6. DISCUSSION

Humans have always relied on natural sources for survival since ancient times, which has been their main source of food, protection, clothing, transportation and remedies. Plants and natural products play an important role in medicine and provide important prototypes for the development of novel drugs (Cragg, 1998). They offer a valuable source of compounds with a wide variety of biological activities and chemical structures. The need for novel plant derived drugs is increasing due to serious side effects and cost of the synthetic drugs. Thus, throughout human history, plants were a fertile source of new drug discovery but the recent competition from combinatory chemistry (Schreiber, 2000) and computational drug design (Clarck, 2000) has limited this dominance of natural products in drug discovery. However, attempts to treat complex multistage diseases, including cancer, with single synthetic molecules did not show great success.

Many individual phytochemicals have been identified and classified in plants. However, still large amount of phytochemicals need to be characterized to comprehend their health benefits. Phenolic compounds constituting one of the most widespread groups of substances are the products of secondary metabolism in plants. More than 8000 phenolic structures have been characterized, which involve essential activities in the reproduction and growth of plants. They also play important role as defense agents against pathogens, parasites, and predators (Dong et al., 2007). Polyphenolic compounds are effective in the prevention of oxidative stress related diseases. Flavonoids are a group of polyphenolic compounds; the therapeutic potential of flavonoids has been determined and is known to have a number of pharmacological properties. Flavonoids also exert the effects of antioxidants, free radical scavengers and are chelators of divalent cations (Cook and Samman, 1996).

6.1. Effects of extraction techniques/solvents on Rubus spp.

Recovery of secondary metabolites from plant materials is typically accomplished through different extraction techniques taking into account their chemistry and uneven distribution in the complex plant matrix. The extraction of various parts of Rubus species was successively done by different solvents such as petroleum ether, chloroform, acetone
and methanol using Soxhlet apparatus. Among the different solvents used for the extraction of the plant materials, methanol and acetone were found to be better extraction media to obtain higher yield. Polar solvents are frequently employed for the recovery of polyphenols from a plant matrix. The most suitable solvents for the extraction of polyphenols are aqueous mixtures containing ethanol, methanol, acetone, and ethyl acetate (Peschel et al., 2006). The results showed that extract yields were better when extraction was done under Soxhlet. The Soxhlet extraction has got many advantages; the displacement of transfer equilibrium by repeatedly bringing fresh solvent into contact with the solid matrix, it can maintain a relatively high extraction temperature with heat from distillation flask and no filtration requirement after leaching. Also, the Soxhlet method is very simple and cheap. The major disadvantages include; it requires long time, more solvent, agitation cannot be provided and possibility of thermal decomposition of few compounds (De Castro and Garcia-Ayuso, 1998).

6.2. Estimation of total phenolic, tannin and flavonoid contents of various *Rubus* extracts

Results of the present study showed that among all the solvent extracts, the acetone and methanol extracts had the highest total phenolic, tannin and flavanoid contents. This may be due to the fact that phenolics are extracted efficiently in high polar solvents (Siddhuraju and Becker, 2003; Anwar et al., 2006). The highest total phenolics and flavanoid contents were observed in acetone extract of *R. fairholmianus* root; 63.53 g GAE/100 g extract and 981.33 mg RE/g extract respectively. The maximum tannins were shown by acetone extract of leaves of *R. ellipticus* (46.47 g GAE/100 g extract). There are no previous reports available on total phenolics and flavonoids contents of *Rubus* species evaluated in this study.

However, previously Yizhong et al. (2004) reported the total phenolic contents of methanol and aqueous extracts of *R. chingii* fruit (4.54 and 4.02 g/100 g extract respectively) and the occurrence of phenolic compounds such as gallic acid and ellagic acid. The phenolics (4.52 mg GAE/g extract) and flavonoid (4.66 mg TE/g extract) contents of *R. sanctus* were reported by Motamed and Naghibi (2010) whereas the phenolic (21 to 225 mg GAE/g extract) and flavanoid (16 to 29 mg RE/g extract) contents
of *R. ellipticus* root extracts have been reported by Vadivelan *et al.* (2009). The methanol extract of *R. idaeus* and *R. fruticosus* had the highest content of total phenolics and flavonoids (Isik *et al.*, 2011). Moreover, Lee *et al.* (2012) reviewed the phenolics from the fruits of various *Rubus* species. With an increased awareness given to the potential health benefits of consuming berries high in phenolics, the review mainly focused on the identified phenolics of *Rubus* fruit, although other edible parts of *Rubus* plants (i.e. leaves, roots) also contain phenolics. Vrhovsek *et al.* (2006) reported that tannins are a major class of phenolics largely responsible for the astringent and antioxidant properties of raspberries and blackberries.

Plant polyphenols, a diverse group of phenolic compounds possess an ideal structural chemistry for free radical scavenging activity. Antioxidative properties of polyphenols arise from their high reactivity as hydrogen or electron donors, from the ability to stabilize and delocalize the unpaired electron and from their potential to chelate metal ions (Rice-Evans *et al.*, 1997). The ability of flavonoids to act as antioxidants are due to several different mechanisms, such as scavenging of free radicals, chelation of metal ions like iron and copper, and inhibition of enzymes responsible for free radical generation. Depending on their structure, flavonoids are able to scavenge practically all known ROS (Benavente-Garcia *et al.*, 1997). A number of authors have found a correlation between the phenolic content and the antioxidant activity (Velioglu *et al.*, 1998).

### 6.3. In vitro antioxidant activities

Acetone and methanol extracts of *Rubus* species showed higher free radical scavenging properties that may be due to the higher extractability and solubility of antioxidant compounds such as phenolics and other metabolites in these solvents.

