7. SUMMARY AND CONCLUSION
In the past couple of decades, approaches to the study of biologically active natural products have changed so dramatically that one is almost tempted to say that a new science has been born. Since, more than 80% of world’s population uses plants as a primary source of medicinal agents, many countries of the world started providing more importance to herbal drug research.

Free radicals or oxidative stress and the damage produced by it is found to be an important pathophysiological event in a variety of diseases including aging, cancer, diabetes, rheumatoid arthritis, atherosclerosis, cardiovascular disorders, neurodegenerative hepatodegenerative disorders (Ajitha and Rajnarayana, 2001; Hogg, 1998; Pong, 2003) and various other pathological effects. Several commercially available synthetic antioxidants such as butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT) are quite unsafe and their toxicity is a matter of concern (Alam et al., 2006). Hence, the development of alternative antioxidants from natural sources, especially plants, has been an area of considerable focus. Many antioxidants from plant sources have been shown to possess preventive action in both primary clinical manifestations and late complications of diseases like cancer, diabetes, inflammation, arthritis, skin diseases etc.

In India, Zingiberaceae is one of the infamously used family of herbs. *Kaempferia galanga* belongs to this family and is widely used as a spice, food flavoring agent and an ingredient in the preparation of a general tonic. *Kaempferia galanga*, particularly its rhizome, is traditionally prescribed for treatment of hypertension, rheumatism and asthma.

A thorough review into the literature revealed that a systematic study on antioxidant, anti-inflammatory, hepatoprotective and cytotoxic activities of rhizome of this plant has not been undertaken yet. Hence, we have investigated above said activities of rhizome extract of this plant.

Dried, powdered *Kaempferia galanga* rhizome was extracted successively with petroleum ether (60-80°C), ethyl acetate and ethanol in a soxhlet extractor. The successive petroleum ether (SKG-1) ethyl acetate (SKG-2) and ethanol (SKG-3) extracts yields were about 3.5, 2.5, and 3 % w/w respectively with respect to dried powdered material of rhizome. Rhizome was also extracted with ethanol (KG) with the yield of about 4% w/w. The preliminary phytochemical studies revealed the presence of steroids, fixed oils, flavonoids, terpenoids and tannins in the extracts.
One compound each was isolated from crude alcoholic extract (KG) and petroleum ether extract (SKG-1). The structural elucidation by various spectroscopic methods suggested the compound as ethyl-\(\beta\)-methoxy cinnamate (ISO-2).

All the extracts, (SKG-1, SKG-2, SKG-3 and KG) and isolated compound (ISO-2) were screened for *in vitro* and *in vivo* antioxidant, hepatoprotective, anti-inflammatory and cytotoxic activities.

All the extracts and ISO-2 were subjected for the evaluation of free radical scavenging potential by seven standard *in vitro* methods viz. 2, 2-diphenyl-1-picryl-hydrazyl (DPPH), nitric oxide, 2, 2'-azino-bis (3-ethylbenzo-thiazoline-6-sulfonic acid) diamonium salt (ABTS), hydrogen peroxide, hydroxyl radical by p-nitroso dimethyl aniline (p-NDA), super oxide radical by alkaline dimethyl sulphoxide (alkaline DMSO) and lipid peroxidation inhibition and compared with standard antioxidants.

DPPH radical scavenging assay is a very important preliminary method for identifying the compounds/extracts with hydrogen donating abilities SKG-2, SKG-3 and KG showed comparable DPPH scavenging ability in steady state experiments.

ABTS\(^{•−}\) scavenging assay is a very powerful method for identifying the compounds/extracts with electron accepting/donating abilities. In this assay SKG-2, SKG-3 and KG exhibited higher ABTS\(^{•−}\) scavenging ability.

