V. DISCUSSION

Medicinal plants occupied an important position in the socio-cultural, spiritual and medicinal arena of rural people of India. According to the World Health Organization (WHO) the definition of traditional medicine may be summarized as the sum total of all the knowledge and practical, whether explicable or not, used in the diagnosis, prevention and elimination of physical, mental or social imbalance and relying exclusively on practical experience and observation handed down from generation to generation, whether verbally or in writing. Traditional medicine might also be considered as a solid amalgamation of dynamic medical known-how and ancestral experience. Interest in medicinal plants as a re-emerging health aid has been fuelled by the rising costs of prescription drugs in the maintenance of personal health, well-being and the bioprospecting of new plant-derived drugs. Based on current research and financial investments, medicinal plants will seemingly; continue to play an important role as a health aid. The demand on plant based therapeutics to increasing in both developing and developed counties due to the growing recognition that they are natural products, being non-narcotic, having no side-effects, easily available at affordable prices and sometimes the only source of health care available to the poor (Report of the Task Force on Conservation and Sustainable use of Medicinal Plants, Government of India Planning Commission, March - 2000, pp. 9).
Medicinal plants are an integral component of research developments in the pharmaceutical industry. Such research focuses on the isolation and direct use of active medicinal constituents, or on the development of semi synthetic drugs or still again on the active screening of natural products to yield synthetic pharmacologically-active compounds. The industrial uses of medicinal plants are many. These range from traditional medicines, herbal teas and health foods such as nutraceutical to galenicals, phytopharmaceuticals and industrially produced pharmaceuticals.

In recent times, there have been increased waves of interest in the field of research in natural products chemistry. This level of interest can be attributed to several factors, including unmet therapeutic needs, the remarkable diversity of both chemical structure and biological activities of naturally occurring secondary metabolites, the utility of novel bioactive natural products as biochemical probes, the development of novel and sensitive techniques to detect biologically active natural products, such as chromatographic techniques-TLC, column chromatography HPTLC, HPLC and GC etc. are invaluable for identifying and isolation of phytopharmaceuticals. Adsorption chromatography has proved particularly important in the isolation and purification of vitamins, hormones, many alkaloids, cardiac glycosides anthraquinones etc. Further, use of physical techniques to establish structures of new compounds and to identify known compounds in plant sources ultraviolet, infrared, mass and nuclear magnetic resonance spectroscopy together with X-ray crystallographic and optical rotatory dispersion methods.
have all played a significant role in these developments. Various modifications of mass spectrometry (MS) have become increasing for the structural characterization and determination of the active constituents of plants (Trease and Evans, 2005). These improved techniques to isolate, purify and structurally characterize active constituents (Soumya, 2009) are contributing significantly in solving the demand for supply of complex natural products (Clark, 1996). The R & D thrust in the pharmaceutical sector is focused on development of new innovative/indigenous plant based drugs through investigation from the traditional system of medicine (Patwardhan, 2004).

Plants produce a wide range of secondary compounds, also referred to as natural products, which may have important functions in the plants adaptation to specific ecological niches or its responses to biotic and abiotic stresses. Some of these secondary metabolites turn out to be beneficial for humans as pharmaceuticals. Because of their unique and often complex chemical structures, synthesis of these natural compounds is frequently unfeasible or not economically justified. The use of plants, plant extracts and plant-derived chemicals in the treatment of diseases, in supplementing foods and in making cosmetics is firmly rooted in the past and still developing. Many drugs used in contemporary medicine have been derived from plants and were originally discovered through the traditional use by indigenous people. Podophyllotoxin, vincristine, vinblastin, camptothecin, taxol, artemisinin, aspirin, atropine, ephedrine, quinine, reserpin and digoxin are well known examples of such drugs. Quite frequently a relatively low yield of active components and
difficulties in standardization are bottlenecks in medicinal plant exploitation. Efforts have been made worldwide to enhance the production of bioactive component using a biotechnology approach.

In India, Department of Biotechnology has launched a Bio-prospecting and Molecular Taxonomy programme which opens avenues for sustainable utilisation and bio-prospecting of medicinal plant genetic resources and commercialization of the drugs originated from the medicinal plants. However, the increasing need for phytochemicals as a safe alternative or an adjunct to modern medicine is seriously felt particularly due to the widely perceived biohazardous side effects of the synthetic drugs.

The Western Ghats of Karnataka is considered as the store house of medicinal plants. It harbors around 4,500 species of higher plants of which 450 species are threatened. Most of the herbal medicinal plants can be cultivated at various parts of the country. The ethnomedical claim of many endemic medicinal plants of this region have been supported by phytochemical and pharmacological research evidences. Extracts obtained from the plants like *Catharanthus roseus*, *Taxus baccata*, *Solanum khassiaum*, *Artemisia annua*, *Digitalis lanata*, *Commifora mukal*, *Cassia angustifolia*, etc. Many companies like CIPLA, Hindustan Lever Ltd., Dabur India Ltd., Himalayas drug House, Vimta Lab., Reddy’s Lab, Tamil Nadu Herbals Ltd., etc were actively engaged in the production of herbal drugs to cater the health needs of the country. In our laboratory also effort was made by the previous investigators on the isolation of bioactive compounds and pharmacological screening of endemic medicinal
plants of the Western Ghats namely, *Andrographis paniculata*, *Celastrus paniculatus*, *Embelia ribes*, *Celastrus paniculatus*, *Pterocarpus marsupium*, *Entada pursetha*, *Caesalpinia bonducella* etc., In the present investigation, sincere attempt was made on phytochemical pharmacological investigation on the medicinal tree *Semecarpus anacardium*

5.1. Phytochemical screening of stem bark of *S. anacardium*

Natural products have played an important role as new chemical entities (NCEs) approximately 28% of NCEs between 1981 and 2002 were natural products or natural product-derived. Another 20% of NCEs during this time period were considered natural product mimics, meaning that the synthetic compound was derived from the study of natural products (Newman *et al.*, 2003). Combining these categories, research on natural products accounts for approximately 48% of the NCEs reported from 1981–2002. Natural products provide a starting point for new synthetic compounds, with diverse structures and often with multiple stereocenters that can be challenging synthetically (Clarky and Walsh, 2004; Nicolaou and Snyder, 2004; Peterson and Overman, 2004; Koehn and Carter, 2005). Many structural features common to natural products (e.g. chiral centers, aromatic rings, complex ring systems, degree of molecule saturation, and number and ratio of hetero atoms) have been shown to be highly relevant to drug discovery efforts (Lee and Schneider, 2001; Feher and Schmidt, 2003; Clarky and Walsh, 2004; Piggott and Karuso, 2004; Koehn and Carter, 2005). Further more, drugs derived from medicinal plants can serve
not only as new drugs themselves but also as drug leads suitable for optimization by medicinal and synthetic chemists.