#### 6.3.1. DPPH• and ABTS** scavenging activities

In the present investigation, the RFRA extract depicted the highest DPPH radical scavenging activity with an IC₅₀ value of 3.55 μg/mL and methanol extract of *R. ellipticus* leaves showed highest ABTS radical cation scavenging activity (53155.94 μM TE/g) in comparison with all other extracts. However, there are reports on related species of *Rubus* which also show significant DPPH radical scavenging activities.
The DPPH• scavenging activities (IC$_{50}$ 52.2±0.9 μg/mL) of the n-butanol extract of *R. parvifolius* have been evaluated by Gao *et al.* (2011). *R. sanctus* was found to scavenge the DPPH radical by 83.27% when compared with Vitamin C (97.15 %) and BHT (96.47%) (Motamed and Naghibi, 2010). Zhang *et al.* (2010) reported that *R. idaeus* extracts possessed DPPH• scavenging activities ranged from 305 to 351 μM TE/g. The antioxidant activity (TEAC value: 3.8±0.3 mM TE; DPPH• EC$_{50}$ value: 5.10±0.5 μg/mL) and total phenolic contents (2.76±0.08 mg/L GAE) of *R. ulmifolius* have been reported by Dall’Acqua *et al.* (2008). Jeong *et al.* (2010) reported the antioxidant activities of the extracts from black raspberry fruits and wines. The ethanol extracts of crushed seeds showed higher antioxidant activities (DPPH• IC$_{50}$ 130 μg/mL) and the lowest ABTS$^+$ (IC$_{50}$ 198 μg/mL) for the black raspberry wine with seeds. DPPH radical scavenging values can be correlated with total phenol content in raspberry (Isik *et al.*, 2011; Deighton, 2000).

Fan *et al.* (2011) reported the antioxidant activities of 11 cold-field fruits in China. Total phenolic contents of the fruit extracts had a positive correlation with antioxidant activity (R$^2$ > 0.7112). Among the 11 fruits, the extracts of *R. kamarowii* had the highest capacities for scavenging DPPH• (EC$_{50}$ 25.6±0.51 μM TE/g) and ABTS$^+$ (EC$_{50}$ 63.6±1.67 μM TE/g). The antioxidant (93% at 42 μg/mL) properties of methanolic extract of *R. ulmifolius* fruits was reported by Fazio *et al.* (2013). Ruiz *et al.* (2013) studied the antioxidant activity of *R. geoides* fruits (TEAC 21.7±0.3 – 40.0±1.3 μM/g). Comparing with the previous reports of DPPH and ABTS radical scavenging activities of various allied *Rubus* species; this study revealed the promising radical scavenging activities, which might be due to the higher phenolic contents.

### 6.3.2. FRAP and phosphomolybdenum assays

Among the various extracts tested in the present investigation for FRAP assay, RELM showed superior activity (7624.44 mM Fe(II)/mg extract). In phosphomolybdenum assay; RFRA showed higher activity (94.09 g AAE/100g extract). The reduction of Fe$^{3+}$ to Fe$^{2+}$ in the presence of TPTZ in FRAP assay and Mo(VI) to Mo(V) in phosphomolybdenum assay by the *Rubus* extracts may be due to the electron transfer or hydrogen ion transfer by the bioactive compounds, specifically phenolics and flavonoids in the extracts.
Related species of *Rubus* also showed considerable ferric and molybdenum reducing activities. Previously, Pantelidis *et al.* (2007) reported the antioxidant capacity (FRAP) of raspberries and blackberries. The highest antioxidant activity was recorded in blackberry and in raspberry x blackberry cultivars. Deighton *et al.* (2000) and Wang and Lin, (2000) found a linear correlation between total antioxidant activity (assessed by FRAP) and phenol content in blackberries. Ruiz *et al.* (2013) reported that the presence of ascorbic acid (43.4-98.5 mg/100g) in the fruits of *R. geoides*. In this investigation, the FRAP and phosphomolybdenum results were higher when compared to the previously reported values and reveals the superior antioxidant potential of tested *Rubus* extracts.

### 6.3.3. Superoxide and nitric oxide radical scavenging activities

Superoxide radical is known to be very harmful to cellular components as a precursor of more reactive oxygen species (Halliwell and Gutteridge, 1985). Although superoxide anion is a weak oxidant, it gives rise to generation hydroxyl radicals as well as singlet oxygen, both of which contribute to oxidative stress (Meyer and Isaken, 1995). The assay is based on the capacity of the samples to enhance the aerobic photochemical reduction of nitroblue tetrazolium (NBT) in the presence of riboflavin. In addition to ROS, nitric oxide is also implicated in inflammation, cancer and other pathological conditions (Moncada *et al*., 1991). The plant products may have the property to counteract the effect of NO formation and in turn may be of considerable interest in preventing the ill effects of excessive NO generation in human body. Further, the scavenging activity may also help to arrest the chain of reaction initiated by the excess generation of NO that is detrimental to the human health (Kumaran and Karunakaran, 2007).

In the present investigation, all the extracts showed good scavenging activity on superoxide and nitric oxide radicals in a concentration dependent manner. Over all, methanol and acetone extracts showed significant scavenging activity than other solvent extracts. Among the extracts; methanol extracts of *R. fairholmianus* leaves showed higher SO radical scavenging (72.88%) and RELM showed higher nitric oxide radical scavenging activities (74.37%). There are very less reports available on SO and NO scavenging properties of the related *Rubus* species. The SO radical scavenging activity of aqueous extract of *R. chingii* was reported by Yau *et al.* (2002). The effect was found to
be dose-dependent with an IC$_{50}$ value of 8.7 μg/mL. The NO scavenging activity (IC$_{50}$ 9.69 μg/mL) of Alocasia indica leaves was reported by Mulla et al. (2009). Nitrite is one of the oxides of nitrogen which was significantly inhibited by plant extract through direct competition with oxygen and other oxides of nitrogen in the reaction medium (Alisi and Onyeze, 2008). The scavenging activity of plant extract against nitric oxide formation was comparable to the standard drugs used in this study. This observation gives an indication of strong antioxidant potential of the extract which is confirmed with SO and NO scavenging assays.

Based on the literature survey, the antioxidant activity and phenolic contents of the extracts have a linear relationship; the results of the present study also support this view and are in strong agreement with the previous reports. In this investigation, all the antioxidant assay results are correlated with total phenolics, flavonoid or tannin contents of the Rubus extracts. The acetone and methanol extracts of all the parts showed higher phenolic contents and antioxidant properties. So it is evident that the commendable free radical scavenging activities of Rubus extracts might be attributed to the appreciable level of phenolic contents.

