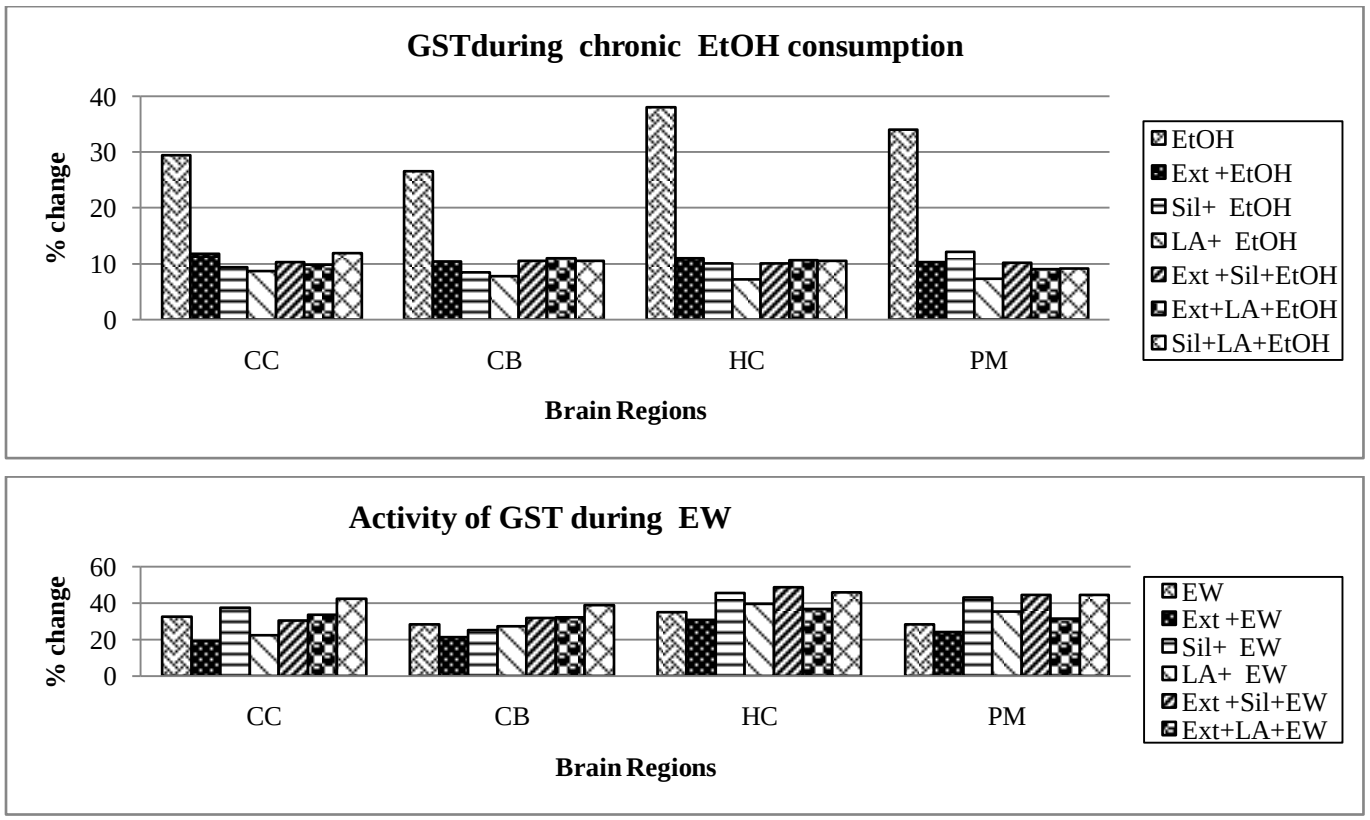
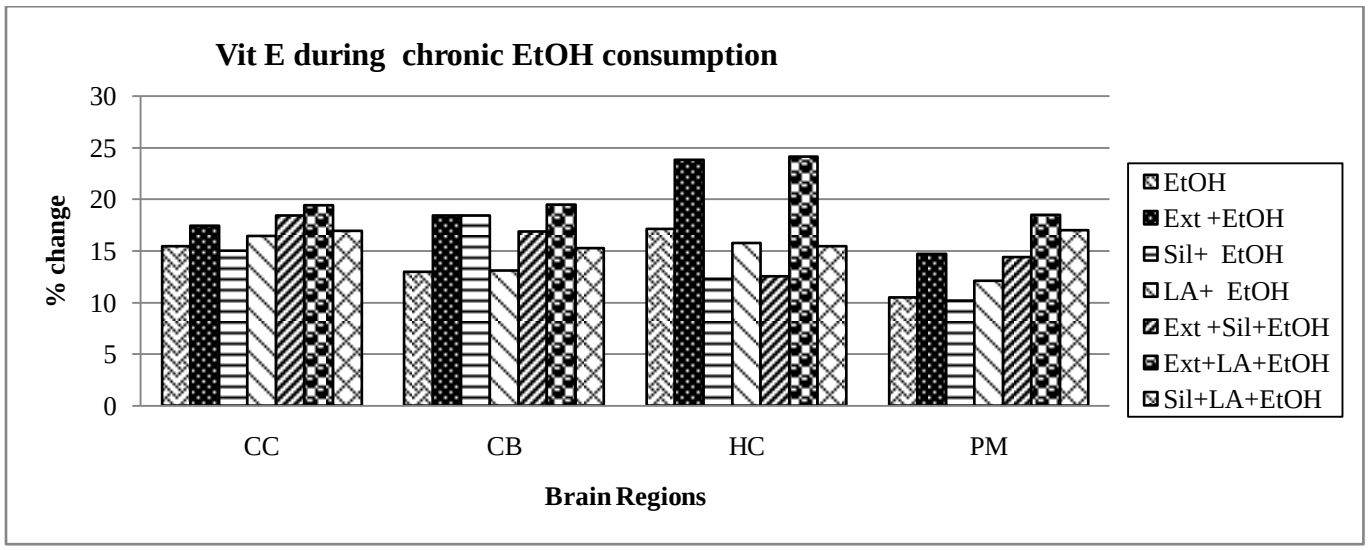