The review of phytochemical screening of *S. anacardium* has showed the presence of biflavonoids, phenolic compounds (Prakasa Rao *et al.*, 1973), bhilawanols, sterols and glycosides (Indap *et al.*, 1986; Nagabhushana *et al.*, 2002) and anacardic acid (Chattopadhyaya and Khare, 1969) in *S. anacardium* nuts. Drug distillation studies of the *S. anacardium* gave rise to catechol and a hydrocarbon (Premalatha, 2000) and monophenolic compounds known as semecarpol (C\textsubscript{17}H\textsubscript{28}O) have also been isolated. Vitamins and Linoleic, Myristic, Oleic, Palmitic and Stearic acids are present (Rai *et al.*, 2000). Vijayalakshmi *et al.* (2000) have reported the presence of carbohydrates, phenols and flavonoids in the milk extract, and it is non-toxic up to 2,000 mg/kg body weight. The presence of the above said compounds was confirmed by the TLC, HPLC and HPTLC analysis of the nut and milk extract (Shin *et al.*, 1999; Aravind *et al.*, 2008; Mythilypriya *et al.*, 2008; Sahoo *et al.*, 2008; Raveedran Nair *et al.*, 2009). On the basis of chemical and spectral data, several biflavonoids have been isolated from the alcoholic fraction of nut shells and characterized namely semecarpus flavanone, jeediflavanone, galluflavanone, nallaflavanone, semecarpitin and anacarduflavanone (Murthy 1992; Murthy, 1983 a; Murthy, 1985 a; Murthy, 1983 b; Murthy, 1983 c; Murthy, 1985 b). The earlier researchers phytochemically investigated the nut and nut milk of *S. anacardium* in the present study only the stem bark of this medicinal tree subjected for phytochemical screening.
In the phytochemical studies, the shade dried powdered stem bark of *S. anacardium* subjected to Soxhlet extraction with different solvents viz. petroleum ether, chloroform and methanol. The yield of petroleum ether and chloroform was very less. The methanol fraction was chromatographed over silica gel column, eluted with the four solvents namely (i) Methanol: Petroleum ether (ii) Methanol: Ethyl acetate (iii) Methanol: Butanol in the ratio of 2:8 of each and finally eluted with (iv) pure methanol. The elutents from column chromatography was monitored by TLC. The fraction C-2 eluted through the solvent system (ii) is an yellow amorphous compound and the yield was 825 mg/ 10g of the crude extract. The compound showed the positive results for Shinoda’s test, ferric chloride test, zinc hydrochloric acid, alkaline test, lead acetate test and it was found to be a flavonoid. The melting point of this compound was found to be 289°C and after subjecting of the compound for IR, $^1$HNMR, and mass spectral studies it was identified as amentoflavone. Ishratullah et al. (1977) also isolated amentoflavone from the leaves of *S. anacardium*. Amentoflavone is a biflavonoid isolated from various plants namely, *Selaginella tamariscina* (Gambhir *et al.*, 1987), *Cupressocyparis leylandii, Taxus baccata, Ginkgo biloba* (Krauz-Baranowska *et al.*, 1999; 2003), *Hypericum perforatum* (Nahrstedt *et al.*, 1997), *Byrsonima crassa, Chrozophora senegalensis, Ginkgo biloba, Taxodium mucronatum* (Xulin Pan, 2005), *Biophytum sensitivum, Arctostaphylos glandulosa* (Hiruma-Lima *et al.*, 2006), *Rheedia edulis* (Acuna *et al.*, 2008), *Semecarpus anacardium* nut and leaves (Ranjith *et al.*, 2010; Rajagopala; 2013), *Ochna pumila, Selaginella*
sinensis (Ma et al., 2001), Rhus succedanea and Garcinia multiflora (Lin et al., 1999), Rhus pyroides, Rhus dentate, Rhus pentheri (Svenningsen et al., 2006), Biophytum sensitivum (Guruvayoorappan et al., (2007), Viburnum lantana (Krzysztof Pawlak et al., 2010), Araucaria angustifolia (Santi-Gadelha et al., 2006). Therapeutic properties of amentoflavone was screened for antiviral activity (Lin et al., 1999; Ma et al., 2001), promote mutagenic activity (Cardoso et al., 2006), inhibit human cathepsin β (Pan et al., 2005), interact at GABA_A receptors (Reena et al., 2005), exhibit strong neuroprotection against cytotoxic insults induced by oxidative stress and amyloid β (Kang et al., 2005), inhibit NF-KB activation in macrophages (Woo et al., 2005), phospholipase C1 (Lee et al., 1996), cAMP-dependent phosphodiesterase (Saponara et al., 1998), release Ca^{2+} in skeletal muscle sarcoplasmic reticulum (Suzuki et al., 1999), anti-inflammatory activity (Gambhir et al., 1987; Kim et al., 1998), antioxidant activity (Huguet et al., 1990), antiplasmodial and leishmanicidal activities (Junert et al., 2008). In the present study amentoflavone was isolated from the stem bark methanol extract and its pharmacological properties have been for the following ailments using rat model.

5.2. Pharmacological evaluation of extracts and the constituent amentoflavone

Traditional healers have from time immemorial exploited the therapeutic properties of plants in traditional medicine. They have always known that a particular plant cures a disease because it contains a bioactive component or some agents responsible for its power to fight the diseases which they treat and
which may not be found in another plant. They however do not know the active components in the plant, the compound(s) that is/are responsible for its medicinal use. Secondary metabolites are sought after because they are known to exhibit numerous biological activities that promote positive health effects. These activities include antibacterial, anticancer, antifungal, and antioxidant that are utilized in the agricultural, food and pharmaceutical industries. As a consequence of these numerous applications, the world market for plant extracts and isolated secondary metabolites exceeds 10 billion US dollars annually (Oomah, 2003). The pharmacological value of plant secondary metabolites is increasing due to constant discoveries of their potential roles in health care and as lead chemicals for new drug development (Wink, 1999).

Although the use of natural products as medicinal agents presumably predates the first recorded history as the earliest humans used various, but specific plants to treat illness, the treatment of diseases with pure pharmaceutical agents is a relatively modern phenomenon. Nevertheless, the role of traditional medicine in the discovery of potent chemicals is quite crucial. Among some of the earliest successes in developing drugs from natural products, one can mention the isolation of the antimalarial agents such as the Cinchona tree alkaloids, pain relievers such as the morphine alkaloids as well as the development of aspirin, Quinine. Solvent extraction is usually used to recover a component from either a solid or liquid. The sample is contacted with a solvent that will dissolve the solutes of interest. Some extraction techniques involve partition between two immiscible liquids, others involve either
continuous extractions or batch extractions. During the present work, dried and pulverized plant materials are soaked in an organic solvent and sequentially extracted by using Soxhlet apparatus.

Natural products are naturally derived metabolites and/or byproducts from microorganisms, plants or animals (Baker et al., 2000). In the field of traditional medicine, natural products have been exploited for human use for thousands of years, and plants have been main source of compounds used for medicine. Traditional healers have from time immemorial exploited the therapeutic properties of plants in traditional medicine. They have always known that a particular plant treats a disease because it contains a bioactive component or some agents responsible for its power to fight the diseases which they treat and which may not be found in another plant. They however do not know the active components in the plant, the compound(s) that is/are responsible for its medicinal use. But they were capable to heal serious ailments in the light of their practical experiences. Nowadays, Indian Ayurveda gaining much importance because for some of the disorders allopathic medical care is incapable to prescribe a specific medical care. Hence, research is going on the rigorous pharmacological evaluation of medicinal plants and its constituents for various ailments. No doubt, the traditional healer is capable to cure various ailments by using combination of different plant extracts for the same ailment and also the same plant extract has been used to treat for various disorders. The therapeutic property of the medicinal plant parts is due to the presence of bioactive radicals which acted upon the disease causing organisms.
or the biomolecules which induced specific symptoms. The scientific mind cannot easily accept the ideology and practical experiences of the medicinal practitioners unless corroborated by preclinical and clinical evidences using various animal models.

Ayurveda describes *S. anacardium* as a potent drug against the variety of ailments and is popularly known as Ardha Vaidhya and commonly known as marking nut. The fruits and oil have been claimed to be highly efficacious in the treatment of neuritis, helmintic infection (Chattopadhyaya and Khare, 1969), rheumatic pain tract and certain dermatologic conditions (Nadkarni, 1954). It has beneficial action on heart, blood pressure, respiration and neurological disorders (Kurup et al., 1979, Raghunath and Mitra, 1982, Sharma et al., 1995).

The fruits of *S. anacardium* are claimed to be useful in treating leprosy, rheumatoid arthritis, piles, asthma and cough, sexually transmitted diseases such as syphilis and gonorrhoea, and skin diseases such as leucoderma (Nadkarni, 1976). The nuts are used for management of rheumatisim, wound healing, diabetes and urinary diseases. The seeds are eaten in certain regions of India and are considered nutritious. Several Ayurvedic preparations such as “Bhallataka rasayana”, “Amritha bhallataki” and “Brihat bhallataka lehya” are marketed in India (Sreenivasacharyulu, 1931).

In the present investigation, pharmacological parameters have been carried out by using rat models to authenticate the medicinal claim this tree species.
5.2.1. Wound healing activity

Wound healing is a complex and dynamic process of restoring cellular structures and tissue layers in damaged tissue as closely as possible to its normal state. Wound healing proceeds via an overlapping pattern of events including haemostasis, inflammation, proliferation, and tissue remodeling (Douglas and Alan, 2003). Tissue injury initiates a response that first clears the wound of devitalized tissue and foreign material, setting the stage for subsequent tissue healing and regeneration. The initial vascular response involves a brief and transient period of vasoconstriction and haemostasis. Around 5-10 minutes period of intense vasoconstriction is followed by active vasodilation accompanied by an increase in capillary permeability. Platelets aggregated within a fibrin clot secrete a variety of growth factors and cytokines that set the stage for an orderly series of events leading to tissue repair.

The second phase of wound healing, the inflammatory phase, presents itself as erythema, oedema, and warmth, and is often associated with pain. The inflammatory response increases vascular permeability, resulting in migration of neutrophils and monocytes into the surrounding tissue. The neutrophils engulf debris and microorganisms, providing the first line of defense against infection. Neutrophil migration ceases after the first few days post-injury if the wound is not contaminated. In the late inflammatory phase, monocytes converted in the tissue to macrophages, which digest and kill bacterial pathogens, scavenge tissue debris and destroy remaining neutrophils.
Macrophages begin the transition from wound inflammation to wound repair by secreting a variety of chemotactic and growth factors that stimulate cell migration, proliferation, and formation of the tissue matrix.

The subsequent proliferative phase is dominated by the formation of granulation tissue and epithelialization. Its duration is dependent on the size of the wound. Around the blood capillary loops is an area of rapidly dividing fibroblasts. This is known as granulation tissue. As the granulation tissue is beginning to form, the body utilizes another strategy in minimizing the risks posed by a wound i.e. the process of wound contraction. Contraction is thought to be mediated by myofibroblasts located around the edges of the wound. By a physical contractile process, the edges of the wound are brought closer together, at the same time stretching the normal skin beyond the wound edge. Chemotactic and growth factors released from platelets and macrophages stimulate the migration and activation of wound fibroblasts that produce a variety of substances essential to wound repair, including glycosaminoglycans (mainly hyaluronic acid, chondroitin-4-sulfate, dermatan sulfate, and heparan sulfate) and collagen (Stadelmann et al., 1998). Collagen is secreted into the intercellular matrix, where it undergoes maturation (cross-linking and coiling) into strong fibers oriented so as to allow stretchability while providing tensile strength. Collagen levels rise continually for approximately three weeks. The amount of collagen secreted during this period determines the tensile strength of the wound. The final phase of wound healing is wound remodeling,
including a reorganization of new collagen fibers, forming a more organized lattice structure that progressively continues to increase wound tensile strength.