6.4. Toxicity studies of Rubus extracts

In acute toxicity study, Rubus extracts did not cause any mortality up to 2000 mg/kg, and hence 1/10$^{th}$ and 1/5$^{th}$ of the maximum dose (i.e. 200 and 400 mg/kg) were fixed for animal experiments. This indicates that the extract has low toxicity when administered orally. According to Clarke and Clarke (1977), substances with LD$_{50}$ of 1000 mg/kg body weight/oral route are regarded as being safe or of low toxicity. The high LD$_{50}$ obtained is an indication that the extract could be administered with a high degree of safety where the absorption might be incomplete due to inherent factors impeding absorption along the gastrointestinal tract (Dennis, 1984). The acute toxicity studies have got some limitations in clinical application since cumulative toxic effects do occur even at very low doses. Hence, repeated dose studies are always invaluable in evaluating the safety profile of plant extracts, sub-acute toxicity study was therefore conducted with doses 200 and 400 mg/kg of Rubus extracts.
Daily clinical as well as the final end point observations are of major importance (Feres et al., 2006) in repeated dose toxicity studies. In water and food consumption, no significant changes were observed. The changes in body weight is an indicator of adverse side effects, as the animals cannot survive lose of more than 10% of the initial body weight (Feres et al., 2006; Raza et al., 2002; Obici et al., 2008; Teo et al., 2002). Changes in organ weights are also indices of toxicity in animals which are readily determined in toxicity tests. There is a very high possibility that plant extracts, when ingested into the body may be toxic to organs such as kidney, liver, spleen and brain because of their diverse roles in body. The absence of any significant differences in the body and organ weight of the treated and untreated animals provides support to the safety of tested Rubus extracts.

The evaluation of histopathological changes in organs remains a cornerstone in safety assessment (Greaves, 2007). In the present study, the histopathological findings of the organs did not indicated toxicity. The kidney, liver, spleen and brain histology of treated animals were normal and did not show any major differences with untreated group, depicts the non-toxicity of Rubus extracts. Blood parameters analysis is relevant in risk evaluation as it has higher predictive value in toxicity studies. Blood forms the main medium of transport for many drugs in body and for that matter the main components of the blood such as WBC and Hb are exposed to toxic compounds. The damage and destruction of the blood cells affects the normal functioning of the body (Olson et al., 2000). Rubus extracts did not produce any significant changes in the haematological parameters, suggesting its very low toxicity. Evaluation of biochemical parameters of organs is important since there are several reports on liver and kidney toxicity related to the use of plant extracts (Obici et al., 2008; Rhiouani et al., 2008; Corns, 2003). In preclinical toxicity studies, renal changes are particularly liable to occur because of the high doses given and the fact that the kidneys eliminate many drugs and their metabolites (Greaves, 2007; Schreiner and Maher, 1965). The creatinine and urea determinations were critical markers of kidney function (Obici et al., 2008; Arneson and Brickell, 2007a). There were no significant differences in serum levels of creatinine and urea in Rubus extracts treated groups compared to normal and vehicle control groups.
Among the biochemical parameters evaluated, ALP, SGPT (ALT), SGOT (AST) and bilirubin are considered as markers of liver function (Feres et al., 2006; Obici et al., 2008; Arneson and Brickell, 2007b). Hepatocellular damage is characterized by a mutual rise in serum levels of AST and ALT. But since ALT is localized primarily in the cytosol of hepatocytes, this enzyme is considered a more sensitive marker of hepatocellular damage than AST (Aniagu et al., 2004). ALP is most often measured to indicate bile duct obstruction (Withawaskul et al., 2003). The serum levels of AST, ALT, ALP and bilirubin were ranged within the normal values; statistically there is not much difference between the treated, normal and vehicle control groups, demonstrating that liver function was preserved in animals exposed to Rubus extracts over 14 days period. This conclusion correlates well with findings from histopathological examination of the liver, spleen, kidney and brain. Since it did not indicate any significant cellular lesions induced by plant extracts. The results of enzymatic (SOD, CAT, GPx and GR) and non-enzymatic (GSH) antioxidants in the livers of animals treated with Rubus extracts for 14 days showed a slight increase indicating that it is non-toxic and safe; since usually these parameters helps to detect the liver toxicities.

6.5. In vivo antioxidant activities of Rubus extracts

The human body has several mechanisms to counteract damage by free radicals and other reactive oxygen species. They act on different oxidants as well as in different cellular compartments. One important line of defence is a system of antioxidant enzymes which includes SOD, CAT, GPx, GST and GR (Firdous et al., 2010). Biological systems protect themselves against the damaging effects of activated species by several means. These include free radical scavengers and chain reaction terminators; enzymes such as SOD and CAT (Proctor and McGinness, 1986). The SOD is a metalloprotein converts superoxide radicals into H$_2$O$_2$ and O$_2$; to eliminate H$_2$O$_2$ which can create highly reactive hydroxyl radicals; thus participating with other antioxidant enzymes in the enzymatic defense against oxygen toxicity. The results of the present study reveals that there is an increase of SOD activity in both blood and liver of the Rubus extracts treated animals suggesting that it has an efficient protective effect in response to ROS.
CAT is a key component of the antioxidant defense system. Inhibition of this protective mechanism results in enhanced sensitivity to free radical induced cellular damage. The reduction in the activity of CAT may therefore result in a number of deleterious effects due to the accumulation of SO radicals and H$_2$O$_2$ (Sampathkumar et al., 2005). In this study, the administration of *Rubus* extracts increases the CAT level in liver and blood of the animals thus preventing the accumulation of excessive free radicals. The extract treated groups significantly ($p < 0.001$) enhanced the enzyme activity.

Glutathione, a major non-protein thiol in living organisms, plays a key role in coordinating the body’s antioxidant defense processes. Excessive peroxidation causes increased glutathione consumption. Reduced thiols have long been reported to be essential for recycling of antioxidants like vitamin E and vitamin C (Constantinescu et al., 1993). GSH is an important substrate for GPx and GST; also which acts by quenching free radicals. In the present study, the administration of extracts has been shown to protect against oxidative stress in humans and animals. The blood and liver tissue glutathione levels were significantly depleted in the control group when compared with the groups treated with *Rubus* extracts.

