4

Results and Discussion

The high cost of drugs and the inability of many developing countries to avail modern drugs have forced communities to look for products from medicinal plants that have proved to be effective, safe, inexpensive and culturally acceptable (Adefa and Abraha, 2011). Pharmaceutical and scientific communities have recently started exploiting medicinal plants and various publications have been documented regarding the therapeutic worth of natural compounds to validate the claims of their biological activity (Das *et al*., 2010).

Hence the present study entitled **"Evaluation of antioxidant and anticancer potential of *Cassia senna* L. using *in vitro* and *in vivo* methods"** was undertaken. The results of the study are furnished and discussed under the following headings:

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PHASE II

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PHASE I

4.1. Preliminary investigation of antioxidants and phytochemicals in the selected parts of *C. senna*

4.1.1. Antioxidant assays in the leaf and pod of *C. senna*

An antioxidant is a compound that inhibits or delays the oxidation of substrates and it scavenges reactive oxygen species (ROS) and reactive nitrogen species (RNS) that may be of major importance in preventing the onset and the progression of oxidative pathologies (Angaji *et al*., 2012). Antioxidants have important preventive roles on tissue damage in various human diseases. They are effective in prevention of degenerative illnesses, such as different types of cancers, cardiovascular and neurological diseases, cataracts and oxidative stress dysfunctions (Sharma *et al*., 2013).

4.1.1.1. Determination of enzymic antioxidant activities

The inevitable generation of reactive oxygen species in biological system and the oxidative damage is counterpoised by an array of enzymic defense system (Nagavani and Rao, 2010). SOD is the key enzyme in the natural defense against free radicals and it inactivates the superoxide ion by transforming it into hydrogen peroxide (H_2O_2). The latter is then quickly catabolised by CAT and POD into dioxygen(O_2) and water (H_2O)(Menvielle-Bourg, 2005). The role of CAT in deterring tumor promotion and enhancing cell survival during periods of oxidative stress supports the antioxidant function of this enzyme (Agar *et al.,* 1986). Increased activity of CAT, POX, and SOD has been suggested as an adaptive mechanism to reduce the H_2O_2 and offer protection against oxidative damage (Agarwal and Pandey, 2004).

GST is an antioxidant, involved in the metabolism of many disease-causing electrophilic substrates that protects the cells against oxidative stress and is also useful in monitoring of cellular induction (Sarkar *et al*., 2010). PPO is otherwise called as catecholase and o-diphenol oxidase. It is an oxidative enzyme (Akond *et al*., 2010) and has been implicated in the formation of pigments, oxygen scavenging and defense mechanism against plant pathogens (Queiroz *et al*., 2008).

The powdered samples of leaf and pod of *C. senna* were extracted with appropriate buffers and the extracts were analyzed for the various enzymic antioxidants

namely superoxide dismutase(SOD), catalase(CAT), peroxidase(POD), glutathione S-transferase(GST) and polyphenol oxidase(PPO) and the results are presented in Table 5.

Table 5 : Enzymic antioxidants of leaf and pod of *C. senna*

Enzymes	Leaf	Pod	't' value
SOD(U/g of tissue)	59.36 ± 0.86	48.63 ± 0.52	15.077**
CAT(U/g of tissue)	283.95 ± 0.66	117.53 ± 0.53	276.6**
POD(U/g of tissue)	1.49 ± 0.14	0.37 ± 0.02	11.19**
GST(U/g of tissue)	0.63 ± 0.02	0.34 ± 0.01	22.72**
PPO(U/g of tissue)	259.03 ± 0.78	250.70 ± 0.70	68.00**

** - Significant at 1% level

SOD - 500 mM potassium phosphate buffer with pH 7.8
CAT - 0.067 M phosphate buffer with pH 7.0
POD, GST and PPO - 0.1 M phosphate buffer with pH 6.5

Units:

a. Superoxide dismutase	Amount of enzyme that cause 50% reduction in NBT oxidation.
b. Catalase	Amount of enzyme required to decrease absorption at 240nm by 0.05 units/min.
c. Peroxidase	Amount of enzyme to cause change in absorption at 430nm/min
d. Glutathione S-Transferase	nmoles of CDNB conjugated / minute
e. Polyphenol oxidase	Amount of catechol oxidase enzyme which transforms 1 unit of dihydrophenol to quinine/min.

It is evident from Table 5 that the leaf and pod extracts of the *C. senna* exhibited remarkable activity of all the enzymic antioxidants. Among the two parts studied, leaves showed significantly($p<0.05$) higher activities SOD, CAT, POD, GST and PPO. In case of SOD activity leaf was found to exhibit 59.36±0.86 U/g while in pod the activity was 48.63±0.52 U/g. A significant ($p<0.05$) two fold increase was found in the activities of remaining enzymic antioxidants namely CAT, POD, GST and PPO.

Many researchers have reported these enzymic antioxidants in plants. Plants evolved variable extent of SOD activity, which is the most indispensable enzyme for protecting the cells from the toxicity of the reactive oxygen species (Halliwell, 2015).

Nanda and Agrawal, (2016), have reported that increased SOD activity in response to Zn and Cu treatment resulted in the detoxification of superoxide radicals into H_2O_2 which is responsible for the increased activity of CAT in *C. angustifolia* seedlings.

Increased activity of SOD, CAT and POD was significantly observed in the pods of *C. tora* and *C. auriculata* (Pant *et al*., 2014b). Considerable activity of SOD, CAT and high POD activity was reported in leaves of *Sesbania grandiflora* of Fabaceae family (Padmaja *et al*., 2011).

The present results are in accordance with the above findings and showed that the leaves of *C. senna* were found to be good source of enzymic antioxidants than pods.

4.1.1.2. Determination of nonenzymic antioxidant levels

Nonenzymic antioxidants are best-known exogenous as well as endogenous antioxidant substances (Venereo, 2002). Non enzymic mechanism comprises antioxidants such as ascorbic acid, α-tocopherol, glutathione, polyphenols and flavonoids. These can contribute to prevent the damage caused by oxidative reactions (Sharma *et al*., 2010).

Ascorbic acid is the strongest water soluble antioxidant (Kumar and Rizv 2012) and it is one of the most extensively studied antioxidants and has been detected in the majority of plant cell types, organelles and apoplast (Wu *et al*., 2007a). α-Tocopherol is the most active form of vitamin E and it is a lipophilic antioxidant synthesized by all plants (Shao *et al*., 2008). It is estimated that a single α-tocopherol molecule can neutralize up to 120 singlet oxygen molecules *in vitro* before being degraded (Sivakumar *et al*., 2005).

Phenolic compounds are called as high level antioxidants (Burda and Oleszek, (2001). Polyphenols are the most significant compounds for the antioxidant properties of plant raw materials. Then antioxidant activity of polyphenols is mainly due to their redox properties, which allow them to act as reducing agents, hydrogen donors and singlet oxygen quenchers (Sharma *et al*., 2013). Flavonoids are one of the largest groups of secondary metabolites and are ubiquitous in photosynthesizing cells. Flavonoids have been reported to quench active oxygen species and inhibit *in-vitro* oxidation (Angaji *et al*., 2012). Glutathione is a tripeptide(α-glutamyl cysteinylglycine), it takes part in the control of H_2O_2 levels. Reduced glutathione (GSH) acts as an antioxidant and is involved directly in the reduction of most active oxygen radicals generated due to stress (Shao *et al*., 2008).

The powdered samples of leaf and pod of *C. senna* were extracted with appropriate buffers and the extracts were analyzed for the various nonenzymic antioxidants such as ascorbic acid (Vitamin C), α-tocopherol (Vitamin E), polyphenols, flavonoids and reduced glutathione and the results are presented in Table 6.

Table 6 : Nonenzymic antioxidants of leaf and pod of *C. senna*

Nonenzymic antioxidants	Leaf	Pod	't' value
Ascorbic acid(mg/g of tissue)	2.48 ± 0.01	1.67 ± 0.01	98.27**
α-Tocopherol(µg/g of tissue)	2.28 ± 0.01	1.58 ± 0.01	130.8**
Polyphenol(mg/g of tissue)	2.51 ± 0.03	1.33 ± 0.02	56.68**
Flavonoids(mg/g of tissue)	0.32 ± 0.01	0.14 ± 0.01	32.35**
Reduced glutathione(nm/g of tissue)	106.10 ± 0.10	80.40 ± 0.02	246.46**

** - Significant at 1% level

Ascorbic acid - 4% Trichloroacetic acid ; α-Tocopherol - 0.1N sulphuric acid

Polyphenol - 80% ethanol; Flavonoids - methanol: water mixture (2:1)

Reduced glutathione - 5% Trichloroacetic acid

It is evident from the Table 6 that, a significantly($p<0.05$) higher levels of ascorbic acid, 2.48 ±0.11 mg/g was seen in leaf sample of *C. senna* while in pods the content of the same was found to be 1.67±0.23 mg/g. The level of α-tocopherol, polyphenols and glutathione of the leaves was found to be significantly($p<0.05$) higher than that of pods. The content of flavonoids was significantly($p<0.05$) two fold higher(0.33±0.15mg/g) in the leaf sample than that of pods(0.14±0.18mg/g). Flavonoids are potent antioxidants followed by ascorbic acid (Sati *et al*., 2010).

The present study indicates that leaves possess higher level of ascorbic acid and α-tocopherol and these results are supported by earlier findings. Ascorbate occurs in all plant tissues, usually higher in photosynthetic cells and meristems (Shao *et al*., 2008). α-tocopherol found in green part of plants particularly in leaves, scavenges lipid peroxy radicals through the concerted action of other antioxidants (Angaji *et al*., 2012). The level of ascorbic acid and α-tocopherol of *C. siamea* leaves was found to be maximum when compared with various other plants studied and both these Vitamins (ascorbic acid and α-tocopherol) are the contributors to the antioxidant activity in the plants as reported by Chanwitheesak *et al*., (2005).

The different extracts of the leaf of *C. tora* were found to be rich in phenolics and might be responsible for the antioxidant capacities (Arya and Yadav, 2010). Antioxidant activities of leaves of four *Cassia* species have been reported by Chanda *et al*., (2012) where a direct correlation between phenol content and antioxidant activity was observed.

The *in vivo* and *in vitro* studies of *C. fistula* revealed that it is a good source of antioxidants and the antioxidant properties of different parts depend on total phenolic component as reported by Rizvi *et al*., (2009).

Liu *et al.* (2004) stated that, when the total flavonoid content increased in the plant, antioxidant activity of the plants also increased. Low antioxidant activity in reproductive parts (flowers) could be due to the existence of prooxidant substances that would exhibit suppressing effects in antioxidant capacity (Siddhuraju *et al*., 2002).

Sreelatha and Padma (2009) reported that the leaves of *Moringa oleifera* possessed higher activities of enzymic antioxidants and a higher level of the nonenzymic antioxidants studied thereby indicating that the mature leaf extract significantly exhibited best values of enzymic and nonenzymic antioxidants.

The present study is in accordance with all the above findings on various *Cassia* species and indicates that leaves of *C. senna* was found to be a rich source of nonenzymic antioxidants than pods.

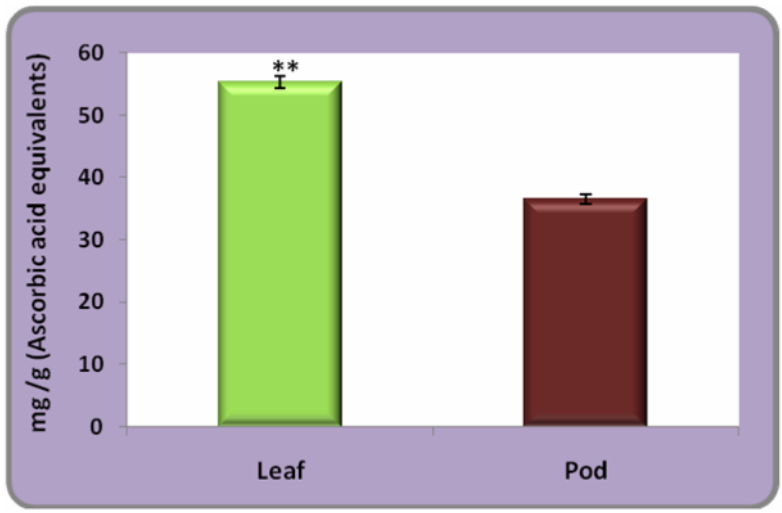
From the present findings of both enzymic and nonenzymic antioxidant assays, leaf of *C. senna* was found to contain higher activity of enzymic antioxidants and elevated levels of nonenzymic antioxidants than pods which might be due to the nature of antioxidants that are present in photosynthetic cells other than reproductive part.

4.1.1.3. Determination of total antioxidant activity

Total antioxidant activity of plant extracts is measured to evaluate both water-soluble and fat-soluble antioxidants totally. Phosphomolybdenum method is widely used to study the antioxidant capacity of plant extracts, wherein the intensity of green phosphomolybdeum complex gives the measure of total antioxidants present in the sample (Ashwini and Krishnamoorthy, 2011).

The total antioxidant activity was measured in the leaf and pod powder of *C. senna* and the results are presented in Figure 5.

Figure 5 : Total antioxidant activity of leaf and pod of *C. senna*



Values are mean ± SD(n=3)

** - statistically significant ($p<0.01$)

From Figure 5, it is clear that leaf sample of *C. senna* exhibited significantly ($p<0.01$) higher (55.36±0.13mg/g of AAE) total antioxidant activity than pod sample (36.56±0.13mg/g of AAE). Recent studies have shown that many flavonoids and related polyphenols contribute significantly to the phosphomolybdate scavenging activity of medicinal plants (Sharififar *et al*., 2009; Khan *et al*., 2012b). This present study is in agreement with the above findings and indicates that presence of significant levels of nonenzymic antioxidants namely flavonoids and polyphenols in leaf sample of *C. senna* might contribute to the phosphomolybdate scavenging activity which is measured as total antioxidant capacity of *C. senna*.

The present study has been supported by earlier findings. Polyphenolic compounds present in *Cassia tora* contributed significantly to the total antioxidant capacity of the fruits (Sirappuselvi and Chitra, 2012). Ashwini and Krishnamoorty, (2011) reported the significant total antioxidant activity of ethanolic extract of *C. tora* leaves and also observed an increasing trend in the total antioxidant activity with increasing concentrations of extract. The total antioxidant activity was observed in the ethanolic extract of *Clerodendrum phlomidis* was higher than that of other solvent extracts namely petroleum ether, chloroform and ethyl acetate (Sathish *et al*., 2011).

Gupta and Prakash (2009) assessed the total antioxidant activity of methanolic extracts of *Amaranthus* species such as *Centella asiatica, Murraya koenigii* and *Trigonella foenum graecum* and also showed the relationship between total antioxidant activity and polyphenol content. Saeed *et al*., (2012) reported that the methanol extract of whole plant of *Torilis leptophylla* (TLM) and its derived fractions [n-hexane (TLH), chloroform (TLC) ethyl acetate (TLE) n-butanol (TLB) and residual aqueous fractions (TLA)] exhibited significant total antioxidant capacity.

Jayaprakasha *et al*., (2003) reported varying degrees of antioxidant capacity (as equivalent to ascorbic acid) in grape seeds. The total antioxidant capacity of the extracts of pineapple was found to decrease in the order, methanol extract > ethyl acetate extract > water extract (Hossain *et al*., 2011). Total antioxidant capacity (equivalent to ascorbic acid) of the three varieties of *Piper betle* leaves and also dried tea leaves have been reported by Dasgupta and De (2004).

The present findings are in accordance with the above literatures cited and indicate that leaves of *C. senna* exhibited higher total antioxidant activity than pods.

4.1.2. Preliminary phytochemical screening of different solvent extracts of leaf and pod of *C. senna*

Preliminary screening of phytochemicals is a valuable step, in the detection of the bioactive principles present in the medicinal plants and subsequently may lead to drug discovery and development (Yadav *et al*., 2014).

The different solvent extracts of leaf and pod of *C. senna* namely petroleum ether, benzene, chloroform, ethyl acetate, successive ethanolic, direct ethanolic and aqueous extract were screened for the presence of various phytochemicals such as carbohydrates, glycosides, proteins, alkaloids, flavonoids, steroids, terpenes, diterpenes, triterpenes, phytosterols, tannins, saponins and phenols. The results obtained are furnished in Table 7 and 8.

Qualitative analysis of phytochemicals in the leaf extracts of *C. senna*

The results of phytochemical screening for leaf extracts of *C. senna* are presented in Table 7.

Table 7 : Qualitative analysis of phytochemicals in the leaf extracts of *C. senna*

S.No	Phytochemical Test	Petroleum Ether Extract	Benzene Extract	Chloroform Extract	Ethyl acetate Extract	Successive Ethanolic Extract (SEE)	Direct Ethanolic Extract (DEE)	Aqueous
I	**TEST FOR CARBOHYDRATES**							
i	Molisch's Test	+++	+++	+++	+++	+++	+++	+++
ii	Fehling's Test	+++	+++	+++	+++	+++	+++	+++
II	**TEST FOR GLYCOSIDES**							
i	Keller-Killani Test	+++	+++	+++	+++	+++	+++	+++
ii	Modified Borntrager's Test	+++	+++	+++	+++	+++	+++	+++
III	**TEST FOR PROTEINS AND AMINOACIDS**							
i	Xanthoproteic Test	-	+++	+++	-	+++	+++	+++
ii.	Ninhydrine Test	-	-	+++	-	+++	+++	+++
IV	**TEST FOR ALKALOIDS**							
i	Mayer's Test	+++	+++	+++	+++	+++	+++	++
ii	Wagner's Test	+++	+++	+++	+++	-	+++	++
iii	Dragendroff's Test	+++	+++	+++	+++	+++	+++	++
V	**TEST FOR FLAVONOIDS**							
i	Alkaline reagent Test	+++	+++	+++	+++	+++	+++	++
ii	Sulphuric Acid Test	-	-	+++	+++	+++	+++	++
iii	Lead acetate Test	+++	+++	+++	+++	+++	+++	++
VI	**TEST FOR TERPENOIDS AND STEROIDS**							
i	Terpenoids	-	-	-	-	++	+++	+
ii	Steroids	+++	+++	+++	+++	+++	+++	+
VII	**TEST FOR DITERPENES**							
i	Copper acetate Test	-	-	-	+++	+++	+++	-
VIII	**TEST FOR TRITERPENOIDS**							
		-	-	-	-	-	+++	-
IX	**TEST FOR PHYTOSTEROLS**							
i	Salkowski's Test	+++	+++	+++	+++	+++	+++	+
ii	Libermann Burchard's Test	+++	+++	+++	+++	++	+++	-
X	**TEST FOR TANNINS**							
i	Lead acetate test	+	++	++	+	+++	+++	+++
XI	**TEST FOR SAPONINS**							
i	Froth Test	-	-	-	-	++	+++	++
XII	**TEST FOR PHENOLS**							
i	Ferric Chloride Test	+++	+++	+++	+++	+++	+++	+++

+++ highly present, ++ moderately present, + slightly present, – absent

Preliminary phytochemical screening of different solvent extracts of leaf of *C. senna* showed the presence of various phytochemicals such as carbohydrates, proteins, glycosides, alkaloids, flavonoids, terpenoids, steroids, phytosterols, tannins, saponins and phenols. As evident from the Table 7, among the various solvent extracts analyzed, DEE followed by SEE of *C. senna* leaves were found to contain maximum number of phytoconstituents. Alkaloids of certain plant species are known to decrease blood pressure and balance the nervous system in case of mental illness. Tannins are known for their astringent, wound healing and anti-parasitic properties. The presence of terpenes suggests its possible use as anti-tumor and anti-viral agent as some terpenes are known to be cytotoxic to tumor cells (Ronan *et al*., 2009). Therefore the presence of terpenes, tannins and alkaloids in the leaf extracts of *C. senna* selected for this study may preliminarily indicate their bioactivity especially antitumour activity.

Many researchers have reported phytochemicals in various *Cassia* species. Panda *et al*., (2011) reported the presence of phytochemicals such as alkaloids, flavonoids, carbohydrates, glycosides, protein and amino acids, saponins and triterpenoids in *C. fistula* leaf extracts. This revealed the presence of most of phytoconstituents in polar extracts (ethanol, methanol and aqueous) compared with nonpolar extracts (petroleum ether and chloroform). The present observations are in concordant with the above findings.