This investigation presented a multiscale modeling framework which allows to analyze the effects of different factors on wound healing, such as contraction of cutaneous wound, its tensile strength, collagen alignment, the hydroxyproline content and the strength of granuloma tissue and scar formation during dermal wound healing. Therefore, in order to examine the above mentioned parameters, three different types of wounds were inflicted on the experimental rats to assess the healing efficacy of various extracts and the isolated constituents of *S. anacardium* stem bark. The standard drug Framycetin is used as a standard reference to assess the healing effect of the drug and the constituents against the controls.

Ethnomedicinally, the roots of *S. anacardium* has been used to heal the wound in Jalgaon District of Maharashtra State, India (Chopda et al., 2009). The results of the present study clearly indicated that methanolic extract enhanced healing of all the three types of cutaneous wounds. Application of ointment base prepared from methanolic extract displayed significant wound healing activity. The healing time required for complete epithelialization of the excision wound was found much earlier (16th post wounding day) and it was on par with that of the standard reference drug Framycetin.

Significant wound healing activity was observed in all the group of animals treated with 5%, 10% and 20% methanol extract ointment, respectively. The percentage of closure of wound was significant in the animals
treated with methanol extract (100 mg/kg ointment) 83.17±5.12 on day 12th and 94.00±2.61 on day 16th, respectively. While in control group animals the duration of epithelialization was delayed by 4 days, it was only 57.87±3.20 and 83.67±4.21, respectively.

The newly formed tissue is known as granulation tissue and the conversion of granulation tissue into fibrous scar tissue is known as cicatrization. The breaking strength of the granulation tissue increases proportionately with the collagen deposition. The tensile strength of the wound is determined by the rate of collagen synthesis and maturation process involving inter and intramolecular crosslinking of collagen fibrils. The breaking strength is the tensile strength of a healing wound and it can be measured practically by the minimum amount of force required to disarticulate it. In the beginning a wound will be having little breaking strength because the clot alone will be holding the edges together. Thereafter breaking strength increases proportionately as collagen deposition increases and cross linkages are formed between collagen fibers. By the 8th to 10th day there is sufficient restoration of breaking strength and stitches can be removed.

A linear resutured incision model was also employed in the present study and the wound breaking strength was determined on 10th post wounding day. By treating the wounded animals with either methanolic extract and we observed consistent and significant stimulation of wound healing. The tensile strength of the resutured incision wound was increased significantly in methanolic extract treated groups in dose depend manner. The tensile strength
of the incision wound was significantly increased in the animals treated with methanol extract (20% ointment) but lesser than the reference drug Framycetin. A moderate gain in tensile strength was observed in (5% ointment) and (10% ointment) treated animals and it was insignificant in control groups.

Dead space wound model provides an opportunity to study the effect on the granulation and collagenation of the healing process. Such wound models have been employed for the quantitative studies on wound healing such as granulation tissue breaking strength and hydroxyproline content (Patil and Kulkarni, 1984).

Granulation tissue formed in the final part of the proliferative phase is primarily composed of fibroblasts, collagen, edema, and new small blood vessels. Collagen is the major component strengthens and supports extra cellular tissue. The increase in the granulation tissue weight suggests higher protein content (Azad, 2002) and increase in the hydroxyproline content of the granulation tissue indicating increased collagen turnover (Gupta and Gupta, 1985), and its measurement could be used as an index for collagen turnover (Madhura and Sushma, 2003). Gain in granuloma breaking strength indicates increased collagen maturation by increased cross-linking. Collagen mainly composed of the amino acid, hydroxyproline, and so the estimation of hydroxyproline content gives the net rate of synthesis and deposition of collagen in healing wound (Kumar et al., 2006).

The presence of foreign body in the subcutaneous area initiates the formation of granulation tissue around it. Initially new blood vessels are
formed accompanied by lymphatics. These arise from the preserved lymphatics at the margins of the wound. In the initial three days of the injury the intercellular spaces are filled with proteinaceous fluid. Later the fluid becomes gelatinous and shows increasing quantities of mucopolysaccharides which are either produced locally by fibroblasts or mast cells or come from the blood. The intercellular fibers are laid down in the wound fluid from the 4th day onwards and the concentration of mucopolysaccharides starts declining. At first these are fine thread like, later they coarsen and thicken. In the beginning, collagen fibers run parallel in one plane but soon their arrangement are remodeled to suit local mechanical stresses. Finally it forms a tough membrane of laminated collagen, which is the essential material for healing of the wound. By this time fibroblasts decrease in number and appear as shrunken in conspicuous fusiform cells in between rows of collagen fibers. The concentration of mucopolysaccharides becomes normal or even low. The wound has now acquired significant tensile strength (Somen Das, 2001).

The effect of oral administration of the *S. anacardium* methanol extract on dead space wound model was evaluated by assessing the weight of granulation tissue, by its tensile strength and hydroxyproline content of the granulation tissue. Methanolic extract significantly augmented the breaking strength of granulation tissue harvested on the 8th day, increased the weight of granulation tissue and hydroxyproline content of the tissue. This may be due to the enhanced collagen maturation by increased cross-linking of collagen fibers.
The increased weight of both wet and dry granulation tissue also revealed the presence of higher hydroxyproline content.

Histological inspection of the granulation tissue showed the infiltration of fibroblasts and monocytes in the subcuticle was significantly greater in the untreated animals. Furthermore, there was significantly decreased the epithelialization and lesser collagen regeneration, indicates the incomplete wound healing. The sections of the granulation tissue harvested from the animals supplemented with the methanol extract and its constituents, provide further evidences on their wound healing efficacy. The sections of granulation tissue obtained from methanol extract treated animals showed complete epithelialization, fibrosis, few macrophages and significantly increased collagen formation and as compare to the control few macrophages were noticed. This indicates the potent wound healing property of the extracts and the constituents.

The effect of oral administration of the *S. anacardium* methanol extract on dead space wound model was assessed by increase in the weight of granulation tissue and increase in its tensile strength. Histology of the wound tissue of the control animals showed the presence of acute inflammatory cells, fibroblastic connective tissue and very little number of blood vessels. The lesser epithelialization and lesser collagen formation indicated incomplete healing of the wound in control animals, whereas in the sections of Framycetin-treated animals showed increased collagen deposition. The sections of the granulation tissue of the animals treated with 50 mg/kg and 75 mg/kg/b.w. of
methanol extract showed moderate epithelialization, fibrosis and collagen formation. In 100 mg/kg methanol extract treated animals, the histology of granulation tissue showed complete healing with more number of fibroblasts within marked increase of collagen tissue and increased number of blood vessels. Many investigators used this model to evaluate the healing property of the indigenous medicinal plants namely, *Elephantopus scaber* (Singh et al., 2005), *Vernonia arborea* (Manjunatha et al., 2005); *Catharanthus roseus* (Nayak and Lexley, 2006), *Allamanda cathartica* L., *Laurus nobilis* L. (Nayak et al., 2006), *Leucas hirta* (Manjunatha et al., 2006) etc.

In our laboratory, Kumara Swamy et al. (2007) reported the wound healing activity of embelin isolated from the ethanol extract of leaves of *Embelia ribes*. The percentage of wound closer on 16th day and the period of complete epithelialization after the topical application of ethanolic extract of *E. ribes* and the constituent embelin on excision wound model was 97% (18.67) and 98.5% (18.17) respectively. In incision wound model, the measurement skin breaking strength for the extract and embelin was 495 and 528 g respectively. They compared these results with the standard reference drug Framycetin. The effect of ethanolic extract and embelin on tissue tensile strength in dead space wound model was 501.67 g and 81.00 g respectively. The dry weight of granulation tissue was significantly increased and for ethanolic extract and embelin it was 70.17 and 81 mg/100g of tissue respectively.
In the present study also, the methanol extract of S. anacardium was found to be the significant in healing of all three wound models. The percentage of wound closer and period of epithelialization for methanol extracts was 94.00% (16th days) respectively. The skin braking strength in incision model was significantly increased to 501.67g respectively. The effect of oral administration of methanol extract on dry weight and tensile strength of granulation tissue was (73.09 mg/100g).