\(\cdot\text{OH}, \text{O}_2^{•−}, \text{H}_2\text{O}_2\) scavenging and inhibition of lipid peroxidation have greater biological relevance than ABTS\(^{•−}\) and DPPH scavenging. \(\cdot\text{OH}\) is most deleterious oxidizing radical among those physiologically present. Its reaction rate constant with most of the biomolecules is diffusion controlled (Buxton et al., 1988). It reacts by addition, hydrogen abstraction and electron transfer, thereby providing the transient radicals of the biomolecules concerned (Spinks and Woods, 1990). SKG-1, SKG-2 SKG-3 and KG showed \(\cdot\text{OH}, \text{O}_2^{•−}\) and \(\text{H}_2\text{O}_2\) scavenging abilities and inhibition of Fe\(^{3+}\)/ascorbate induced lipid peroxidation. However, nitric oxide scavenging activity was shown by SKG-2 and SKG-3 only. Further nitric oxide radical scavenging by ISO-2 was not significant with IC\(_{50}\) 850 ± 1.4 \(\mu\)g /ml and failed to inhibit lipid peroxidation as well as scavenging of the DPPH, ABTS, \(\cdot\text{OH}, \text{O}_2^{•−}\) and \(\text{H}_2\text{O}_2\) free radicals even up to 1000 \(\mu\)g/ml.
The *ex-vivo* study has shown that SKG-2, SKG-3, KG and ISO-2 can protect hemoglobin from oxidation by sodium nitrite in hemolysate but not in intact erythrocytes. However, these did not reverse the effect of nitrite if added at a later stage. It is well established that oxidation of hemoglobin takes place in two stages. There is a slow initial stage followed by a rapid autocatalytic stage, which carries the reaction to completion. (Doyle et al., 1982.) SKG-2, SKG-3, KG and ISO-2 were able to prevent the onset of autocatalytic stage. Since superoxide is implicated in the autocatalytic stage (Doyle et al., 1982) and the experimental evidence that SKG-2, SKG-3 and KG are potent scavenger of superoxide, the protective action is by scavenging superoxide generated during oxidation. (Table 5.1.1.)

The inability of all the extracts and ISO-2 to protect intact erythrocytes suggested that the active ingredient responsible for activity may not cross the membrane of erythrocytes.

The elevation of TBARS and decrease in the activity of free radical scavenging enzymes, viz., catalase, reduced glutathione and total thiols which are the indications of cellular damage due to high oxidative stress as a result of CCl₄ assault in the intoxicated rats. A significant prevention of these changes and restoration of normalcy was observed with SKG-1 (300 and 500 mg/kg), SKG-2, SKG-3, KG (300 mg/kg) and ISO-2 (100mg/kg) treated animals and also a significant reduction in TBARS of liver tissues was observed. This supports the antioxidant property of SKG-1, SKG-2, SKG-3, KG and ISO-2 (Table 5.1.3.).

Carbon tetrachloride is one of the most commonly used hepatotoxins. It is well documented fact that carbon tetrachloride is biotransformed under the action of cytochrome P450 in the microsomal compartment of liver to trichloromethyl radical which readily reacts with molecular oxygen to form trichloromethylperoxy radical (Raucy et al, 1993). Both these radicals can bind covalently to the macromolecules and induce peroxidative degradation of the membrane lipids of endoplasmic reticulum rich in polyunsaturated fatty acids (Recknagael, 1967). This leads to the formation of lipid peroxides followed by pathological changes such as depression of protein synthesis (Faroon et al, 1994), elevated levels of serum marker enzymes such as AST, ALT, ALP, GGT, LDH, total bilirubin, and direct bilirubin (Zimmerman and Seeff, 1970). Although serum enzyme levels are not a direct measure of hepatic injury and the status of liver the
elevated levels of serum enzymes are indicating of cellular leakage and loss of functional integrity of cell membrane in liver (Zimmerman and Seeff, 1970).