Preliminary phytochemical screening of ethanolic extract of *C. tora* indicated the presence of alkaloids, phenolics, glycosides, fatty alcohols and triterpenoids which might be responsible for the antioxidant activity of the extract and the leaf extract of *C. tora* may serve as a substitute for synthetic antioxidants and can be used to boost the antioxidanat status of the body (Aswhini and Krishnamoorthy, 2011).

Jose *et al*., (2012) have reported that phytochemical screening of fresh juice and ethanolic extract of leaves of *C. alata* showed the presence of steroids, alkaloids, tannins, flavonoids, glycosides, phenolic compounds, diterpenes, triterpenes and saponins. Phytochemical screening of the ethanol extract of *Cassia sophera* leaves revealed the presence of steroids, alkaloids, tannins, saponins, and flavonoids (Mondal *et al*., 2012).

Phytochemical screening leaf of *Cassia occidentalis* using various solvents like ethanol, methanol, ethyl acetate and water revealed the presence of carbohydrate glycosides, alkaloids, phenols, flavanoid, aminoacid, coumarine and phytosterols. Since it contains high proportion of phenols and alkaloid, it is reliable to possess antioxidant and anticancer activity (Ranjithkumar *et al*., 2010). The water–ethanolic leaf extract of *C. occidentalis* showed the presence of alkaloids, tannins, saponins and phlobatannins. (Ogunkunle and Ladejobi, 2006).

Comparative studies of secondary metabolites using qualitative tests performed on ethyl acetate, methanol and ethanolic extracts of leaves of *Cassia spectabilis, C. siamea, C. fistula, Cassia biflora* and *Cassia hirsuta* revealed the presence of phytoconstituents like alkaloids, tannins, saponins, flavonoids, carbohydrates, proteins, steroids, terpenoids, cardiac glycosides and phlobatannins (Veerachari and Bopaiah, 2011).

Phytochemical screening of leaf extracts of *C. tora* and *C. sophera* using different solvents such as petroleum ether, ethyl acetate and methanol revealed the presence of carbohydrates, proteins, glycosides, phenolics, alkaloids, tannins, saponins, flavonoids and phytosterols (Rao and Suresh, 2013).

From the above literature, it is clear that *Cassia* species are good sources of beneficial phytoconstituents and the present study indicate that the ethanolic leaf extracts of *C. senna* are good sources of phytochemicals than other solvent extracts.

Qualitative analysis of phytochemicals in the pod extracts of *C. senna*

The results of phytochemical screening for pod extracts of *C. senna* are shown in Table 8.

Table 8 : Qualitative analysis of phytochemicals in the pod extracts of *Cassia senna*

S.No	Phytochemical Test	Petroleum Ether Extract	Benzene Extract	Chloroform Extract	Ethyl acetate Extract	Successive Ethanolic Extract	Direct Ethanolic Extract	Aqueous
I	**TEST FOR CARBOHYDRATES**							
i	Molisch's Test	+++	+++	+++	+++	+++	+++	+++
ii	Fehling's Test	---	---	+++	---	+++	+++	+++
II	**TEST FOR GLYCOSIDES**							
i	Keller-Killani Test	+++	+++	+++	+++	+++	+++	+++
ii	Modified Borntrager's Test	---	---	---	---	---	---	---
III	**TEST FOR PROTEINS AND AMINOACIDS**							
i	Xanthoproteic Test	+++	+++	+++	+++	+++	+++	+++
ii.	Ninhydrine Test	+++	+++	+++	+++	+++	+++	+++
IV	**TEST FOR ALKALOIDS**							
i	Mayer's Test	+++	+++	---	++	+++	+++	++
ii	Wagner's Test	+++	+++	+	+++	+++	+++	++
iii	Dragendroff's Test	+++	+++	---	++	+++	+++	++
V	**TEST FOR FLAVONOIDS**							
i	Alkaline reagent Test	+	+	+	---	+++	+++	+++
ii	Sulphuric Acid Test	+	+	+	---	+++	+++	+++
iii	Lead acetate Test	+	+	+	+	+++	+++	+++
VI	**TEST FOR TERPENOIDS AND STEROIDS**							
i	Terpenoids	---	---	---	---	+	+	---
ii	Steroids	---	---	---	---	+	++	+
VII	**TEST FOR DITERPENES**							
i	Copper acetate Test	---	---	---	---	+	+	---
VIII	**TEST FOR TRITERPENOIDS**							
		---	---	---	---	++	++	---
IX	**TEST FOR PHYTOSTEROLS**							
i	Salkowski's Test	---	---	+	+	+	++	
ii	Libermann Burchard's Test	---	---	+	+	+	++	
X	**TEST FOR TANNINS**							
i	Lead acetate test	+	+	+	+	+	++	+++
XI	**TEST FOR SAPONINS**							
i	Froth Test	+++	---	+++	---	+++	+++	+++
XII	**TEST FOR PHENOLS**							
i	Ferric Chloride Test	---	+	+	+	+	++	++

+++ highly present, ++ moderately present, + slightly present, - absent

Analysis of various solvent extracts of the pod sample of *C. senna* showed the presence of primary metabolites such as carbohydrates and proteins and the secondary metabolites namely glycosides, alkaloids, flavonoids, terpenoids, phenols, steroids, phytosterols, tannins and saponins(Table 8). Among the various phytochemicals, terpenoids and diterpenes were found to be absent in all selected solvents except a trace amount in the two ethanolic extracts of pod.

Similar observations have been reported in many research findings. The qualitative phytochemical investigations of *C. fistula* Linn. pod extract showed the presence of steroids, coumarins, saponins, alkaloids and tannins in the methanol extracts (Sumi and Saj, 2012). *C. fistula* fruit pulp and seed extracts have a significant amount of phenolic compounds like flavonoids, rhein, anthraquinone and tannins (Irshad *et al*., 2011). The 50% methanol extract of root, leaves and seeds of *C. sophera* showed the presence of glycosides, flavonoids, tannins and mucilage (Chetan *et al*., 2011)

The results of the phytochemical screening of the whole pulverized plant of *C. occidentalis* indicates the presence of carbohydrates, saponins, sterols, flavonoids, resins, alkaloids, terpenes, anthraquinones and glycoside. The presence of these metabolites suggests great potential for the plant as a source of useful phytomedicines (Egharevba *et al*., 2010).

Based on the observations made so far, it is clear that major phytoconstituents have been reported in leaf than in pod among *Cassia* species. The present study also revealed that leaf of *C. senna* was found to be positive for most of the phytochemicals than pod indicating that leaf may be a rich source of secondary metabolites when compared to pods.

4.1.3. Quantitative estimation of phytochemicals in the various extracts of leaf and pod of *C. senna*

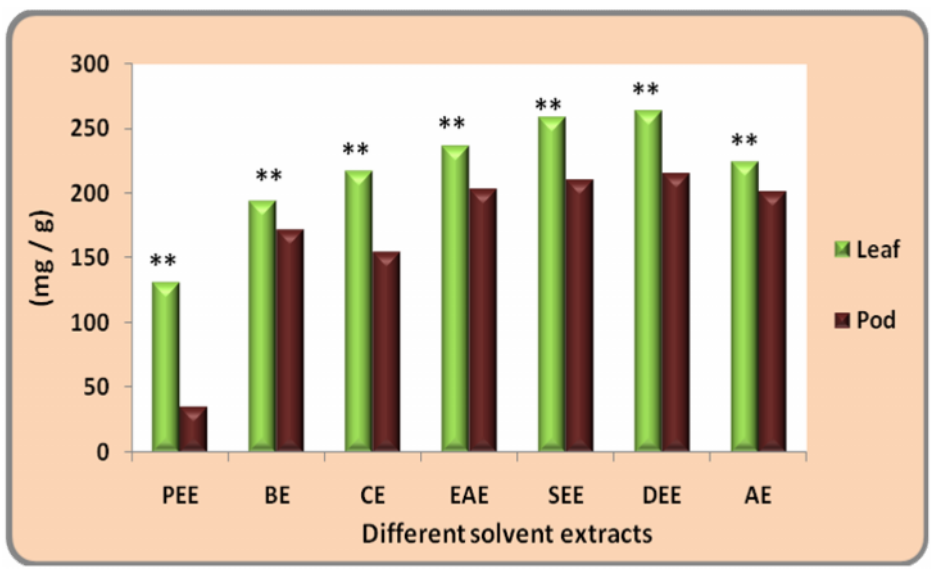
The therapeutic potentials including antioxidant and anticarcinogenic properties of higher plants are due to the presence of secondary metabolites (Kaur and Arora, 2009). The medicinal values of plants lie in bioactive phytochemicals namely flavonoids, phenolic compounds and tannins which produce definite physiological actions in the human body (Akinmoladun *et al*., 2007).

Quantitative estimation of alkaloids, flavonoids, phenols, steroids and tannins was carried out in the selected solvent extracts namely petroleum ether, benzene, chloroform, ethyl acetate, successive ethanolic, direct ethanolic and aqueous extracts of leaf and pod of *C. senna* The results of quantitative estimation of selected phytochemicals are elaborated in Figure 6, 7, 8, 9 and 10.

Estimation of total alkaloids

The alkaloid extracts obtained from medicinal plant species have multiplicity of host-mediated biological activities including antimalarial, antimicrobial, antihyperglycemic, anti-inflammatory and pharmacological effects (Tackie and Schiff, 1993; Boakye-Yiadom, 1979). The results of estimation of alkaloids for the extracts of leaves and pods of *C. senna* are presented in Figure 6.

Figure 6 : Total Alkaloid content in leaves and pods of *C. senna*



PEE-petroleum ether extract, BE -benzene extract, CE-chloroform extract, EAE-ethyl acetate extract, SEE-successive ethanolic extract, DEE-direct ethanolic extract and AE-aqueous extract

Values are mean ± SD(n=3)

** - statistically significant ($p<0.01$);

From the results shown in Figure 6, it is evident that successive and direct ethanolic extracts of both leaf and pod of *C. senna* showed maximum level of alkaloids followed by ethyl acetate extract. Between leaf and pod, leaf possessed considerably($p<0.01$) the rich source of alkaloids than pods in all the extracts.

The results are supported by previous research findings. Veerachari and Bopaiah, (2011) have also observed the presence of higher level of alkaloids in ethanol, methanol and ethyl-acetate extracts of leaves of *C. spectabilis, C. siamea* and *C. hirsuta.* Quantitative analysis of methanloic extract of leaf and stem of *Senna siamea* showed higher content of alkaloids in the leaves than in stem as reported by Mohammed *et al*., (2013a).

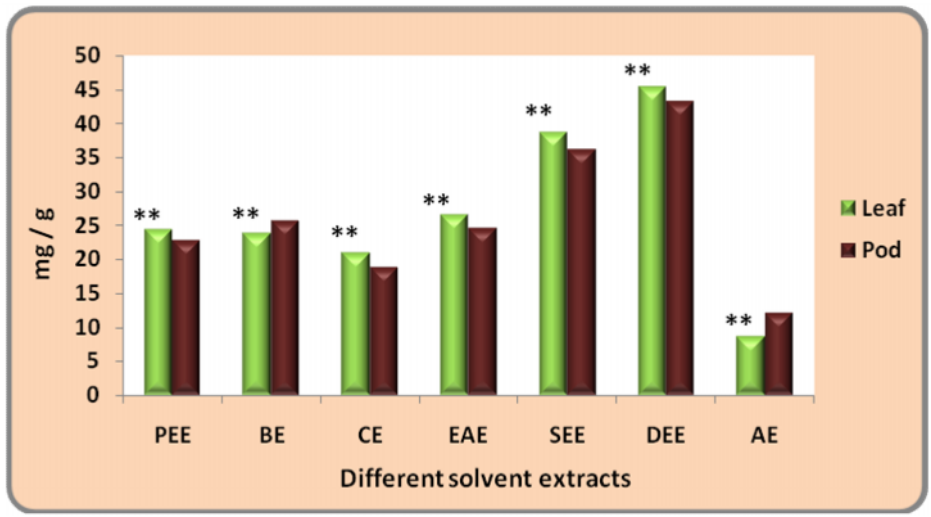
Thus, the present findings revealed that ethanolic extracts of *C. senna* leaves exhibited higher content of alkaloids than all the other extracts studied which is in agreement with earlier observations.

Estimation of total flavonoids

Flavonoids (polyphenols) are also known as Vitamin P or plant modifiers (Veerachari and Bopaiah, 2011). Flavonoids play some important pharmacological roles against diseases, such as cardiovascular diseases, cancer, inflammation and allergy. Epidemiological studies have indicated the relationship between flavonoid intake and reduced risk of certain cancer (Chandler and Dodds, 1993). It may be useful to estimate the total flavonoid content that are highly considered to be responsible for the antioxidant activity (Priyadharshini and Sujatha, 2013). The quantity of flavonoids observed in the various extracts of leaves and pods of *C. senna* is depicted in Figure 7.

From Figure 7, it is evident that high content of flavonoids was observed in polar solvent extracts such as DEE followed by SEE in both leaf and pod as compared to other solvent extracts. Further, it is noteworthy to mention that leaf was found to exhibit significantly($p<0.01$) maximum level of flavonoids than pods of *C. senna* in all the extracts except benzene and aqueous extracts.

Figure 7 : Total flavonoid content in leaves and pods of *C. senna*



PEE-petroleum ether extract, BE -benzene extract, CE-chloroform extract, EAE-ethyl acetate extract, SEE-successive ethanolic extract, DEE-direct ethanolic extract and AE-aqueous extract

Values are mean ± SD(n=3)
** - statistically significant ($p<0.01$);

Many researchers have reported *Cassia* species as good source of flavonoids. Mehta *et al*., (2013) reported that leaf of *Cassia javanica* showed higher content of flavonoids than seeds and among the various solvent extracts ethanolic extract was found be best than other solvents. From the previous findings, it has been reported that the total flavonoid content and identified flavonoids in the leaves of *Cassia angustifolia* make it a better alternative to the synthetic antioxidants and flavonoid source (Laghari *et al*., 2011).

A high quantity of flavonoids was reported in the ethanol extract of *Cassia hirsuta* leaves whereas a moderate amount in ethanol extract of *C. siamea* leaves (Veerachari and Bopaiah, 2011). *C. tora* methanol extract showed higher content of phenols and flavonoids with the standards gallic acid and quercetin respectively (Rao and Suresh, 2013).

Priyadharshini and Sujatha, (2013) reported that the total flavonoid content in the flower extracts of *C. auriculata* and *C. siamea* was in the order of methanol(polar solvent) > ethyl acetate > water > chloroform > hexane(non polar). The present findings are in accordance with the above results that ethanol(polar solvent) was found to contain

high quantity of flavonoids and petroleum ether(non polar solvent) was found to contain less quantity of flavonoids in both leaf and pod of *C. senna*.

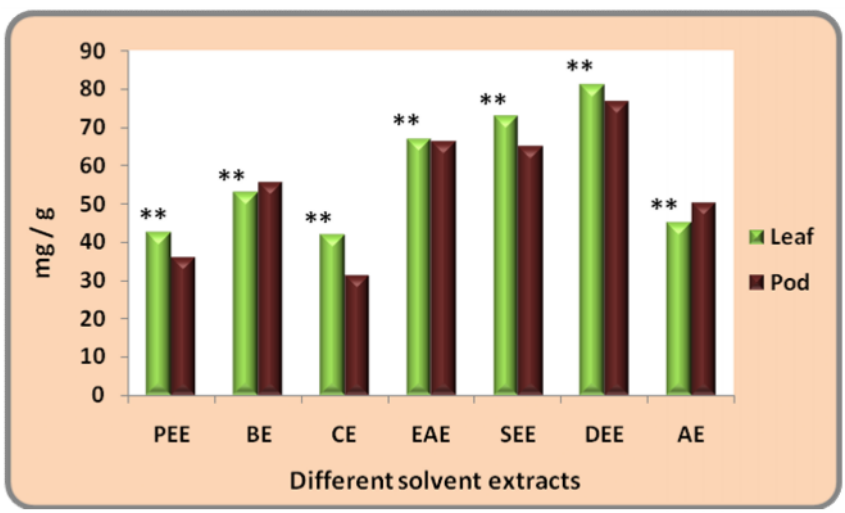
One of the most prominent properties of flavonoids is their excellent radical scavenging ability. It is also a valuable aspect for therapeutic and prophylactic applications of flavonoids (Sarkar *et al*., 2014).

The present study is in accordance with the above literature and shows that leaf extracts of *C. senna* were found to be rich sources of flavonoids than pod extracts. The higher content of flavonoids in leaf extracts of *C. senna* could be attributed to their antioxidant activity.

Estimation of total phenols

Phenolic compounds are one of the largest and most ubiquitous groups of plant metabolites (Singh *et al*., 2007) and the natural antioxidants mainly come from plants in the form of phenolic compounds (Ali *et al*., 2008). The phenolic content quantified in the different solvent extracts of leaf and pod samples of *C. senna* is shown in Figure 8.

Figure 8 : Total phenolic content in leaves and pods of *C. senna*



PEE-petroleum ether extract, BE -benzene extract, CE-chloroform extract, EAE-ethyl acetate extract, SEE-successive ethanolic extract, DEE-direct ethanolic extract and AE-aqueous extract

Values are mean ± SD(n=3)

** - statistically significant ($p<0.01$);

It is evident from Figure 8 that phenolic content of two the ethanolic extracts, SEE and DEE of leaves and pods of *C. senna* was found to be maximum followed ethyl acetate and aqueous extracts indicating that both the leaf and pod are the good sources of phenols. Among the various solvent extracts of leaf and pod, except in benzene and aqueous extracts, leaf exhibited significantly($p<0.01$) higher quantity of phenols than pods.

Similarly Mehta *et al*., (2013) reported that the order of phenolic content obtained from three solvent extracts was found to be ethanol > methanol> water for both *C. siamea* and *Cassia javanica* and all the three solvent extracts namely ethanol, methanol and aqueous extracts of leaves of *C. javanica* had higher phenolic content than seeds. The present study which is in accordance with the above findings showed the leaf ethanolic extracts of *C. senna* as the rich sources of phenols than all the other extracts.

In the present study, it is also noteworthy to mention that phenolic content was higher than flavonoid content in leaf and pod of *C. senna.* The present findings are supported by earlier findings. Nassr-Allah *et al*., (2009) have reported that the amount of phenols was higher than the flavonoids in both the water and ethanolic extract of *Cassia acutifolia*. Chanda *et al*., (2012) also reported that leaves and stem of four *Cassia* species namely *C. fistula*, *C. auriculata*, *C. siamea* and *C. tora* showed the presence of greater quantity of total phenols than flavonoids.

The comparative study of secondary metabolites investigation performed by Ranjithkumar *et al*., (2010) on *C. occidentalis* leaf as well as stem using various solvents like ethanol, methanol, ethyl acetate and water showed high proportion of phenols and alkaloids which was reliable to possess antioxidant activity. Likewise in the present study also, high proportion of phenols and alkaloids was found in leaf extracts of *C. senna* which may be possibly attributed for their antioxidant activity.

According to Arya and Yadav, (2011), the maximum total phenolic content was in the methanolic extract(polar solvent) of leaves of *C. occidentalis* than corresponding extract of leaves of *C. tora.* However, in contrast, chloroform, benzene and petroleum ether extracts(non polar solvents) of leaf of *C. occidentalis* showed less total phenolic content than the corresponding extracts of *C. tora*. The present results are in agreement

with the above report and ethanol being a polar solvent showed high phenolic content than non polar solvent extracts.

The study by Luximon-Ramma *et al*., (2002) indicated that the highest levels of phenolics were found in the pod of *Cassia fistula*. Ayo, (2010) have reported that *Cassia* species are rich sources of polyphenols, anthroquinone derivatives, flavonoids and polysaccharides. Antioxidant activity of the methanol extracts of pulp and seed of *C. fistula* was also compared for total phenolic content and it was found that the radical scavenging effects of extracts were directly proportional to the phenolic content (Irshad *et al*., 2012).