5.2.2. Antioxidant activity

Free radicals have created significant interest among scientists in the past decade. Their broad range of effects in biological systems has drawn the attention of many experimental works. It has been proved that these mechanisms may be important in the pathogenesis of certain diseases and ageing. There are many reports that support the use of antioxidant supplementation in reducing the level of oxidative stress and in slowing or preventing the development of complications associated with diseases (Rose, 1982). Many synthetic antioxidant components have shown toxic and/or mutagenic effects, which have shifted the attention towards the naturally occurring antioxidants. Numerous plant constituents have proven to show free radical scavenging or antioxidants activity (Aruoma, 1997).
i. *In vitro* Antioxidant activity

a. DPPH Radical Scavenging Activity

The present study demonstrated that, DPPH is a free radical, stable at room temperature, which produces a purple colour solution in methanol. It is reduced in the presence of an antioxidant molecule, giving rise to uncoloured methanol solutions. DPPH radical scavenging activities were increased with an increased content of total phenolics. The methanol extract of *S. anacardium* showed strong inhibition activities and positively correlated with total phenolic content. Sirwardhana *et al.* (2003) have also reported higher DPPH scavenging activities for a water and methanol extract of *Hizikia fusiformis* (a brown alga), But, isolated constituent amentoflavone showed lesser DPPH scavenging activities, similar to the report of Nabaweya *et al.* (2007) with 15.53 % of inhibition of DPPH scavenging activity. Therefore, the present work showed significant activity in methanol extract against DPPH scavenging, when compared standard ascorbic acid.

b. Superoxide anion scavenging activity

The superoxide anion derived from dissolved oxygen by Phenazine methosulphate/NADH coupling reaction reduces nitro blue tetrazolium. The decrease the absorbance at 560 nm with the plant extract thus indicates the consumption of superoxide anion in the reaction mixture. In the PMS/NADHNBT system, superoxide anion derived from dissolved oxygen by PMS/NADH coupling reaction reduces NBT. The decrease of absorbance at 560 nm with antioxidants thus indicates the consumption of superoxide anion.
in the reaction mixture. The methanolic extract and the constituent amentoflavone of *S. anacardium* showed decrease in absorbance indicating potent superoxide anion activity than BHA (standard).

**ii. In vivo antioxidant activity**

Many investigators demonstrated that the development of toxin-induced chronic liver injury in rats by administering potent hepatotoxin CCl$_4$. The chronic liver injury is a result of prolonged process with persistent hepatic injury due to various causes. A period of seven successive days of CCl$_4$ administration was based on our previous study by Krishna *et al.* (2005). The CCl$_4$ injected is readily converted into trichloromethyl radical (CCl$_3^-$) and by reacting with oxygen molecules to form trichloromethyl peroxy radical (CCl$_3$OO$^-$) (Zhu and Fung, 2000). These free radicals cause hepatocellular damage by reacting with cellular molecules through the initiation of lipid peroxidation. The toxic effect of CCl$_4$ is due to free radical generation (CCl$_3^-$), which leads to the formation of lipid peroxides, which in turn give products like malondialdehyde (MDA) that cause damage to the membrane. This lipid peroxidative degradation of biomembranes is one of the principal causes of hepatotoxicity of CCl$_4$ (Cotran *et al.*, 1994; Kaplowitz *et al.*, 1986), by encouraging the auto-oxidation of the fatty acids present in the cytoplasmic membrane phospholipids and causes functional and morphological changes in the cell membrane, thus altering the permeability of the liver cell membranes (Handa and Sharma, 1990). This is evidenced by an elevation in the serum
marker enzymes namely AST and ALT after CCl₄ administration in experimental rats. The rise in serum levels of AST and ALT has been attributed to the damaged structural integrity of the liver because these are cytoplasmic in location and are released into circulation after cellular damage (Ahmed and Khater, 2001). This suggests disturbance in the transport function of hepatocytes resulting in leakage of enzymes from cells due to altered permeability of membranes.

In the present investigation, it was observed that the levels of the serum marker enzymes was significantly increased in the animals treated with CCl₄. Concomitant administration of the methanolic extract with CCl₄ showed significant reduction in the serum enzyme levels. While the animals supplemented with amentoflavone displayed reduction in the levels of serum enzymes up to some extent. This effect was comparable to that of the standard drug silymarin.

ALP is a membrane bound glycoprotein enzyme and has been shown using histochemical techniques to be present in high concentration in the sinusoids and the endothelium of the central and periportal veins (Aruna et al., 2002). It has been reported that ALP involved in the transport of metabolites across the cell membranes, protein synthesis, synthesis of certain enzymes, secretory activities and glycogen metabolism. Thus the rise in serum ALP activity in rats induced with CCl₄ damaged may be due to a disturbance in the secretory activity or in the transport of metabolites or may be due to altered synthesis of certain enzymes as in the other hepatotoxic conditions. The same
observation, as in case of AST and ALT, was noticed. The level of the serum ALP was significantly increased in the animals treated with CCl₄ alone. On the contrary, concomitant administration of the methanolic extract and its constituent, amentoflavone with CCl₄ showed significant restoration in the elevated level of serum ALP. This effect was comparable to that of the standard drug silymarin.

Jaundice refers to the yellow coloration of the skin, conjunctiva of the eyes and mucous membranes caused by hyperbilirubinemia. Bilirubin is produced by the normal breakdown of red blood cells. Normally bilirubin passes through the liver and is excreted as bile through the intestines. Jaundice occurs when bilirubin builds up faster than the liver can break it down and pass it from the body. The causes of hepatic jaundice include acute hepatitis, hepatotoxicity, biliary cirrhosis and alcoholic liver disease, whereby cell necrosis reduces the liver’s ability to metabolize bilirubin and the excess bilirubin regurgitated back to the blood. The increase in the levels of serum bilirubin reflected the depth of jaundice. Measurement of serum bilirubin has been reported to be sensitive indicator of liver injury (Molander et al., 1955). Therefore in the present investigation the CCl₄ intoxicated rats displayed hyperbilirubinemia, a common sign of hepatic jaundice. In the meanwhile, decrease in serum bilirubin after treatment with the methanolic extract in liver damage indicated the effectiveness of the extract in normal functional status of the liver. The result of animals pretreated with amentoflavone showed
reduction in the serum level of bilirubin less equal to that of CCl₄ group of animals.

After CCl₄ is injected, it is readily converted into CCl₃⁺ radical by the activity of cytochrome P₄₅₀ oxygenase system of the endoplasmic reticulum (McCay et al., 1984; Nelson and Harrison, 1987). This leads to prominent disintegration of the rough endoplasmic reticulum and polyribosomes in livers of CCl₄ intoxicated rats. Protein synthesis is a function of the ribosomes and the associated membranes of the endoplasmic reticulum. The striking dilation and swelling of the rough endoplasmic reticulum and dislocation of the ribosomal particles from the membranes of the rough endoplasmic reticulum in liver cells of rats treated with CCl₄ was noted by a profound investigation of Edward et al. (1962). Since the rough endoplasmic reticulum is imperative in protein synthesis, the alterations produced by carbon tetrachloride suggested that there might be a defect in protein synthesis process (Smuckler et al., 1961). In general, the formation of polysomes is an essential prerequisite for protein synthesis and the defect in the both, rough endoplasmic reticulum and polyribosomes causes the diminished protein synthesis in hepatocytes. The CCl₃⁺ formed in turn reacts with nucleotides, nucleic acids and amino acids (Weber et al., 2003) in hepatocytes causing further impairment in transcription and translation processes leads to complete obstruction of hepatic protein synthesis. Therefore, the characteristic changes in the levels of serum proteins occur in CCl₄ hepatotoxicity and their estimation serves as an important index in the diagnosis of liver diseases (Kagan, 1943 and Henry, 1986).
In CCl₄ treated animals total protein level was decreased due to the alteration of albumin:globulin ratio. The serum albumin level was slightly decreased, while the globulin level was moderately increased. Administration of rats with methanol extract and its isolated constituent of *S. anacardium* elicited immediate recovery in the levels of serum total protein. Stimulation of protein synthesis has been advanced as a contributory hepatoprotective mechanism, which accelerates the regeneration process and the production of liver cells (Awang, 1993). This probably, reveals the immediate recovery and regeneration of polysomes and endoplasmic reticulum membranes, thus emphasizing the crucial importance of antioxidants in protection of liver. The methanol extract was found to be most potential in maintaining the normal concentration of total protein as its effect was greater than the standard reference drug silymarin. But the observations in the serum total protein levels of animals treated with amentoflavone indicate the impotency of these tested compounds in protection of CCl₄ hepatic damage.

Krishna and Shanthamma (2004) reported the hepatoprotective activity of root extracts of *Boerhaavia erecta*. The 50% ethanol extract at the dose of 100 mg/100g body weight (b.w) significantly reversed the toxicity induced by the CCl₄. It was evidenced by the decreased level of serum bilirubin (1.35 mg/dl), significantly elevated concentration of total proteins (6.68 mg/dl) in serum and the depletion in the levels of serum markers such as, AST (11.53 IU/L), ALT (13.82 IU/L) and ALP (12.73 IU/L).
In the present study, the administration of methanol extract of stem bark of *S. anacardium* protect the liver from the toxic effects of CCl₄ by restoring the levels of total bilirubin (1.27 mg/dl), serum total protein (6.62 gm %) and subsequent decrease in the levels of serum hepatic enzymes, like AST (188.76 IU/L), ALT (122.74 IU/L) and ALP (172.07 IU/L) and isolated constituent amentoflavone exhibit, total bilirubin (2.32 mg/dl), serum total protein (6.48 gm %) and subsequent decrease in the levels of serum hepatic enzymes, like AST (214.66 IU/L), ALT (208.81 IU/L) and ALP (261.34 IU/L)) The hepatoprotective activity of methanolic extract may probably due to the presence of amentoflavone. These constituents exhibited significant hepatoprotective activity at the dose of 50 mg/kg b.w.

