6. Discussion
In the present study, tibial & sural nerve transection (TST); chronic constriction injury of sciatic nerve (CCI) and vincristine (VIN) administration have significantly induced painful neuropathy manifested in the terms of behavioral; electrophysiological, biochemical as well as histopathological changes. These observations are in line with our earlier findings (Muthuraman et al., 2008; 2008a) and reports from the other laboratories (Bennett and Xie., 1988; Lee et al., 2000; Siau and Bennett, 2006).

Tibial and sural nerve transection (TST) is a common method for induction of painful peripheral neuropathy (multiplex mononeuritis type of neuropathic pain model) in experimental animals (Jain et al., 2009). In response to an injurious insult to a nerve, initial steps of inflammatory reactions, involve the release of pro-inflammatory mediators from the resident macrophages and the Schwann cells (Marchand et al., 2005). It is then followed by an infiltration with the inflammatory cells including the macrophages from the blood capillaries to an area adjacent to nerve lesion. It has been documented that the sustained activation of peripheral nociceptors leads to the hypersensitivity of the primary afferent neurons and central sensitization of the dorsal horn neurons (Woolf and Mannion, 1999). The experimental evidence suggested that, in TST condition, animals exhibit increased levels of nerve tissue superoxide anions; total calcium; tissue myeloperoxidase (MPO) activity along with significant histopathological changes. Myeloperoxidase is an enzyme released from neutrophils and is used as a specific marker of inflammation (Grisham et al., 1994). Neuropathic pain (including TST induced) has further been demonstrated to produce a rise in tissue total calcium levels (Cecile et al., 2005; Jain et al., 2009; Siau and Bennett, 2006). Calcium ion accumulation has been documented to trigger the secondary messengers i.e., activation of calcium binding protein (calpain, calmodulin etc) and calcium dependent kinase and phosphatase action, which undergo an auto-destruction including long term potentiation and neuronal hyper-excitation (Young, 1992). Calcium-induced activation of calpain has been reported to produce the axonal degeneration by degradation of the axonal cytoskeleton (Glass et al., 2002). Further calcium induced activation of calpain is demonstrated to be associated with generation of reactive oxygen species form mitochondria, therefore indicating a prominent role of calcium in tissue oxidative stress (Carriedo et al., 2000). Therefore, it looks quite evident that inflammation, increased calcium levels and oxidative stress together play a vital role in TST induced peripheral neuropathic pain which is also supported by histological studies (Fig. 38).
Chronic constriction injury of sciatic nerve (CCI) is another common method for induction of painful peripheral neuropathy (entrapment type of neuropathic pain model) in experimental animals (Muthuraman et al., 2008a) which clinically resembles to complex regional pain syndrome (CRPS). Nerve injury induced development of neuropathic pain is either sequential and/or collective function of cellular events. The primary steps are alteration of ion homeostasis (especially calcium ion concentration); release of pro-inflammatory mediators (i.e., myeloperoxidase and tumor necrosis factor-alpha etc) from the resident macrophages; Schwann cells followed by generation of free radical and enzymatic changes eventually leading to enhancement of the neuronal damage and progress of nociceptive pain sensation (Marchand et al., 2005; Li et al., 2011). It has also been demonstrated that sustained activation of peripheral nociceptive receptor leads to the sensitization of secondary neuron, dorsal horn neurons and central nervous system (Woolf and Mannion, 1999). CCI induced neuropathic pain has been demonstrated to produce a rise in tissue total calcium and free radical (Muthuraman and Singh, 2011; 2008a). As indicated above calcium ion accumulation has been reported to trigger the various secondary messengers i.e., MPO enzymes, inflammatory cytokine (TNF-α), activation of calcium binding protein, calcium dependent kinase & phosphatase and expression of toxic proteins leading to long term potentiation, long term depression and neuronal hyper-excitation (Muthuraman and Singh, 2011; Muthuraman et al., 2011; 2011a; 2008b; Young, 1992). Calcium-induced activation of calpains has been shown to be responsible for the axonal degeneration by alteration of stability of axonal cytoskeleton protein (Glass et al., 2002; Muthuraman and Singh, 2011; Muthuraman et al., 2008a). Therefore, CCI induced biochemical and other alternations noted in this study are in line with previous reports (Muthuraman and Singh, 2011; Muthuraman et al., 2011) (Fig. 38).

Vincristine has been widely used as a chemotherapeutic agent for the management of various cancer disorders including Hodgkin's disease (Villani et al., 2008). However, its clinical application has been limited due to unavoidable painful neuropathy. It possesses the property of high binding affinity towards neuronal cytoskeleton protein i.e., β-tubulin, subsequently it has been documented to cause disruption of microtubule polymerizations eventually leading to neurotoxicity as well as cancer preventive actions (Swain and Arezzo, 2008). Molecular mechanism of vincristine has also been reported to modulate the cellular Ca\textsuperscript{2+} levels; free radical generation; TNF-alpha expression and myeloperoxidase activation (Muthuraman et al., 2008a; Jaggi and Singh, 2010; Kesik et al., 2010; Muthuraman and Singh, 2011b). Crucially, axonal degeneration has been reported due to
these events, suggesting that cellular oxidant and inflammatory mediators play a key role in the pathogenesis of painful neuropathy (Muthuraman et al., 2008, 2008a; Siau and Bennett, 2006). Massive intracellular calcium accumulation has been implicated to play a pivotal role in neuronal and tissue injury in various types of neuropathic disorders such as post-traumatic; axotomy; anti HIV drugs; tibial sural transaction; chronic constriction injury; ischemic-reperfusion injury and vincristine induced neuropathy (Muthuraman et al., 2008a; Muthuraman et al., 2011; Muthuraman et al., 2010; Jaggi and Singh, 2010; 2011). Vincristine treatment in our study produced a rise in the levels of TNF-alpha; calcium (Ca^{2+}) ion; superoxide anion concentration as well as MPO activity, these observation supports the contention that release of inflammatory cytokines; calcium (Ca^{2+}) ions accumulation; free radical generations and resident immune cell activation play a key role in vincristine induced neuropathic pain (Fig. 39).