Figure 6: Activity of Glutathione- S- Transferase (GST) in the brain regions of rats (n=6), treated with aqueous ginger extract (AGE) and with Silymarin and ALA during chronic ethanol ingestion and ethanol withdrawal induced oxidative stress



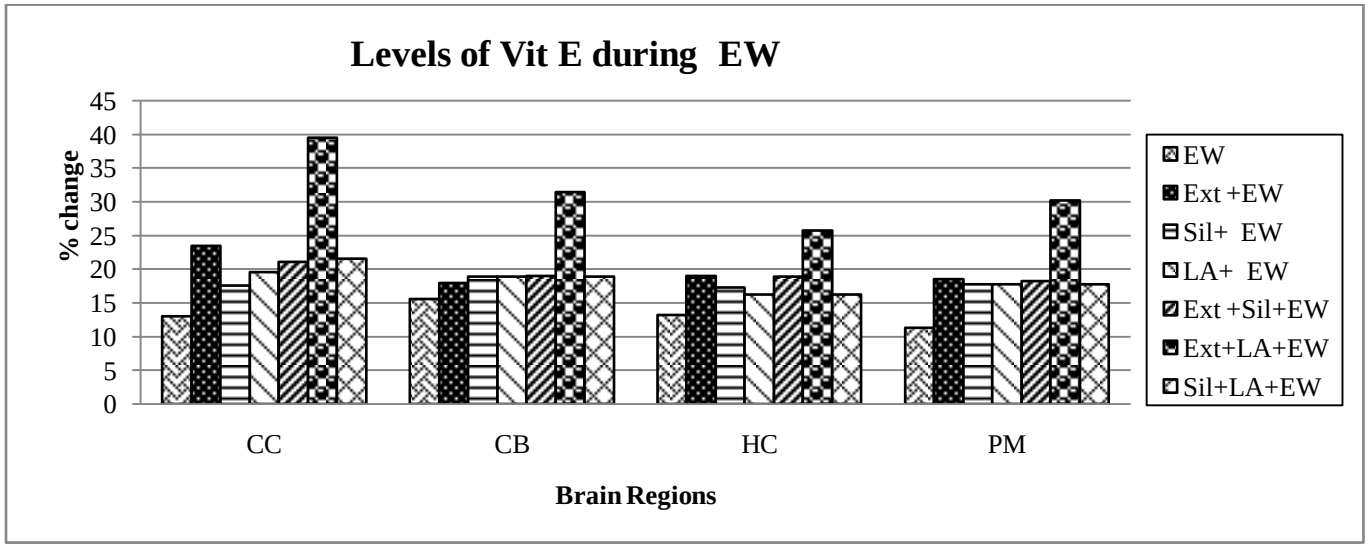
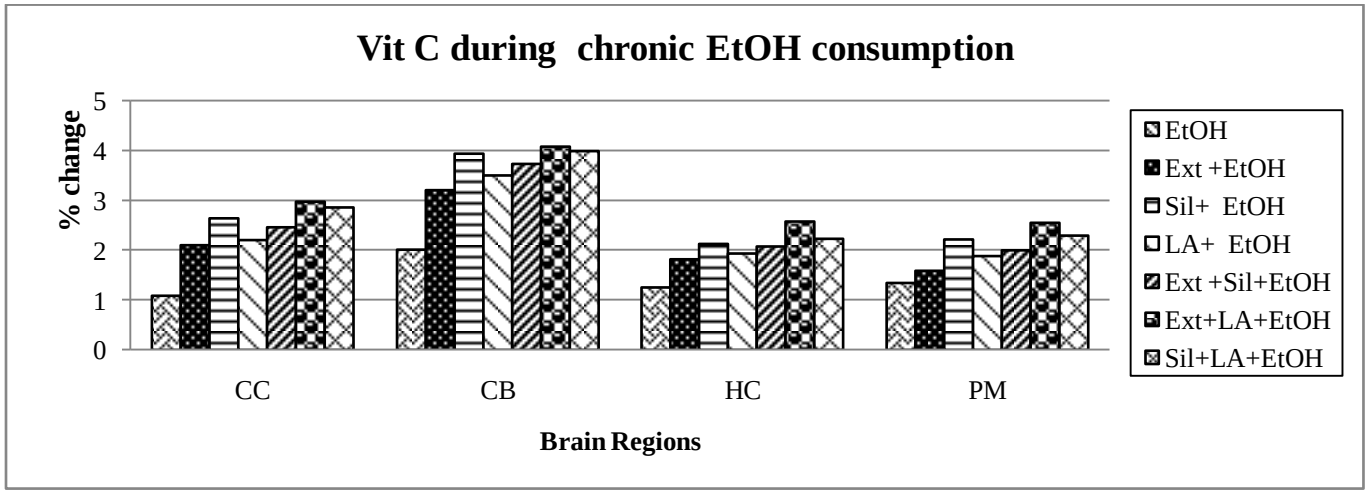