Siddhuraju *et al*., (2002) reported that *C. fistula* leaves have higher phenolic content, reducing power and free radical inhibition activity than flowers(reproductive organ) due to the existence of several groups of polyphenol compounds such as anthraquinones, xanthones, proanthocyanidins and flavonol which could be contributing to the antioxidant activity measured.

Leaves of *C. siamea* was found to exhibit appreciable quantity of phenols and a correlation between phenolic content and antioxidant activity was also found out (Chandiwitheesuk *et al*., 2005).

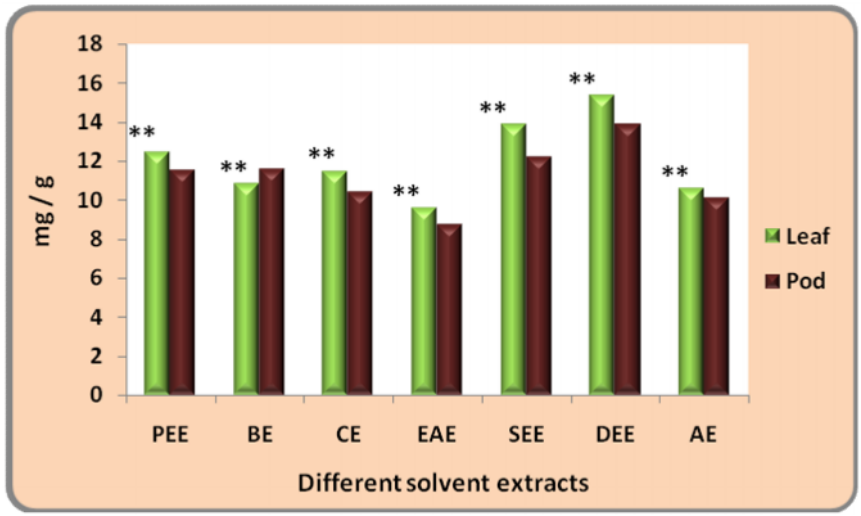
With the above literature cited, it is clear that the results of the present study showed that leaf ethanolic extracts of *C. senna* were found to be good sources of phenols than all the other extracts including pod extracts. The higher content of phenols in DEE and SEE of leaves could probably be attributed to their antioxidant activity.

Estimation of total tannins

Tannins reduce the risk of coronary heart diseases (Ranjithkumar *et al*., 2010). The results of quantitative estimation of steroids observed in the various extracts of leaves and pods of *C. senna* are presented in Figure 9.

From the results shown in Figure 9, it is clear that the level of tannins was maximum in DEE and SEE followed by chloroform and petroleum ether extracts of leaf and pod of *C. senna*. Leaf samples of *C. senna* recorded significantly($p<0.01$) a higher level of tannins than that of pods.

Figure 9 : Total tannins content in leaves and pods of *C. senna*



PEE-petroleum ether extract, BE -benzene extract, CE-chloroform extract, EAE-ethyl acetate extract, SEE-successive ethanolic extract, DEE-direct ethanolic extract and AE-aqueous extract

Values are mean ± SD(n=3)

** - statistically significant ($p<0.01$);

The present findings have been supported by earlier studies. Leaves of *C. siamea* have been reported to contain appreciable quantity of tannins (Chandiwitheesuk *et al*., 2005). Ethanolic extract of leaves, stem, seeds and roots of *Cassia obtusifolia* and *C. auriculata* have also revealed the presence of tannins (Deshpande and Bhalsingh, 2011). The chloroform and methanolic extracts of both flower and seed of *C. occidentalis* were found to contain tannins and steroids (Kathirvel and Sujatha, 2012).

Phytochemical analysis of leaves and seeds of *Senna alata* revealed the presence of alkaloids, flavonoids and tannins in all the solvent extracts(Petroleum ether, Benzene, Chloroform, Water) in varying quantities. Since the plant contains high amounts of these bioactive compounds, it is reliable to possess large number of medicinal values like anticancerous, antimutagenic, antioxidant, antifungal, laxative and antibacterial activities (Archana *et al*., 2012).

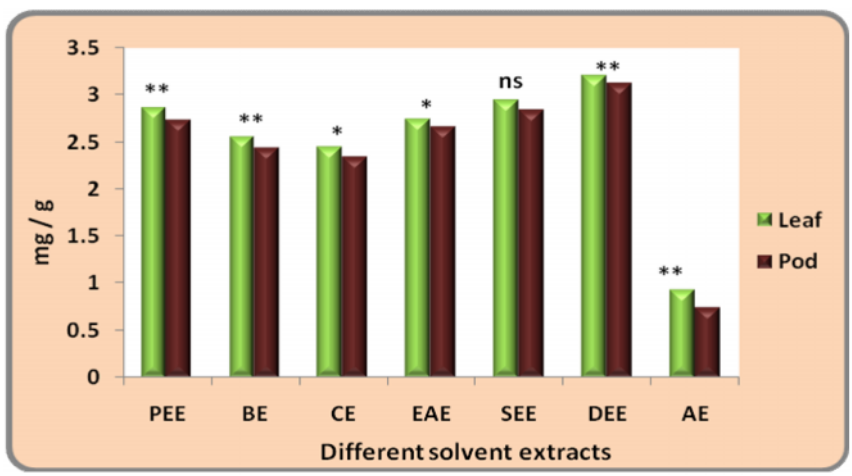
The present results have been supported by above findings cited and demonstrate that DEE and SEE of *C. senna* leaves are good sources of tannins than the other extracts studied.

A previous study by Sirappuselvi and Chitra, (2012) suggested that the antioxidant potential of methanolic extract of leaves of *C. tora* may be attributed to the presence of , alkaloids, flavanoids, phenols, tannins and steroids and it can be considered as a model herbal drug for experimental studies including free radical induced disorders like cancer, diabetics and atherosclerosis. The leaves would be useful as an antioxidant and free radical scavenging agent and it helps in the treatment of many diseases that was mediated by reactive oxygen species. Similarly in the present study leaves of *C. senna* also revealed the presence of appreciable quantity of the various phytochemicals namely alkaloids, flavonoids, phenols, tannins and steroids and than that of pods and it can be concluded that *C. senna* leaves may be used as an accessible source of natural antioxidants with consequent health benefits.

Estimation of total steroids

Steroids in modern clinical studies have been supported for their role as anti-inflammatory and analgesic agents (Singh, 2006). The quantity of steroids observed in the various extracts of leaves and pods of *C. senna* is depicted in Figure 10.

Figure 10 : Total steroids content in leaves and pods of *C. senna*



PEE-petroleum ether extract, BE -benzene extract, CE-chloroform extract, EAE-ethyl acetate extract, SEE-successive ethanolic extract, DEE-direct ethanolic extract and AE-aqueous extract

Values are mean ± SD(n=3)

** - statistically significant ($p<0.01$);

* - statistically significant ($p<0.05$);

ns – not Significant

From Figure 10, it is indicated that except SEE all the extracts of leaf of *C. senna* contain significantly($p<0.05$) higher level of steroids than pod extracts, while in SEE the steroid content of leaf was not significantly different from that of pod. In leaf, SEE, DEE and petroleum ether extracts showed high content of steroids whereas in pod, both the ethanolic extracts and ethyl acetaete extracts showed high content of steroids.

The present observations are supported by earlier findings. The ethanolic and methanolic extracts of *Cassia* species namely of *C. siamea*, *C. fistula* and *C. hirsuta* were found to contain higher quantity of steroids while a moderate level was observed in *C. spectabilis* and *C. biflora* extracts (Veerachari and Bopaiah, 2011).

Higher levels of β-sitosterol (total) was found in *Cassia renigera* with the maximum concentration in pods and minimum in *Cassia pumila* roots. Similarly maximum concentration of lanolsterol (total) was observed in *C. renigera* pods and minimum in *Cassia nodosa* roots. Total levels of stigmasterol was in higher concentration in *C. pumila* with the maximum levels in pods. A minimum concentration was recorded in stem of *C. nodosa* (Singh *et al*., 2013).

The present findings are in accordance with the above literatures cited and indicate that DEE and SEE of *C. senna* leaves are better sources of steroids than all the other extracts studied.

At the outset, in the present study, between the various solvent extracts of leaf and pod analysed, the polar solvent extracts DEE and SEE were found to contain more quantity of phytoconstituents than the other solvent extracts. This is in accordandance with some of the earlier findings. The use of nonpolar solvents is comparatively less indicating that the active components are soluble in polar solvents only (Chanda and Dave, 2009). Additionally, ethanol was found to be easier to penetrate the cellular membrane to extract the intracellular ingredients from the plant material (Wang *et al*., 2010).

Nearly all of the identified phytocomponents from plants are aromatic or saturated organic compounds and they are most often obtained through initial ethanol extraction (Cowan *et al*., 1999).

From the quantitative estimation of phytochemicals for different extracts of leaf and pod samples of *C. senna*, it is clear that leaf extracts are rich sources of the above phytochemicals quantified than pod extracts of *C. senna*. Between the leaf extracts, the two ethanolic extracts namely DEE and SEE were found to contain higher quantity of phytochemicals namely alkaloids, flavonoids, phenols, tannins and steroids than all other extracts of *C. senna* leaves.

Based on the results obtained for antioxidant assays, qualitative and quantitative analysis of phytochemicals performed in the selected parts of *C. senna*, namely leaf and pod, it is evident that leaf was found to be rich source of antioxidants and secondary metabolites than pod. Hence, for the next phase of studies only leaf extracts of *C. senna* were used.

PHASE II

4.2. Screening of effective extract of *C. senna* leaves by radical scavenging activity, chromatographic analysis and *in vitro* anticancer activity.

The results of phase I study for preliminary investigation of antioxidants and phytochemicals in the selected parts of *C. senna*, namely leaf and pod, showed that leaf was a rich source of antioxidants and secondary metabolites than pod. Hence, only the leaf extracts were used for Phase II studies.

4.2.1. Evaluation of free radical scavenging activity of the leaf extracts of *C. senna*

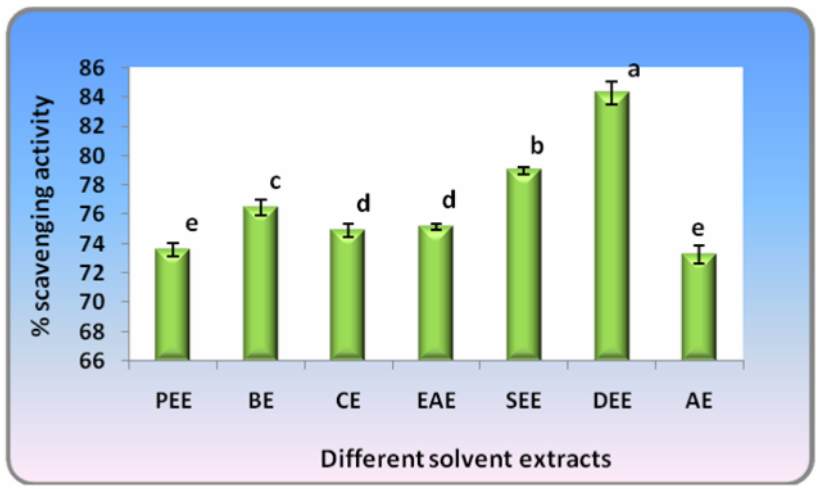
Generally, any part of the plant can be used for antioxidant studies but most commonly used part is leaf followed by fruit (Chanda and Dave, 2009).

The solvent extracts of leaf of *C. senna* such as petroleum ether extract(PEE), benzene extract (BE), chloroform extract(CE), ethyl acetate extract(EAE), successive ethanolic extract(SEE), direct ethanolic extract(DEE) and aqueous extract(AE) were analysed for its ability to scavenge free radicals namely DPPH, hydroxyl and ABTS and the non radical hydrogen peroxide. The percent inhibition of radical generation such as super oxide and nitric oxide radical was also determined. The results are presented in Figure 11, 12, 13, 14, 15 and 16.

4.2.1.1. Determination of DPPH radical scavenging activity

The DPPH model provides a method for evaluating antioxidant activity of a compound or plant extract in a relatively shorter time as compared to other methods. The disappearance of DPPH radical is directly proportional to the amount of antioxidant present in the reaction mixture (Ghanta *et al*., 2007). The proton radical scavenging action is known to be one of the important mechanisms for measuring antioxidant activity. This assay determines the scavenging of stable radical species DPPH by antioxidant compounds present in plant extracts (Irshad *et al*., 2012). The results of DPPH radical scavenging activity for the different solvent extracts of *C. senna* leaves are presented in Figure 11.

Figure 11 : DPPH radical scavenging activity of leaf extracts of *C. senna*



PEE-petroleum ether extract, BE -benzene extract, CE-chloroform extract, EAE-ethyl acetate extract, SEE-successive ethanolic extract, DEE-direct ethanolic extract and AE-aqueous extract

Values are Mean ± SD(n=3)

$^{a-g}$ mean values indicated in a each bar sharing no common supercript differ significantly at 5% by DMRT

Figure 11 shows the DPPH radical scavenging activity of the different solvent extracts of *C. senna* leaves. From the present results, it was observed that the two ethanolic extracts(SEE and DEE) of the leaves of *C. senna* exhibited significantly($p<0.05$) higher percentage of scavenging activity than that of other extracts

studied. The scavenging activity of DEE of the leaves reached a value of 84.25±0.8% while that of the SEE was 79±0.25%.

The present findings are supported by earlier studies. The leaf ethanolic extracts of *C. occidentalis* and *C. hirsuta* were also shown to posses the highest scavenging activity of DPPH radical (Michael and Oyeronke, 2013; Rahman and Ahmed, 2013). Khan *et al*., (2012b) reported DPPH radical scavenging activity in the ethanolic extracts of various parts of *C. fistula* which was in the decreasing ordrer of leaves>bark>flowers>pods. DPPH scavenging activity for various parts of *C. fistula* was low for hot percolation as compared to the simple maceration technique. The above findings support the present results where DEE prepared by simple maceration technique was found to exhibit highest DPPH radical scavenging activity as compared with other solvent extracts that were prepared by hot percolation. It is well known that free radical scavenging activity of plant botanicals is chiefly due to polyphenols (Bushra *et al*., 2009). The significant($p<0.05$) low radical scavenging activity of the SEE than DEE of *C. senna* leaves may be attributed to the thermal decomposition of polyphenolic compounds present in SEE that was prepared by hot percolation using soxhlet apparatus.

Siddhuraju *et al*., (2002) calculated the DPPH radical scavenging activity of *C. fistula* in order, stem bark, leaves, butylated hydroxytoluene (BHT), flowers, pulp and noted that it was directly proportional to total phenolic content in the extract. It indicates the ability of *Cassia fistula* extracts to act as radical scavenger and metal quencher thereby, protecting against free radical mediated damage.

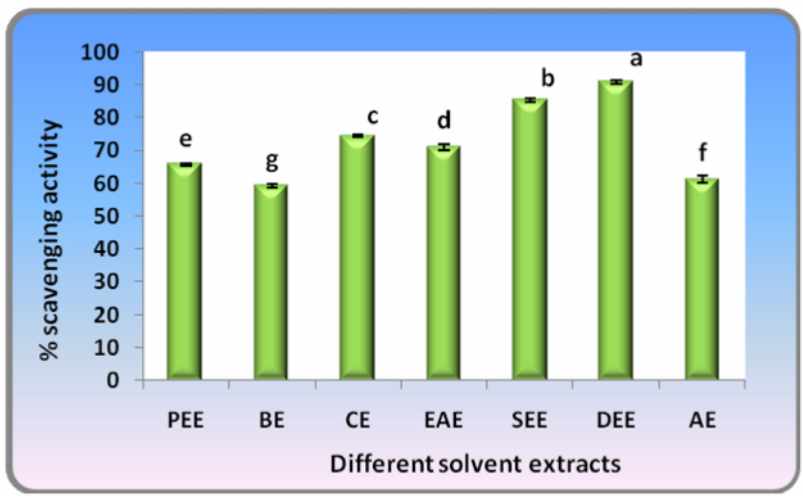
In a study by Mak *et al*., (2013) both ethanolic and aqueous extracts of *Senna bicapsularis* flowers have shown to exhibit rich scavenging effects on DPPH radicals and it was reported that higher radical scavenging activity might be attributed to the presence of high phenolics, tannins or flavonols in the sample extracts. The quantity of phytochemicals namely flavonoids, phenols and tannins in the leaf extracts of *C. senna* selected for the present study were found to be high and might be responsible for the DPPH radical scavenging activity which is in accordance with the above findings. The antioxidant activity of *C. spectablis* was evaluated by Sangetha *et al.,* (2008) using the DPPH radical-scavenging assay and it was reported that the flower, stem, leaf and pod extracts exhibited radical-scavenging activities.

Thus the present observations are in accordance with the above findings cited and also demonstrate that ethanolic extracts namely DEE and SEE of *C. senna* leaves showed efficient DPPH scavenging activity than the other solvent extracts.

4.2.1.2. Determination of ABTS radical scavenging activity

The ABTS method is known to be a rapid method for the determination of the antioxidant activity and could be a useful tool to screen samples in order to obtain high content of natural antioxidants (Silva *et al*., 2006). The results of ABTS(2,2'-azino-bis-3-ethyl benzthiazoline-6-sulphonic acid) radical scavenging effect of the various extracts of *C. senna* leaves is shown in Figure 12.

Figure 12 : ABTS radical scavenging activity of leaf extracts of *C. senna*



PEE-petroleum ether extract, BE -benzene extract, CE-chloroform extract, EAE-ethyl acetate extract, SEE-successive ethanolic extract, DEE-direct ethanolic extract and AE-aqueous extract

Values are Mean ± SD(n=3)
a-g mean values indicated in a each bar sharing no common supercript differ significantly at 5% by DMRT

The various extracts of *C. senna* leaves significantly($p<0.05$) exhibited(Figure 12) varying degree of ABTS radical scavenging activity. Both the ethanolic extracts(DEE and SEE) of *C. senna* leaves showed significantly($p<0.05$) maximum scavenging effect when compared to the other extracts tested. It is evident that the leaf *C. senna* are good sources of antioxidants particularly polyphenols and flavonoids which are responsible for the ABTS radical scavenging activity.

While comparing the scavenging capacities of the various extracts for ABTS and DPPH radicals(Figure 11 and 12) DEE exhibited significantly($p<0.05$) the highest antiradical property followed by SEE for both radicals and the order of ABTS radical scavenging activity of all extracts was similar to that observed for DPPH. The differences in the ABTS scavenging activity exhibited by the various solvent extracts of *C. senna* leaves indicated that the nature of extracting solvents and extraction methods influences the antioxidant ability of the extracts.

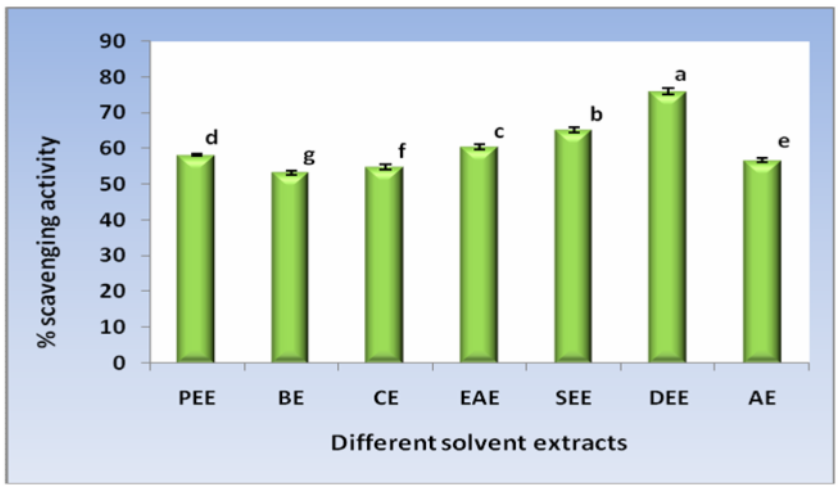
C. fistula was identified as potentially novel source of free radical scavenging compound and was effective against free radical mediated diseases (Bhalodia, 2011). Results of free radical scavenging activity showed that the leaf extracts obtained by soxhlet extraction(hot percolation) exhibited low radical scavenging activity as compared with DEE that was obtained by simple maceration technique. DEE was found to exhibit highest radical scavenging activity. This may be attributed to the thermal decomposition of phytocomponents during hot percolation (Bushra *et al*., 2009).