When compare to the works on different medicinal plants mentioned above, the work carried out by Krishna *et al.* (2005) on *Diospyros cordifolia* reported more hepatoprotective activity, since it’s petroleum ether extract at the dose of 25 mg/kg body weight showed very significant protection. Whereas the methanolic extract of stem bark of *S. anacardium* and its amentoflavone, displayed the significant hepatoprotection at the doses of 250, 500 and each 50 mg/kg body weight respectively. This justifies the frequently use of *S. anacardium* bark in jaundice and other liver related diseases by the traditional healers.

Although, serum AST, ALT and ALP activities are used as indicators for liver injury, the elevations of these enzymes in the serum are not specific to liver damage. For example, elevated serum AST activity has also been
observed during myocardial damage (Nekrassova, 1963). An additional parameter is required to confirm the presence of liver injury. Several studies have demonstrated or suggested that the pathological effects of CCl₄ are mediated by induction of oxidative stress (Cotran et al., 1994; Kaplowitz et al., 1986). Among the various mechanisms involved in hepatotoxic effect of carbon tetrachloride, one is oxidative damage through free radical generation and antioxidant property is claimed to be one of the mechanism of hepatoprotective effect of indigenous substances (De Leve and Kaplowitz, 1995; Farrel, 1998). They can react with reactive lipids including cholesterol, unsaturated fatty acids, and glycolipids, leading to lipid peroxidation (Girotti, 1998). Lipid peroxides are commonly found in oxidative stress-mediated liver injury (Jayatilleke and Shaw, 1998; Sadrzadeh et al., 1994). In order to probe the possible mechanism by which active constituents prevent hepatic damage caused by CCl₄ and to examine the presence of oxidative stress in CCl₄ treated rat livers, investigation on lipid peroxidation and glutathione were carried in the liver homogenate. CCl₄ is capable of generating highly reactive free radicals, inhibiting glutathione (GSH) synthesis, producing glutathione loss from the tissue, increasing malondialdehyde (MDA) levels and impairing antioxidant defense systems in humans and experimental animals.

Glutathione (GSH) content in the liver plays a primary role in the protection against trichloromethyl radical-induced liver damage (Recknagel et al., 1989; Campo et al., 2001). GSH is widely distributed in cells and is an important constituent of intracellular protective mechanisms against a number
of noxious stimuli, including oxidative stress. It is an intra-cellular reductant and plays major role in catalysis, metabolism and transport. It protects cells against free radicals, peroxides and other toxic compounds. Indeed, GSH depletion increases the sensitivity of cells to various aggressions and also has several metabolic effects, for example, a decrease in the rate of gluconeogenesis or an increase in glycogenolysis. The concept of a glutathione-SH threshold for drug detoxification was discussed by Jollow (1980). GSH is a naturally occurring substance that is abundant in many living creatures. It is widely known that a deficiency of GSH within living organisms can lead to tissue disorder and injury. For example, liver injury included by consuming alcohol or by taking drugs like acetaminophen, lung injury by smoking and muscle injury by intense physical activity are known to be correlated with low tissue levels of GSH (Leeuwenburgh and Ji, 1995). From this point of view, sufficiently high levels of GSH levels should be there in the cells to prevent tissue from disorders and injuries caused by the free radicals. The present study, we have demonstrated the effectiveness of methanolic extract and its constituents amentoflavone, by using CCl₄ induced rats, which is known model for both hepatic GSH depletion and injury.

GSH is a non-enzymic mode of defense against free radicals (Tripathi and Sharma, 1998). The GSH antioxidant system consists of an array of non-enzymic reaction pathways involving the neutralization of free radical species. Perturbation of the GSH status of a biological system has been reported to cause serious consequences (Sreepriya et al., 2001). Glutathione peroxidase
utilizes GSH for the decomposition of lipid hydroperoxides and other reactive oxygen species (ROS) and glutathione-S-transferase maximizes the conjugation of free radicals and various lipid hydroperoxides to GSH to form water-soluble products that can be easily excreted out (Ahmed et al., 2000). It has been suggested that the lipid peroxidases generated after CCl₄ intoxication is eliminated by glutathione peroxidase in the presence of glutathione, thus curbing the propagation of lipid peroxidation (Recknagel et al., 1989). The present study found a significant decrease in hepatic glutathione levels following CCl₄ exposure. However, our results found that the hepatic glutathione was higher in rats receiving either methanol extract or silymarin than in rats administered with CCl₄ alone. The GSH levels in CCl₄ administered rats (group II) and recovery to near normalcy in animals treated with methanol extract and its constituent amentoflavone revealed that oxidative stress elicited by CCl₄ intoxication has been nullified due to the antioxidant effect of these tested compounds.

The discovery (Packer et al., 1978), that CC1₃⁻ reacts very rapidly with molecular O₂ to give the much more reactive CC1₃OO species allows the interpretation that it is this peroxyl species that initiates lipid peroxidation, whilst CC1₃⁻ is largely responsible for covalent binding (Slater, 1982). Subsequent studies showed that CC1₃OO reacts very rapidly with polyunsaturated fatty acids to initiate lipid peroxidation (Forni et al., 1983). Malondialdehyde (MDA) formed as a result of lipid peroxidation induced by CCl₄ in liver, is an important parameter to assess the oxidative stress generated
in liver. In the present investigation, a significant elevation in the levels of end product of lipid peroxidation, MDA in the liver of rats treated with CCl₄ was observed when compared to normal control. The increase in MDA levels in liver suggests enhanced lipid peroxidation leading to tissue damage and failure of antioxidant defense mechanisms. It is of interest to note, therefore, that constituent amentoflavone inhibits CCl₄-mediated lipid peroxidation. Pretreatment with methanol extract as well as silymarin significantly (P<0.01) reversed toxic effect caused by CCl₄. Treatment with methanol extract and its constituent amentoflavone, significantly prevented these changes. Hence, the mechanism of hepatoprotection is due to the antioxidant effect.

5.2.3. Antidiabetic activity

Numerous animal models have been developed for the past few decades for studying diabetes mellitus and testing anti-diabetic agents that include chemical, surgical and genetic manipulations (Srinivasan et al., 2007; Etuk, 2010). Alloxan and streptozotocin are the prominent diabetogenic chemical compounds in experimental diabetes research. Both compounds are cytotoxic glucose analogues. But the mechanisms of cytotoxic action of the two compounds are different. Alloxan is a well-known diabetogenic agent that is used to induce Type I diabetes and streptozotocin induced Type 2 Diabetes mellitus in experimental animals (Nurten et al., 2011).

Alloxan induces a multiphasic blood glucose response when injected into the experimental animals, which is accompanied by corresponding inverse changes in the plasma insulin concentration followed by sequential
ultrastructural beta cell changes ultimately leading to necrotic cell death. The first phase that comes into view within the first minutes after alloxan injection is transient hypoglycemic phase that lasts maximally for 30 minutes (Lenzen, 2008; Wrenshall et al., 1950). The hypoglycemic response has been noted to be the result of a transient stimulation of insulin secretion that was confirmed by an increase of the plasma insulin concentration (Kliber et al., 1996). The underlying mechanism of this transient hyperinsulinemia may be attributed to a temporary increase in ATP availability due to inhibition of glucose phosphorylation through glucokinase inhibition. The 2\textsuperscript{nd} phase appearing one hour after administration of alloxan leads to rise in blood glucose concentration. Moreover, the plasma insulin concentration has been noted to decrease at the same time. This is the first hyperglycemic phase after the first contact of the pancreatic beta cells with the toxin (Lenzen, 2008; Goldner et al., 1944; Tasaka et al., 1988; West et al., 1996). This hyperglycemic phase lasts for 2-4 hours which is accompanied by decreased plasma insulin concentrations. These changes are a result of inhibition of insulin secretion from the pancreatic beta cells that is attributed to the induction due to their beta cell toxicity.

The 3\textsuperscript{rd} phase is again a hypoglycemic phase that is noted 4-8 hours after the alloxan injection, which lasts for several hours (Lenzen, 2008; Tasaka et al., 1988; West et al., 1996; Jacobs et al., 1937). The flooding of circulation with insulin occurs as a result of the alloxan-induced secretory granule and cell membrane rupture resulting in severe transitional hypoglycemia (Banerjee,
1945; Banerjee and Bhattacharya, 1948). In addition, other subcellular organelles are also ruptured that include cisternae of rough endoplasmic reticulum and the golgi complex. Moreover, the outer and inner membranes of the mitochondria loose structural integrity in this particular phase (Jorns et al., 1997; Mythili et al., 2004). These changes are irreversible and highly characteristic for a necrotic cell death of pancreatic islets. The last and the 4th phase of the blood glucose response is the final permanent diabetic hyperglycemic phase during which complete degranulation and loss of the integrity of the beta cells within 24-48 h after administration of the alloxan takes place (Lenzen, 2008; Mythili et al., 2004). Surprisingly, the non-beta cells and other endocrine and non-endocrine islet cell types along with extra pancreatic parenchyma remain intact, providing the evidence of selective toxic action of alloxan. Thus, alloxan injection has been noted to induce an insulin-dependent type I like diabetes syndrome and all the morphological features of beta cell destruction are characteristic for a necrotic cell death (Lenzen, 2008; Mythili et al., 2004; Lenzen et al., 1996; Peschke et al., 2000). Administration of methanol extract and amentoflavone at doses (250, mg, 500 mg and 50 mg/kg/b.w) caused significant (p < 0.01) decrease in serum glucose levels at the end of the study. Of the two doses, methanol extract of 500 mg/kg showed maximum decrease which was comparable to glibenclamide. Glibenclamide is often used as an insulin stimulant in many studies and also used as a standard antidiabetic drug in alloxan-induced moderate diabetes to compare the antidiabetic properties of a variety of hypoglycemic compounds.
(Bhatia et al., 1970). Ali et al. (2012) evaluated the antidiabetic effects of ethanolic extract of *Semecarpus anacardium* (SA) stem barks in normal and alloxan induced diabetes rats. Metformin (150 mg/kg) was used as reference drugs for comparison. The phytochemical screening of the plant *S. anacardium* stem bark showed the presence of steroids, triterpenoids, flavonoids, glycosides, saponins and tannins. However a glucose tolerance hypoglycemic test is comparable to diabetic control group and effect is a dose dependent.

