CHAPTER IV
Discussion
DISCUSSION

Synthetic pesticides, while valued for effectiveness and convenience can pose certain problems, including phytotoxicity and toxicity to non-target organisms, environmental persistence and health hazards to humans. Natural pesticides are active principles derived from plants for the management of human and animal pest organisms or it can be said to be biologically active ingredients, principally derived from plants, for the management of human and animal pest organisms. With the growing global demand for environmentally sound pest management strategies; there is a need to develop alternative pesticides with minimal health hazards. *Streblus asper*, is a traditionally used medicinal plant in India, various parts, particularly the stem bark possess several beneficial effects such as anti-inflammatory, anti-microbial, and anti-filarial activity. Insecticidal effects of extracts of the stem of this plant have been reported. It is a folk medicine used for the treatment of several diseases like diarrhea, dysentery, lymphoderma, and chyluria. Our results indicated that the polyphenolic fraction from the stem bark of *S. asper* possess promising insecticidal activity against *D. cingulatus*, and this fraction affect acetylcholine esterase enzyme and induce oxidative stress in insect may be the probable reason for the death of insects. Our toxicological study revealed that polyphenolic fraction from *S. asper* was less toxic compared with an organophosphorous insecticide malathion and a neem based formulation vepacide.

1. Preparation of various extracts from stem bark of *Streblus asper* and evaluation of insecticidal action on red cotton bug, *Dysdercus cingulatus*

The results of the present study indicated that various extracts of the stem bark of *S. asper* possess insecticidal activity against *D. cingulatus*. The LC$_{50}$ value of the crude methanolic extract is 5.56 $\mu$g. Four fractions obtained from methanolic extract of *S. asper* showed insecticidal action with increasing order of water (LC$_{50}$ 10.15 $\mu$g) < n- butanol (LC$_{50}$ 8.97 $\mu$g) < n- hexane (LC$_{50}$ 5.89 $\mu$g) < chloroform (LC$_{50}$ 2.01 $\mu$g). The most active chloroform fraction of *S. asper* on
further partial purification by silica gel column chromatography and testing the insecticidal action clearly showed that the fractions rich in polyphenolic content (fraction C, D & E) exhibited the highest insecticidal action. Among the three fractions, chloroform fraction possesses better insecticidal action in both topical application, and residue film technique. Partially purified component of the active chloroform fraction (fraction C) of *Streb/us asper* is named as “PBSA” i.e., polyphenolic bioinsecticide from *Streb/us asper*. The LC$_{50}$ values of PBSA were observed to be 1.82 µg, and 2.52 µg in topical application, and residue film technique respectively. Chloroform fraction (C) was subjected to preparative TLC using silica gel (30% acetic acid as solvent system), two spots (compound I and Compound II) were obtained upon UV examination. The mortality rate was very high in *Dysdercus cingulatus* (red cotton bug) exposed to compound I by topical application and residue film technique. The maximum insecticidal activity was shown by the compound I (spot I: Rf - 0.482) with an LD$_{50}$ of 0.894 µg/insect by residual film technique and 0.595 µg/insect by topical application.

Plants are virtually inexhaustible sources of structurally diverse and biologically active substances (Istvan 2000). The use of plant products as insecticides is gaining importance in recent years in view of the environmental and health hazards posed by synthetic organic insecticides. Conventional pesticides are generally synthetic materials that directly kill or inactivate the pests. Bioinsecticides are certain natural plant products that belong to the so-called secondary metabolites, which include thousands of alkaloids, terpenoids, phenolics and minor secondary chemicals. It is reported that the methanolic extract of *A. oxyphylla* possess insecticidal action against larvae of *D. melanogaster* (Miyazawa, Nakamura et al. 2000). The chloroform extract of *Piper guanacastensis* was also reported to have insecticidal action against *A. atropalpus* mosquito larvae (Pereda, Bernard et al. 1997).

Chloroform fraction from crude methanolic extract as well as the fractions C, D, & E obtained from silica gel column chromatography showed
maximum polyphenolic content. The insecticidal action of the fractions obtained from *S. asper* may be due to the action of polyphenolic compounds. The increase in insecticidal action upon further fractionation of crude methanolic extract may be due to the accumulation of the polyphenolic compounds in some of the fractions.

Secondary metabolites from plants such as polyphenolic compounds can affect insects in several ways. They may disrupt major metabolic pathways; act as deterrents and antifeedants and cause rapid death of insects (Harborne 1988; Houghton 1996). It has become evident in recent years that polyphenolic compounds play a widespread role in natural plant protection against phytophagous insects and phytopathogenic fungi. Several publications devoted to plant polyphenols underline their role in plant resistance (Catherine, Michel et al. 2004; Romanelli, Virla et al. 2010). There are so many reports available which show that polyphenolic compounds possess insecticidal activity (Martin, Daniel et al. 1993; Shripad, Hemlata et al. 2003). Polyphenols extracted from the root of Tephrosia spp. (Fabaceae) showed antifeedant activities against the spotted stalk borer (*Chilo partellus*) (Machocho, Lwande et al. 1995). Polyphenol derivatives identified from the leaves and stems of *Flourensia thurifera* displayed high levels of antifeedant activity in *Spodoptera littoralis* (Faini, Labbe et al. 1997). Kotkar, Mendki et al. (2002) reported that polyphenols isolated from aqueous extracts of *Annona squamosa* (Custard apple) showed 80% insecticidal activity against the stored grain pest pulse beetle, *Callosobruchus chinensis*. Numerous polyphenolic compounds are reported to be involved in the antifeedant activity (Tandon, Shukla et al. 1998). Polyphenolic compounds appear to be involved in the toxic effect exhibited by Mediterranean aromatic plants against *Acanthoscelides obtectus* (Catherine, Michel et al. 2004). Also in our study, the insecticidal action showed by various extracts of the plant *S. asper* may be due to the presence of polyphenolic compounds.
3. Mode of action of insecticidal action of partially purified polyphenolic compound (spot I) from *Streblus asper*.