Thus the present study is in agreement with the above findings cited by researchers and indicates that the DEE and SEE of *C. senna* leaves possess good ABTS radical scavenging activity.

4.2.1.3. Determination of hydroxyl radical scavenging activity

Hydroxyl radical($OH^{\bullet}$) is implicated as one of the most damaging free radicals in the body and it is the important mediator of damage to cell structures, nucleic acids, lipids and proteins. The highly reactive hydroxyl radicals can cause oxidative damage to DNA, lipids and proteins (Jayaprakasha *et al*., 2003). The hydroxyl radical scavenging activity of seven different extracts of *C. senna* leaves was assessed and the results are presented in Figure 13.

Figure 13 : Hydroxyl radical scavenging activity of leaf extracts of *C. senna*



PEE-petroleum ether extract, BE -benzene extract, CE-chloroform extract, EAE-ethyl acetate extract, SEE-successive ethanolic extract, DEE-direct ethanolic extract and AE-aqueous extract

Values are Mean ± SD(n=3)

[a-g] mean values indicated in a each bar sharing no common supercript differ significantly at 5% by DMRT

The results(Figure 13) suggest that DEE was found to be significantly($p<0.05$) the most powerful scavenger of the hydroxyl radical, with an scavenging activity of up to 75.92%. All the other extracts exhibited moderate scavenging activity. The weakest scavenger was found to be benzene extract(53.2%).

Studies reported by Arya and Yadav, (2010), showed that the methanolic extract of leaves of *C. tora* was found to scavenge hydroxyl radical maximally. The most reactive of the ROS that attacks almost every molecule in the body is the hydroxyl radical. It initiates the peroxidation of cell membrane lipids yielding malondialdehyde, which is mutagenic and carcinogenic. Generally molecules that inhibit deoxy-ribose degradation are those that can chelate ferrous(Fe^{2+}) ions and thereby prevent them from complexing with deoxyribose and render them inactive in a Fenton reaction.

Earlier observations have demonstrated that *C. occidentalis* seed extracts reduced the DNA degradation caused by iron (II)-driven Fenton reaction and also noted that inhibition of DNA damage may be due to their strong ferrous ion chelation capability. In

addition, they also proposed that it may be due to their good scavenging activity towards free radicals (Bhattacharyya *et al*., 2003).

Ethanolic extracts from the roots of *Glycyrrhiza lepidota*, *Echinacea angustifolia*, *Polygala senega*, leaves of *Arctostaphylos uva-ursi* (bearberry) and aerial parts of two varieties of *Equisetum* species(horsetail) exhibited hydroxyl radical scavenging activity and the polyphenolic constituents appeared to be responsible, at least in part, for the their radical-scavenging capacity (Amarowicz *et al*., 2004).

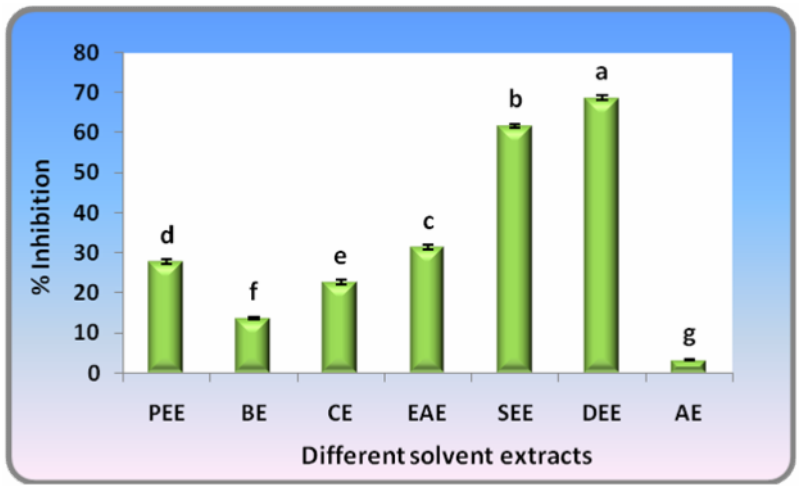
The present study is in agreement with the above supportive findings and the two ethanolic extracts (DEE and SEE) of *C. senna* leaves being effective in scavenging hydroxyl radicals reveal their antioxidant potential.

4.2.1.4. Determination of inhibition of super oxide radical generation

Superoxide anion ($O_2^{\bullet -}$) is produced by electro leakage from the mitochondrial electron transport chain, by activated phagocytes and from chemical conversion of xanthine to uric acid. Superoxide anion ($O_2^{\bullet -}$) is an oxygen–centered radical with selective reactivity (Bast *et al*., 1991). Superoxide is biologically important since it can be decomposed to form stronger oxidative species such as singlet oxygen and hydroxyl radicals and is very harmful to the cellular components in a biological system (Okhawa *et al*., 1979). The extent of inhibition of *in vitro* generation of superoxide radicals by different extracts of *C. senna* leaves was analyzed and results are shown in Figure 14.

The inhibition of superoxide radical generation by DEE was significantly($p<0.05$) superior(68.71±0.58%)(Table 14) to all other extracts. Between the various solvent extracts studied, the ethanolic extracts were found to exhibit significantly($p<0.05$) maximum inhibition on superoxide radical generation when compared to other extracts. In nonpolar solvent extracts namely petroleum ether and benzene, minimum inhibitory action on superoxide radical generation was noted to be 27.82±0.65% and 13.71±0.21% respectively. The maximum inhibition of superoxide radical generation exhibited by DEE followed by SEE might be due to their polar nature and this ability of DEE and SEE to inhibit superoxide radical generation can be well correlated with the maximum phenolic content in these extracts.

Figure 14 : Inhibition of Super oxide radical generation by leaf extracts of *C. senna*



PEE-petroleum ether extract, BE -benzene extract, CE-chloroform extract, EAE-ethyl acetate extract, SEE-successive ethanolic extract, DEE-direct ethanolic extract and AE-aqueous extract

Values are Mean ± SD(n=3)
[a-g] mean values indicated in a each bar sharing no common supercript differ significantly at 5% by DMRT

Similar observations have been reported by many researchers. Phenolic compounds and flavonoids have been reported to be associated with antioxidant action in biological systems, mainly due to their redox properties, which can play an important role in absorbing and neutralizing free radicals, quenching singlet and triplet oxygen or decomposing peroxides (Sarkar *et al*., 2014). Polyphenolics in the plant extracts appear to function as good electron and hydrogen donors and therefore are able to terminate radical chain reaction by converting free radicals to more stable products (Arya and Yadav, 2011).

Polyphenols and flavonoids are well known scavengers of superoxide radical, hydroxyl radical and hydrogen peroxide (Jyothi *et al.,* 2012). The present study is in accordance with the above finding and indicates that the remarkable quantity of phenols and flavonoids detected in the quantitative estimation of leaf extracts may be responsible for free radical neutralization especially quenching superoxide radical, hydroxyl radical, singlet and triplet oxygen.

The investigation by Siddhuraju *et al*., (2002) suggested that 90% ethanolic extract of leaves, and 90% methanolic extract of stem bark of *C. fistula* recorded maximum antioxidant activity in terms of superoxide radical ($O_2^{\bullet -}$) and DPPH radical scavenging ability. Suresh *et al*., (2008) also reported that stem bark and leaf extracts of *C. fistula* exhibited highest superoxide radical scavenging and reducing power than other parts in a dose-dependent manner. This may be due to presence of fistucacidin (flavan 3, 4-diol), the main compound present in stem bark of *C. fistula* (Jawahar and Gupta, 1972). It contains a 4'ortho-position hydroxyl which provides active hydrogen to take part in reaction to scavenge superoxide radical($O_2^{\bullet -}$).

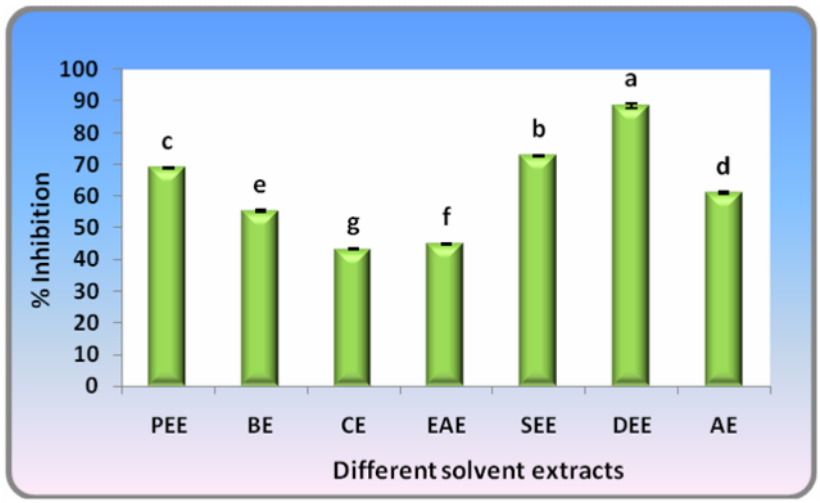
Flower extracts of *C. alata*, *C. auriculata*, *C. fistula* and *C. siamea* prepared using various solvents namely hexane, chloroform, ethyl acetate, methanol and hydroalcohol were evaluated for inhibition of superoxide radical generation in which, the methanol extracts of *C. fistula* and *C. auriculata* have demonstrated their excellence whereas *C. alata* and *C. siamea* have exhibited satisfactory values for the above assay (Priyadharshini and Sujatha, 2013). The methanolic leaf extract of *C. tora* showed maximum inhibitory potential on superoxide radical generation as compared to other extracts tested in a dose dependent manner (Arya and Yadav, 2010).

The present findings showed that DEE and SEE of *C. senna* leaves possess maximum inhibition on superoxide radical generation and are in agreement with the above literatures cited.

4.2.1.5. Determination of inhibition of nitric oxide radical generation

Nitric oxide (NO) is a potent pleiotropic mediator of physiological processes such as smooth muscle relaxation, neuronal signaling, inhibition of platelet aggregation and regulation of cell mediated toxicity (Sirappuselvi and Chitra, 2012). Nitric oxide is a diffusible free radical that plays many vital roles in diverse biological systems. However, excess production of nitric oxide is associated with several health hazards. Hence in this study the extent of inhibition of nitric oxide radical generation by different solvent extracts of *C. senna* leaves was observed and the results are shown in Figure 15.

Figure 15 : Inhibition of nitric oxide radical generation by leaf extracts of *C. senna*



PEE-petroleum ether extract, BE -benzene extract, CE-chloroform extract, EAE-ethyl acetate extract, SEE-successive ethanolic extract, DEE-direct ethanolic extract and AE-aqueous extract

Values are Mean ± SD(n=3)

$^{a-g}$ mean values indicated in a each bar sharing no common supercript differ significantly at 5% by DMRT

It is evident from Figure 15 that the leaf extracts showed a varied level of inhibitory activity on nitric oxide radical generation and a significant($p<0.05$) highest activity being initiated by DEE as 88.62±0.67% while chloroform extract recorded a significant($p<0.05$) lowest value, 43.34±0.38%.

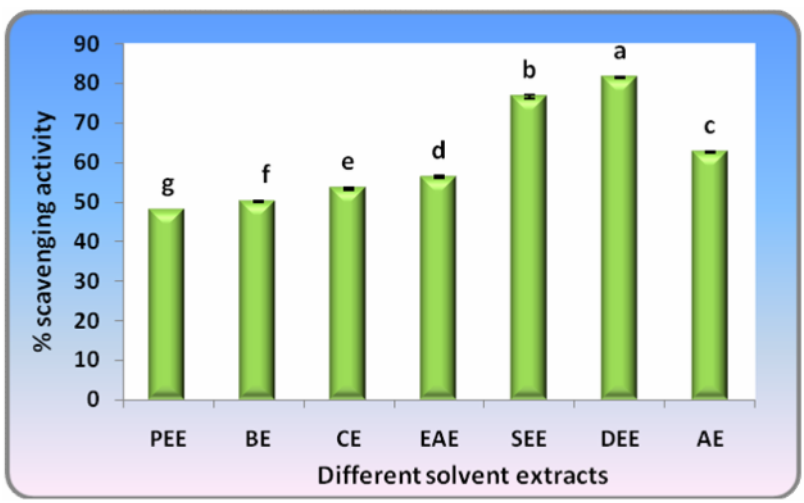
DEE of leaf extract was found to exhibit high quantity of phenols as well as maximum inhibitory activity on nitric oxide radical generation and this is in accordance with earlier studies by Arya and Yadav, (2011) who reported that the methanolic extract of leaves of *C. occidentalis* and *C. tora* recorded maximum nitric oxide radical scavenging activity and the antioxidant activity of leaf extracts of both *C. occidentalis* and *C. tora* are due to antioxidative principles(chiefly phenolics) present in the extracts which compete with oxygen to react with nitric oxide thereby inhibiting the generation of nitrite.

The present study supported by the above literatures suggests that DEE and SEE of *C. senna* leaves were found to be effective in inhibiting nitric oxide radical generation than the other extracts analysed.

4.2.1.6. Determination of hydrogen peroxide scavenging activity

Though H_2O_2 itself not very reactive, it generates highly reactive molecule such as $OH^{\bullet}$ by reacting with metals (Fe^{2+} or Cu^{2+}) and superoxide anions in the Haber-Weiss reaction. Therefore, removal of H_2O_2 is very essential from the cell. The extent of hydrogen peroxide scavenging potential of the various extracts of *C. senna* leaves is shown in Figure 16.

Figure 16 : Hydrogen peroxide scavenging activity of leaf extracts of *C. senna*



Values are Mean ± SD(n=3)

a-g mean values indicated in a each bar sharing no common supercript differ significantly at 5% by DMRT

Results of the present study(Figure 16) showed that H_2O_2 scavenging potential of DEE of *C. senna* leaves was found to be significantly($p<0.05$) the highest among the various solvent extracts and it was in the order of DEE>SEE>Aq>EA>Ch>B>PE. Electron donors in the ethanolic extracts of *C. senna* leaves might accelerate the conversion of H_2O_2 to H_2O which could possibly scavenge H_2O_2. This fact is in accordance with the report given by Ruch *et al*., (1984).

Many researchers have reported the H_2O_2 scavenging activity of different plants. Methanolic extract of leaf of *C. tora* demonstrated hydrogen peroxide scavenging activity in a concentration dependent manner (Sirappuselvi and Chitra, 2012). Michael and Oyeronke, (2013) reported the hydrogen peroxide scavenging activities of the ethanolic leaf extract of *C. occidentalis*.

Priyadharshini and Sujatha, (2013) reported that maximum hydrogen peroxide scavenging activity was produced by methanolic extract of flowers of *C. fistula* and *C. auriculata* and ethyl acetate extract of flower of *C. alata*. Nahak and Sahu, (2011) reported that the ethanol extract of *Piper cubeba* had a higher antioxidant activity than both methanol and aqueous extracts.

Thus the present observations are in agreement with the above findings cited by many researchers and also it is clear that the ethanolic extracts namely DEE and SEE of *C. senna* leaves were good hydrogen peroxide scavengers.

The findings of free radical scavenging activity of various extracts of *C. senna* leaves clearly show that the polar organic solvent extracts such as DEE and SEE were found to exhibit maximum radical scavenging activity against all the free radicals tested than the other solvent extracts.

4.2.2. Analysis of selected phytoconstituents in the leaf extracts using HPTLC and HPLC methods

4.2.2.1. HPTLC analysis for alkaloid and flavonoid profiles in the different solvent extracts of *C. senna* leaves

HPTLC is an invaluable quality assessment tool for the evaluation of botanical materials and it allows for the analysis of a broad number of compounds both efficiently and cost effectively. HPTLC studies have shown that it is more versatile than ordinary TLC methods, as the spots were well resolved (Chavan *et al*., 2016). HPTLC fingerprint of a plant species helps in the proper identification and quality control of a particular plant species and also provides basic information regarding isolation, purification, characterization and identification of marker chemical compounds of the species (Seasotiya *et al*., 2014).

The results of qualitative and quantitative phytochemical analysis of various solvent extracts of leaf of *C. senna*confirmed the presence of alkaloids and flavonoids. However, to ensure the presence of these phytocomponents and their derivatives HPTLC was performed with the various extracts of *C. senna* leaves namely petroleum ether extract(PEE), benzene extract (BE), chloroform extract(CE), ethyl acetate extract(EAE), successive ethanolic extract(SEE), direct ethanolic extract(DEE) and aqueous extract(AE)

along with the standard marker compound colchicine for alkaloids and quercetin for flavonoids.

HPTLC analysis for flavonoid profile in the leaf extracts of *C. senna*

Since the plant extracts are the potential sources of natural polyphenols with promising antioxidant activity and a wide range of biological activities, research is needed for the isolation and identification of these active components which can serve as beneficial sources for herbal therapies in future. The results of HPTLC analysis for alkaloid profile of various extracts of *C. senna* leaves are shown in Table 9, Plates 2-3 and Figure 17.

CSL1 - Petroleum ether extract(PEE)

CSL2 - Benzene extract(BE)

CSL3 - Chloroform extract(CE)

CSL4 - Ethyl acetate extract(EAE)

CSL5 - Successive ethanolic extract(SEE)

CSL6 - Aqueous extract(AQE)

CSL7 - Direct Ethanolic extract(DEE)

STD – Quercetin – flavonoid standard as reference marker

Table 9 – HPTLC peak table for flavonoids in the leaf extracts of *C. senna*

Track	Peak	Rf	Height	Area	Assigned substance
STD	1	0.77	376.7	8518.9	Quercetin standard
CSL 1	1	0.07	25.7	803.8	Unknown
CSL 1	2	0.12	27.8	1235.2	Unknown
CSL 1	3	0.81	263.4	15686.3	Flavonoid 1
CSL 1	4	0.95	464.2	19783.1	Unknown
CSL 2	1	0.05	23.1	675.1	Unknown
CSL 2	2	0.16	25.8	445.7	Unknown
CSL 2	3	0.76	428.2	8344.8	Flavonoid 1
CSL 2	4	0.78	431.4	10168	Unknown
CSL 2	5	0.81	340.0	13795.3	Unknown
CSL 2	6	0.94	404.9	11245.2	Unknown
CSL 3	1	0.08	22.1	737.9	Unknown
CSL 3	2	0.13	24.9	859.7	Unknown
CSL 3	3	0.34	10.5	375.2	Unknown
CSL 3	4	0.46	111.7	3483.9	Flavonoid 1
CSL 3	5	0.63	46.0	1412.5	Unknown
CSL 3	6	0.76	542.7	25484.8	Flavonoid 2
CSL 3	7	0.81	412.4	16351.4	Unknown
CSL 3	8	0.97	55.3	808.8	Unknown
CSL 4	1	0.18	21.1	409.6	Unknown
CSL 4	2	0.33	14.7	402.0	Unknown
CSL 4	3	0.46	268.9	9137.1	Flavonoid 1
CSL 4	4	0.64	83.4	2641	Unknown
CSL 4	5	0.78	734.0	33028.1	Flavonoid 2
CSL 4	6	0.81	473.2	21358.7	Unknown
CSL 4	7	0.95	401.7	11171.5	Unknown
CSL 5	1	0.05	380	7770.7	Unknown
CSL 5	2	0.16	105.2	4093.1	Unknown
CSL 5	3	0.20	59.0	985.1	Flavonoid 1
CSL 5	4	0.29	15.6	412.3	Unknown
CSL 5	5	0.41	48.1	2138.9	Unknown
CSL 5	6	0.45	137.7	4267.7	Flavonoid 2
CSL 5	7	0.58	20.3	517.4	Unknown
CSL 5	8	0.64	45.1	1357.5	Unknown
CSL 5	9	0.80	396.8	17515.1	Flavonoid 3
CSL 6	1	0.12	40.7	1008.3	Flavonoid 1
CSL 6	2	0.20	16.8	384	Unknown
CSL 6	3	0.78	342.1	10323.1	Unknown
CSL 6	4	0.84	300.3	23328.8	Unknown
CSL 6	5	0.96	77.1	2073.7	Unknown
CSL 7	1	0.01	22.0	215.7	Unknown
CSL 7	2	0.07	268.7	7020.2	Flavonoid 1
CSL 7	3	0.18	42.1	1584.0	Flavonoid 2
CSL 7	4	0.23	21.8	415.4	Unknown
CSL 7	5	0.49	38.8	1525.8	Unknown
CSL 7	6	0.63	15.2	585.1	Unknown
CSL 7	7	0.77	323.8	12381.6	Flavonoid 3

Track	Peak	Rf	Height	Area	Assigned substance
CSL 7	8	0.85	360.4	17736.4	Flavonoid 4
CSL 7	9	0.96	142.3	4693.7	Flavonoid 5
CSL 7	10	0.82	342	21748.2	Unknown
CSL 7	11	0.95	201.4	5720.6	Unknown

STD - Quercetin – flavonoid standard as reference marker

CSL1 - Petroleum ether extract(PEE) CSL2 - Benzene extract(BE)

CSL3 - Chloroform extract(CE) CSL4 - Ethyl acetate extract(EAE)

CSL5 - Successive ethanolic extract(SEE) CSL6 - Aqueous extract(AE)

CSL7 - Direct Ethanolic extract(DEE)

From the Peak Table(Table 9), it is clear that HPTLC analysis of leaf extracts of *C. senna* confirmed segregation of different types of flavonoids and other unknown compounds in the chromatogram(Plate 2 and 3) and densitogram(Figure 17) with individual Rf values and peak area. The range of Rf values of these compounds was between 0.01 to 0.96(Table 9). The leaf extracts showed good resolution at both 366nm and 254nm before and after derivatization(Plate 2 and 3). Blue and brown coloured zones at visible light mode(Plate 3) were observed in the tracks of the chromatogram after derivatization, which confirmed the presence of different derivatives of flavonoids with individual Rf values in leaf extracts.