Kothai et al. (2005) reported the ethanolic extract of dried nuts of *S. anacardium* on blood glucose level in both normal and alloxan induced diabetic rats. The blood glucose levels were measured at 0, 1, 2 and 3 hours after the treatment. The ethanolic extract of *S. anacardium* (100 mg/kg) reduced the blood glucose of normal rat from 84±1.4 to 67±1.7 mg/dl, 3 hours after oral administration of the extract (P < 0.05). It also significantly lowered blood glucose level in alloxan induced diabetic rat from 325±2.2 to 144±1.4 mg/dl, 3 hours after oral administration of the extract.

Goutam Brahmacari (2001) reported the flavonoid compounds namely, luteolin 7-O-glucoside, luteolin and amentoflavone evaluated their effect in reducing the blood glucose levels by inhibiting the absorption of glucose in intestinal brush border or flavonoids may act on tyrosine phosphorylation of the insulin receptor and insulin receptor substrate to mimic the insulin.

In the present study, results was obtained after 5 h supplementation of amentoflavone (130.23±1.26) from methanol stem bark extract of *S. anacardium* showed significant diminution of fasting blood glucose level in
respect to diabetic rat (298.86±1.6). Moderate significant alteration of fasting blood glucose level was observed in different concentration (250 mg/kg and 500 mg/kg) of methanol extract (152.43±1.27, 119.29±1.51) supporting the Ali et al. (2012) reported which revealed the ethanol extract of the stem bark of S. anacardium exhibited significant activity at 200 mg/kg (55%) and 400 mg.kg (68%).

5.2.4. Antipyretic activity

Antipyretics are the agents which reduce the elevated body temperature. Yeast-induced pyrexia is called pathogenic fever and its etiology involves production of prostaglandins, which set the thermoregulatory centre at a lower temperature. The production of prostaglandins, mainly the most potent pyretic agent, PGE_{2} appears to be a final pathway responsible for fever production induced by several pyrogens. The antipyretic activity is generally exhibited as one of the properties of non-steroidal anti-inflammatory drugs, resulting from their inhibitory effect on prostaglandin biosynthesis in the central nervous system (Howard, 1993).

Most of the antipyretic drugs inhibit COX-2 expression to reduce the elevated body temperature by inhibiting PGE2 biosynthesis (Cheng et al., 2005). These synthetic agents irreversibly inhibit COX-2 with a high selectivity and are toxic to the hepatic cells, glomeruli, cortex of brain, and heart muscles. Natural COX-2 inhibitors have lower selectivity with fewer side effects (Cheng et al., 2005).
Both the methanol extracts and its isolated amentoflavone of *S. anacardium* exhibited significant (p < 0.05) antipyretic activity in yeast-induced elevation in body temperature in rats and the effects are comparable to the reference antipyretic drug (paracetamol). The methanol extract was found to be slightly potent than the amentoflavone. It appears that the observed antipyretic activity of *S. anacardium* may be due to inhibition of prostaglandin synthesis. Again the extracts contain flavonoids and saponins, the antipyretic potential of which have been reported in various studies (Martinez-Vazquez *et al.*, 1996; Singh *et al.*, 2000; Reanmongkol *et al.*, 2007). Therefore, the activity may be due to presence of the above group of phytoconstituents in *S. anacardium*.

5.2.5. Analgesic activity

In the present investigation, petroleum ether, chloroform and methanol crude extracts of *S. anacardium* were evaluated for their analgesic activity by the tail-flick and abdominal writhing methods to determine the therapeutic efficacy of *S. anacardium*. Tail-flick and writhing methods are the most common tests for evaluating the analgesic efficacy of drugs/compound/crude extracts in rodents. The abdominal constriction response induced by glacial acetic acid is a sensitive procedure to establish peripherally acting analgesics. This response is thought to involve local peritoneal receptors. We used the tail-flick and the writhing method to assess of the analgesic activity. The methanol extract of *S. anacardium* possessed a significant analgesic activity by
increasing the delayed onset of writhing and decrease of the number of writhes as compared with the control group. However, the petroleum ether and chloroform extracts showed a moderate activity. On the other hand, the hot tail-flick method is shown here as incapable of differentiating between opiate and non-opiate analgesics, or between peripherally acting and centrally acting substances. In the report of Jansen and Prast (1988), stated that nalorphine did not antagonize the effect caused by alkaloid from *Mitragyna speciosa*. Consistent analgesic activity was elicited by all the three *S. anacardium* extracts by both the methods used in the present study. The methanol extract showed consistent results than the petroleum ether and chloroform extracts. But, this was less effective than the standard reference acetyl salicylic acid.

The model which evaluates peripheral antinociceptive (analgesic) activity is very sensitive and able to detect analgesic effects of compounds at dose levels that may appear inactive in other methods (Bentley *et al.*, 1981). A similar report revealed that acetic acid-induced writhing response in mice is a simple, rapid and reliable model to evaluate peripheral type of analgesic action of herbal and other drugs (Shinde *et al.*, 1991). Reports from Le Bar *et al.* (2001) and Malec *et al.* (2008) however, showed that the acetic acid induced abdominal writhing in mice is a nonspecific which test response to both central and peripheral analgesia. It is therefore used for evaluating analgesic properties.

In this research, the methanol extract of *S. anacardium* exhibited a significant inhibitory effect on acetic acid induced writhing. The results
suggested that the extracts possess peripheral analgesic properties and their mechanisms of action may be mediated through inhibition of local peritoneal receptors as similarly reported by Mbiaticha et al. (2011).

On mode of action, local peritoneal receptors are suspected to be partly involved in abdominal contraction response Bentley (1983). The injection of acetic acid is reported to induce the release of mediators of pain such as prostaglandins and other cyclokinase (Nkeh-Chungag et al., 2010; Divya et al., 2009). This suggests that the extracts of *S. anacardium* acted by inhibiting the actions of cycloxygenase, the enzyme responsible for producing prostaglandins from arachidonic acids (Nkeh-Chungag et al., 2010). Analgesic activities have earlier been observed in glycosides, tannins and flavonoids compounds (Ahmadiani et al., 2000). The phytochemical screening of methanol extracts of *S. anacardium* showed the presence of glycosides, tannins and flavonoids. It is therefore possible that the analgesic effects observed may be attributed.

### 5.2.6 Anticonvulsant activity

Epilepsy is one of the major neurological disorders. It can be treated by synthetic antiepileptic drug therapy. But, due to its complexity by side-effects, teratogenic effects and long-term toxicity, a safe therapy remains a challenge. Medicinal plants and plant based phytoconstituents used for the therapy of epilepsy shown to possess promising anticonvulsant activities in animal models.
Maximum electroshock (MES) is also one of the commonly used models for preliminary testing of anticonvulsant drugs that produces generalized tonic-clonic seizures i.e. hind limb tonic extensor, tonic flexion and clonic convulsion. It has often been stated that antiepileptic drugs that block MES induced tonic extension act by blocking seizure spread (Macdonald et al., 1995).

MES-induced convulsion the *S. anacardium* extract significantly protected the animals against seizures, increased the onset and reduced the duration of seizures (Table 3). The current available antiepileptic drugs (AEDs) that are clinically effective in the management of generalized tonic–clonic and partial seizures such as carbamazepine, phenytoin, primidone, phenobarbital, valproate and lamotrigine all suppress hind limb tonic extension (HLTE) in MES (Browning, 1992; Rho and Rho, 1999). Protection against HLTE also indicates the ability of a testing material to inhibit or prevent seizures discharge within the brainstem seizure substrate (Browning, 1992). The ability of the methanol extract to inhibit the HLTE in MES as compared to phenytoin (100% protection) in the model suggests anticonvulsant activity for generalized tonic–clonic and partial seizures. The isolated constituent amentoflavone acts as a potent anticonvulsant agent by slowdown the HLTE and this report was supported by Nielsen et al. (1988) and Baureithel et al. (1997) focused their investigations on the biflavone amentoflavone, which bound to the brain benzodiazepine receptors with an affinity comparable to diazepam.
AEDs effective in the therapy of generalized seizures of (absence or myoclonic) petit mal type such as phenobarbitone, valproate, ethosuximide and benzodiazepines exhibit dose-dependent suppression of various seizure pattern induced by PTZ (Loscher et al., 1991). PTZ-induced seizures are similar to the symptoms observed in the absence seizures and drugs such as valproate and ethosuximide which are useful in the management of absence seizures inhibit PTZ-induced seizures (McNamara, 2001). At cellular level, one of the basic mechanisms of actions of AEDs such as ethosuximide and valproate is the suppression of T-type calcium currents in thalamic neurons (Macdonald and Kelly, 1994; Meldrum, 1996; Rho and Sankar, 1999).