Pesticide-induced oxidative stress as a possible mechanism of toxicity has been a focus of toxicological research for the last decade. Pesticides induced an excessive production of free radicals which are responsible for several cell alterations in the organism (Saafi, Louedi et al. 2011). Pesticide-induced oxidative stress is the final manifestation of a multi-step pathway, resulting in an imbalance between pro-oxidant and antioxidant defense mechanisms. The level of oxidative stress in cells and the increase in concentration of molecular products of oxy-radical reactions is a possible cause of death in mammals as well as insects (Sohal and Brunk 1992). Intracellular defense systems that protect cells from reactive oxygen species (ROS) induced damage include glutathione reductase (GR), superoxide dismutase (SOD), and catalase (CAT). As a consequence, they are important effectors in the life span determination of the insects (Missirlis, Phillips et al. 2001). The intracellular concentration of ROS is a consequence of both their production and their removal by various antioxidants. It has been established that many pesticides are capable of inducing oxidative stress by overwhelming or modulating cellular drug metabolizing systems (Olgun and Misra 2006; Zhenquan and Hara 2007). Insecticides have been reported to induce production of reactive oxygen species and oxidative tissue damage (Bagchi, Bagchi et al. 1995). Malkovics (1995) reported that organophosphorous insecticides, besides their inhibitory effect on AChE, they can also induce oxidative stress. The activities of the antioxidant enzymes like SOD, GPx and GST and the level of antioxidant GSH were significantly reduced and the level of lipid peroxidation was significantly increased, indicating an oxidative stress upon by fenitrothion (OPI) treatment (El-Shenawy 2010). Studies showed that the most important factor in the insect’s defensive system is an increased capacity to detoxify the insecticides, most likely as a result of the production of additional enzymes of detoxification (Syvanen, Zhou et al. 1996).
Study on the mode of action of the most active compound isolated from the stem bark of *Streblus asper* revealed that they significantly reduced the activities of acetyl cholinesterase, glutathione S-transferase, superoxide dismutase and catalase and decreased glutathione content and significantly elevated the TBARS content in red cotton bugs. The antioxidant enzymatic defense of insects consists of SOD, catalase (CAT), GR, and GST (Ahmad and Pardini 1990). GST is one of the most general and efficient xenobiotic detoxification systems in all animals (O'Brien and Tew 1996). In insects GST have been induced and is becoming recognized for their importance in the metabolic detoxification of insecticides (Yu 1996), and allelochemicals from host plants (Yu 1994), in protecting insects from the toxic effects of reactive oxygen species (Ahmad and Pardini 1990; Parkes, Hilliker et al. 1993; Zaman, MacGill et al. 1994) and for enhancing the defense machinery, speeding the development of resistance and causing cross-tolerance to other pesticides (Anspauch, Rose et al. 1994; Carlini, McPheron et al. 1995; Hinkle, Wadleigh et al. 1995). The inhibition of acetyl cholinesterase and antioxidant activity, elevated levels of lipid peroxidation and decreased level of GSH in polyphenol bioinsecticide exposed red cotton bugs was in agreement with the following reports which state that there were significantly reduced GSH levels in all tissues after pesticide administration (Fontan, Picollo et al. 1994; Ozden and Alpertunga 2010) and decreased activities of acetyl cholinesterase, superoxide dismutase and glutathione S-transferase activities (Kostaropoulos, Papadopoulos et al. 2001) and increased level of lipid peroxidation (Ahmad, Zaman et al. 1995; Gabriel, Juraj et al. 1999; Mansour, Mossa et al. 2009). The probable mode of the death of insects may be due to the inhibitory effect on the activity of acetyl cholinesterase and antioxidant enzymes and significant increase in the level of lipid peroxidation.
4. Acute toxicity evaluation of polyphenolic bioinsecticide from *Streblus asper* (PBSA)

In the present study the most active polyphenolic bioinsecticide from *Streblus asper* (PBSA), was subjected to acute toxicological testing to document its safety for use as an insecticide. It did not reveal any changes in the general behavior of the tested animals and did not produce any signs of toxicity in rats upto 25g/Kg body weight. The acute toxicity, along with subacute toxicity, is considered important for the assessment of risk posed by new chemical substances, and for a better control of natural and synthetic agents in the environment. The acute toxicity of an insecticide refers to its ability to do systemic damage as a result of a one-time exposure to relatively large amounts of the insecticide. A insecticide with a high acute toxicity may be deadly if even a very small amount is absorbed. The commonly used term to describe acute toxicity is LD$_{50}$. The test animals are given specific amounts of the insecticide in one oral dose and are then observed for a specified time. Single- dose toxicity can contribute initial biological information for the classification of chemicals. Lower the LD$_{50}$ value, the more acutely toxic the insecticide.