Peak densitogram recorded after scanning at 254nm displayed varying number of peaks for both flavonoids and unknown compounds in each extract of the leaves(Figure 17). Among the seven extracts of leaf analysed, CSL6-SEE showed three peaks and CSL7-DEE showed five peaks for flavonoids(Table 9) as compared with other extracts and this might be due to the ability of ethanol to extract more number of flavonoids. The Rf value of flavonoid 3(peak 7) present in CSL7-DEE matched with standard quercetin with a peak height of 323.8 and peak area of 12381.6 which may be due to the presence of quercetin in DEE of *C. senna* leaves. The use of markers ensures that the concentration and ratio of components in the herbal mixture are present in reproducible levels in raw materials and in the final dosage form (Pattanaya *et al*., 2010). The present results are on par with the findings of Gowda and Veerabhadrappa, (2013) who reported the presence of flavonoid, quercetin in the methanolic extract of *C. auriculata* leaves using HPTLC analysis.

Plate 2

HPTLC Chromatogram for flavonoids in the leaf extracts of *C. senna* before derivatization

Visible light **UV 366nm** **UV 254nm**

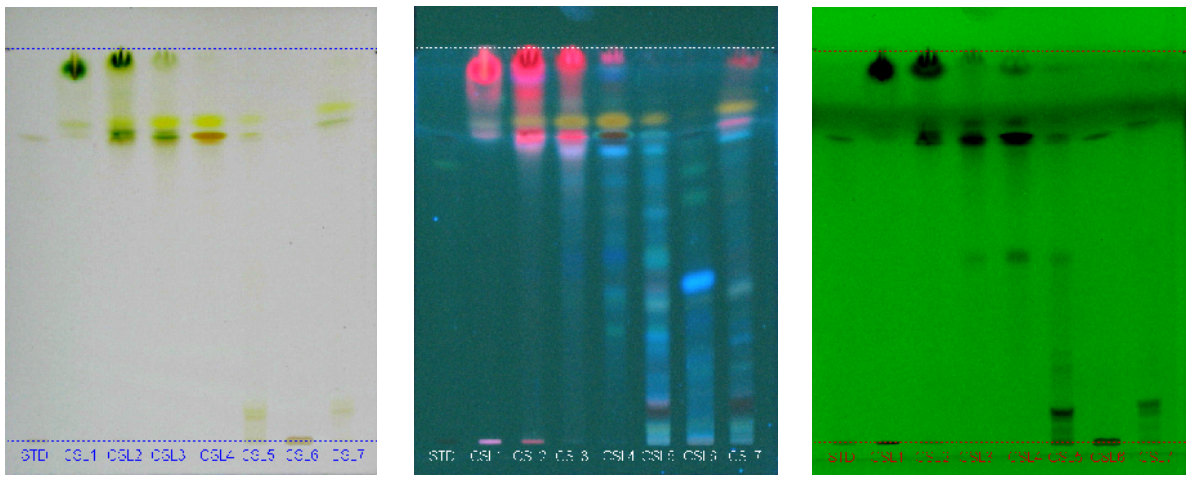
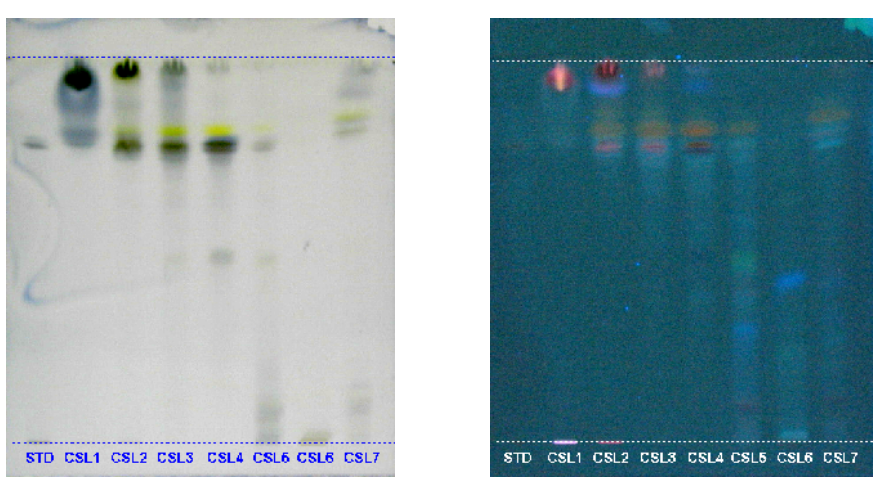


Plate 3

HPTLC Chromatogram for flavonoids in the leaf extracts of *C. senna* after derivatization

Visible light **UV 366nm**



STD - Quercetin – flavonoid standard as reference marker

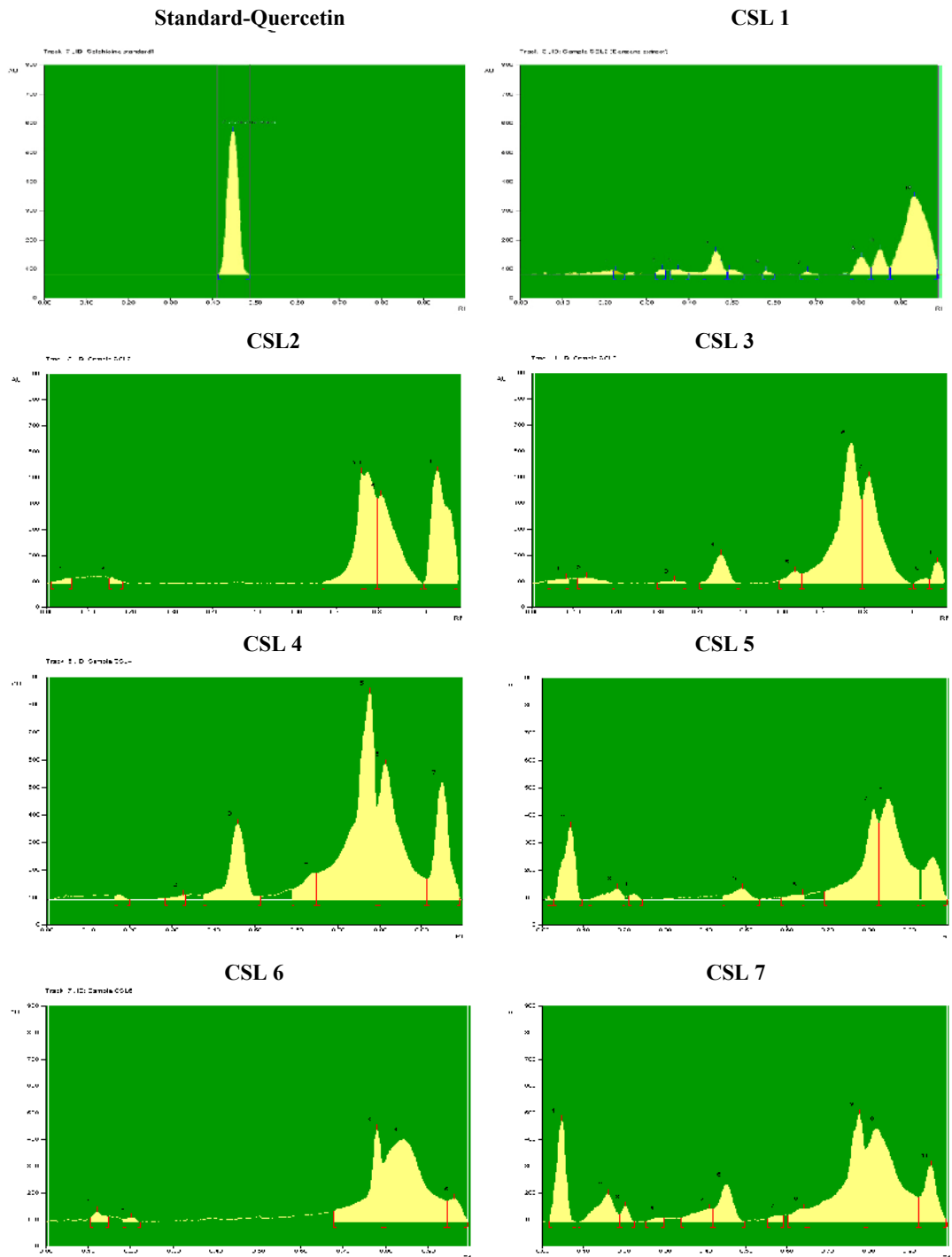
CSL1 - Petroleum ether extract(PEE) CSL2 - Benzene extract(BE)

CSL3 - Chloroform extract(CE) CSL4 - Ethyl acetate extract(EAE)

CSL5 - Successive ethanolic extract(SEE) CSL6 - Aqueous extract(AE)

CSL7 - Direct Ethanolic extract(DEE)

Figure 17 : HPTLC peak densitogram for flavonoids in the leaf extracts of *C. senna*



STD - Quercetin – flavonoid standard as reference marker

CSL1 - Petroleum ether extract(PEE) CSL2 - Benzene extract(BE)

CSL3 - Chloroform extract(CE) CSL4 - Ethyl acetate extract(EAE)

CSL5 - Successive ethanolic extract(SEE) CSL6 - Aqueous extract(AE)

CSL7 - Direct Ethanolic extract (DEE)

Many researchers have reported flavonoids in various *Cassia* species. Dhandapani and Kadarkarai, (2011) have reported four different types of flavonoids in ethanolic leaf extract of *C. Occidentalis* using rutin as flavonoid standard. Patidar *et al*., (2012) quantified a flavonoid called (-) epicatechin in methanolic crude extract of *C. fistula*. HPTLC analysis of the methonolic extracts of stem, leaf, flower, seed and root of *C. tora* along with a mixture of standard solution containing gallic acid and quercetin revealed that leaf extract displayed maximum number of components (Sahadeo *et al*., 2014).

The leaf, seed and root extracts of *C. sophera* was subjected for HPTLC and the selected scanning wavelength 280nm and the mobile phase(Toluene-Ethyl acetate-Methanol-Formic acid) produced highly symmetrical peaks showing good resolution between each other indicating the presence of flavonoids (Chetan *et al*., 2011). In the present study the use of Folin-Ciocalteu's spraying reagent demonstrated active blue colour bands indicating the presence of flavonoid(polyphenolic compound). This observation has also been made by Jothy *et al*., (2011a) who noted active blue colour bands of phenolic compounds on chromatogram visualized after spraying the plate with Folin-Ciocalteu's in HPTLC screening of *C. fistula* seed extract. All these findings indicate that *Cassia* species are good sources of flavonoids.

Thus the present findings which are in accordance with the above findings cited suggest that the two ethanolic extracts of *C. senna* leaves revealed the presence of several unknown active components and known flavonoids which may be responsible for their biological activity.

HPTLC analysis for alkaloid profile in the leaf extracts of *C. senna*

The results of HPTLC analysis of *C. senna* leaf extracts for alkaloid profile are presented in Table 10, Plates 4-5 and Figures 18.

1 – Petroleum ether extract(PEE)

2 – Benzene extract(BE)

3 – Chloroform extract(CE)

4 – Ethyl acetate extract(EAE)

5 - Successive ethanolic extract(SEE)

CSL6 - Aqueous extract(AE)

CSL7 - Direct Ethanolic extract(DEE)

COL – Colchicine - alkaloid standard as reference marker

Table 10 - HPTLC peak table for alkaloids in the leaf extracts of *C. senna*

Track	Peak	Rf	Height	Area	Assigned substance
COL	1	0.44	499.2	11956.0	Colchicine standard
1	1	0.47	11.9	249.8	Alkaloid 1
1	2	0.90	528.6	39644.6	Alkaloid 2
2	1	0.23	14.7	203.7	Unknown
2	2	0.34	17.7	270.9	Alkaloid 1
2	3	0.37	17.9	480.6	Alkaloid 2
2	4	0.46	80.3	1824.5	Alkaloid 3
2	5	0.50	16.3	339.4	Unknown
2	6	0.58	14.5	149.0	Alkaloid 4
2	7	0.68	11.2	168.4	Unknown
2	8	0.81	60.7	1464.1	Unknown
2	9	0.85	87.4	1882.8	Unknown
2	10	0.93	268.4	13980.8	Alkaloid 5
3	1	0.30	11.9	168.7	Alkaloid 1
3	2	0.35	37.2	1113.6	Alkaloid 2
3	3	0.47	164.8	4323.4	Alkaloid 3
3	4	0.50	156.8	3595.9	Alkaloid 4
3	5	0.64	28.6	722.2	Alkaloid 5
3	6	0.67	20.9	371.7	Unknown
3	7	0.80	127.7	5406.6	Unknown
3	8	0.84	122.5	2554.6	Unknown
3	9	0.93	277.2	16808.1	Alkaloid 6
4	1	0.03	13.0	84.0	Unknown
4	2	0.14	79.4	2651.2	Unknown
4	3	0.22	25.4	689.0	Unknown
4	4	0.28	29.5	383.1	Unknown
4	5	0.32	37.2	963.7	Alkaloid 1
4	6	0.36	46.3	1272.2	Unknown
4	7	0.40	58.8	1346.3	Alkaloid 2
4	8	0.48	412.4	19238.7	Alkaloid 3
4	9	0.56	67.0	2559.7	Unknown
4	10	0.65	28.1	703.9	Unknown

Track	Peak	Rf	Height	Area	Assigned substance
4	11	0.67	35.8	1231.7	Unknown
4	12	0.89	520.7	48962.0	Alkaloid 4
5	1	0.03	41.2	735.7	Alkaloid 1
5	2	0.05	42.8	704.6	Alkaloid 2
5	3	0.09	14.1	168.0	Unknown
5	4	0.13	131.5	3654.2	Unknown
5	5	0.20	135.4	2664.9	Unknown
5	6	0.23	331.1	7943.6	Unknown
5	7	0.26	317.0	8543.3	Alkaloid 3
5	8	0.37	151.2	4861.5	Unknown
5	9	0.39	139.9	2412.8	Alkaloid 4
5	10	0.47	110.2	2345.3	Alkaloid 5
5	11	0.51	224.6	5605.7	Alkaloid 6
5	12	0.56	56.3	2139.3	Unknown
5	13	0.65	13.1	378.1	Unknown
CSL 6	1	0.04	10.4	84.6	Alkaloid 1
CSL 6	2	0.13	19.3	600.2	Unknown
CSL 6	3	0.21	36.6	898.6	Unknown
CSL 6	4	0.50	23.8	620.9	Unknown
CSL 6	5	0.95	229.7	10787.6	Unknown
CSL 7	1	0.03	29.7	386.8	Unknown
CSL 7	2	0.07	21.6	485.1	Alkaloid 1
CSL 7	3	0.13	86	2440	Alkaloid 2
CSL 7	4	0.26	252.2	5244	Unknown
CSL 7	5	0.29	268.5	5179.4	Alkaloid 3
CSL 7	6	0.39	45.4	1300.9	Alkaloid 4
CSL 7	7	0.48	30.4	572.4	Unknown
CSL 7	8	0.50	155.9	4355.3	Alkaloid 5
CSL 7	9	0.58	130.6	3035.5	Unknown
CSL 7	10	0.63	29.7	1163.1	Alkaloid 6
CSL 7	11	0.77	12.2	248.5	Alkaloid 7
CSL 7	12	0.79	21.9	431	Alkaloid 8
CSL 7	13	0.93	298.3	17099.2	Unknown
CSL 7	14	0.90	245.6	15803.4	Alkaloid 9

COL – Colchicine - alkaloid standard as reference marker

1 – Petroleum ether extract(PEE) 2 – Benzene extract(BE)

3 – Chloroform extract(CE) 4 – Ethyl acetate extract(EAE)

5 - Successive ethanolic extract(SEE) CSL6 - Aqueous extract(AE)

CSL7 - Direct Ethanolic extract(DEE)

From the Peak table(Table 10), it is clear that HPTLC analysis of leaf extracts confirmed segregation of different types of alkaloids and other unknown compounds in the chromatogram(Plates 4 and 5) and densitogram(Figure 18) with individual Rf values and peak area. The range of Rf values of these compounds was between 0.03 to 0.95(Table 10). The leaf extracts showed good resolution at both 366nm and 254nm before and after derivatization(Plates 4 and 5). Yellow, Brownish-Yellow and Brownish blue coloured zones at visible light mode(Plate 5) were observed in the tracks from the chromatogram after derivatization, which confirmed the presence of different derivatives of alkaloids with individual Rf values in leaf extracts.

Peak densitogram recorded after scanning at 254nm displayed a number of peaks for both alkaloids and unknown compounds in each extract of the leaves(Figure 18). Among the seven extracts of leaves analysed, CSL6-SEE showed six peaks and CSL7-DEE showed nine peaks of alkaloids(Table 10) as compared with other extracts which further confirms the ethanolic extracts as the good source of alkaloids. It also shows the ability of ethanol to extract more number of alkaloids.

Thus the HPTLC profile for alkaloids and flavonoids of the leaf extracts revealed the presence of more number of bands and peaks for alkaloids than flavonoids which was also observed in the quantitative estimation where the total alkaloid content was higher than that of flavonoids.