GPx is a seleno-enzyme two third of which is present in the cytosol and one third in mitochondria. It catalyses the reaction of hydroperoxides with reduced GSH to form glutathione disulphide (GSSG) and the reduction product of the hydroperoxide. GPx is found throughout the tissues, being present as different isoenzymes (Zaltzber et al., 1999). GPx measurement should also be considered in particularly with patients who are under oxidative stress for any reason; low activity of this enzyme is one of the early consequences of a disturbance of the prooxidant/antioxidant balance (Benabdeslam et al., 1999; Yang et al., 1984; Paglia et al., 1967). In this study the levels of GPx in the liver homogenate increased significantly in treated groups.

GST is thought to play a physiological role in initiating the detoxication of potential alkylating agents (Habig et al., 1974), including pharmacologically active compounds. GST is GPx like enzyme and its function is to eliminate various hydroperoxides. These enzymes catalyze the reaction of such compounds with the -SH group of glutathione, thereby neutralizing their electrophilic sites and rendering the products more water-soluble.
GR is a member of the disulphide oxidoreductase family, catalyses the NADPH-dependent reduction of GSSG to GSH. This ubiquitous tripeptide glutathione is the most abundant low molecular weight thiol in all cells (Mann et al., 1932) and is involved in enzymatic reactions. A heat labile system capable of reducing GSSG was discovered in liver (Rall and Lehninger, 1952). The enzyme directly involved in reduction of GSSG, glutathione reductase was demonstrated in rat liver (Conn and Vennesland, 1951). GR levels in the blood and liver, GST levels in liver of the extract treated animals found to be increased significantly in the present investigation, indicating the high antioxidant potential.

6.6. Anti-inflammatory, analgesic and antipyretic activities of *Rubus* extracts

The antioxidant activity of *Rubus* spp., as well as the traditional use encouraged to extend the research using *in vivo* models of anti-inflammation, analgesic and antipyretic studies. It is well known that all the pharmaceutical companies are now interested in developing more effective plant derived drugs to treat inflammation, pain and fever.

Carrageenan-induced hind paw edema has been widely used as an experimental model of acute inflammation and is believed to be biphasic (early phase up to 2 hr and late phase up to 6 hr) (Saha et al., 2007). The early phase was associated with severe inflammation, whereas the late phase observed to have slow increase in volume of paw edema. The early phase has been attributed to the action of mediators such as histamine, serotonin and bradykinin on vascular permeability (Niazi, 2009). The late phase edema has been shown to be a result of over production of PG (Gurrero et al., 2001). Park et al. (2006) reported the *in vitro* anti-inflammatory activity of ethanol extract of *R. coreanus* fruit, which suppressed NO and PGE2 production. The test of erythema in mouse ears induced by croton oil is used for the evaluation of the effect of new anti-inflammatory drugs (Swingle et al., 1981; Tragni et al., 1985; Falodun et al., 2008). Dermatitis induced by croton oil represents a model of acute inflammatory response; the edema is mediated by COX metabolism of arachidonic acid (Galey et al., 1985; Inoue et al., 1986; Young and Young, 1989). In croton-oil induced edema, the groups treated with 400 mg/kg doses of RNRA showed statistically significant results when compared to control, which were not statistically different from the effect of
indomethacin (10 mg/kg), obstructing the generation of these mediators. The data obtained from the present research revealed that the RFRA possessed potent anti-inflammatory effect in the carrageenan-induced acute model of inflammation in the late phase of inflammation, which might be due to the inhibition of PG over production.

Previous studies have shown that berries possess anti-inflammatory properties (Juranic, 2005; Lau et al., 2007; Seeram, 2008; Szajdek and Borowska, 2008; Bralley et al., 2007). The findings of Fazio et al. (2013) revealed that the phenolic contents of the extracts are mainly responsible for their free radical scavenging and anti-inflammatory abilities, the present study results are in good agreement with this view and thus confirming the anti-inflammatory activities by the phenolic rich extracts such as RFRA, RNRA and RELM.

Acetic acid-induced writhing is a non-specific pain model and many compounds belonging to diverse pharmacological categories including opioids, NSAIDs etc. show analgesic activity in this test (Vogel and Vogel, 1997). Acetic acid test is a visceral pain model produces a painful reaction and acute inflammation in the peritoneal area. Release of arachidonic acid and biosynthesis of prostaglandin via cyclooxygenase pathway plays a role in the nociceptive mechanism of this test (Franzotti, 2000). The analgesic effect of the plant derived bioactive compounds may be mediated through inhibition of COX (Vogel and Vogel, 1997; Koster et al., 1959). Aspirin offers relief from inflammatory pain by suppressing the formation of pain mediators in the peripheral tissues, where proinflammatory mediators PG and bradykinins were suggested to play an important role in the pain process (Vinegar et al., 1969). PG elicits pain by the direct stimulation of sensory nerve endings.

The hot plate model has been found suitable to investigate central antinociceptive activity because of several advantages, particularly the sensitivity to antinociceptives and limited tissue damage (Das and Ahmed, 2012). The results obtained from this study confirmed that, in acetic acid induced writhing test, higher dose of RFRA showed maximum activity (62.69%), which was comparable with aspirin (73.13%). On the other hand, in hot plate method RELM showed superior activity. The analgesic effect of *R. fairholmianus* might be attributed to the inhibition of synthesis of pro-inflammatory
mediators, such as PG and cytokines. It is evident from the study that *R. fairholmianus* exhibits significant peripheral analgesic effect in mice. There are no such previous reports on the analgesic properties of related *Rubus* species.

Antipyretics are drugs, which reduces the elevated body temperature. Regulation of body temperature requires a delicate balance between production and loss of heat and the hypothalamus regulates the set point at which body temperature is maintained. In fever this set point is elevated and a drug like paracetamol does not influence body temperature when it is elevated by factors such as exercise or increases in ambient temperature (Goodman, 1996). Fever is thought to be produced by several endogenous substances including interleukins (IL), TNF-α and PG. Brewer’s yeast induces both TNF-α and PG synthesis (Kluger, 1991). An antipyretic drug reduces fever by depressing inflammatory messages at both peripheral sites of tissue inflammation and within CNS thermoregulatory sites. These agents suppress peripheral production of pyrogenic cytokines such as TNF-α and IL-1β, while lowering the thermoregulatory set point by blocking central COX production of PGE2 (Aronoff and Neilson, 2001). The present results showed that RNRA extract was highly efficient in suppressing the cytokines thereby reducing fever in yeast induced rats; followed by RFRA and RELM. The antipyretic effects of the *Rubus* extracts were comparable with paracetamol. This is the pioneer report on the antipyretic activity *Rubus* species.