5. Sub-acute toxicity studies of PBSA in comparison with malathion, and vepacide

5. A. Effect on haematological parameters

In sub-acute toxicity study there was no significant change was observed in haematological parameters in PBSA treated rats when compared to control animals at a dose of 250 mg/Kg BW/ day & 500 mg/Kg BW/ day in 0.5ml 10% DMSO. But malathion and vepacide treated rats showed significant decrease in RBC count, hemoglobin content, mean corpuscular hemoglobin concentration (MCHC), hematocrit (Ht) value and increase in mean corpuscular volume (MCV) when compared to DMSO control animals in duration dependant manner. Neskovic, Karan et al. (1991) have reported that malathion showed significant decrease in
hemoglobin concentration when compared with control animals in a subchronic test conducted on male rats. Pesticides induce regenerative anemia which is accompanied by an increase in reticulocytes and possibly higher mean corpuscular volume (MCV) and lower mean corpuscular hemoglobin concentration (MCHC) values. Pesticides may also cause increased destruction of red blood cells by directly damaging red blood cell membranes or by oxidizing hemoglobin (Bloom and Brandt 2001). Mean total RBCs, WBCs counts, PCV, Hb, MCHC values were lower in malathion treated *Tilapia nilotica* fish (Khalaf-Allah 1999). Administration of malathion residues (10 and 100 ppm) for 90 days showed changes in hemoglobin concentration in male rats (Neskovic, Karan et al. 1991). Administration of a single oral dose of the malathion to the rat resulted in hemostatic disorders like prolongation of blood clotting, prothrombin and thrombin time (Keadtisuke, Dheranetra et al. 1990). Neemrich-100 (30% solution of neem oil) at 600 mg/kg after prolonged dermal application produced depression of erythropoiesis and increased mean liver weight which are indicative of its potential to produce tissue-selective toxicity (Quadari, Usha et al. 1984). Male and female albino rats administered with “azadirachtin technical” (12% azadirachtin) in peanut oil at 1000 and 1500 mg/kg bw/day for 90 days produced reductions in RBC and Hb values (Raizada, Srivastava et al. 2001). According to Afshar, Heidari et al. (2008) a significant dose dependent decrease was observed in some hematological parameters like RBC counts, Hb content, Ht and MCH values in fenitrothion, an OPI treated rats. *In vitro* administration of an OPI, dursban caused cell lysis in erythrocytes of human and fish blood (Laji and El-Elaimy 1991). Acute effects of sublethal concentrations of malathion (5.76 mg/L) is evidenced by the decreased Ht value and significantly increased value of MCV (Ghazaly 1995).

5. B. Effect on acetyl cholinesterase activity and neurotransmitters

The experiment on the effect of insecticides in male albino rats revealed that bioinsecticide from *Streblus asper* (PBSA) did not play any significant role in the levels of neurotransmitters as well as in AChE activity while malathion and vepacide inhibited the activity of acetyl cholinesterase in plasma and increased the content of
acetyl choline in brain. The mechanism of toxic effect of organophosphates compounds is based on the acetylcholinesterase inhibition in the nervous system. The organophosphates after entering the body of an organism reach the cholinergic sites of the nervous system and inhibit the activity of AChE by binding at its active sites. It has been conclusively shown that OPI can exert significant adverse neurotoxic effects in nontarget species, including humans (Sekar Babu, Uma Devi et al. 2011). Organophosphorus compounds show long-lasting impairment in neurobehavioral performance (reduction of verbal attention, memory, visual attention, flexibility of thinking) caused by either direct cholinergic-mediated or noncholinergic neurotoxicity (Vaishwanar and Mallik 1984; Liu, Kao et al. 1994; Beauvais, Jones et al. 2000; Colosio, Tiramani et al. 2009). Subchronic exposure of rats to malathion at an increasing doses equivalent to 1/50 LD_{50}, 1/25 LD_{50} and 1/10 LD_{50}, respectively for 45 days resulted in statistical dose-dependent decrease in acetylcholinesterase (AChE) activity (Aboul-Soud, Al-Othman et al. 2011).

AChE, a sensitive marker of neurotoxicity is widely distributed within the Central Nervous System (CNS). The AChE inhibition, thus leads to the accumulation of acetylcholine at nerve endings which in turn cause the disruption of the nervous activity resulting in excitation, paralysis and finally the death of the organism (Gaines and Linder 1986; Satoskar, Bhandarkar et al. 1999). Hence recently new combination of pesticides have been introduced to reduce environmental pollution and at the same time to have a maximum action in killing the pest animals (Dede and Dogara 2004; Dinham 2005). Chlorpyrifos (OPI) induces toxicity through inhibition of acetyl cholinesterase (AChE) but also involves multiple mechanisms besides the inhibition of AChE (Slotkin, Olivier et al. 2005). Honnegowda and Garg (1984) showed that malathion at a low dose of 23 mg/kg for a period of 60 days significantly increased the levels of catecholamines in rats when compared to control animals. The levels of catecholamines during malathion treatment were elevated which indicate either increased synthesis of these catecholamines i.e. epinephrine, norepinephrine and dopamine or their reduced
Discussion

release. According to Zahran, Abdel-Aziz et al. (2005) acetyl cholinesterase activity was significantly decreased and epinephrine, norepinephrine, dopamine and acetylcholine concentrations were significantly increased in mice following treatment with Nuvacron (an OPI).

5. C. Effect on liver function enzymes

In sub-acute toxicity study conducted with malathion, vepacide and PBSA, malathione and vepacide showed significant increase in the activity of liver function enzymes, whereas PBSA did not produce any significant change in the levels of liver function enzymes like AST, ALT, ALP and LDH. AST and ALT activity is regarded as a specific indicator for hepatocytes damage (Zhang, Noordin et al. 2000). Elevation in the activity of liver marker enzymes AST and ALT is particularly useful in measuring hepatic necrosis, especially in small animals (Adedapo, Abatan et al. 2004). Alkaline phosphatase is a marker enzyme, and their increase in serum, with parallel increases in different tissues, might be due to the increased permeability of plasma membranes. The changes observed in this enzyme activity could be a useful biomarker of exposure to Vepacide (Rahman and Siddiqui 2004).