In PTZ-induced convulsions, the extract of *S. anacardium* had increased the latency and the incidence of seizures as compared to diazepam. This extract might not be useful in the management of absence seizures. In PTZ-induced studies may probably be due to possible interaction between constituents of the crude extract. The majority of currently available antiepileptic drugs fall into one of two pharmacological classes, those that modulate neuronal voltage-gated sodium channels (e.g. carbamazepine, phenytoin, lamotrigine, and topiramate) and those that modulate inhibitory GABAergic neurotransmission (e.g. benzodiazepine, vigabatrin and tiagabine). While, small number of AEDs such as ethosuximide, gabapentin and possibly levetiracetam, may exert their effects via an interaction with voltage-operated calcium channels (Wickenden, 2002). The ability of the extract to exhibit activity against these two types of seizures suggests that it may act through different mechanisms to elicit its
anticonvulsant effects, such as voltage-gated sodium, calcium, and potassium or GABAergic pathway. The results of the study have demonstrated that S. anacardium possessed anticonvulsant activity on the animal models.

Basavaraj et al. (2011) evaluated the anticonvulsant activity of S. anacardium (L.) nut extract. In maximal electroshock induced convulsion, a phenytoin (25 mg/kg) was possessed anticonvulsant activity by decreased duration of tonic extension phase of the animals but not with chloroform extract of S. anacardium. Similarly, pentalenetetrazol (PTZ)-induced convulsion (PTZIC), strychnine-induced convulsion (SIC), picrotoxin induced convulsion (PIC), isoniazid (INH)-induced convulsion (IIC) and 4-amino pyridine (4-AP)-induced convulsion (4-APIC) models, a diazepam exhibited anticonvulsant effect, the chloroform extract of S. anacardium was ineffective among all those paradigms. But, in the present investigation, methanol extract and its isolated amentoflavone from stem bark of S. anacardium showed significant anticonvulsant activity in maximal electroshock and pentalenetetrazol induced animal models. This, may be prevented either by drugs that inhibit voltage dependant Na+ channels (phenytoin, valproate) Ragwaski, et al., 1995; Macdonald et al., 1995) or by drugs that block glutaminergic excitation mediated by the N-methyl- D-aspartate (NMDA) receptor (felbamate) (Subramaniam et al., 1995; Macabe et al., 1993).

Locomotor activity is considered as an index of alertness and decrease is considered as indication of sedative activity (Maharudra et al., 2010). Significant reductions in locomotor scores were recorded with a diazepam
(5 mg/kg) but not with chloroform extract of *S. anacardium* (100, 200 and 400 mg/kg) (Basavaraj *et al.*, 2011). In our investigation, locomotor activity of *S. anacardium* was studied, there was insignificant difference in methanol extract and its isolated amentoflavone with standard drug Diazepam which decreased the frequency and magnitude of the movements.

5.2.7. Antimicrobial activity

For a long period of time, plants have been a valuable resource for medical problems, including ailments caused by microbial infection. Numerous studies have been reported in different parts of the world to screen antimicrobial activity from plants products (Ravikumar *et al.*, 2005). Many of these substances are important in the defense against herbivores and contribute to the resistance to disease (Cowan, 1999). Due to the increasing prevalence of antibiotic-resistant pathogens in hospital and homes, deliberate search is in progress for alternative treatments to combat further spread of antibiotic resistant-pathogens (Olukoya *et al.*, 2003).

Antibacterial drugs exert their action by interfering with either the structure or metabolic pathways of bacteria. The molecular mechanisms of action of specific antibiotics and synthetic antibacterial are considered in more detail when individual groups of antibacterial agents are discussed in the following aspects (Oreste, 2003).
The most common mechanisms of action are

- Inhibition of bacterial cell wall biosynthesis
- Inhibition of protein, RNA, or DNA synthesis
- Damage of membranes

**Inhibition of bacterial cell wall biosynthesis:** Since bacterial cells have a high internal osmotic pressure, they require a rigid peptidoglycon polymer to protect themselves from lysis. Some of the antibiotics that interfere with the biosynthesis of peptidoglycon are β-lactams (penicillin, cephalosporins, cephamycins, monobactams, and carbapenems), glycopeptides (vancomycin and teicoplanin), D-cycloserine, fosfomycin, and bacitracin. These drugs have a high therapeutic index (the ratio of the toxic dose to the therapeutic dose) because they interfere with a biosynthetic process that is unique to prokaryotic cells (cell wall biosynthesis).

**Inhibition of protein synthesis:** Although the mechanism of protein synthesis is similar in prokaryotes and eukaryotes, prokaryotic ribosomes differ substantially from those in eukaryotes. This explains the effectiveness of several clinically important antibiotics. There are many clinically useful antibiotic classes that interact with rRNA or interfere with some steps in bacterial protein synthesis. Examples include aminoglycosides and tetracyclines, which bind conserved sequences within the 16S rRNA of the 30S ribosomal subunit; macrolides, ketolides, lincosamides, quinupristin-dalfopristin, and chloramphenicol, which act at the level of the 23S rRNA of
the 50S ribosomal subunit; oxazolidinones, which are potent inhibitors of bacterial protein biosynthesis and prevent the formation of the N-formyl-
methionyl-tRNA-ribosome-mRNA ternary complex; and mupirocin, which inhibits the isoleucyl-tRNA synthetase.

**Cell membrane disruption:** The bactericidal activity of the polymyxin and cationic antimicrobial peptides results from their interaction with the bacterial cytoplasmic membrane causing gross disorganization of its structure.

**Inhibition of nucleic acid synthesis:** The growth and division of the bacterial cell depend on, among other factors, DNA and RNA synthesis. Antibacterial drugs can disrupt nucleic acid synthesis in a variety of ways, both direct (e.g., the action of fluoroquinolones on DNA gyrase and topoisomerase IV) and indirect (e.g., the action of sulfonamides on folic acid metabolism).

In the present antibacterial study, we have selected ciprofloxacin as a standard drug to compare the therapeutic efficacy of test compounds. This is because of the specific characteristics of ciprofloxacin which contribute to a high therapeutic efficacy. Ciprofloxacin has a broad spectrum bactericidal activity (Wolfson and Hooper, 1985; Zeiler, 1985). Another important characteristic of ciprofloxacin is a large volume of distribution and a high capacity of tissue penetration (Schlenkhoff et al., 1986; Wise et al., 1984).

*S. anacardium* is used in traditional medicine to treat bacterial infections, diarrhea, itching, etc. These traditional claims have been supported by the current bioassay results, which have shown activity against human
pathogenic bacteria (Mohanta et al., 2007; Nair et al., 1996; Sharma et al., 2010).


Generally, the gram-positive bacteria should be more susceptible having only an outer peptidoglycan layer which is not an effective permeability barrier (Scherrer and Gerhardt, 1971). Where as, the gram-negative bacteria possess an outer phospholipidic membrane carrying the structural lipopolysaccharide components. This makes the cell wall impermeable to drug constituents. Because of the presence of multilayered peptidoglycan and a phospholipidic bilayer wall most of the gram-negative bacteria showed multi drug resistant characteristics. In spite of these barriers the constituents of *S. anacardium* were effective in controlling the growth of these pathogenic strains. A good activity of the isolated compounds amentoflavone indicates that those compounds alone are solely responsible for antibacterial activity of the *S. anacardium*.

Since, the present study is purely a preliminary one, we cannot be ascertain the reason for the efficient activity of the isolated compounds, since the drug-pathogen interaction studies are still to be confirmed by profound
investigation. However, our findings ratify the bactericidal property of S. anacardium and provide a supportive scientific evidence for its medicinal use.

From our research laboratory, several investigators have reported the bactericidal activity of some medicinal plants and their bioactive constituents on the same bacterial isolates for instance, Harish et al. (2007) reported the antibacterial activity of Celapanin isolated from the leaves of Celastrus paniculatus; Raja Naika et al. (2007) isolated that the taraxerol and β-sitosterol from petroleum ether extract of Naravelia zeylanica leaves; Venkatesh et al., (2013) reported that the antibacterial activities of plant Musa paradisiaca cv. Puttabale and Musa acuminate var. grandnaine and Pradeepa et al. (2012) reported that the Ethanol extract of Litsea glutinosa stem bark.

In S. anacardium, the previous investigators worked on the antibacterial property of nut shell and leaf extracts. Mohanta et al. (2007) reported that the antimicrobial activity (disc diffusion method) of S. anacardium with different extract. The petroleum ether and aqueous extract fractions of S. anacardium showed inhibitory activity against Staphylococcus aureus (10 mm) and Shigella flexneri (16 mm) at 100 mg/ml concentration. While chloroform extract showed inhibition against Bacillus licheniformis, Vibrio cholerae and Pseudomonas aeruginosa, the ethanol extract showed inhibition to Pseudomonas aeruginosa and S. aureus.