Inflammation is the host defense mechanism, an immediate response of the body to tissue injury caused by microbial infection and other noxious stimuli. Acute inflammation is characterized by vasodilation and infiltration of leukocytes into the site of infection to destroy invading pathogens and is followed by a rapid resolution phase and repair of the damaged tissue. Thus, acute inflammation plays a beneficial role against infection and injury. However, inadequate resolution of inflammation and uncontrolled inflammatory reactions can evoke a state of chronic inflammation, which is a common etiologic factor for various human ailments including cancer (Aggarwal *et al.*, 2006). Since 19th century proposition of an inflammation–cancer connection (Balkwill and Mantovani, 2001), the persuasive role of inflammation in carcinogenesis has become more and more evident (Itzkowitz and Yio, 2004; Kundu and Surh, 2008; O'Byrne and Dalgleish, 2001). Numerous laboratory and population-based studies suggest that certain
malignancies arise at tissues severely damaged by chronic inflammation. For example, cancers of stomach, liver, gall bladder, prostate and pancreas are causally linked to gastric inflammation, chronic hepatitis and inflammatory atrophy of the prostate respectively (Kundu and Surh, 2008). Present investigation proved the commendable anti-inflammatory property of Rubus extracts which will encourage the researchers to engage their research in finding out an anticancer drug in the light of this study.

6.7. Wound healing activities of Rubus extracts

In the wound healing studies, RFRA extracts showed superior wound healing activity in excision, incision and infected wound models. The epithelization period of 2% RFRA and betadine treated excision wound was 13.02 and 12.62 days respectively. The tensile strength of the 2% RFRA treated mice was found to be significant (95.31 N/cm²) in the incision model. In infected model, the epithelization period of 2% RFRA and neomycin treated wound was 15.02 and 12.06 days respectively. Previously, Pesin and coworkers (2011) reported that the aerial parts of R. sanctus promote incision and excision wound healing; the present results are in strong agreement with their work. Flavonoids and their derivatives are known to decrease lipid peroxidation by improving vascularity and by preventing or slowing down the progress of cell necrosis. Hence, any drug that inhibits lipid peroxidation is supposed to increase the viability of collagen fibrils by increasing the circulation and the strength of collagen fibres, by encouraging the DNA synthesis and preventing cell damage (Getie et al., 2002; Shetty et al., 2008). Flavonoids are known to endorse the wound healing process due to their antimicrobial and astringent properties (Tsuchiya et al., 1996), which appears to be responsible for wound contraction and elevated epithelization.

The process of wound healing is promoted by several natural products which are composed of active principles like triterpenes, alkaloids, flavonoids and biomolecules (Sumitra et al., 2005). In this investigation the extracts which showed significant wound healing properties also showed higher levels of phenolics and flavonoids, which may be the reason behind the wound healing activities and supports previously published reports. Oxidative stress also plays an important role in impaired wound healing; botanicals with antioxidant or free radical scavenging activity thus can play a significant role in healing.
of wounds (Gutteridge, 1995). Present results can also be correlated strongly with this view; the tested *Rubus* extracts which is having very good antioxidant properties also showed significant wound healing activities.

The breaking strength is the strength of a healing wound and is measured experimentally by the amount of force required to disrupt 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 rapidly as collagen deposition increases and cross linkages are formed between the collagen fibers. Similar to the work of Shirwaikar *et al.* (2003) and Singh *et al.* (2005), an increase in the tensile strength of the *Rubus* extract treated wounds were observed in incision model, and this may be due to the increase in collagen concentration and stabilization of the fibres. Wound strength is acquired from both remodeling of collagen and the formation of stable intra and inter molecular cross link (Udupa *et al.*, 1995). Free radicals and oxidative reaction products produce tissue damage and encounter the wound healing process, the overproduction of ROS results in oxidative stress thereby causing cytotoxicity and delayed wound healing. Therefore, elimination of ROS could be an important strategy in wound healing (Dissemend *et al.*, 2002). Hence, the estimation of antioxidants like SOD in granulation tissue is relevant, because this will fasten the wound healing process by destroying the free radicals (Halliwell *et al.*, 1988). In this study, SOD levels of the granuloma tissue of the standard and *Rubus* extract treated groups possessed significant increase when compared to the control animals, which would help to prevent oxidative damage and promote wound healing. The wound healing potential of *Rubus* extracts may be attributed to the presence of phytoconstituents, which may be either due to their individual or additive effect that speeds up the process most probably the proliferation phase of wound healing.

6.8. Cytotoxic and antitumor properties of *Rubus* extracts

Plant derived extracts which contain antioxidant compounds usually shows cytotoxicity towards tumor cells. The present study indicates that *Rubus* extract is a strong antioxidant and is cytotoxic towards EAC and DLA cells in trypan blue dye exclusion method. RELM induced a 70% death in DLA cells and 78% death in EAC cells
at 200 μg/mL concentration. At the same concentration RNRA showed 58% and 70% toxicity to DLA and EAC cells respectively, whereas RFRA extract was highly toxic to both the cells (100% at 200 μg/mL). The most active RFRA extract was also checked for its cytocidal activity in various cancer cell lines like Jurkat, MCF-7 and HeLa by MTT assay. It showed a varying level of cytotoxicity to each cell line. The extract was highly toxic to MCF-7 cells with an IC$_{50}$ of 29.36 μg/mL. When treated with Vero cells, the normal kidney cell, interestingly it was comparatively less toxic (IC$_{50}$: 57.14 μg/mL). The RFRA extract (20 μg/mL) significantly induced apoptotic process in the EAC cells and it was evident from the morphological examination of the treated EAC cells and from the DNA ladder formation.