Alkaline phosphatase is a marker enzyme, and its increase in serum, with parallel increase in different tissues, might be due to the increased permeability of plasma membranes. The significant elevations in enzyme activity of LDH indicate damage to liver or kidneys in the experimental animals (Amacher 2002). This is in support with the finding that feeding of malathion residues to mice for 90 days resulted in an increased levels of AST and ALP (Zayed, Farghaly et al. 1992). In subchronic test on rats fed malathion residues (10 and 100 ppm) for 90 days caused an increase in the activity of serum ALT and ALP. Short term (24 hr.) and long term (4 weeks) treatment with malathion and methyl-parathion resulted in an increased specific activities of liver enzymes, ALP, AST and ALT and malathion had greater effect than methyl-parathion on the biochemical parameters studied (Jabbar,
Khawaja et al. 1990). A significant increase in the levels of various serum and liver marker enzymes viz. ALP, AST, and ALT was observed following 8 weeks treatment with chlorpyrifos, an OPI (Goel, Chauhan et al. 2000). When compared to the control group, the malathion-treated rats had significantly higher ALP, ALT, AST, and LDH levels (Kalender, Uzun et al. 2010). Animals kept on low protein diets (5% and 10%) when exposed to malathion (400 mg/kg) showed significant increase in the activities of AST, ALT and ALP in liver and kidney of animals when compared to their respective control animals (Vaishwanar and Mallik 1984). Daily dermal spray of malathion (0.5, 1.0 and 5.0 %) for four weeks showed significant elevation in the activities of serum AST, ALT, ALP and the extent of various biochemical changes were dose and time dependent (Gupta and Paul 1978). A single oral dose of malathion (687.5 mg/kg) to adult male albino rats, resulted in an increase in intestinal alkaline phosphatase activity (Saigal, Bhatnagar et al. 1982). Prolonged exposure of rats to profenofos, an OPI (26.53 and 53.07 mg/kg body weight /day for 28 days) caused a significant increase in the activity of LDH (Mogda, Afaf et al. 2009). Irfan, Namik et al. (2002) has reported that the activities of AST, ALP, and LDH were significantly increased in methidathion (an OPI) treated groups when compared with the control group.

When rats were treated with azadirachtin, increased serum AST and ALT activities were observed (Abdel Megeed, Radwan et al. 2001). Intramuscular injection of neem seed oil at 250 and 500 mg/kg bw/day for 8 days in male rats caused substantial and highly statistically significant increase in the activity of ALP (Manoranjitham, Prem et al. 1993; Sampathraj, Badri et al. 1993). Rats treated with vecapide, a neem based formulation showed a significant increase in the activity of ALT and AST in serum and kidneys, generally in a dose and time related manner (Rahman, Siddiqui et al. 2001). Significantly elevated levels of liver function enzymes in this study may indicate hepatic damage caused by malathion and vecapide treated rats. Malathion induced significant alterations in AST, and ALT activities in rats maintained on 16, 6, and 1% protein diets for a period of 3 weeks.
Discussion

LDH and ALP activities in various tissues were increased after 24 days of administration of aqueous extract of powdered neem leaf treated rats (Joshi, Ahamed et al. 1996). In the present study, the PBSA from Strebulus asper showed no significant toxicity to rats compared to malathion and vepacide as evidenced by its effect on liver enzymes in albino rats.

5.D. Effect on antioxidant defense system

The present study examined the effect of an OPI malathion, a neem based formulation vepacide and PBSA, a bioinsecticide from Strebulus asper on antioxidant enzymes SOD, GPx, GR, GST, and CAT in the liver and kidney of rats. Pro-oxidant and antioxidant balance is vital for normal biological functioning of the cells. If any of the complex components such as environmental contaminants affecting this balance can provoke excessive production of ROS that is effectively scavenged by endogenous antioxidant defense system (Jamieson 1989). The antioxidant enzyme activity was monitored and significant decrease was observed in malathion and vepacide treated rats. According to (Johnson, Rosenberg et al. 2002) malathion has been shown to induce oxidative stress in experimental animals. In vitro administration of an OPI chlorpyrifos-ethyl resulted in the induction of erythrocyte lipid peroxidation and significant changes in antioxidant enzyme activities, suggesting that ROS/ or free radicals may be involved in the toxic effects of chlorpyrifos-ethyl (Gultekin, Ozturk et al. 2000). Pesticide intoxication induces a derangement of certain antioxidant mechanisms in different tissues, including alterations in antioxidant enzymes and the glutathione redox system (Banerjee, Seth et al. 2001). Kataria, Gupta et al. (1998) reported that Tric Vet Care, a neem based formulation has the potential to influence the oxidant defense mechanism of RBC and brain. Exposure to chlorpyrifos (OPI) can modify endogenous antioxidant enzymes like SOD, and GPx and antioxidant like GSH, which can lead to the development of oxidative stress in some tissues (Bebe and Panemangalore 2003). Concomitantly, pesticide intoxication induces a derangement of certain antioxidant
Discussion

mechanisms in different tissues, including alterations in antioxidant enzymes and the glutathione redox system (Banerjee, Seth et al. 2001).