Plate 4

HPTLC Chromatogram for alkaloids in the leaf extracts of *C. senna* before derivatization

Visible light **UV366nm** **UV254nm**

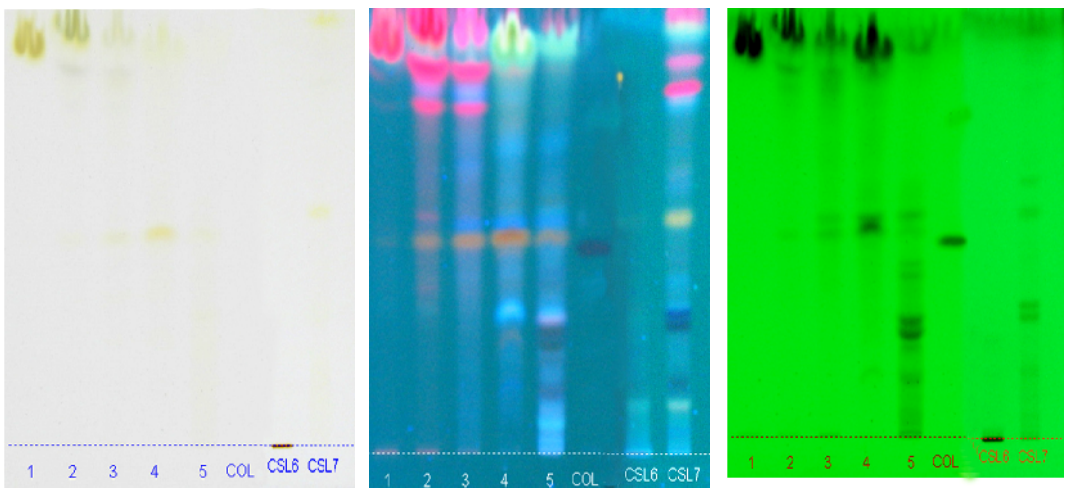
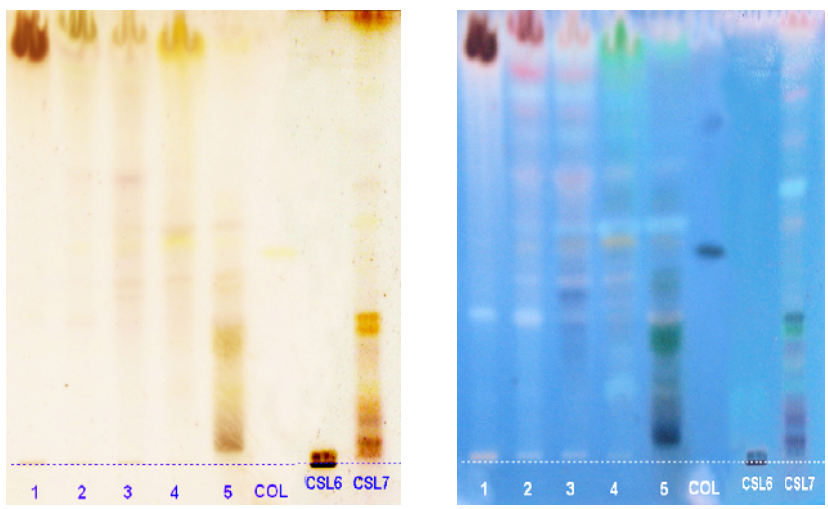


Plate 5

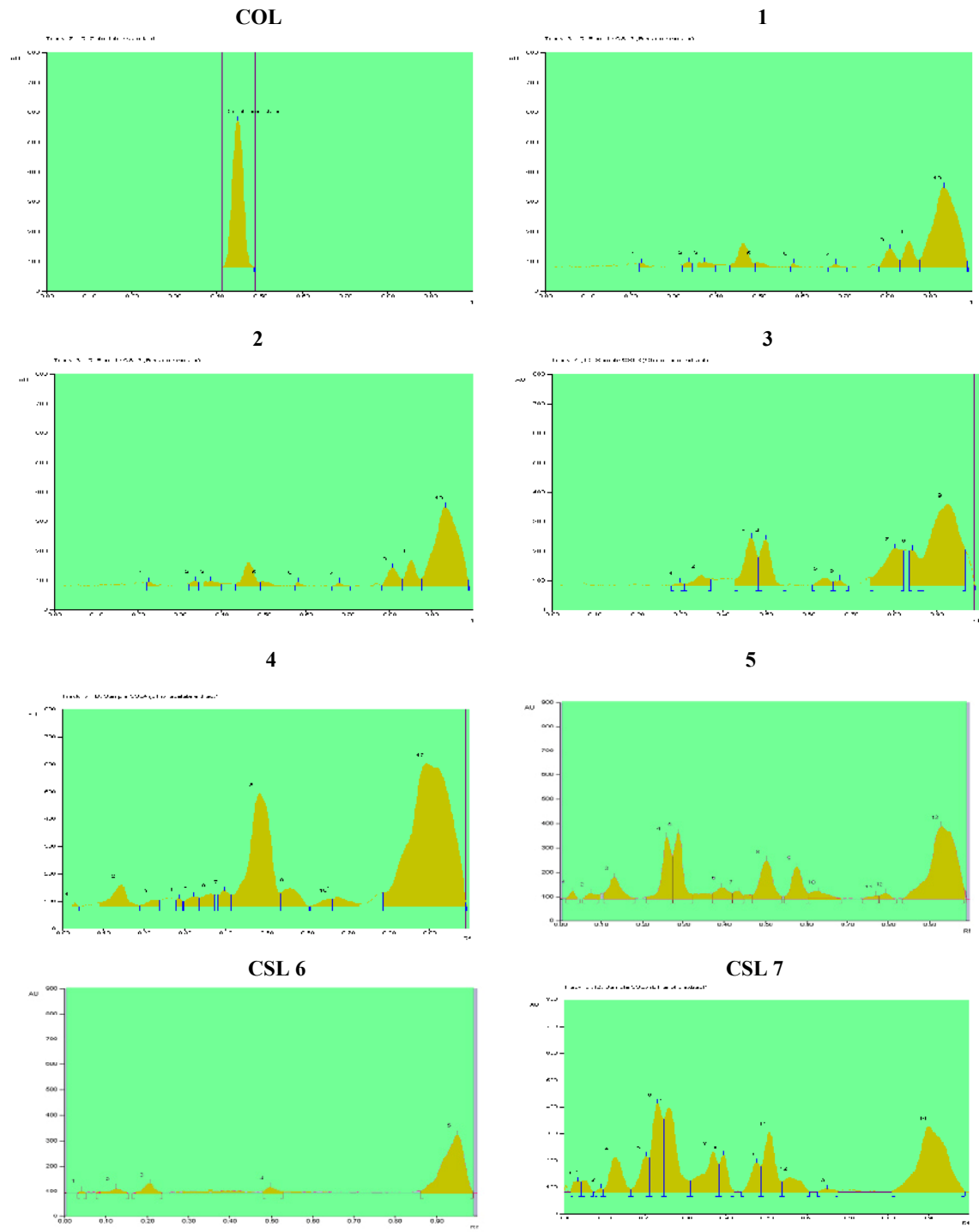
HPTLC Chromatogram for alkaloids in the leaf extracts of *C. senna* after derivatization

Visible light **UV366nm**



COL – Colchicine - alkaloid standard as reference marker
1 – Petroleum ether extract(PEE)
2 – Benzene extract(BE)
3 – Chloroform extract(CE)
4 – Ethyl acetate extract(EAE)
5 - Successive ethanolic extract(SEE)
CSL6 - Aqueous extract(AE)
CSL7 - Direct Ethanolic extract(DEE)

Figure 18 : HPTLC peak densitogram for alkaloids in the leaf extracts of *C. senna*



COL – Colchicine - alkaloid standard as reference marker
1 – Petroleum ether extract(PEE)
2 – Benzene extract(BE)
3 – Chloroform extract(CE)
4 – Ethyl acetate extract(EAE)
5 - Successive ethanolic extract(SEE)
CSL6 - Aqueous extract(AE)
CSL7 - Direct Ethanolic extract(DEE)

The present results are supported by earlier studies. Chavan *et al*., (2016) reported that the HPTLC analysis of methanolic extract of leaves of *C. fistula* L. confirmed that flavonoids and alkaloids as the major group of chemicals in leaf extract. Panda *et al*., (2011) has also reported the same result. The TLC-densitometric and TLC-image analysis methods provided a good reproducibility, accuracy and selectivity for the quantitative determination of barakol(3,4-dihydroxy-2,5-dimethyl-1,4-dioxyphenalene) which is the major compound found in *Senna siamea* leaf extract and is biologically active. Pharmacological studies imply that barakol possessed anxiolytic activity (Padumanonda *et al*., 2007).

HPTLC finger print scanned at 400 nm for methanol and ethyl acetate leaf extracts of *C. fistula* revealed fifteen and sixteen polyvalent phytoconstituent peaks with varying Rf values. It also revealed presence of greenish, purple, pink and light yellowish orange bands showing the presence of steroids, terpenoids and saponins after spraying with anisaldehyde sulphuric acid reagent (Seasotiya *et al*., 2014).

From the HPTLC densitometric quantification of sennosides in leaves of *C. angustifolia*, it was noted that the percentage of active principles varied significantly in the samples procured from different solvents namely ethyl acetate, chloroform, ethanol and methanol and it was found to be best in polar solvents namely methanol followed by ethanol. (Upadhyay *et al*., 2011). The present findings are in agreement with the above results indicating that ethanol being polar solvent exhibits higher ability to extract maximum phytoconstituents than nonpolar solvents.

From all the above literatures of HPTLC analysis of various *Cassia* species, it can be explained that the ethanol and methanol(polar solvents) extracts of leaf of *Cassia* species were found to be effective in the segregation of bands of active principles.

The present findings are in accordance with the above reports. DEE and SEE of *C. senna* leaves revealed the presence of several unknown active components and known alkaloids which can be attributed to their biological activity.

Between the two ethanolic extracts used in the study CSL7-DEE was found to separate more number of alkaloids and flavonoids than CSL5-SEE. The lower ability to segregate bands of active principles by SEE than DEE might be due to the sequential

extraction procedure, where ethanol was used after petroleum ether, benzene, chloroform and ethyl acetate. This has been previously reported by Bhatt and Negi, (2012) who noted lower total phenolic content in methanol that is used after ethyl acetate and acetone.

From the HPTLC analysis of various extracts of *C. senna* leaves, it could be stated that DEE followed by SEE was found to contain more number of alkaloids, flavonoids and the other unknown active components which may be responsible for their biological activity

4.2.2.2. HPLC analysis for alkaloids and flavonoids in the ethanolic extracts (DEE-Direct ethanolic extract and SEE-Successive ethanolic extract) of *C. senna* leaves

HPLC has been established as a premier technique for phytochemical analysis and purification of a wide range of molecules. The inherent features of HPLC, such as high recovery, reproducibility and ease of selectivity manipulation, have made this technique extensively applied in many studies (Aguilar, 2003).

Based on the results of free radical scavenging effect and HPTLC analysis of leaf extracts of *C. senna*, the two ethanolic extracts namely DEE and SEE of *C. senna* leaves were found to contain more number of active principles and to be effective than other solvent extracts of *C. senna* leaves. Hence only DEE and SEE were selected for HPLC study. Inorder to further screen and determine the phytoconstituents of DEE and SEE, HPLC analysis was carried out using the reference standards caffeine for alkaloid and quercetin and kaempferol for flavonoids.

A Shimadzu HPLC system with C18 column, temperature control module and UV detector was used. The mobile phase was acetonitrile:water and flow rate and injection volume were 1ml /min and 20µl. The identification of phytoconstituents was determined by comparing their retention time(RT) and UV spectra of the chromatographic peaks of the analytes detected in the extracts with those of the reference standards. All chromatographic operations were carried out at ambient temperature.

HPLC analysis for alkaloids in the ethanolic leaf extracts of *C. senna*

The HPLC chromatograms of caffeine, DEE and SEE for alkaloid profile at 254nm are shown in Figures 19, 20 and 21.

Figure 19 - HPLC chromatogram of caffeine(alkaloid standard) at 254nm

Figure 20 - HPLC chromatogram of DEE for alkaloid profile at 254nm

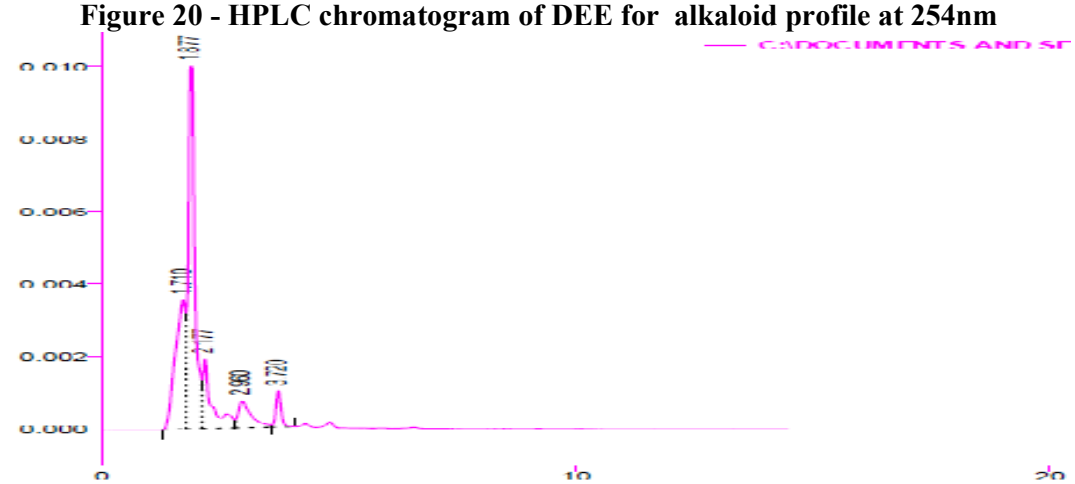


Figure 21 - HPLC chromatogram of SEE for alkaloid profile at 254nm

From Figure 19, it is clear that HPLC chromatogram of standard caffeine shows one peak at retention time 1.457 with peak area 102.923 at 260nm. Five peaks(two major and three minor) were observed from HPLC chromatogram (Figure 20) of DEE of *C. senna* leaves, whereas SEE showed only three peaks (Figure 21) with individual retention times(RT) indicating the presence of active principles. The range of retention times was between 1.7 to 3.7 for DEE and 1.9 to 2.9 for SEE. Each of the peaks may represent one or more compounds present in the extracts.

The RT (1.710) of peak 1 in chromatogram for DEE was found to be closer to RT (1.457) of alkaloid standard, caffeine indicating the possible presence caffeine in DEE of *C. senna* leaves. The remaining peaks with various RT in chromatogram for DEE and SEE might be indicatives of the other unknown compounds.

Silva *et al.,* (2010) reported the presence of the caffeine in the leaf extracts of *Senna spectabilis*. Isolation and structural elucidation of different types of alkaloids namely spectalinine and iso-6-carnavaline from the seeds of *C. spectabilis* have been reported by Christofidis *et al*., (1977). Jothy *et al*., (2011b) reported that HPLC analyses of the active fraction isolated from crude methanolic extract of *C. fistula* seeds showed the presence of eight peaks with one distinct peak that indicated the presence of major bioactive compound called roseanone which showed a high antiyeast activity.

The present findings of HPLC analysis for alkaloid profile suggest that DEE of *C. senna* leaves was found to be good source of alkaloids and other active principles than SEE.

HPLC analysis for flavonoids in the ethanolic leaf extracts of *C. senna*

The HPLC chromatograms of kaempferol, DEE and SEE for flavonoid profile at 215nm are shown in Figures 22, 23 and 24.

Figure 22 - HPLC chromatogram of kaempferol(flavonoid standard) at 284nm

Figure 23 - HPLC chromatogram of DEE for flavonoid profile at 284nm

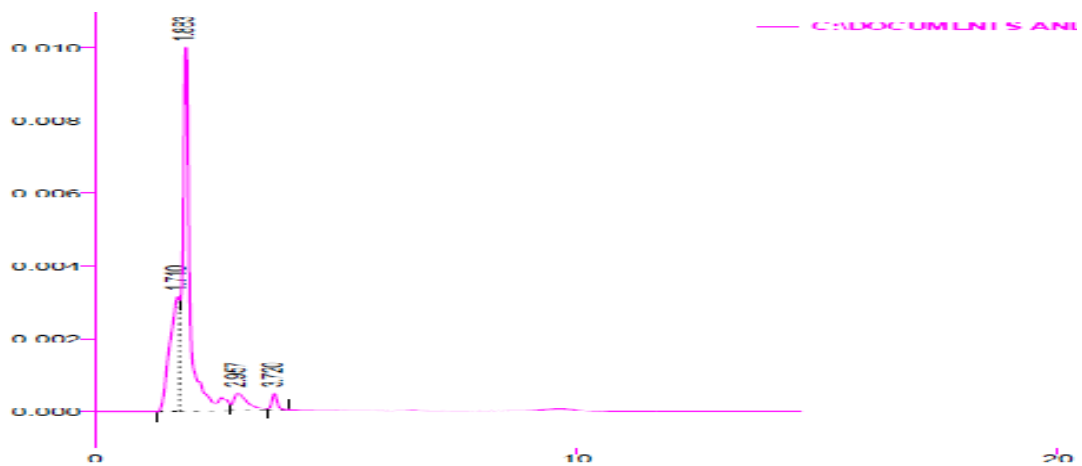
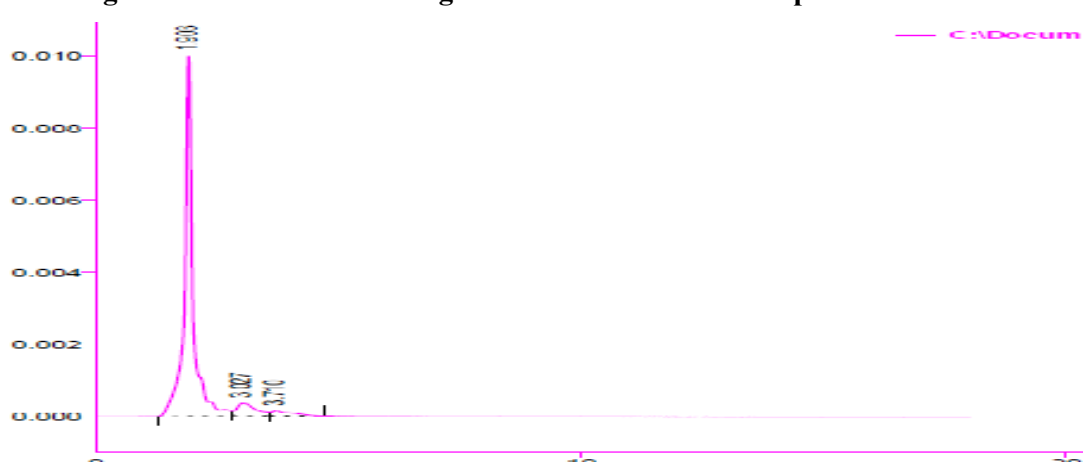


Figure 24 - HPLC chromatogram of SEE for flavonoid profile at 284nm



From Figure 22, it is clear that HPLC chromatogram for standard kaempferol shows one peak at retention times 2.983 peak area 103.535 that was detected at 254nm. Four peaks (two major and two minor) were observed from the HPLC chromatogram(Figure 23) for DEE of *C. senna* leaves, whereas SEE showed only three peaks(Figure 24) with individual retention time(RT) indicating the possible presence of active principles. The range of retention time was between 1.710 to 3.720 for DEE and 1.903 to 3.720 for SEE repectively. Each of the peaks may represent one or more compounds present in the extracts.

The RT(2.957) of peak 3 with peak area 9.748 in chromatogram for DEE exactly matches the RT(2.983) of kaempferol and RT(3.027) of peak 2 for SEE with peak area 9.669 was found to be closer to RT of kaempferol that indicates the possible presence kaempferol in those extracts. The remaining peaks with various RT in chromatogram for DEE and SEE might be indicatives of the other unknown compounds.

The HPLC chromatograms for quercetin, DEE and SEE are shown in Figure 25, 26 and 27.