Figure 7: Levels of Vitamin E (Vit E) in the brain regions of rats (n=6), treated with aqueous ginger extract (AGE) and with Silymarin and ALA during chronic ethanol ingestion and ethanol withdrawal induced oxidative stress



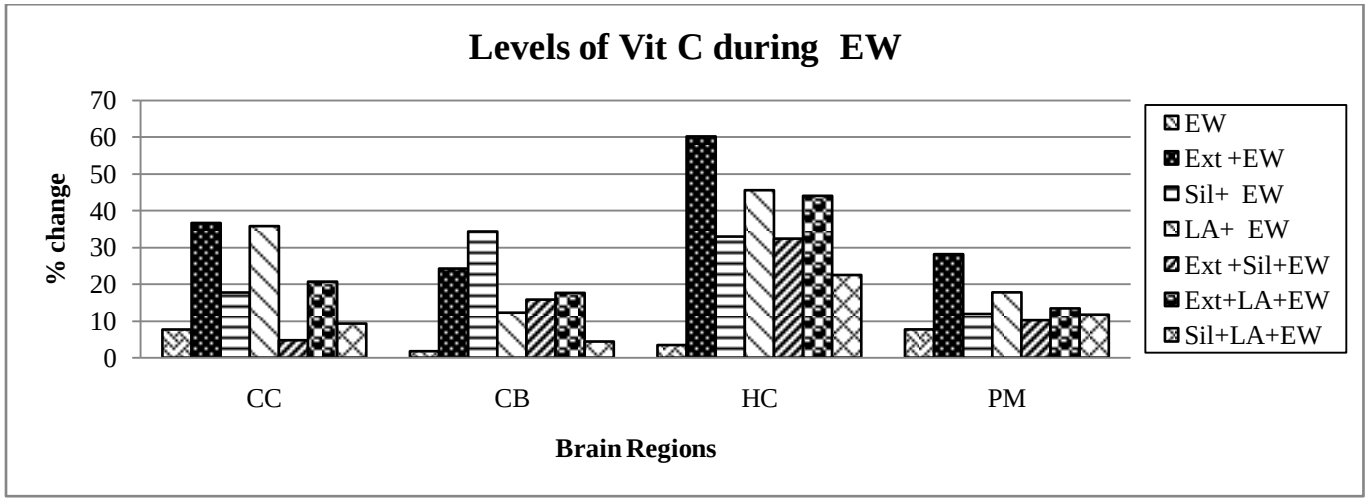


Figure 8: Levels of Vitamin C (Vit C) in the brain regions of rats (n=6), treated with aqueous ginger extract (AGE) and with Silymarin and ALA during chronic ethanol ingestion and ethanol withdrawal induced oxidative stress