In addition to strong antioxidant properties, previously many works have been done in vitro cytotoxic activities of Rubus species against various cell lines (Liu et al., 2002; Seeram et al., 2006; Fan et al., 2011; Jeong et al., 2010; Skupien et al., 2006; Kim et al., 2005; Shiow et al., 2008). McDougall et al. (2008) suggested that the key component that related to the inhibition of cancer cell growth could be ellagitannins from the Rubus family, whereas; Fresco et al. (2010) investigated that phenolic-rich extracts have been widely used due to their potential antiproliferative properties. The Rubus extracts with higher levels of tannins and phenolics also showed higher cytotoxic activity which supports the previous findings.

Bowen-Forbes et al. (2010) analyzed the antiproliferative activities of fruits of wild Jamaica-grown; R. jamaicensis, R. rosifolius and R. racemosus, and Michigan-grown; R. acuminatus and R. idaeus. The hexane extracts of the Jamaican Rubus spp. exhibited the greatest potential to inhibit cancer cell growth; inhibiting colon (HCT-116), breast (MCF-7), lung (NCI-H460) and gastric (AGS) human tumor cells by 50, 24, 54 and 37% respectively. Bowen-Forbes et al. (2009) also reported the antiproliferative activity of compounds isolated from R. rosifolius fruit. Some of the compounds displayed significant growth inhibition (40-56%) and were specific to HCT-116 colon tumor cells. Whereas moderate levels of cytotoxicity observed in MCF-7, SF-268, NCI H460, and AGS human tumor cells. Contradictory to these reports, RFRA extract possessed greater potential to inhibit MCF-7 cell lines (IC$_{50}$: 29.36 μg/mL). Ross et al. (2007) reported that R. idaeus extract reduced proliferation of HeLa cells in vitro in a dose-dependent manner.
RFRA extract tested in this study also had a moderate activity for HeLa cell inhibition ($IC_{50}: 33.88 \mu g/mL$). The short term and long term cytotoxic activities of the tested Rubus extracts showed significant antiproliferative activity, when compared to the previous findings.

There are many reports that extracts from plants such as Emblica officinalis (Jeena et al., 2011), Phyllanthus amarus (Rajeshkumar et al., 2002), Curcuma longa (Ruby et al., 1995), Picrorrhiza kurroa (Joy et al., 2004), Piper longum (Sunila and Kuttan, 2004) etc. have antitumor activities, all these plants have also shown better antioxidant potentials. These observations correlate with the present study in a way that the ability of Rubus extracts to inhibit the incidence and progression of tumor would essentially due to the antioxidant property. Stoner et al. (2007) reported that dietary freeze-dried berries were shown to inhibit chemically induced cancer. The berries are effective at both initiation and progression stages of tumor development. Berries inhibit tumor initiation by influencing carcinogen metabolism and inhibit progression by reducing the growth rate of pre-malignant cells and thereby promoting apoptosis.

Antitumor properties of the Rubus extracts in animal models have not been reported previously. The results of the present study showed an increase in life span of the tumor bearing mice treated with Rubus extracts by inhibiting the ascitic fluid accumulation in the peritoneal cavity and by decreasing the volume of solid tumor. These results clearly demonstrated that the antitumor effect of these extracts is by a direct cytotoxic effect. From scientific evidences, many of the natural herbs have anticancer activity and the active compounds can inhibit cell growth, proliferation and induce apoptosis. Antitumor activity of antioxidants is either through induction of apoptosis or by inhibition of angiogenesis (Ruby et al., 1995). Lee and coworkers, (2003) suggested that apoptosis plays a critical role in tissue homeostasis and cancer development and thus it has become an interesting target for both cancer therapeutic and preventive approaches. Apoptosis is regulated by certain events in the cell which include morphological changes, production of apoptotic bodies and damage to genetic material (Sunila et al., 2009). The present study results strongly agrees these facts and suggests that the significant antitumor activity of RFRA extracts might be due to induction of apoptosis. The cascade of events taking place within the cell produces an ultimate cell death which is evident
from the morphological changes and DNA laddering of EAC cells treated with 20 μg/mL of RFRA extract, so it could be an external agent for the treatment of cancer by inducing apoptosis.

6.9. Bioactive compounds isolation from acetone extract of *R. fairholmianus* root

The process that leads to the discovery of biologically or pharmacologically active pure constituents from the plant is tedious and requires multidisciplinary approach. Thus, the process of bioactivity guided separation is very important for the new drug development. Activity guided fractionation involves the pharmacological evaluation of a crude drug followed by extraction using solvents. Each of these individual extracts is then tested for biological activity; those which are not active are discarded and the active extracts are further separated into fractions. This process is repeated till the pure active compound is obtained (Dey and Harbone, 1991).

The present study reports the bioactivity guided isolation, structure elucidation and antioxidant activities of 6 compounds; 1-((2-hydroxy phenyl)-4- methyl pentan-1-one (1), cis 2-(isopentyloxy carbonyl) benzoic acid (2), 2-(5-methylhexyl) benzoic acid (3), 4- methylpentyl benzoate (4), 3-(iminomethylene)-2,4-dimethylphenol (5) and isopentyl benzoate or 3- methyl benzoate (6) from the root acetone extract of *R. fairholmianus*. There are no previous reports available on this plant about the compound isolation and other bioactivity studies. However there are many phytochemical reports available on the related species of *Rubus*.

Porter *et al.* (2012) reported minor flavonoid components in *R. idaeus* such as; quercetin 3-O-a-rhamnosyl-(1-2)- [(6-O-3-hydroxy-3-methylglutaryl) -b-galactoside], kaempferol 3-O-a-rhamnosyl-(1-2)- [(6-O-3-hydroxy-3-methylglutaryl) -b-galactoside], quercetin 3-O-[(6-O-3-hydroxy-3-methylglutaryl)-b-galactoside] and kaempferol 3-O-[(X-O-3 hydroxy-3-methylglutaryl)-b-galactoside]. Bowen-Forbes *et al.* (2009) reported the presence of hydroxyursane type compounds in the ethyl acetate extract of *R. rosifolius* fruits. These compounds were highly effective in the inhibition of cancer cell proliferation and have *in vitro* anti-inflammatory properties. Li *et al.* (2009) isolated ten new triterpenoid saponins named rubusides A-J, and 21 known saponins from the roots of *R. ellipticus*. Mertz *et al.* (2007) analysed the phenolic compounds in *R. glaucus* and
*R. adenotrichus* in which ellagitannins were the major compounds with sanguin H-6 and lambertianin C being the predominant ones. The anthocyanin composition as well as the presence or absence of kaempferol glycosides can be used to distinguish the *Rubus* species. They also identified flavonol hexoside-malonates in both berries. Vrhovsek *et al.* (2006), Wada and Ou (2002) and Kahkonen *et al.* (2001) studied the *Rubus* ellagitannins, which constitutes a complex mixture of monomeric and oligomeric tannins. They reported high contents of ellagitannins and ellagic acids in strawberry, cranberry, blueberry and blackberry extracts. Cuevas-Rodriguez *et al.* (2010) suggested the presence of anthocyanins and proanthocyanidins in wild and domesticated Mexican blackberries.