Our present study indicated that malathion and vepacide treatments significantly inhibit the activities of SOD, CAT, GPx and GST in both kidney and liver of rats, where as TBARS were significantly elevated. Meanwhile PBSA treated rats showed significant increase in the activities of the above enzymes and TBARS level was not significantly altered when compared with control animals. These findings were compatible with the study of Yarsan, Tanyuksel et al. (1999), which reported that chronic administration of malathion in mice showed a significant decrease in the activity of SOD, GPx and catalase in erythrocytes. Phosalone, an OPI at high concentrations caused an increase in TBARS formation and a decrease in the activities of SOD, GPx and CAT (Altuntas, Delibas et al. 2003). One of the important aspects of the antioxidant enzymes is their synergistic functioning. Any impairment in one member of the system will influence the activities of the other enzymes. A decrease in SOD activity will favour the accumulation of superoxide radicals, which is known to inhibit CAT (Kono and Fridovich 1982). Syrovatskaia, Sereda et al. (1993) reported that deltametrin and dichlorvos (OPI) intoxication in rats lowered SOD activity and stimulate lipid peroxidation.

TBARS is a major degradation product of lipid hydro peroxides, and measurement of TBARS concentration is generally accepted as a marker for assessing the extent of lipid peroxidation in vivo (Kalender, Kalender et al. 2004; Abdulaziz, Khaled et al. 2011). The elevated concentration of TBARS clearly indicated the presence of oxidative injury in liver and kidney. Pedrajas, Peinado et al. (1995) reported that malathion treatment in fish enhanced significantly the TBARS content, while decreased the glutathione-S-transferase activity. Human blood sample obtained from malathion poisoning showed an increase in the level of TBARS (Banerjee, Seth et al. 1999). Healthy agricultural sprayers, exposed to pesticides for 5 years, caused significant increase in TBARS content (Prakasam, Sethupathy et al. 2001). Administration of malathion (20 ppm) for 4 weeks increased the TBARS
levels in serum of rats (Ahmed, Seth et al. 2000). Lipid peroxidation was significantly increased in brain of rats treated with 4.5 ml/Kg of Tric Vet Care, a neem based formulation, in a period of 60 days (Katari, Gupta et al. 1998). Treatment with Rotenone, a bioinsecticide increased lipid peroxidation in liver tissue of male albino rats (Terzi, Iraz et al. 2004). In vitro administration of an OPI chlorpyrifos-ethyl resulted in the decrease in the activity of CAT and increase in the concentration of TBARS (Gultekin, Ozturk et al. 2000). Profenofos, an organophosphate insecticide, treatment result in a significant increase in TBARS concentrations but a significant decrease was obtained in GSH levels of brain and liver tissues (Saeed, Al-Koly et al. 1995; Fortunato, Feier et al. 2006; Güney, Demiin et al. 2007). Oral administration of profenofos (an OPI) at a dose of 17.8 mg/Kg body weight/day for 15 days caused a significant increase in TBARS in male albino rat liver (Mohamed, Metwally et al. 2010). From this study it appears that malathion and vepacide induced oxidative stress and interferes with the antioxidant enzyme system where as polyphenolic fraction did not produce any oxidative stress as it is evidenced by the normal level of lipid peroxide content. The maintaining of the normal levels of lipid peroxides in PBSA treated rats may possibly be due to the increased activities of antioxidant enzymes.

The present data shows that liver and kidney GSH content and specific activities of glutathione reductase (GR), and glucose-6-phosphate dehydrogenase (G6PD) were found to be decreased after exposure to malathion as well as vepacide. Activities of GR and G6PD were significantly elevated and GSH content was well comparable with control values in PBSA administered rats. Glutathione is the cell's natural antioxidant, which destroys free radicals formed in cells. Many xenobiotics exert their toxicity by decreasing cellular glutathione below certain threshold values (Ketterer, Coles et al. 1983; Imberti, Mapelli et al. 1990; Reed 1990). Significant dose-dependent depletion of GSH levels and perturbations in antioxidant enzyme levels confirmed the ability of the insecticide, fenvalerate to induce oxidative stress in hepatic tissue (Prasanthi and Rajini 2005). Subchronic
Discussion

exposure to an insecticide, dimethoate, an OPI, (6 and 30 mg/kg) resulted in a decrease in glutathione levels in both liver and brain tissues of male wistar rats (Sharma, Bashir et al. 2005). Interaction of GSH with pesticide-derived free radicals caused the reduction of GSH content (Portig, Kraus et al. 1979). Malathion caused a time and dose dependent depletion of hepatocellular glutathione levels in rats (Malik and Summer 1982). GSH content was significantly decreased in human blood sample obtained from malathion poisoning (Banerjee, Seth et al. 1999). Administration of malathion (20 ppm) for 4 weeks in albino rats significantly decreased the glutathione (GSH) level by 35% in whole blood (Ahmed, Seth et al. 2000). In the subchronic study, rats treated with vepacide (12% azadirachtin) for 90 days showed a significant reduction in GSH levels in the liver and kidney after 45 and 90 days (Mahboob, Siddiqui et al. 1995). Significant dose-dependent depletion of GSH levels confirmed the potential of the profenofos (OPI) to induce oxidative stress in brain and hepatic tissue (Rajeswary, Kumaran et al. 2007). A decreased level of G-6-PD in liver and kidney under subacute pesticide exposure indicates less operation of hexose mono phosphate pathway, resulting in decreased generation of NADPH (Rao and Rao 1987). Methyl parathion, an OPI, administration significantly decreased hepatic GSH content and the activities of hepatic GPx and G-6-PD. The decreased level of GSH in malathion and vepacide treated rats induced stress may be due to the decreased activity of either GR or G-6-PD activity. But there was a significant elevation in G-6-PHD, GPx and GR enzyme activities and GSH content in liver and kidney of rats treated with PBSA which indicate that glutathione enzyme system is involved in the alleviation of oxidative stress. The present data has indicated the manner of OPI poisoning and given strong evidence of the nontoxic effect of PBSA from *Streblus asper* in albino rats in comparison with a biopesticide formulation vepacide in the experimental animals.