Figure 25 - HPLC chromatogram for quercetin(flavonoid standard) at 215nm

Figure 26 - HPLC chromatogram of DEE for flavonoid profile at 215nm

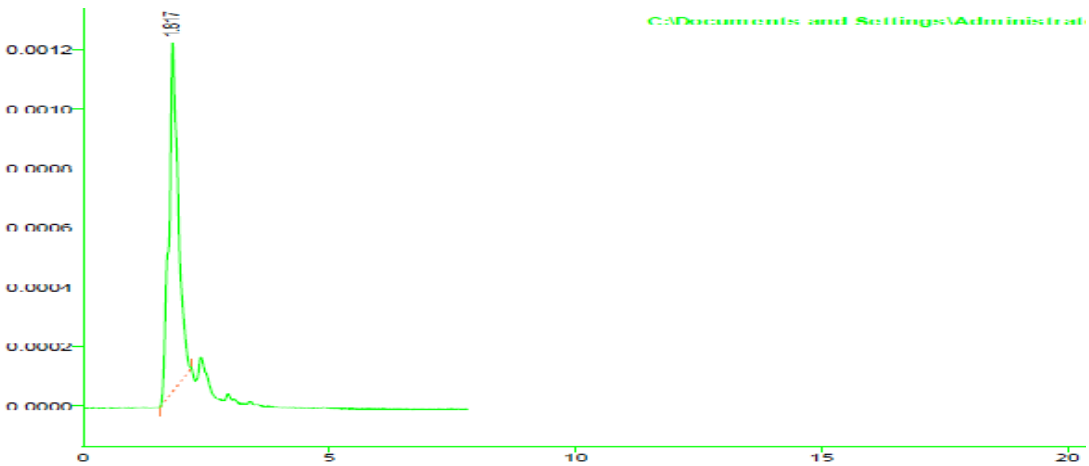
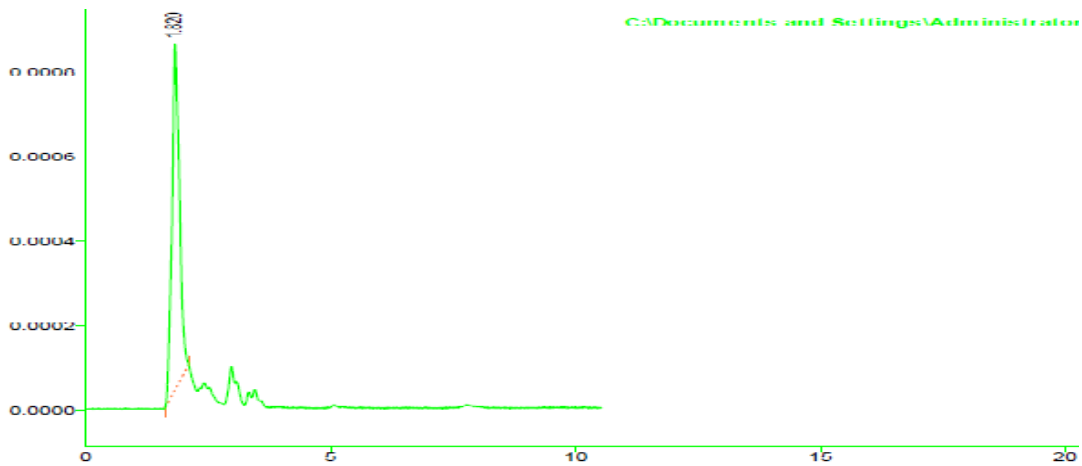


Figure 27 - HPLC chromatogram of SEE for flavonoid profile at 215nm



From Figure 25, it is clear that HPLC chromatogram for standard, quercetin shows one peak at 1.500RT with peak area 185.345 at 215nm. Two peaks (one major and one minor) were observed from the HPLC chromatogram (Figure 26) for DEE, whereas SEE also showed two peaks (one major and one minor (Figure 27) with individual retention time(RT) indicating the possible presence of active principles. The range of retention time was between 1.817 to 2.5 for DEE and 1.817 to 3.0 for SEE repectively.

The RT(1.817) of peak 1 with peak area 15.511 in chromatogram for DEE and RT (1.820) of peak 1 for SEE were found to be closer to RT (1.500) of quercetin that indicates the possible presence quercetin in those extracts. The remaining peaks with various RT in chromatogram for DEE and SEE might due to the presence of other unknown compounds. Quercetin is thought to have potent antioxidant, antidiabetic, anti tumour, antiviral and anti inflammatory benefits (Spencer, 2008). American cancer society has promoted quercetin as a effective compound against wide variety of diseases including cancer (Sousa *et al*., 2007).

Mahesh *et al*., (1984) reported that phytochemical investigation of six species of *Cassia* namely *C. fistula*, *Cassia grandis*, *C. nodosa*, *C. renigera*, *C. javanica* and *Cassia marginata* were found to contain kaempferol and a mixture of anthraquinones. Mehta, (2012) identifid the three medicinally important anthraquinone derivatives such as rhein, emodin and chrysophanic acid in the seed extract from *C. fistula* and each analyte was identified by using standards. Major types and representative components of phenolic and flavonoid compounds were identified in leaf and flower of *C. auriculata* and in pod of *C. fistula* through HPLC analysis (Surveswaran *et al*., 2007).

Extracts of aerial parts of *Senna alata* were investigated for antioxidant phenolic compounds using HPLC. Two major phenolic compounds Naringin and Apigenin were identified in some of the solvent fractions of *S. alata*. Major phytoconstituents in *S. alata* (guajava) include kaempferol, kaempferol 3-O-gentiobioside, naringenin, quercetin, and rhamnetin-3-O-(2"-O-beta-D-mannopyranosyl)-beta-D-allopyranoside. The presence of these flavoniods in *S. alata* may explain its wide use in ethnomedicine practice for the treatment of hypertension, sickle cell anemia and diabetes in Southwestern Nigeria(Okpuzor *et al*., 2009).

The concentration of six phenolic compounds, five anthraquinones(rhein, aloe-emodin, emodin, chrysophanol and physcion) and a flavonoid(kaempferol) was determined in the ethanolic root extracts of *C. alata* using HPLC with ultraviolet detection at 260 nm (Fernand *et al*., 2008). Compounds such as flavonoids, phenols and chromone alkaloids(barakol, cassiarin A-B) are the main constituents that are reported in *C. siamea* and barakol was identified as the major constituent of *C. siamea* leaves and flowers (Kamagaté *et al*., 2014). HPLC analysis of hexane fraction derived from methanolic extract of *C. alata* leaves identified kaempferol as a phytoconstituent by comparing the relative retention time (RRT) of hexane fraction with that of the chemical standard kaempferol (Levy and Carley, 2012).

The present findings which are in accordance with the above literatures indicate that DEE of *C. senna* leaves was found to be good source of flavonoids and other active principles than SEE.

From the results of HPLC analysis, DEE gave more peaks for alkaloid and flavonoid profiles than SEE at respective wavelength. The RT of DEE was found to match exactly with that of kaempferol while in case of caffeine and quercetin the RT of DEE was found to be closer to that of standards. Hence HPLC confirmed that DEE was an effective extract containing essential active principles than SEE. As discussed in HPTLC results the lower ability to separate the peaks of active principles by SEE than DEE might be due to the sequential extraction procedure, where ethanol was used after petroleum ether, benzene, chloroform and ethyl acetate.

4.2.3. Assessment of *in vitro* cytotoxic activity of the two ethanolic extracts (DEE and SEE) of *C. senna* leaves in selected cancer and noncancer cell lines

Cell lines are useful models for doing research since they provide large amounts of consistent cells for prolonged used. Because most cellular characteristics are maintained in cell lines, they provide reliable in experimental results (Bretagnol, 2008).

MTT assay

Several rapid colorimetric assays have been described for *in vitro* chemosensitivity testing of tumour cell lines. In the recent years, the MTT[3-(4,5-

dimethylthiazol-2-yl)-2,5-diphenyltetrazolium Bromide] assay has been the most widely used assay. This assay can be used to assess cytotoxicity because only metabolically active cells can reduce the water soluble MTT(salt) to its formazon product and therefore, dead cells do not interfere with MTT absorbance levels (Mosmann T, 1983).

This colorimetric assay is based on the capacity of mitochondrial succinate dehydrogenase enzyme in living cells to reduce the yellow coloured water soluble substrate 3- (4, 5-dimethyl thiazol- 2-yl)-2, 5-diphenyl tetrazolium bromide (MTT) into an insoluble purple colored formazan product which is measured spectrophotometrically. This assay depends on the mitochondrial activity per cell and number of cells present and the reduction of MTT can only take place in metabolically active cells and the level of activity is a measure of the viability of the cells (Wilson and John, 2000).

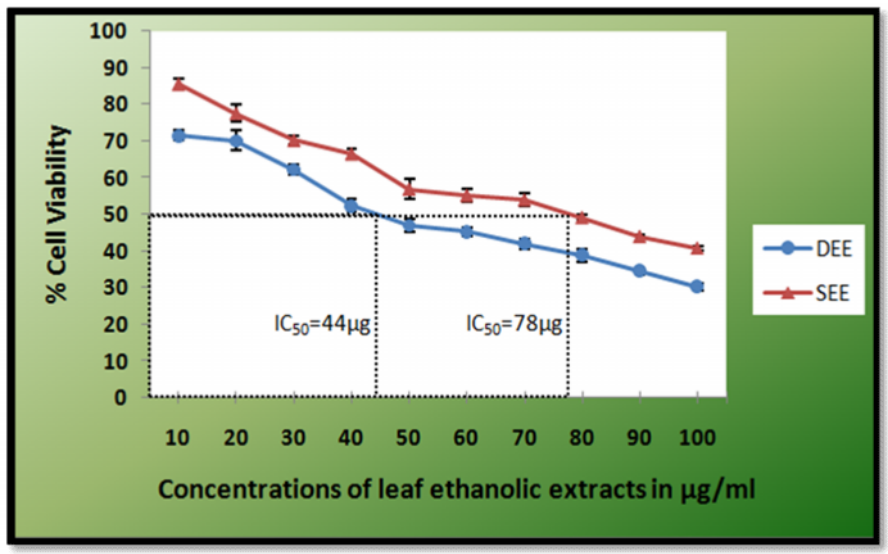
In vitro cytotoxic activity of DEE and SEE of _C. senna_ leaves against cancer and noncancerous cell lines

To evaluate the cytotoxic effect of DEE and SEE on different cancer cell lines namely Ehrlich ascites carcinoma(EAC), Breast adenocarcinoma(MCF7), Colon cancer(HCT116) cell lines and L929 cell line(noncancerous cells), the cells were exposed at various dose levels of both the ethanolic extracts for 24 hours in the presence of MTT.

MTT assay in EAC cells

The results of cytotoxicity exerted by the leaf ethanolic extracts(DEE and SEE) of *C. senna* are presented in Figure 28.

Figure 28 : Cytotoxic effect of DEE and SEE of *C. senna* leaves on EAC cell line



Values are Mean ± SD(n=3)

In this bioassay, as shown in Figure 28, a dose dependent lethality was observed for different concentrations of ethanolic extracts of *C. senna* leaves and it was observed that cell viability was markedly decreased as the concentration increases. The extracts DEE and SEE killed 50 percent of EAC tumor cells at a concentration (IC50 values) of 44µg and 78µg respectively. These results demonstrated that DEE and SEE mediated a concentration dependent increase in toxicity towards EAC cells. The efficient cytotoxic effect of DEE on cancer cell lines might be due to the presence of more number of antitumour compounds than that of SEE. The cytotoxicity of plant material is considered to be due to the presence of antitumor compounds (Kawsar *et al.,* 2008).

Bhattacharya *et al*., (2011) reported that the hydroalcoholic extract from *Trichosanthes dioica* root increased the percent cytotoxicity on ehrlich ascites carcinoma cell lines thus suggesting the feasibility of its possible promise as natural anticancer agent. In a study by Mohamed, (2014), the cytotoxic effect of a new cycloartane triterpene isolated from methanolic extract of *C. italica* tested against L5178Y(mouse lymphoma cells) and PC12(brain tumour cells of the rats) cell lines displayed a remarkable percentage of growth suppression against both the cell lines. The ethanolic

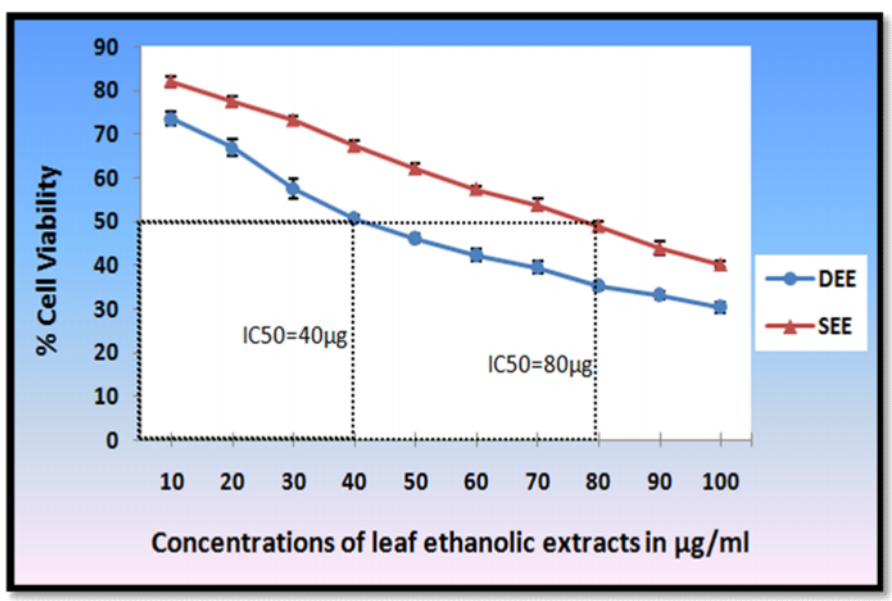
extracts of aerial parts of *Hyptis suaveolens* and *Leonotis nepetaefolia* were subjected to anticancer screening against EAC cell line using MTT assay and both the ethanolic extracts showed potent cytotoxicity against Ehrlich Ascites carcinoma (Gurunagarajan and Pemaiah, 2011).

The present results are in accordance with the above literatures cited and DEE of *C. senna* leaves was found to be highly cytotoxic to EAC cells than SEE.

MTT assay in colon cancer(HCT 116) cells

The cytotoxic effects of DEE and SEE *C. senna* leaves exerted on HCT 116 cells are presented in Figure 29.

Figure 29 : Cytotoxic effect of DEE and SEE of *C. senna* leaves on HCT 116 cell line



Values are Mean ± SD(n=3)

From Figure 29, the present results clearly showed that cell viability in HCT116 cell line was markedly decreased after exposure to both the ethanolic extracts in a dose-dependent manner. The IC50 values of extracts DEE and SEE were found be as 40µg and 80µg respectively. These results demonstrated that DEE and SEE mediated a dose dependent increase in toxicity towards HCT116 cells.

Similar observations have been reported by many researchers in various *Cassia* species. From the previous study by Aviello *et al*., (2010) it was reported from the MTT assay that rhein, the active metabolite of senna(isolated from some *Cassia* species), on human colon adenocarcinoma cells(CaCo-2) was found to significantly reduce cell proliferation as well as mitogen-activated protein (MAP) kinase activation even at very low concentration. It may be due to the fact that rhein prevents the DNA damage probably through anti-oxidant mechanism.

Methanolic extract of leaf of *Cassia uniflora* showed >70% growth inhibitory effect against Du145(Prostate carcinoma) and FaDu(squamous cell carcinoma of pharynx) cell lines whereas in DLD1(Colorectal adenocarcinoma) cell line it showed >65% growth inhibition. Methanolic extracts of stem and root of *C. auriculata* also showed >70% growth inhibitory effect against Du145(Prostate carcinoma) cell lines whereas the methanolic extracts obtained from the root of *C. siamea and C. auriculata* were able to inhibit growth in both DLD1(Colorectal adenocarcinoma) and FaDu(squamous cell carcinoma of pharynx) cell lines, in the assay(SRB) of *in vitro* anti-proliferative activity(Chandra *et al*., 2015).

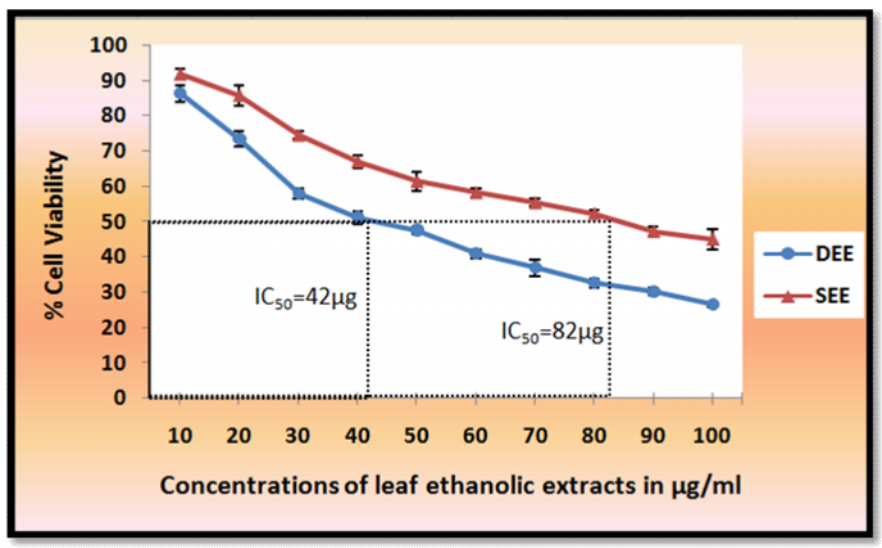
Glucuronoxylan, a hemicellulosic polysaccharide(COB1B1S2-Sul) was isolated from an alkaline extract of *C. obtusifolia* seeds and the sulfated derivative of Glucuronoxylan exhibited a strong anti-angiogenic activity by inhibiting tube formation of human microvascular endothelial cells(HMEC-1) and exhibited a significant inhibition against human hepatocellular carcinoma cell lines namely Bel7402, SMMC7721 and Huh7 and colon cancer cell lines such as HT-29 and Caco-2 in MTT assay(Cong *et al*., 2014).

The present findings which are supported by earlier observations suggest that DEE of *C. senna* exhibited maximum cytotoxicity towards breast cancer(MCF 7) cells than SEE.

MTT assay in breast cancer (MCF 7) cells

The extent of cytotoxicity on MCF 7 cells by the two ethanolic extracts of *C. senna* leaves is shown in Figure 30.

Figure 30 : Cytotoxic effect of DEE and SEE of *C. senna* leaves on MCF 7 cell line



Values are Mean ± SD (n=3)

It is evident from Figure 30, percentage cell viability of MCF 7 cell lines was inversely proportional to the concentration of DEE. A similar trend was also noticed with SEE. The IC50 values for DEE and SEE were 42µg and 82µg respectively. These results demonstrated that DEE and SEE mediated a concentration and time dependent increase in toxicity towards MCF7 cells.

Similar observations were reported in many research findings. Prasanna *et al*., (2009) evaluated the *in vitro* anti-cancer effect of *C. auriculata* leaf extract in human breast adenocarcinoma(MCF-7) and human larynx carcinoma(Hep-2) cell lines. Sermakkani and Thangapandian, (2010) revealed the *in vitro* anticancer activity of leaves of *C. italica* against two types of cell lines such as Hep-2 and Vero and it was evident that the magnitude of the cytotoxicity was predominant in methonolic extract of leaves in both the cell lines and confirmed the cytotoxic property of *C. italica*.

Polyphenolic extracts(flavonoids) from *Vaccinium macrocarpon* inhibited the growth and proliferation of breast, colon, prostate, lung, and other tumors (Neto, 2007). The cytotoxic effects of hexane extracts of *C. alata* and *P. guajava* leaves at various concentrations were evaluated in OV2008 ovarian and Kasumi-1 leukemia cancer cell lines for 24 h, and it was found that the cytotoxicity exhibited by *C. alata* might be attributable to the flavonoid, kaempferol and that of *P. guajava* could be attributable to the flavonoids, quercetin and kaempferol which were identified in those extracts. Both kaempferol and quercetin have been reported to exhibit cytotoxic activities in various cancer cell lines (Levy and Carley, 2012). The present study which is supported by above findings suggests that DEE was found to possess high content of flavonoids which may be responsible for the inhibition of growth and proliferation of selected cell lines namely MCF7(breast cancer), HCT116(colon cancer) and EAC cell lines.

Chandra *et al*., (2015) reported that seed and stem of *C. auriculata* were able to inhibit growth in A549(lung carcinoma) and MCF-7(breast adenocarcinoma) cell lines in the assay(SRB) of *in vitro* anti-proliferative activity. Eight types of isolated flavonoid compounds namely furanoflavones from the stem of *C. fistula* were evaluated for their cytotoxicity against five human tumor cell lines such as NB4, A549, SHSY5Y, PC3 and MCF7 and it was found that one of the compounds called 3,4'-dimethoxy-5-hydroxy-7,8-[2"-(2-hydroxyethyl)furan]-flavone showed potent cytotoxicity against SHSY5Y and MCF7 cells (Gao *et al*., 2013).

Imam *et al*., (2013) reported that the cytotoxic effect of different concentrations of *Cassia arereh* stem bark methanolic extract after 24hrs of incubation revealed the highest mortality on both K562S and HepG2 cell lines and also reported the presence of flavonoids, terpenes, sterols and tannins. Mohammed *et al*., (2013b) have reported the significant cytotoxic effect of petroleum ether extract of the leaves of *Cassia roxburghii* against HCT-116 and MCF-7 cell lines. *In vitro* antiproliferative and cytotoxic potentials of alkaloids such as (−)-cassine(1) and (−)-spectaline(2) derived from *Senna spectabilis*, tested on six tumor cell lines reduced the cell viability in a concentration-dependent manner (Pereira *et al*., (2016).