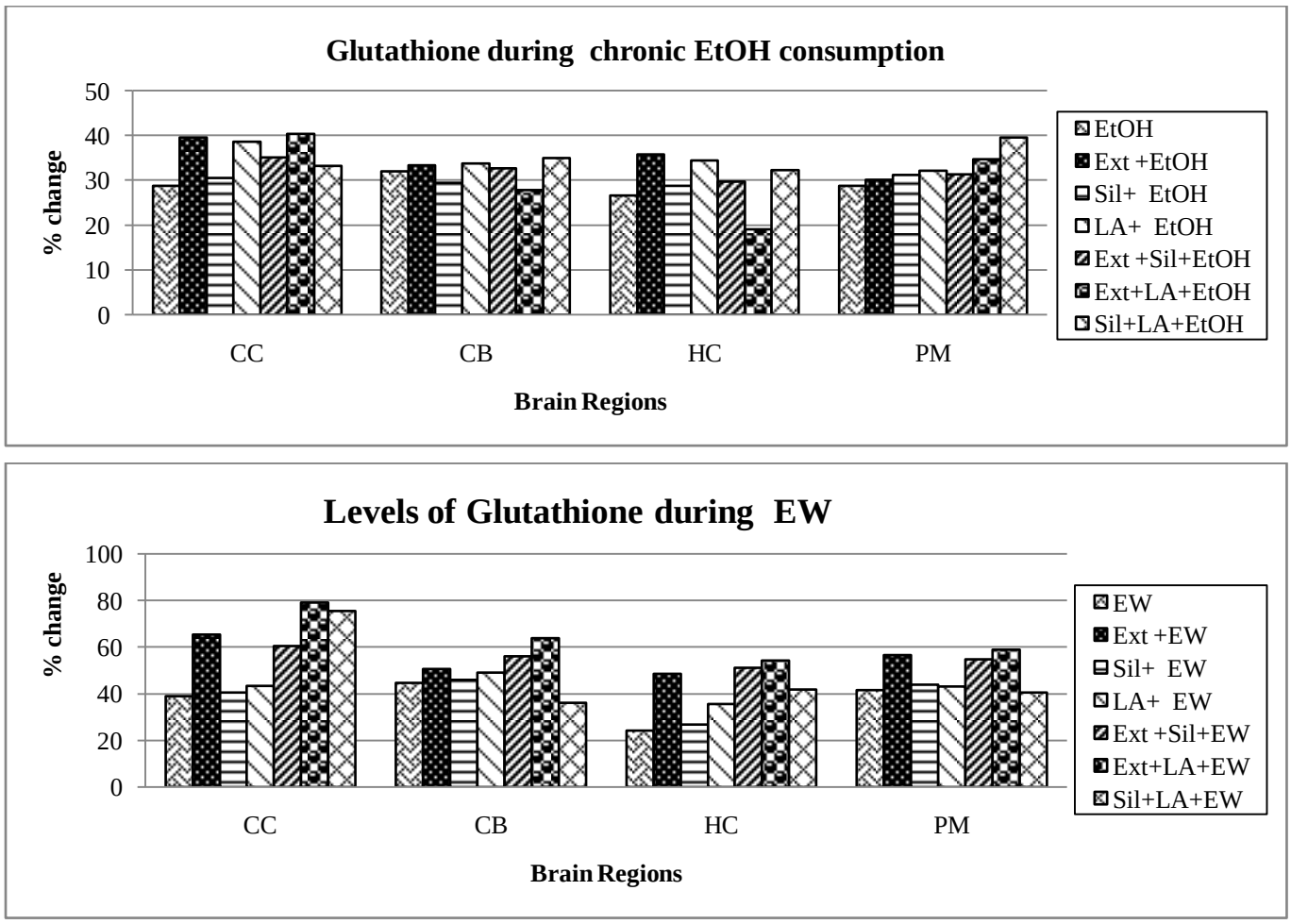