5. Effect on xenobiotic metabolizing enzymes

The human body is exposed to a wide array of xenobiotics in one’s lifetime, from food components to environmental toxins to pharmaceuticals, and has
developed complex enzymatic mechanisms to detoxify these substances. Common detoxification enzymes that metabolize pesticides and other xenobiotics in mammalian system are carboxyl esterases, GST, and UDPGT. The scientific literature suggests an association between impaired detoxification and certain diseases, including cancer, Parkinson’s disease, and immune dysfunction syndrome. Data regarding these hepatic detoxification enzyme systems and the body’s mechanisms of regulating them suggests the ability to efficiently detoxify and remove xenobiotics can affect these and other chronic disease processes (DeAnn and Liska 1998). Dietary factors influence xenobiotic metabolism by inducing specific enzymes responsible for detoxification of xenobiotics. Among them the GST is a family of detoxification enzymes involved in cellular protection (Yang 2006). They catalyze the conjugation of electrophilic compound with reduced glutathione.

The present study showed that the activities of drug metabolizing enzymes and cytochrome P₄₅₀ were significantly decreased in malathion and ve pacide treated rats when compared to control animals. This is in support with the finding that sub-acute toxicity study of ve pacide at a dose of 320 mg/kg/day for 45 and 90 days treatment in male wistar rats caused a significant decrease in cytochrome P₄₅₀ concentration in liver and kidney (Mahboob, Siddiqi et al. 1998). But PBSA treated rats showed no significant change in enzyme activities at low concentrations whereas there was a significant increase in activity was observed at high dose treated rats. Chlorpyrifos, an OPI, intoxication causes a significant decrease in the reduced glutathione (GSH), and glutathione- S-transferase (GST) activities (Goel, Danni et al. 2005). General esterases, which are capable of degrading or sequestering pesticides, can play a significant role in the detoxification of organopesticides pesticides (Anspaugh, Kennedy et al. 1995; Argentine, Lee et al. 1995; Valles 1998). The blockage of glutathione-dependent route of detoxification can lead to increased toxicity of malathion and increased toxic load may lead to inhibition of detoxification of a number of compounds by simply overwhelming the systems and competing for detoxification enzyme activities. Daabees, El-Domiaty et
al. (1992) reported that glutathione S-transferase activity was decreased in malathion treated animal and they found that malathion is highly hepatotoxic. Several reports have suggested that GST provide a protective mechanism against OPI that binds probably to the active site of the enzyme inhibiting its activity towards CDNB in a competitive manner, but is not conjugated with GSH (Parkes, Hilliker et al. 1993; Kostaropoulos, Papadopoulos et al. 2001; Fujioka and Casida 2007). Zhang, Qiao et al. (2004) reported that paraoxon- and chlorpyrifos (OPis) inhibited carboxylesterase in experimental animals. Report suggested that the oxidative stress due to dimethoate, an OPI, is ascribed to the induction of cytochrome P450, inhibition of AChE and disturbance in the activities of GST enzymes and GSH content causing lipid peroxidation (Sharma, Bashir et al. 2005). Paraoxon, an OPI, inhibit carboxyl esterases and can be used as a marker of pesticide poisoning severity (Petroianu, Kärcher et al. 2001). Malathion induced significant alterations in beta-glucuronidase activities in rats maintained on 16, 6, and 1% protein diets for a period of 3 weeks (Bulusu and Chakravarty 1984). Malathion caused a dose-dependent increase in the activity of beta-glucuronidase in the blood of rats (Keadtisuke, Dheranetra et al. 1990).

5. F. Histopathological evaluation

Histological study in liver of malathion and vepacide treated rats showed shrunken cells with disruption of the chords and dilated sinusoids. Fibrosis was also seen in liver cell of malathion and vepacide treated rats. Malathion and vepacide induced severe hepatic and renal damages as shown in histopathological examination which coupled with markedly elevated levels of liver hematobiochemical markers (AST, ALT, and ALP). But in polyphenolic fraction treated rats did not show any alteration in the architecture of liver and kidney. Rutong, Tian et al. (2010) has reported that omethoate, a systemic organophosphorous insecticide induced liver injury in Sprague Dawley rats. Furthermore, histopathological studies ofliver in the rats which received malathion at an increasing doses equivalent to 1/50 LD$_{50}$, 1/25 LD$_{50}$ and 1/10 LD$_{50}$, respectively for 45 days exhibited, moderate to
severe degenerative and necrotic changes in the hepatocytes (Aboul-Soud, Al-Othman et al. 2011). Tos-Luty, Obuchowska et al. (2003) showed that malathion intoxication led to severe effects on the structures of the liver and kidney including the presence of fine subcapsular infiltrations, diffused parenchymatous degeneration of single hepatocytes, and the presence of fine foci constructed of plasmatic cells, and histiocytes located between hepatic plates In the submicroscopic structure of hepatocytes, there occurred lucent areas of cytoplasm containing the residues of cell organelle and lipid vacuoles. Additionally, they showed that the histopathological changes in the kidneys occurred in all animals. These changes covered parenchymatous degeneration of the cells of renal tubules and hyperemia of the cortical part of the kidney, especially of renal glomeruli, as well as infiltrations were noted. There are various reports available which show that malathion and other pesticides induced liver and kidney histopathological alterations in experimental animals (Yavasoglu, Sayim et al. 2006; Abdel Razik, Farrag et al. 2007; Kerem, Bedirli et al. 2007; Afshar, Farshid et al. 2008; Saadi, Lebaili et al. 2008). Experimental histopathological studies in albino rats revealed that oral administration of profenofos (an OPI) at a dose of 17.8 mg/Kg body weight/day for 15 days caused degenerative changes in the liver and kidney accompanied with infiltration of mononuclear inflammatory cells (Abdel Razik, Farrag et al. 2007). High doses of the profenofos induced tissue vacuolization, haemorrhage and hyperplasia of kupffer cells in the liver and swelling of Bowman's capsules and tubular degeneration in the kidney (Fawzy, Iman et al. 2007). The oral administration of Azadirachtin of neemix-4.5 for 11.5 weeks has resulted in some histopathological changes in the liver and kidney of treated animals compared with the control group (Abou-Tarboush, El-Ashmaouib et al. 2009). Histopathological studies showed significant changes in the liver in terms of congestion, hydropic degeneration, necrosis and lymphocytic infiltration in rats treated with azadirachtin (Abdel Megeed, Radwan et al. 2001).
Discussion