Maatta-Riihinen *et al.* (2004) reported cyanidin-3-glucoside, cyanidin-3-rutinoside and pelargonidin-3-glucoside in *R. parviflorus*; also reported the phenolic compounds such as hydroxycinnamic acids, flavonols, anthocyanidins flavon-3-ols, cyanidin-3-glucoside, cyanidin-3-rutinoside, pelargonidin-3-glucoside, pelargonidin-3-rutinoside and ellagitannins in *R. arcticus*. Sahar *et al.* (2003) isolated natural caffeoyl esters; 3, 6-di-O-cafeoyl-(a/b)-glucose, 1-O-cafeoyl-b-xylene and a natural tannin; 2, 3-O-hexahydroxydiphenoyl-4, 6-O-sanguisorboyl-(a/b)-glucose from the aerial parts of *R. sanctus*. Flaminia *et al.* (2002) reported new anthrones and rubanthrone from aerial parts of *R. ulmifolius*.

Ruiz *et al.* (2013) reported the presence of total anthocyanins (0.34-0.92 μM/g), monomeric anthocyanins (0.31-1.16 μM/g) and cyanidin-3-sambubioside in *R. geoides* fruits. Bowen-Forbes *et al.* (2010) reported cyanidin-3-glucoside and cyanidin-3-rutinoside in *R. acuminatus*; cyanidin-3-glucoside and cyanidin-3-malonylglicoside in *R. jamaicensis*; cyanidin-3-glucoside, pelargonidin-3-glucoside, and pelargonidin-3-rutinoside in *R. rosifolius*; cyanidin-3-glucoside and cyanidin-3-rutinoside in *R. racemosus*. Montoya *et al.* (2010), Gancel *et al.* (2010) and Mertz *et al.* (2007) reported cyanidin-3-glucoside, cyanidin-3-malonylglicoside, lambertianin C and sanguin H-6 in *R. adenotrichos*. Their studies concluded that tropical highland blackberry is a good source of antioxidants and contains appreciable levels of phenolic compounds, mainly ellagitannins and anthocyanins.

the presence of cyanidin-3-glucoside, cyanidin-3-sambubioside, cyanidin-3-xylosylrutinoside, cyanidin-3-rutinoside, pelargonidin-3-glucoside, and pelargonidin-3-rutinoside, cyanidinglycoside, lambertianin C and sanguin H-6 and 2 ellagittannins in *R. glaucus* and *R. idaeus*. Tian *et al.* (2006), Byamukama *et al.* (2005), Koponen *et al.* (2007), Maatta-Riihinen *et al.* (2004), Netzel *et al.* (2006) and Kubota *et al.* (2012) reported the occurrence of cyanidin based anthocyanins in various *Rubus* species such as *R. occidentalis*, *R. pinnatus*, *R. rigidus*, *R. chamaemorus*, *R. moluccanus*, *R. croceacanthus* and *R. sieboldii*. Wyzgoski *et al.* (2010); Dossett *et al.* (2008 and 2010); Ling *et al.* (2009), Tulio *et al.* (2008), Kim *et al.* (2011), Ku and Mun (2008), Bae *et al.* (2007) and Deighton *et al.* (2000) reported cyanidin-3-sambubioside, cyanidin-3-glucoside, cyanidin-3-xylosylrutinoside, cyanidin-3-rutinoside, pelargonidin-3-rutinoside and peonidin-3-rutinoside in *R. occidentalis* and *R. coreanus*.

The fractions and isolated compounds showed strong DPPH• and ABTS radical cation scavenging activities; among the different fractions, F9 fraction showed highest scavenging activity against DPPH• with a least IC_{50} value (3.61 µg/mL), whereas F15 showed maximum ABTS radical cation scavenging activity (4974.72 µM TE/g extract). The IC_{50} values of the isolated compounds ranged between 12.35 and 3.23 µg/mL. Compound 1 showed higher DPPH• scavenging activity with an IC_{50} of 3.23 µg/mL. The highest ABTS radical cation scavenging was observed for compound 2 (9233.20 µM TE/g extract). The phenolic compounds in berries have been well characterized as natural antioxidants, which are believed to play a major role in certain health benefits. The naturally occurring phenolic antioxidants encompass a diverse range of chemical classes that protect against the damage caused by ROS to DNA, membrane and cellular components (Shahidi, 1997). The strong antioxidant activities of RFRA extract, fractions and isolated compounds might be due to the phenolic compounds. Previously, Vadivelan *et al.* (2009) reported the antioxidant properties of *R. ellipticus* root extracts. The methanol extracts had the strongest radical scavenging activity (IC_{50}: 12.2 µg/mL) against DPPH• and (IC_{50}: 2.5 µg/mL) against ABTS radical. The DPPH radical scavenging activity of allied species; *R. sanctus* has also been reported, which found to scavenge the DPPH radical by 83.27% when compared with Vitamin C and BHT (97.15 and 96.47%) (Motamed and Naghibi, 2010).
6.10. Molecular Docking of bioactive compounds against BRCA and COX proteins

Molecular docking is an approach to help researchers to screen a large set of small molecules by orienting and scoring them in the binding site of target proteins. Top ranked compounds may be tested for binding affinity and activity in vitro and further it may become lead compounds for drug development.