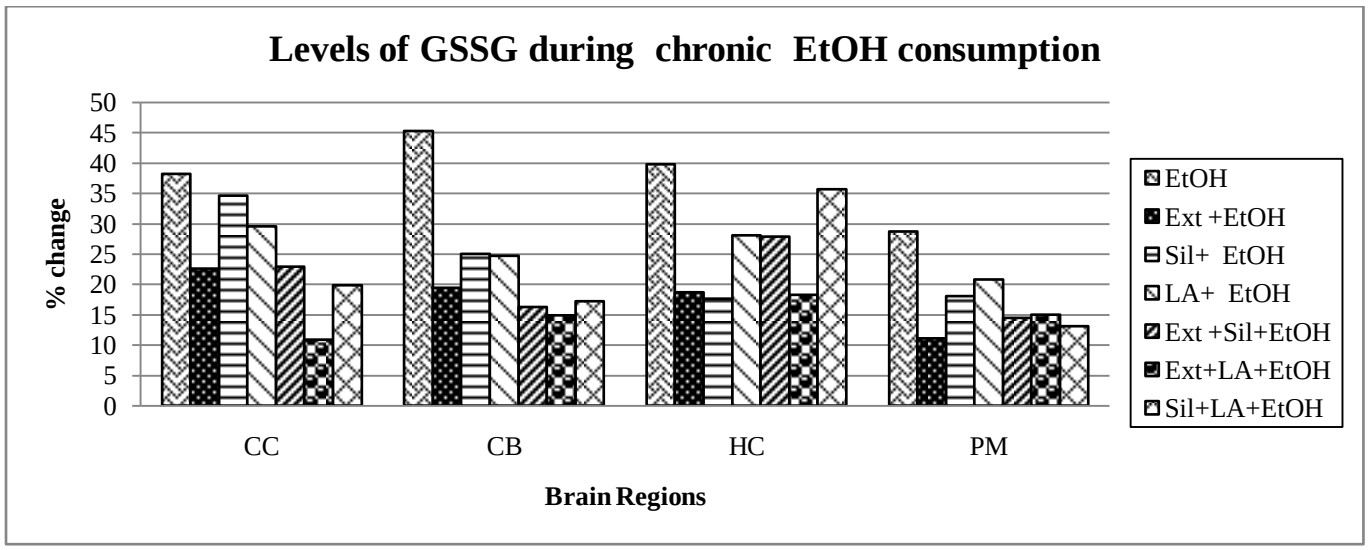