Treatment of hydroalcoholic extract (HAE-AC) and saponin rich extract (SAE-AC) of rhizome part of Acorus calamus and that of pregabalin (serving as positive standard) significantly ameliorated TST; CCI and vincristine induced behavioral; electrophysiological; biochemical as well as histopathological changes hence painful neuropathy. Acorus calamus has been reported to exert various beneficial pharmacological actions (Mittal et al., 2009). Ethanolic extract of Acorus calamus has been reported to exert immunosuppressive effects along with inhibition of nitric oxide, interleukin-2 and tumor necrosis factor-alpha productions (Mehrotra et al., 2003; Gilani et al., 2006). Acorus calamus is also reported to act by blocking voltage activated calcium channels (Mehrotra et al., 2003; Gilani et al., 2006; Muthuraman et al., 2011). Additionally, it has also shown to possess neuroprotective actions against the hypoxic event and chemically induced severe insult in the nervous system (Shukla et al., 2002, 2006). Moreover, ethanolic extract of Acorus calamus has also been demonstrated to possess potential anti-oxidative and anti-inflammatory actions (Shukla et al., 2006; Muthuraman et al., 2011; Muthuraman and Singh, 2011).

Acorus calamus has various phytochemical constituents such as saponins, glycosides, flavonoids, tannins, polyphenolic compounds, mucilage, volatile oil and bitter principles (Mittal et al., 2009; Raja et al., 2009). Saponins are glycosylated plant secondary metabolites found in food and many major medicinal plants. Saponin is one of the major constituents in hydroalcoholic extract of Acorus calamus (Mittal et al., 2009; Muthuraman and Singh, 2011). Saponins play a numerous functional and pathophysiological role in the biological systems such as immune responses (Zhai et al.,
2011), phagocytic action (Kang et al., 2008), anti-convulsant (Jalsrai et al., 2010), neuroprotective (Kaur et al., 2010). Triterpenoids saponins of Ocimum sanctum have been reported to produce anti-neuralgic effect (Kaur et al., 2010a). Acorus calamus is also known to possess the triterpenoids saponins (Raja et al., 2009). The saponin constituent of Acorus calamus has been reported to possess the neuropharmacological activities (Jayaraman et al., 2010; Parap and Mengi, 2003). Saponins in various studies have been shown to exert beneficial effect in relieving nociceptive as well as neuropathic pain (Kaur et al., 2010; Wang et al., 2008). Further, saponin rich extracts are also well demonstrated to process anti-oxidative; anti-inflammatory (Wang et al., 2008); anti-neuralgic (Kaur et al., 2010) effects in animal studies. Similarly, saponins have also been reported to possess free radical scavenging action in addition they also cause inhibition of calcium accumulation and reduction of TNF-α levels (Kaur et al., 2010; Shah et al., 2009). Saponin rich extract of AC in our study not only alleviated neuropathic pain but also exhibited significant anti-oxidative; anti-inflammatory and calcium inhibitory actions.

Therefore, with support from literature and data in hand it appears quite evident that Acorus calamus has ameliorative potential in neuropathic pain. The saponins may be the main constituents in Acorus calamus responsible for its beneficial effects and these effects are attributed to potential anti-oxidative, anti-inflammatory, neuroprotective and calcium inhibitory actions of Acorus calamus.

Pregabalin is a well-known agent being currently used clinically to manage neuropathic pain of various etiologies (Navarro et al., 2011; Plested et al., 2010; Pérez et al., 2010). Pregabalin mediated beneficial effects are proposed to be potentially mediated via inhibition of voltage gated calcium [Cav 2.2 (α2–δ subunit)] channels. In addition pregabalin has also been shown to possesses good anti-oxidative, anti-TNF-alpha as well as anti-inflammatory actions (Ha et al., 2011; Muthuraman and Singh, 2011, 2011a; Ha et al., 2008; Beyreuther et al., 2007). Therefore, pregabalin was selected as positive standard in this investigation.

Hence it is concluded that Acorus calamus has ameliorative potential in TST; CCI and vincristine induced painful peripheral neuropathy; and it can be of enormous value in conditions like painful neuropathy.
Fig. 38. Possible mechanism of hydroalcoholic extract (HAE-AC) and saponin rich extract of *Acorus calamus* (SRE-AC) mediated ameliorative effect in tibial & sural nerve transection (TST) and chronic constriction injury of sciatic nerve (CCI) induced painful peripheral neuropathy.
Fig. 39. Possible mechanism of hydroalcoholic extract (HAE-AC) and saponin rich extract of Acorus calamus (SRE-AC) mediated ameliorative effect in vincristine induced painful peripheral neuropathy.