Nair et al. (1996) reported that the alcoholic extract of dry nuts of S. anacardium showed bactericidal activity in vitro against three gram negative strains (Escherichia coli, Salmonella typhi and Proteus vulgaris) and two gram
positive strains (*Staphylococcus aureus* and *Corynebacterium diphtheriae*). Subsequent studies have shown that the alcoholic extracts of different parts of the plant (leaves, twigs and green fruit) also possess anti-bacterial properties, especially the leaf extract.

Sharma *et al.* (2010) reported nut oil of *S. anacardium* shows significant anti-microbial activity against some gram positive (*Bacillus subtilis, Staphylococcus aureus*) and gram negative bacteria (*Proteus vulgaris, Escherichia coli*) and Zabin *et al.* (2012) depicted the petroleum ether nut extract of *Semecarpus anacardium* against organisms like *Escherichia coli* (19 mm), *Micrococcus luteus* (23 mm), *Salmonella typhi* (26mm), *Bacillus subtilis* (14 mm) and *Klebsiella pneumoniae* (22 mm). In the present antimicrobial activity, stem bark methanol extract of *S. anacardium* showed a highly significant level of bacterial inhibition *K. pneumonia, P. aeruginosa, S. typhi, S. aureus, S. paratyphi* and *P.vulgaris*. The petroleum ether extract showed moderate activity against *P. aeruginosa, K. pneumoniae, S. typhi*, and very less against *S. aureus*. The chloroform extract of showed less activity in *P.vulgaris* and moderate activity in *K. pneumoniae*. The isolated amentoflavone from methanol extract showed significant activity in *K. pneumonia* and moderate activity in *P. vulgaris, S. aureus, S. typhi* and *P. aeruginosa*.

In antifungal assay, the zone of inhibition of methanol extracts of *S. anacardium* was found to be maximum against *Candida albicans* and moderate effect on *Aspergillus fumigatus, Aspergillus niger* and *Aspergillus flavus* respectively. In isolated flavonoid, amentoflavone showed maximum
inhibition zone in *Candida albicans* and moderate activity on *Aspergillus flavus, Aspergillus niger, Aspergillus fumigatus*. Sharma *et al.* (2002) also found that due to presence of flavonoid, alcoholic extract of dry nuts of *S. anacardium* showed antifungal activity (*Aspergillus fumigatus* and *Candida albicans*) at 400 mg/ml concentration. Both the fungi were show inhibition in growth, reduction in size of cells and sporulation also decreased.

5.2.8. **Anticancerous activity**

Interest in higher plants as potential source of tumor damaging agents was stimulated with finding that colchicine, an alkaloid derived from *Colchicum luteum*, was capable of producing regression and cytolysis in tumors. Later this interest was enhanced with the demonstration that podophyllotoxin *a* - and P-peltatin were capable of inflicting considerable damage on experimental tumors (Belkin, 1948 and Greenspan *et al.*, 1950).

Many herbs have been evaluated in clinical studies and are currently being investigated to understand their tumouricidal actions against various cancer cell lines. *S. anacardium* is categorized in Ayurveda as one of the toxic plant (Sharma *et al.*, 1975). Many references are available on the anticancer properties of *S. anacardium* nuts (Sharma *et al.*, 1966). An extensive review also describes the phytochemical and pharmacological properties of *S. anacardium* (Premalatha, 2002). The chloroform extract of *S. anacardium* nut possess antitumour action with increased life span against leukaemia, melanoma and glioma (Cassady *et al.*, 1981). The milk extract of
*S. anacardium* produces regression of hepatocarcinoma by stimulating host immune system (Premalatha, 1998; 1999) and normalizing tumour markers including alpha- and fetoprotein levels. This preparation stabilizes the lysozomes, and normalizes glycoprotein and mineral content in the body during cancer progression (Premalatha, 2002). It also corrects hypoglycaemia (Premalatha, 1998) and controls abnormal lipid peroxidation by the maintenance of antioxidant defense status (Premalatha, 1997). In the microsomes, it acts as a bifunctional inducer of both phase I and II biotransformation enzymes and prevents tumour initiation by preventing carcinogen activation (Premalatha, 2000). Histologically, on treatment with the *S. anacardium* extract to hepatocarcinoma animals, the liver sections showed almost a normal architecture. The nodules become completely regressed and further cell necrosis was prevented. *Anacartin forte*, another preparation from *S. anacardium* has been used for several decades as an anticancer drug since it is giving health improvement with alleviation or disappearance of troublesome symptoms. It provides clinical benefit with an extension of survival time in various cancers including oesophageal, chronic myeloid leukaemia, urinary bladder and liver cancer (Premalatha, 1999). Another Ayurvedic drug containing *S. anacardium, Amura rohitaka, Glycyrrhiza glabra* and copper powder were reported to inhibit breast tumour development in mice by significantly extending the survival period. This drug was also found to be efficient in clinical trials (Prasad, 1987).
In the present investigation, the evaluation of anticancerous activity of the chloroform extract, methanol extract and the amentoflavone isolated from methanol extract of *S. anacardium* stem bark has been carried out *in vitro* by SRB assay. The sulforhodamine B (SRB) assay, which was developed in 1990, remains one of the most widely used methods for *in vitro* cytotoxicity screening (Skehan, 1990). The assay relies on the ability of SRB to bind to protein components of cell that have been fixed to tissue culture plates by trichloroacetic acid (TCA). SRB is a bright-pink aminoxanthene dye with two sulfonic groups that bind to basic amino-acid residues under mild acidic conditions, and dissociate under basic conditions (Lillie, 1977) As the binding of SRB is stoichiometric, the amount of dye extracted from stained cells is directly proportional to the cell mass.

The strong intensity of SRB staining allows the assay to be carried out in a 96-well format. Skehan *et al.* (1990) showed that the assay can detect densities as low as 1000-2000 cells per well, and with a signal-to-noise ratio of 4.83 at a density of 5000 cells per well. This level of sensitivity is comparable to those of fluorescent dye-staining methods, and is superior to those of other protein-staining methods using conventional visible dyes (McCaffrey *et al.*, 1988). Results from the SRB assay exhibit a linear dynamic range over densities of 7500-180000 cells per well, corresponding to 1-200% confluence. Furthermore, the SRB method has proven to be practical, because after the TCA-fixed and SRB-stained cell monolayer are dried they can be stored indefinitely. Color extracted from SRB-stained cells is also stable. With its
high level of sensitivity, adaptability to the 96-well format and endpoint stability, the SRB assay is well suited to large-scale screening applications, as well as research. This assay has been widely used for drug-toxicity testing against different types of cancerous and non-cancerous cell lines (Monks, 1991).

The present study of anticancerous activity on human cancerous cell lines namely, Human Neso pharangeal Cancer Cell Line KB, Human Colon Cancer Cell Line HCT15, Human Leukemia Cell Line Molt-4, Human Leukemia Cell Line MDA-MB-435 and Human Leukemia Cell Line Jurkat. It revealed that the Chloroform extract of S. anacordium exhibited significant anticancerous activity against the human leukemia cell line Jurkat. Where as, isolated compound and the methanol extract showed not significant activity against the leukemia cell line Jurkat. The LC$_{50}$ values on Jurkat cell line and the tested compounds were >80μg/ml. Where as, the TGI values of methanol extract, chloroform extract, isolated constituent and the standard ADR were > 80, 53.2, 80 and 62 μg/ml respectively. This showed that the chloroform extract exhibited significant anticancerous activity against the leukemia cell line Jurkat and at the concentration of 32μg/ml its anticancerous activity is similar to that of the standard drug ADR. According to NCI, USA guidelines for extracts, GI$_{50}$$\leq$20 μg/ml is considered as active against the cancerous cell lines. But the amentoflavone exhibited not significant against Jurkat cell line. In the remaining tested cancerous cell lines (as mentioned above) the anticancerous activity of the stem bark extracts and the isolated constituent was nil. This
showed that the anticancerous effect of the phytoconstituent is cell line specific.

The presence of amentoflavone has been reported in many plant species such as, *Selaginella tamariscina* (Gambhir *et al.*, 1987), *Biophytum sensitivum*, *Ochna pumila*, *Selaginella sinensis* (Ma *et al.*, 2001), *Rhus succedanea* and *Garcinia multiflora* (Lin *et al.*, 1999), *Rhus pyroides*, *Rhus dentate*, *Rhus pentheri* (Svenningsen *et al.*, 2006), *Biophytum sensitivum* (Guruvayoorappan *et al.* (2007), *Viburnum lantana* (Krzysztof Pawlak *et al.*, 2010), *Araucaria angustifolia* (Santi-Gadelha *et al.*, 2006).

Pharmacological properties of amentoflavone have been reported for anti-inflammatory, anti-ulcerogenic activities (Gambhir *et al.*, 1987). Kamil *et al.* (1987). Hee Kee Kim *et al.* (1998) reported that amentoflavone possess a potent anti-inflammatory activity by intraperitoneal injection on animal models of acute inflammation. Nahrstedt *et al.* (1997) reported principle biflavonoid constituents namely, quercetin, quercitrin, amentoflavone from *Hypericum perforatum*. Similarly, Nielsen *et al.* (1988) and Baureithel *et al.* (1997) focused their investigations on the biflavone amentoflavone, which bound to the brain benzodiazepine receptors with an affinity comparable to diazepam.