Figure 9: Levels of Glutathione (GSH) in the brain regions of rats (n=6), treated with aqueous ginger extract (AGE) and with Silymarin and ALA during chronic ethanol ingestion and ethanol withdrawal induced oxidative stress



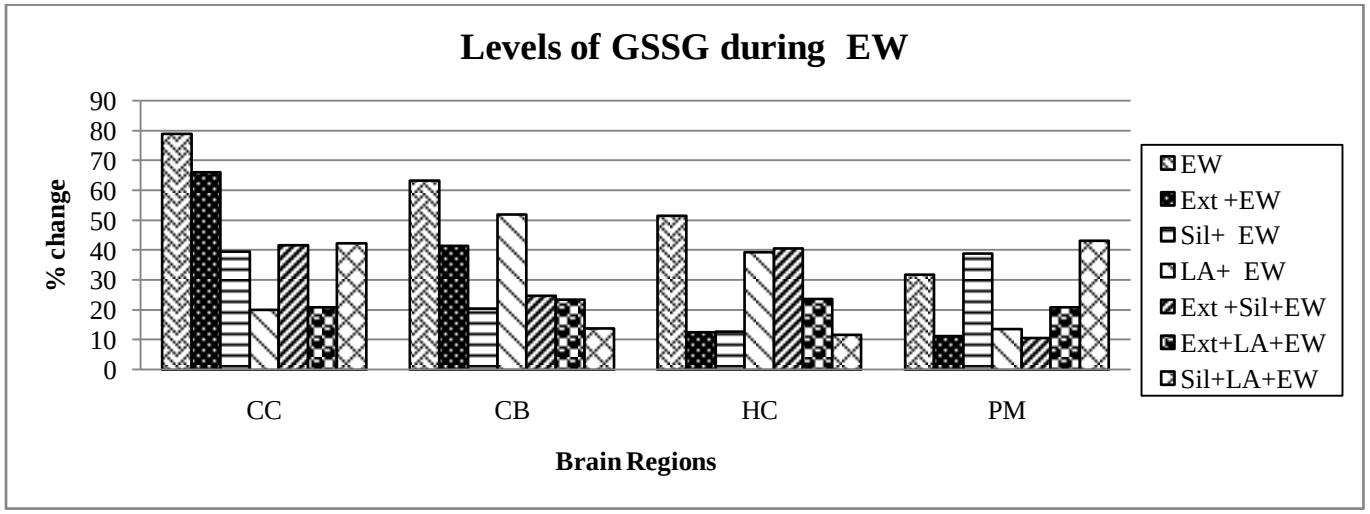
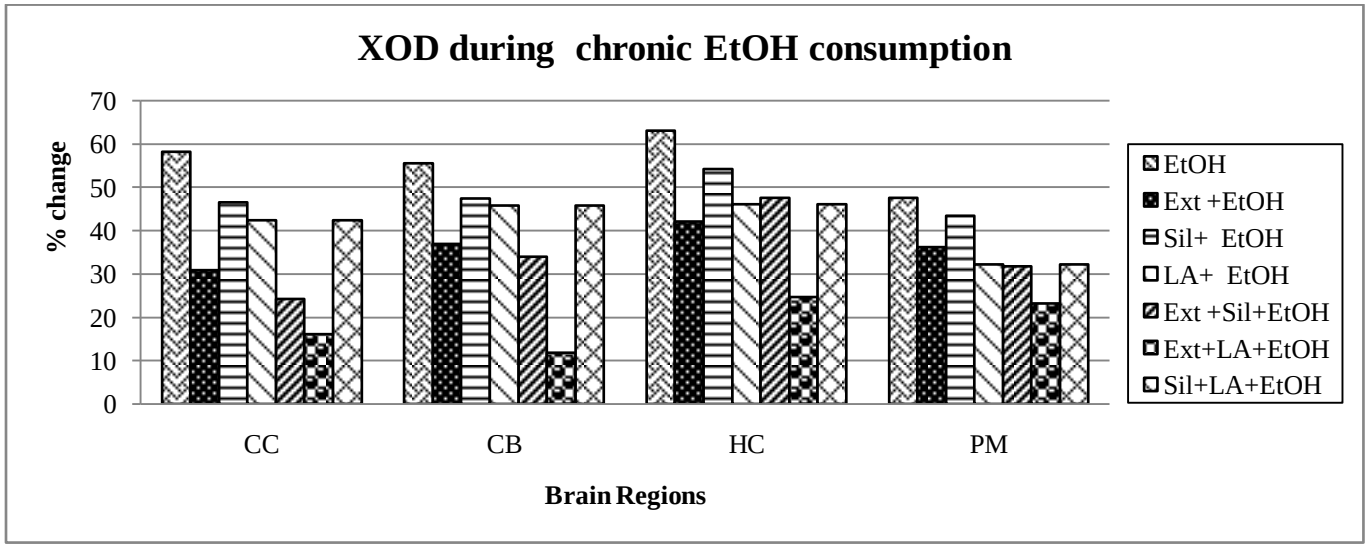


Figure 10: Levels of Oxidized Glutathione (GSSG) in the brain regions of rats (n=6), treated with aqueous ginger extract (AGE) and with Silymarin and ALA during chronic ethanol ingestion and ethanol withdrawal induced oxidative stress