The molecular docking results showed that the isolated compound 1-(2-hydroxyphenyl) -4-methylpentan-1-one is highly significant in binding (G-score -4.7) and formed three hydrogen bonds against SER 6: H (HG), GLY 1656: (H)H and LYS 1702 (H) HZ2 residues of BRCA1. The residues involved in binding were more or less similar with that of standard drugs. The docking scores of 2-[(3-methylbutoxy) carbonyl] benzoic acid, 4-methylpentyl benzoate, 2-(5-methylhexyl) benzoic acid, 3-(iminomethyl)-2,4-dimethylphenol and 3-methylbutyl benzoate compounds ranged between -3.4 to -3.83 and the docking score of the standard drugs falls between -2.0 to -4.82. Docking score and H bond interaction of all 6 isolated compounds were closely related to standard drugs.

The docking results of BRCA2 with the isolated compounds 1-(2-hydroxyphenyl) -4-methylpentan-1-one and 4-methylpentyl benzoate are found to be significant. Besides, 1-(2-hydroxyphenyl)-4-methylpentan-1-one formed three hydrogen bonds with the ALA 874: (O) O, ALA874: (H) H and GLY1166: (H) H residues of BRCA2 protein and 4-methylpentyl benzoate formed 2 hydrogen bonds with the ALA 1063: (H) H and LYS 1062 (H) HZ2 residues of BRCA2. Some of the binding residues were common in both standard drugs and the isolated compounds. The G-scores of isolated compounds ranged between -4.5 and -3.1, where as the standard drugs were in between -11.1 and -3.2. BRCA1 and BRCA2 predispose individuals to breast, ovarian and other cancers. These proteins are required for the maintenance of genetic stability and have function in DNA damage (Venkitaraman, 2001). Previously, Raja et al. (2011) studied the docking of mangrove-derived compounds and revealed that triterpenoid, stigmasterol and pyrethrin were efficient in destroying BRCA1. Saravanakumar and coworkers (2012) proposed the docking of fungal metabolites against BRCA1. The docking score of phthalic acid was lowest (-13.71 kcal/mol) followed by 2, 3-dihydro-1H-inden-2-yl acetate and 3-methylcyclopentan-1-ol having the value of -11.11 and -9.08 respectively. However,
there is no report on the docking of isolated compounds from *R. fairholmianus* against BRCA proteins. So the information gained from this study will be useful for further development of novel breast cancer inhibitors.

The results of the COX1 docking with the isolated compounds found to be significant. The G-score of the three isolated compounds (-3.23 to -5.28) were higher than standard drugs such as diclofenac, paracetamol and morphine (-3.77, -3.41 and -3.36). The hydrogen bond interaction of 2-[(3-methylbutoxy) carbonyl] benzoic acid and diclofenac were seems to be similar. The compound 1-(2-hydroxyphenyl)-4-methylpentan-1-one formed two hydrogen bonds with the ASN 375: (H) H, ASN 375: (O) O residues of COX1 and 2-[(3-methylbutoxy) carbonyl] benzoic acid formed three hydrogen bonds with the ARG 374: (H) HH21, ARG 374 (H) HH12 and ASN 375: (H) H residues of COX2. The docking results of isolated compounds with COX2 are significant when compared with the standard drugs. The G-score of the compounds ranged between -2.9 to -0.4 in which the highest G-score was obtained for 2-[(3-methylbutoxy) carbonyl] benzoic acid which bound with HID 228: (H) HD1, LYS 543: (H) HZ1 and LYS 543: (H) HZ1 residues of the COX2 protein. The binding residues (LYS 543: (H) HZ3, LYS 543: (H) HZ2, and THR 547: (H) HG1) of the diclofenac was also similar to 2-[(3-methylbutoxy) carbonyl] benzoic acid.

Recent studies have shown that, stellatin have COX-1 and COX-2 inhibitory activities which was isolated from *Dysophylla stellata*. Docking study revealed the binding orientations of stellatin and its derivatives into COX-1 and COX-2 and thereby helps to design potent inhibitors (Gautam et al., 2011). Olgen et al. (2001) reported the COX inhibitory activities of indomethacin derivatives (*N*-substituted indole-2-carboxylic acid esters). All the derivatives were shown to be docked at the site where intact flurbiprofen was embedded for COX-1 and s-58 for COX-2. Three series of Spiro derivatives have been synthesized and docked to COX-2 by Amin et al. (2010). The results showed nearest value of indomethacin. Abdel-Aziz et al. (2011) designed a group of cyclic imides (1-13) and evaluated its selective COX-2 inhibition. The Molecular docking study showed that the CH$_3$O substituents of 5b inserted firmly to COX-2, where the O-atoms of such group underwent a H-bonding interaction with HIS90 (2.43, 2.83 Å), ARG513 (2.89 Å) and TYR355 (3.34 Å). This revealed a similar binding mode to
SC-558, a selective COX-2 inhibitor. Basile and coworkers (2012) have synthesized 14 sulfonilamidothiopyrimidone derivatives. The compounds 2-5 were able to fit into the active site of COX-2 with highest scores and interaction energies. Furthermore, compound 2, which showed an inhibition of around 50% on PGE2 production, was best scored. El-Sayed et al. (2011) designed and synthesized new arylhydrazone derivatives and a series of 1,5-diphenyl pyrazoles from 1-(4-chlorophenyl)-4,4,4-trifuorobutane-1,3-dione 1. The designed compounds docked into the COX-2. Docking study of the synthesized compounds 2f, 6a and 6d into COX-2 revealed a similar binding mode to SC-558.

The present study describes, the isolated compounds from acetone extract of *R. fairholmianus* root docked to the active pockets of BRCA and COX target proteins to predict if these compounds have analogous binding mode to the BRCA and COX inhibitors. The results revealed that the compounds were docked more firmly and inhibited the breast cancer related BRCA1 and BRCA2 oncoproteins, and COX-2 and COX-1 inflammatory proteins. Among the 6 compounds, 1-(2-hydroxyphenyl)-4-methylpentan-1-one and 2-[(3-methylbutoxy) carbonyl] benzoic acid were found to be more active in inhibiting BRCA and COX proteins. Therefore, these compounds may be considered as the lead compounds for the development of drugs against BRCA and COX proteins.