6. Toxicity evaluation of PBSA in pregnant rats and in comparison with malathion and vepacide

PBSA did not produce any reproductive abnormality when administered orally to pregnant rats during 6-13th day of gestation, whereas malathion and vepacide treated rats showed significant reproductive abnormalities. Administration of a teratogen on day 6-13th of gestation in rats caused high levels of malformations of brain, eye, heart, skeletal and urogenital organs (Andreas, Brigitta et al. 1990). Asmatullah, Mufti et al. (1993) described the morphometric studies of the fetuses recovered following different concentrations of malathion showed reduction in body weight and crown rump length which was basically dose dependent. Malathion was tested for its embryotoxic and teratogenic properties in mice embryos following relative high doses (125, 250 and 500mg/g) of the insecticide. It was observed that along with a decrease in body weight and crown rump length, the embryos showed a significant lag in the development of main body parts such as brain, snout, external pinnae and limbs while significant increase in uncovered area of eyeball (Asmatullah, Mufti et al. 1993). Chlorpyrifos, an acetyl cholinesterase (AChE) inhibitor is a widely used organophosphate pesticide and is potentially dangerous to pregnant mice and developing fetuses and a significant decrease in body weight and crown rump length of embryos were noted (Muhammad, Muhammad et al. 2010).

Administration of 827 mg/kg/day malathion (98% pure) to pregnant Sprague-Dawley rats on gestation day 6–13 induced abortions after the 5th dose, but this dose level also induced lethal effects on dams (Mathews and Devi 1994). A slight but significant decrease in the number of implants was observed on gestation day 20 in Sprague-Dawley rats administered doses of 500 mg/kg/day of malathion (98% pure) on gestation day 6, 10, and 14; this level of malathion exposure also reduced maternal body weight gain by 22% (Prabhakaran and Devi 1993). Treatment of female Sprague-Dawley rats with 50 mg/kg/day of malathion for 3 months prior to mating and during gestation day 1–20 did not affect the ability to mate or conceive or
Discussion

the number of total implants or number of implants per dam (Lechner and Abdel-Rahman 1984). Etephon, an OPI administered to swiss albino pregnant mice with three doses level (50, 100 and 150 mg/ kg bw/ day) for 3 weeks produced certain mutagenic, teratogenic and biochemical effects on mouse dams and fetuses (Abd El Raouf and Girgis 2011).

Administration of technical malathion (98% pure) by gavage (0, 138, 276, or 827 mg/kg) from gestation day 6- 13th to Sprague-Dawley rats resulted in inhibition of brain cholinesterase in a dose dependent manner both in dams and pups on postnatal day 21. This treatment significantly increased the activities of carboxylesterase, glutathione-S-transferase, and cytochrome P450 content in the liver from both dams and pups. Malathion also reduced the glutathione content and the activities of glutathione reductase and glutathione peroxidase and increased lipid peroxide content in the liver from both dams and pups (Mathews and Devi 1994). 30 or 100 mg/kg malathion (98% pure) administration to mice by gavage in corn oil from 1- 14th day of lactation found that glutathione-S-transferase activity was increased in the liver from male pups from both treated groups and in high-dose female pups (Chhabra, Hashim et al. 1993). Glutathione reductase activity was increased only in high-dose male pups. Glutathione peroxidase activity was significantly increased (dose-related) in both dose groups of pups. Vepacide, an enriched neem based formulation containing 12% azadirachtin, upon oral administration of 80, 160, and 320 mg/kg daily doses for 90 days in male rats caused significant decreases in cytochrome P450 concentration in the liver and kidneys (Mahboob, Siddiqui et al. 1998). Possible adverse effects of neem used as a pesticide is the reproduction disturbance in both male and female mammals upon subacute and chronic exposure (Boeke, Boersma et al. 2004; Brahmachari 2004). Oral administration of neem seed extract to female rats from days 8 to 10 of pregnancy caused complete resorption of embryos by day 15 of pregnancy (Mukherjee and Talwar 1996).
7. Mutagenicity evaluation of PBSA and comparison with malathion and vepace by micronucleus test

One of the methodologies currently utilized for the evaluation of the harmful effects caused by genotoxic substances in organisms is the micronucleus (MN) assay. The micronucleus test, is an *in vivo* and *in vitro* short-time screening cytogenetic test, introduced by Heddle (1973) and Schmid (1975) is a widely used method for assessing genotoxicity of chemicals in organisms (Meier, Wemsing et al. 1999). MN is small, extranuclear bodies that are formed during mitosis from acentric chromosomal fragments or chromosomes that are not included in each daughter nucleus. Thus, a micronucleus will contain either a chromosomal fragment or a whole chromosome (Carrano and Natarajan 1988; Sonam and Agrawal 2010). This test can predict the induction of structural aberrations, which is most specific for assessing the clastogenic potential (Heddle 1973). Cyclophosphamide (CP) yields clearly positive results in the micronucleus test (Tinwell, Bandara et al. 1990) and therefore is used as a positive control by several investigators (Adler and Kliesch 1990; Cihak and Vontorkova 1990; Rohit and Kalyani 2010). Among the potential secondary biological consequences of these pesticides, genotoxicity and carcinogenicity are of special importance.