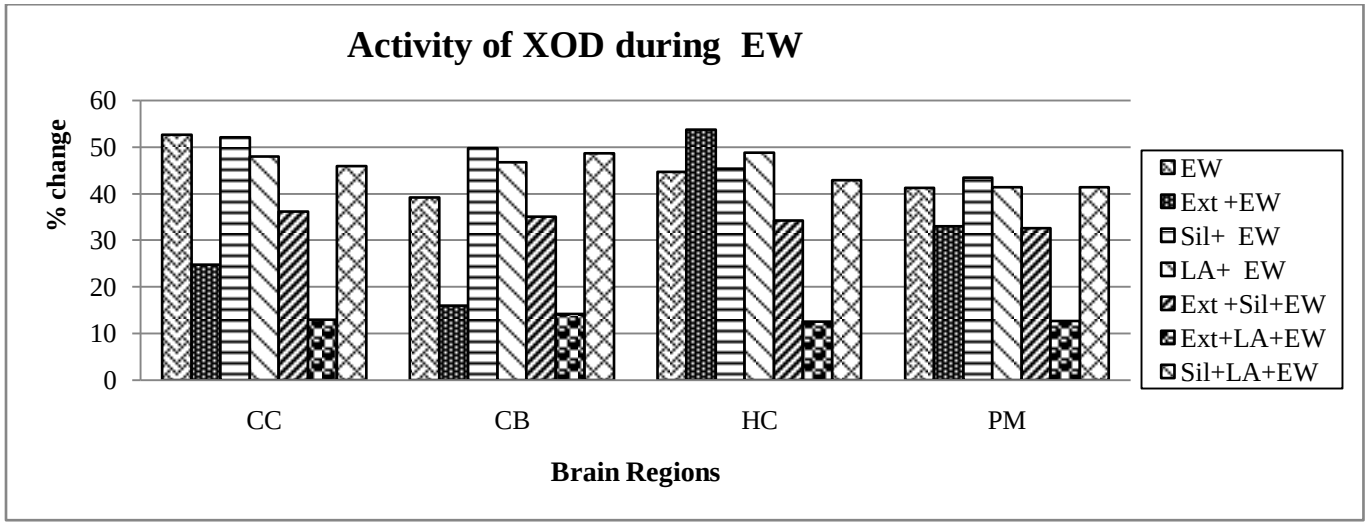


Figure 11: Activity of Xanthine Oxidase (XOD) in the brain regions of rats (n=6), treated with aqueous ginger extract (AGE) and with Silymarin and ALA during chronic ethanol ingestion and ethanol withdrawal induced oxidative stress

Ethanol Withdrawal caused oxidative stress in brain areas vulnerable to ethanol, such as cerebellum and cortex [33,34]. During EW, excessive glutamate-induced neuronal excitation increases intracellular concentrations of Ca^{+2} and reactive oxygen species (ROS), factors that provoke PTP opening [22, 35-40]. While ethanol itself is pro-oxidant because it directly generates reactive oxygen species during its metabolism[41], not only chronic ethanol exposure, but also abrupt withdrawal can produce significant neurotoxicity due to oxidative stress [42-44]. Ethanol withdrawal (EW) produces oxidative stress indirectly through the activation of excitatory neurotransmitter receptors and the concomitant alteration of intracellular calcium levels [45]. It has been shown that sudden EW results in an increase in glutamate levels/glutamate receptor activity and a compensatory up-regulation of *N*-methyl-D-aspartate (NMDA) receptors [1,46]as well as in a decrease in GABA levels/GABA-receptor activity[47,48]. These events will eventually lead to oxidative stress. Therefore, free-radical scavenging antioxidants may be beneficial to alleviate oxidative stress-initiated neuronal cell death during EW.

Under normal physiological conditions, the mitochondrial inner membrane is impermeable to all but a few, selected metabolites and ions. However, under conditions of stress, a nonspecific pore known as the mitochondrial permeability transition pore can open in the mitochondrial inner membrane that allows free passage of any molecule of <1.5 kDa[49-51]. When the MPTP opens, the permeability barrier of the inner membrane becomes disrupted with two major consequences. First, although all small molecular weight solutes move freely across the membrane, proteins do not and, as a result, they exert a colloidal osmotic pressure that causes mitochondria to swell.

The unfolding of the cristae allows the matrix to expand without rupture of the inner membrane, the outer membrane will break and lead to the release of proteins in the intermembrane space such as cytochrome *c* and other factors that play a critical role in apoptotic cell death. Second, the inner membrane becomes freely permeable to protons. This uncouples oxidative phosphorylation, causing the proton-translocating ATPase to reverse direction and so actively hydrolyse ATP, rather than synthesis it. Under such conditions, intracellular ATP concentrations rapidly decline, leading to the disruption of ionic and metabolic homeostasis and the activation of degradative enzymes such as phospholipases, nucleases, and proteases. Unless pore closure occurs, these changes will cause irreversible damage to the cell, resulting in necrotic death. Even if closure does occur, the mitochondrial swelling and outer membrane rupture may be sufficient to set the apoptotic cascade in motion.

The key factor responsible for MPTP opening is mitochondrial calcium overload (i.e., when mitochondrial matrix [Ca^{2+}] is greatly increased), especially when this is accompanied by oxidative stress, adenine nucleotide depletion, elevated phosphate concentrations, and mitochondrial depolarisation[49, 52]. Ginger is found to prevent MPTP opening due to oxidative stress during ethanol withdrawal as evidenced from the increase in the time for 50% PTP opening during EW (Figure 1).