An increased level of AST indicates the liver damage due to viral hepatitis. ALT catalyses the conversion of alanine to pyruvate and glutamate and is released in a similar manner. Therefore ALT is more specific to the liver and a better parameter for detecting liver damage (Willianson et al, 1996). SKG-1(300 and 500 mg/kg) SKG-2, SKG-3, KG (300 mg/kg) and ISO-2 (100 mg/kg) significantly decreased the levels of AST ALT and LDH (Table 5.1.4.). Thus, the lowering of enzyme content in serum is a definite indication of hepatoprotective action of the drug.

Serum ALP, GGT, total bilirubin and direct bilirubin levels are also related to the status and function of hepatic cells. In the present study serum ALP and GGT increased and bilirubin decreased in treated groups compared with untreated group (Table 5.1.4).

Since, anti-inflammatory activity can be associated with antioxidant activity, we have tested anti-inflammatory potential of all the extracts and ISO-2 using two standard rat models viz., a) evaluation of acute anti-inflammatory activity by carrageenan induced rat hind paw edema and b) evaluation of chronic anti-inflammatory activity by Freund’s Complete Adjuvant induction paw inflammation model and myeloperoxidase estimation.

The SKG-1 showed greater acute anti-inflammatory activity compared with other extracts. ISO-2 failed to reduce the inflammation in rats. The ability of SKG-1 to exhibit anti-inflammatory activity could be associated with its antioxidant activity. Hence, SKG-1 was taken up for chronic anti-inflammatory studies.

In chronic inflammation SKG-I (100 mg/kg) and diclofenac (5 mg/kg) produced a significant reduction in paw volume on day 28 from the mean volume on day 21. This means that the test drug has significantly reduced inflammation in this model, which is comparable with that of the standard drug, diclofenac.

In the present work, treatment with diclofenac sodium and SKG-1 has prevented the elevation in myeloperoxidase, showing anti-inflammatory activity. SKG-1, at 100 mg/kg b.w. was able to keep the myeloperoxidase at the same level as in normal rats. Between diclofenac treated and SKG-1 treated groups, there was a significant difference in
myeloperoxidase, with the former group having the higher level. Since the paw edema volumes in the two groups were similar, it seems other mechanisms, in addition to myeloperoxidase inhibition, could have contributed to anti-inflammatory effect.

It is well understood that antioxidants can be potent anticancer agents. Hence, in vitro cytotoxic properties of all the extracts and ISO-2 was carried out against several cancer and normal cell cultures using standard Microculture tetrazolium (MTT) and Sulphorhodamine B (SRB) assays. Cell lines representing SW 620 (human colorectal adenocarcinoma cells), PA-1 (human ovarian cancer cells), DU-145 (human prostate cancer cells), B16-F10 (mouse skin melanoma cells) and Vero (normal, African green monkey kidney) were selected for study. The SKG-2 showed selective toxicity towards cancer cells and showed less toxicity towards normal cells. The SKG-1 showed potent activity against SW 620 with CTC_{50} 0.55 \mu g/ml and was comparatively non toxic towards normal Vero cell cultures.

Use of standardized extract for biological study is one of the main criteria in the recent years in the pharmacological evaluation of herbal drugs. Standardization of Kaempferia galanga rhizome was carried out by RP-HPLC technique using ethyl-p-methoxy cinnamate as marker compound. The proposed standardization method was found to be simple, rapid, accurate, precise, linear, rugged and robust. The method can be used for quantitative analysis of Kaempferia galanga.

The results have clearly revealed that amongst four extracts studied, petroleum ether extract showed potent antioxidant, hepatoprotective and anti-inflammatory activities. This study has indicated a bioactive marker ethyl-p-methoxy cinnamate for future standardization of Kaempferia galanga. It showed good antioxidant (in vivo) and moderate hepatoprotective activity. Further studies of the extracts are needed to establish its mechanism of action of the bioactive molecule. This will help in understanding and designing of potent antioxidant, hepatoprotective, anti-inflammatory and anticancer drug from this plant.