Our results indicated that, in the tested condition, both concentrations of PBSA did not influence the induction of micronucleus in different time intervals, whereas malathion caused a significant micronucleus induction in mice at both 24 and 48 hours exposure period. The single i.p. administration of *B. variegate* bark extract the dose of 125, 250 and 375 mg/kg body weight, 24 hours prior the administration of cyclophosphamide (at the dose of 50 mg/kg) have significantly prevented the micronucleus formations in dose dependent manner in bone marrow cells of mice as compared to cyclophosphamide group (Sonam and Agrawal 2010). Several reports indicated that organophosphorus pesticides malathion and methyl parathion are genotoxic and carcinogenic (Walter, Czajkowska et al. 1980; Chen, Sirianni et al. 1982; Flessel, Quintana et al. 1993; Sunil Kumar, Ankathil et al. 1993;
Pluth, Nicklas et al. 1996). Malathion showed a dose dependent increase in the frequency of chromosomal aberration as well as sister chromatid exchanges in in vitro culture of human peripheral blood (Balaji and Sasikala 1993). (Salvadori, Ribeiro et al. 1988) reported the clastogenic effect of malathion on somatic and germ cells of mice. Increased number of chromosomal aberrations, sister chromatid exchange frequency, micronucleus frequency, and values of comet assay parameters were observed in the blood samples of workers after they spent eight months in the production of malathion (Garaj-Vrhovac and Zeljezic 2002).

The results of our study indicated that the micronucleus induction of neem based formulation, vepacide was significant after 24 hour exposure but no significant induction was shown after 48 hours. This may be due to the rapid metabolism of vepacide to less active metabolite. Similar results of micronucleus induction were obtained in okadaic acid exposed mussels (Carvalho Pinto-Silva, Ferreira et al. 2003) and domoic acid exposure in a hepatocyte mediated assay with V79 Chinese hamster lung cells (Rogers and Boyes 1989). In mice, crude ethanol extract of neem showed dose-dependent increase in both individual (breaks and gaps) and gross (aneuploidy and polyploidy) types of abnormalities (Awasthy, Churasia et al. 1995). Akudugu, Gade et al. (2001) reported that 28 µM azadirachtin reduced the proportion of dividing cells and induced formation of micronuclei in TP53 mutant cell lines. Oral administration of a crude ethanol extract of the leaves of neem (Azadirachta indica A juss) to adult Swiss albino mice for 7 days at 5 mg, 10 mg or 20 mg/10 g bw/day significantly increased the incidence of structural and mitosis disruptive changes in metaphase chromosomes of bone marrow cells on days 8, 15 and 35th of observation (Awasthy, Chaurasia et al. 1999). These results suggest that neem leaf extract and azadirachtin can be genotoxic to mammalian cells.
8. Carcinogenicity evaluation of PBSA and comparison with malathion and vepacide by in vitro microsomal degranulation technique

The rationale for carrying out this experiment was to predict the carcinogenicity potential of pesticides by using microsomal degranulation technique. Microsomal degranulation is an efficient and well established technique for the detection of carcinogenicity of chemicals (Purchase, Longstaff et al. 1978; Gupta and Dani 1979; Jagota and Dani 1985; Puri, Dani et al. 1998). Williams and Rabin (1971) suggested that carcinogenicity of chemicals could be screened by measuring the detachment of ribosomes from rough endoplasmic reticulam after treating it with suspected compounds under in vitro conditions. Chemical carcinogens are known detach more than 5% ribosomes from microsomal preparations when incubated in the presence of 1mM NADPH (Purchase, Longstaff et al. 1978; Gupta and Dani 1979; Gupta and Dani 1987). The interference in the binding of the ribosomes to reticular membrane by O-dianisidine and benzidine (Jagota 1991) tannic acid, (Sharma and Dani 1994) and tobacco metabolites (Puri, Dani et al. 1998) has already been reported. The carcinogen, benzopyrene affects the binding of ribosomes to degranulated microsomes when either degranulated microsomes or ribosomes were pretreated with this carcinogen (Sharma and Dani 1997).

Our present study indicated that malathion, an organophosphorus pesticide and vepacide, a neem based formulation showed a remarkable increase in the percentage of degranulation and decrease in binding of ribosomes to reticular membranes. Both malathion and vepacide treatment cases treated polysome showed impairment of ribosome reconstitution than treated degranulated microsomes. Polyphenolic compound did not affect the binding of ribosomes to degranulated microsomes and failed to influence the alteration of degranulation of ribosomes. These facts seem to indicate that malathion and vepacide could be potentially carcinogenic, whereas polyphenolic compound did not induce any carcinogenic potential.
The damage effect of malathion and vepacide could be due to the changes in the conformation of proteins involved at the binding sites. The above data suggests that malathion and vepacide may damage the binding sites more when compared to polyphenolic compound. Among the insecticides, greatest degranulation effect was exhibited by malathion followed by vepacide, while polyphenolic compound did not produce microsomal degranulation. From this microsomal degranulation study, it can be concluded that polyphenolic compound from *Streblus asper* at 40µg/ml concentration failed to influence the induction of microsomal degranulation indicating that it is a non carcinogenic insecticide. Based on the above findings we concluded that polyphenolic compound from *Streblus asper* is safer than malathion, an organophosphorus insecticide and vepacide, a neem based insecticide and it can be considered as a best candidate instead of synthetic insecticides.