The altered balance of the antioxidant enzymes caused by decrease in CAT, SOD, GP_X, GST and GSH activities may be responsible for the inadequacy of the antioxidant defenses in combating ROS mediated damage. The decreased activities of CAT and SOD may be a response to increased production of H_2O_2 and O_2 by the metabolism of ethanol. These enzymes have been suggested as playing an important role in maintaining physiological levels of oxygen and hydrogen peroxide by hastening the dismutation of oxygen radicals and eliminating organic peroxides and hydroperoxides generated from inadvertent exposure to EW. Treatment with AGE increased the activity of enzymes and may help to control free radicals, as *ginger* has been reported to be rich in flavonoids and polyphenols, well-known antioxidants, which scavenge the free radicals. The increase in SOD activity may protect CAT and GPx against inactivation by $O_2^{\bullet-}$ anions as these anions have been shown to inactivate CAT and GPx.

We have observed significant decrease in GSH levels in brain during withdrawal. The decrease in GSH levels represents increased utilization due to oxidative stress [53]. The depletion of GSH content may also lower the GST activity[54]. Depression in GPx activity was also observed brain of withdrawal rats. GPx has been shown to be an important adaptive response to condition of increased peroxidative stress[55]. The increased GSH content in the brain of the rats treated with

silymarin, LA and ginger extract may be a factor responsible for inhibition of lipid peroxidation. The elevated level of GSH protects cellular proteins against oxidation through glutathione redox cycle and also directly detoxifies reactive oxygen species generated from exposure to ethanol and withdrawal. The significant increase in GSH content and GSH dependent enzymes GPx and GST in withdrawal rats treated with AGE indicates an adaptive mechanism in response to oxidative stress.

Exposure of cells to ethanol and its metabolism have been demonstrated to cause a myriad of alterations in cellular physiology and mitochondrial function[56]. An important consequence from this cascade of malfunctions is an increased sensitivity to cell death exhibited by cells exposed to ethanol. Increased oxidative stress has been shown to be a prominent and early feature of vulnerable neurons. Exposure to oxidative stress induces the accumulation of intracellular reactive oxygen species (ROS), which in turn causes cell damage In the form of protein, lipid, and DNA oxidations. If enhanced ROS exceeds the basal level of cellular Protective mechanisms, oxidative damage and cell death will result.

The observed decrease in the levels of some antioxidants like vitamin C and E in rats treated with ethanol over longer periods as well as withdrawal rats reflected the lipid peroxidation as a consequence of oxidative stress. The non-enzymic antioxidants such as vitamin C and E play excellent role in protecting the cells from oxidative damage [57]. It has been shown that vitamin E (α-tocopherol) scavenges peroxyl radicals quicker than vitamin C (ascorbic acid); the α-tocopheroxyl radical derived from tocopherol is reduced back to regenerate α-tocopherol by ascorbic acid [57, 58]. Ginger extract treatment either individually or in combination with silymarin and ALA is found to restore the levels of these non-enzymic antioxidants in almost all the brain regions under study.

Ginger worked as an antioxidant and increased the level of non-enzymatic antioxidant GSH, enzymatic antioxidants CAT, SOD, GPx, GST, GR, and QR and the protein level in animals exposed to dichlorvos and lindane[59]. Ginger reduces the oxidative stress in the animals, by its high ROS scavenging capacity and protecting the antioxidant enzymes from being denatured. Protective role of ginger has also been reported by Nirmala*et al*., 2010 [60] and Nabil *et al*.,2009[61]. Our results were in consistent with the earlier reports that demonstrated the neuroprotective effect of *Z. officinale* extract at dose of 200 mg/kg body weight that could mitigate the brain infarct volume and could decrease oxidative stress by increasing the activity of SOD in cerebral cortex, hippocampus, and striatum and increased the activities of CAT and GSH-Px in cerebral cortex and hippocampus resulting in the decrease of lipid peroxidation level in all areas

mentioned before due to its antioxidant effect [62]. XOD is reported to play an important role in cellular oxidative status, detoxification of aldehydes and oxidative injury [63]. The extract exhibited an inhibitory effect on the XOD activity thus preventing free radical generation during chronic EtOH administration and ethanol withdrawal.

CONCLUSION

The present study implies that the total extract for ginger enhanced brain antioxidants such as glutathione peroxidase, reduced glutathione, glutathione reductase, superoxide dismutase and catalase and also Vit E and C, significantly prevented withdrawal-induced oxidative stress in the investigated brain regions. Furthermore, AGE prevented histological damage. Therefore, ginger could offer a useful support therapy to addiction related disorders by acting as a neuroprotective antioxidant and protecting neural tissue from histological damage. The investigation about the antioxidative protection during EW by the AGE may provide new insights into endogenous defense mechanisms against EW. Only, further studies may elucidate the exact molecular mechanisms of actions of various constituents of AGE in the role of neuroprotection.

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