The present findings are in agreement with all the above reports and DEE of *C. senna* exhibited higher cytotoxicity towards colon cancer(HCT 116) cells than SEE.

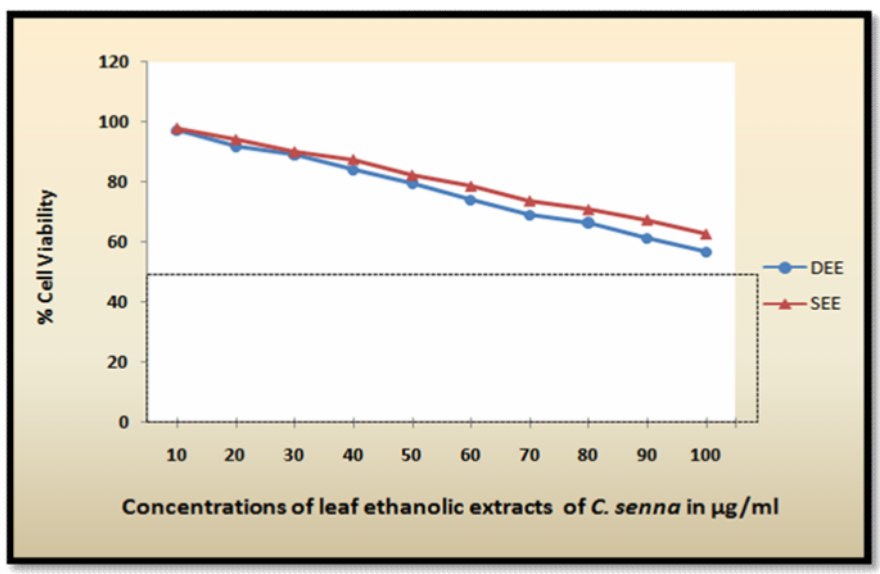
The higher percentage of cytotoxicity against all the tested cancer cell lines observed with DEE of *C. senna* leaves than that of SEE might be due to the extraction of more number of essential phytoconstituents namely alkaloids and flavonoids in DEE. SEE being a product of sequential extraction procedure might contain lesser concentration of phytoconstituents than DEE.

MTT assay in L929 cell line(normal cells)

Mouse fibroblast L929 is a normal cell line, which is recommended by international standards for the testing of medical devices (ISO, 1992) and responds more sensitively than primary cells (Schedle *et al*., 1995). L929 cell line is popular in many experimental aspects such as drug cytotoxicity testing (Faria *et al*., 2009) and cell biology studies (Roelofs *et al*., 2006).

To evaluate the cytotoxic effect of DEE and SEE of *C. senna* leaves on normal mouse fibroblast L929 cell line, the L929 cells were exposed to various doses(10-100µg) of both the ethanolic extracts for 24 hours in the presence of MTT. The percent cell viability was calculated and results are shown in Figure 31.

Figure 31 : Cytotoxic effect of DEE and SEE of *C. senna* leaves on L929 cell line



In this bioassay, as shown in Figure 31, a dose dependent minimum decrease in % cell viability on L929 cells was observed for different concentrations of both the ethanolic extracts of *C. senna* leaves. The IC50 values of both DEE and SEE were being >100µg and thus the extracts were found to be less toxic to L929 cells. The results of MTT assay for both cancer and noncancer cell lines demonstrated that DEE and SEE mediated a specific cytotoxicity towards only cancer cells, but those extracts were not toxic to untransformed cells(normal cells). This is accordance with the report given by Lacroix *et al*., (2006) who showed that the plant extract will act successfully as an anti-cancer drug by killing cancer cells without causing excessive damage to normal cells, such as L929.

Many researchers have tested the cytotoxicity of various plant extracts on noncancer cell lines such as L929. Graidist *et al*., (2015) reported that out of the seven fractions derived from methanolic crude extract of *Piper cubeba* a single fraction was more cytotoxic and inhibited cell growth on breast cancer cell lines namely MCF-7, MDA-MB-231 and MDA-MB-468(high-grade cancer) whereas all the fractions were appeared to be less toxic in L929 cells(normal fibroblast cells). Khairunnisa and Karthik, (2014) reported that the methanolic bark extract of *Hymenodictyon excelsum* (Roxb) was found to be cytotoxic towards L-929 cells at 72 hrs by MTT assay and also was found to cause 50% cell death even at very low concentration.

MTT assay for ethanolic extract of *Citrullus colocynthis*(bitter melon) showed a dose dependent cytotoxicity effect on human caucasian larynx carcinoma(Hep2) cell line but showed no effect on L929(normal mouse fibroblast) cell line. The percentage cell viability was higher in L929 and lower in Hep2 thereby making that extract useful for inhibition of growth of cancer cells such as human larynx carcinoma (Afshari *et al*., 2005). The present results are in accordance with the above findings and indicate that both the ethanolic extracts(DEE and SEE)of *C. senna* leaves showed higher cytotoxicity for the tested cancer cell lines namely EAC, MCF7 and HCT116 and showed lower cytotoxicity for normal cell line, L929. This validates DEE and SEE of *C. senna* as effective cytotoxic agents to cancer cells but nontoxic to normal cells.

The ethanolic extracts of *Prosopis laevigata* (Mesquite tree) and *Quercus ilex* (Oak tree) showed high protective effect on normal cell lines namely L929(mouse, subcutaneous connective tissue) and RAW 264.7(mouse leukaemic monocyte macrophage cell line) and less protective effect in cancer cell lines namely A549(human caucasian lung carcinoma), NCl-H1395(human lung adenocarcinoma) and 22Rv1(human prostate carcinoma thereby suggesting nontoxic effect of plant extracts on normal cell lines, L929 and RAW 264.7 (Assanga *et al*., 2013). Parizadeh *et al*., (2011) observed that the aqueous saffron extract did not cause any morphologic modifications at any concentrations and was very less toxic to L929 cell lines. The extract at noncytotoxic concentration detected on L929 cell lines could causes growth control and cytotoxic effect on carcinoma cells namely HepG-2 and Hep-2 cell lines.

The cytotoxic effect of ginger essential oil (*Zingiber officinale* roscoe) was lower in L929 and Vero cell lines while in EAC cell line the cytotoxicity was higher. The low cytotoxic effect on non-cancerous cell lines indicates that ginger essential oil exhibits cytotoxicity specifically to cancer cells only but not in normal cells (Jeena *et al*., 2015). It was observed by Cherng *et al*., (2008) that four anthraquinones of *C. tora* namely aloeemodin, emodin, chrysophanol and rhein at non-cytotoxic concentrations were effective in stimulating the proliferation of human peripheral blood mononuclear cells (PBMC) during the bioassay called lymphoproliferation test.

Thus, the observations of the above studies support that the plant extracts that are nontoxic or less toxic to normal cells such as L929 cells but more toxic to cancer cells may become effective anticancer drug. The present findings of MTT assay for both cancer and noncancer cell lines indicate that DEE of *C. senna* leaves was more toxic to cancer cell lines, but less toxic to noncancer cells and this is in accordance with the above findings cited. This may validate DEE as the successful an anticancer source.

PHASE III

4.3. Evaluation of *in vivo* anticancer activity and characterization of the active principles of the Direct Ethanolic Extract (DEE) of *C. senna* leaves

4.3.1. Assessment of *in vivo* anticancer activity of DEE (Direct Ethanolic Extract) of *C. senna* leaves in Ehrlich Ascites Carcinoma (EAC) induced Swiss albino mice

The phase II studies on the free radical scavenging effect, chromatographical analysis and *in vitro* cytotoxicity studies have clearly indicated that among the various extracts of leaf, DEE of *C. senna* leaves was found to be a rich source of active principles like alkaloids and flavonoids and also found to be more effective than SEE. Therefore DEE was selected for Phase III studies to further authenticate its use as a promising antioxidant and anticancer agent under *in vivo* conditions. The *in vivo* study was focused on the tumour growth response, tumour markers, liver marker enzymes, antioxidants, lipid peroxidation, lipid content and histopathological studies in the Swiss albino mice challenged with the EAC tumour cells and compared with the standard anticancer drug methotrexate.

Adult male Swiss albino mice weighing approximately 20-25g were used as experimental model. After acclimatization of mice to laboratory conditions for 10 days the mice were divided into six groups of six each. Ehrlich ascites carcinoma (EAC) cells were injected into tumour control(Group–II) and experimental controls(Groups-III, IV, V, VI) of mice intraperitoneally at concentration of $1X10^6$ cells/mouse. After 24 hour of the tumor cell induction treatment with DEE and standard anticancer drug(methotrexate) was started. Group I and Group II animals received saline only. Group III was administered with the standard drug and the remaining groups(IV, V and VI) were treated with various concentration of DEE of *C. senna* leaves for 14 days as given below.

Group – I **:** Normal control(Negative control)-Saline only

Group – II **:** Tumor control(Positive control)- EAC cells + Saline

Group – III **:** EAC cells+Standard anticancer drug-Methotrexate 10mg/Kg b.w.

Group – IV : EAC cells+DEE 100mg /Kg b.w.

Group – V : EAC cells+DEE 200mg /Kg b.w.

Group – VI : EAC cells+(1X10^{6}cells/mouse)+DEE 200mg /Kg b.w.

After the experimental period, mice were sacrificed by cervical decapitation, the blood was collected from the controls and the experimental groups of mice and the serum was separated out for the biochemical analysis. The liver and kidney samples were washed with ice cold saline. After washing, samples from each group were homogenized with phosphate buffer for the analysis of antioxidants and lipid content while few samples from each group were fixed with 10% formalin for histopathological studies.

4.3.1.1. Effect of DEE of *C. senna* leaves on tumour growth response

Body weight

The body weight (BW) of the control and treated group mice was measured at 0th day, 5th day, 10th day and 14th day and was depicted in Table 11.

Table 11 : Effect of DEE of C. senna leaves on body weight of control and experimental animals

Body weight (g)				
Groups	**0th day**	**5th day**	**10th day**	**14th day**
I	**25.17**	**25.83**	**26.27**	**26.77**
II	**25.57**	**29.63**	**32.33**	**37.53**
III	**27.3**	**28.17**	**28.8**	**29.3**
IV	**26.57**	**27.33**	**28.53**	**30.17**
V	**27.23**	**27.83**	**28.5**	**29.27**
VI	**25.53**	**25.87**	**26.6**	**27.23**
SEd	**0.11**			
CD p(<0.05)	**0.22**			

When compared with normal control, significant($p<0.05$) substantial gain in body weight was observed(Table 11) in the tumor-bearing mice with a maximum value of 37.53g which is due to the accumulation of ascites fluid. On treatment with DEE at different concentrations(100mg - 300mg/kg b.w.), gain in body weight was found to be partially maintained as observed in normal control and standard drug treated groups.

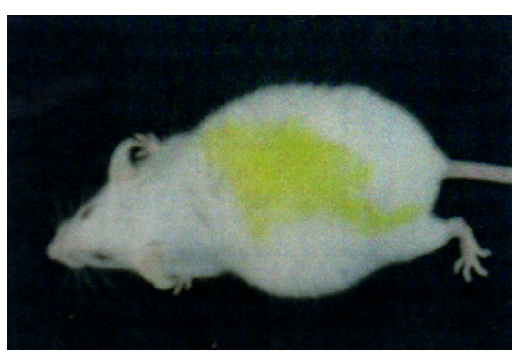
When compared to tumour control, a significant($p<0.05$) reduction in the body weight gain was noted which might be due to the reduction in the ascites fluid accumulation. This observation could be attributed to the efficiency of DEE in bringing down the progression of cancer.

Plate 6

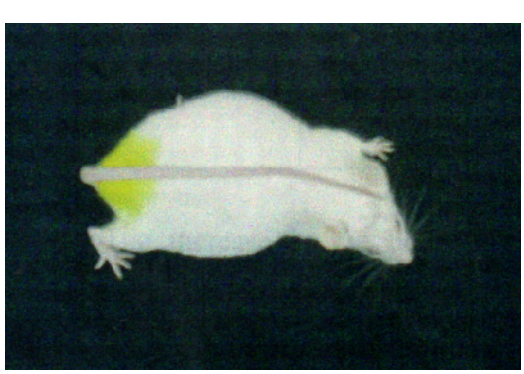
Effect of DEE of *C. senna* leaves on body weight of control and experimental animals

GpI - Normal control(Saline only)

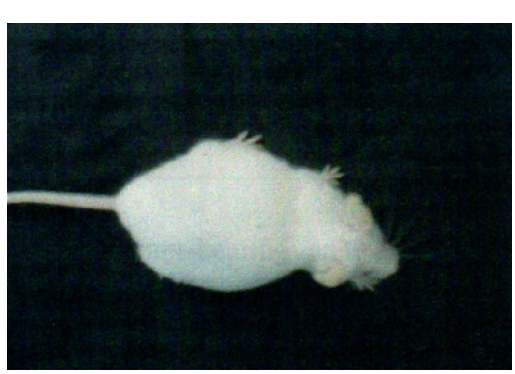
GpII - Tumour control(EAC cells+ Saline)



Gp III - Standard drug (Methotrexate - 10mg/Kg b.w.)



Gp VI – High dose administration of DEE of *C. senna* leaves (300mg/Kg b.w.)



Mean survival Time (MST) and Increased Life Span(ILS)

Figure 32 : Effect of DEE of *C. senna* leaves on Mean survival Time (MST)

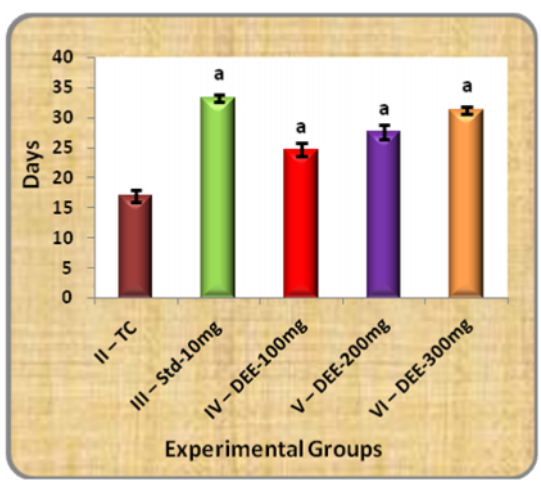
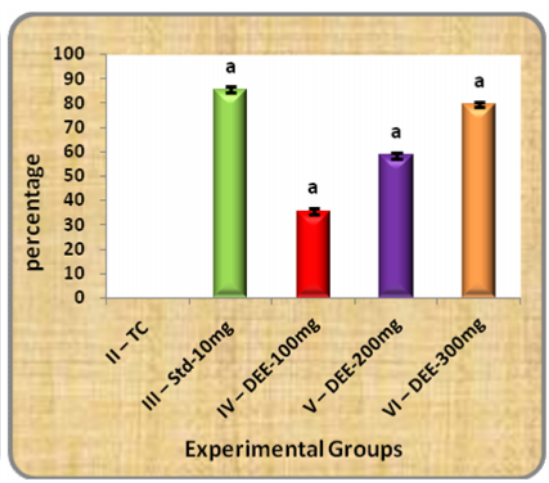


Figure 33 : Effect of DEE of *C. senna* leaves on Increased Life Span(ILS)



Values are mean ± SD (n=6)

a - GpII Vs GpIII, GpIV, GpV, GpVI

a - statistically significant ($p<0.05$)

From Figures 32 and 33, it is clear that there was a significant($p<0.05$) increase in the life span of DEE and standard drug treated animals. The mean survival time of tumour control groups were found to be 17 days while in the standard drug treated animals it was increased upto 33 days. MST of DEE treated animals at high dose(300mg/kg body wt.) was comparable with that of standard drug. The percentage of Increased Life Span(ILS%) was also found to be significantly($p<0.05$) enhanced in standard drug and DEE treated animals. Hence the administration of DEE resulted in maintenance of body weight near to normal and also remarkable($p<0.05$) improvement in the MST and ILS%. These observations clearly indicate the ability of DEE in reducing tumour burden.

Many researchers have reported these parameters of tumour growth response. Sakthivel *et al*., (2012) reported that administration of methanolic extract aerial parts of Acacia nilotica at concentration of 10 mg/kg.bw showed increase in mean survival time and percentage increase in life span, decrease in percentage of increase in body weight (due to reduction of tumor burden) when compared to control DAL bearing ascitic tumor group.

Anti-tumor activity of methanolic extract of seed of *C. fistula* against Ehrlich ascites carcinoma has been reported and it was found that the treatment with the same showed an increase of life span and a decrease in the tumour volume in experimental animals (Gupta *et al*., 2000). Alcoholic extract of *Biorhythms sensitivum* tested for antitumor activity, could inhibit the solid tumor development on mice induced with Dalton's lymphoma ascites (DLA) cells and increase the life span of mice bearing Ehrlich ascites carcinoma (EAC) tumors (Guruvayoorappan and Kuttan, 2007)

Administration of ethanolic extract *C. italica* (Senegal senna) significantly delayed the death, inhibited the tumor growth and increased the life span of mice transplanted with EAAC tumour compared with that of untreated animals. It has also been reported that the extract appeared to activate EACC tumor cell death possibly by immunosuppression (Nassr-Allah *et al*., 2009).

The antitumor, antimetastatic and anti-angiogenic actions of a new anthrone-c-glycoside named cassialoin isolated from of *Cassia garratiana* heartwood has been reported in mice induced with highly metastatic colon 26 cell line. Cassialoin inhibited tumor growth, metastasis to the abdomen and the expression of angiogenesis marker in the tumors. The percentage of tumor invasion to abdomen and the percentage of tumour weight were decreased in colon 26 bearing mice with cassialoin administration (Kimura *et al*., 2008).

Kumar *et al*., (2007) reported that ethanol extract of *Indigofera trita* increased the MST, ILS % and decreased the average weight gain of EAC bearing mice to near normal and the antitumor activity of that extract may be due to the presence of flavonoids which have a chemopreventive role in cancer through their effects on signal transduction in cell proliferation (Weber *et al*., 1996) and angiogenesis (Fotis *et al*., 1997).

Thus the present study is in agreement with the above findings cited by researchers. From the present observations of tumour growth response, it is clear that the DEE of *C. senna* leaves was found to be effective in reducing tumour burden.

4.3.1.2. Tumour Markers in serum

Gamma glutamyl transferase (GGT), an enzyme involved in cellular glutathione homeostasis which is often increased in tumors. Nitric oxide (NO) is an important regulator of tumor growth and involved in various pathophysiological process includes inflammation and carcinogenesis (Hong *et al*., 2002).

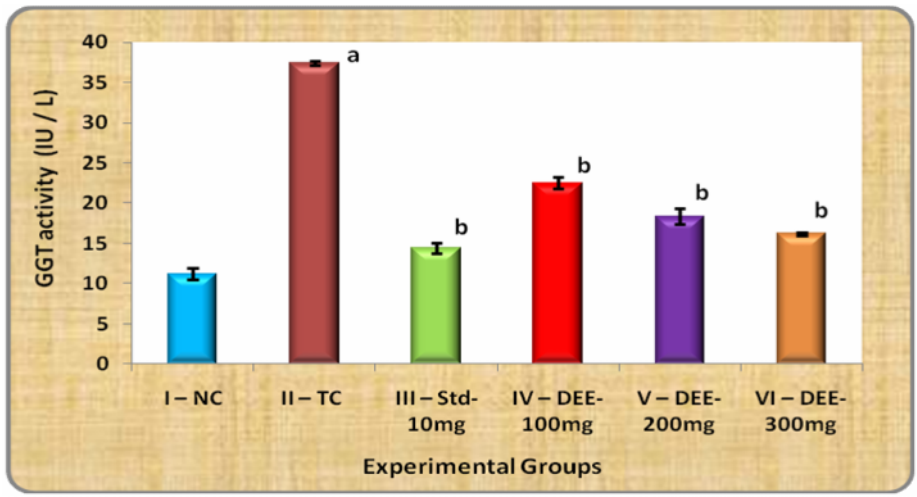
Hence, in the present study these tumour markers were analysed in serum of experimental group mice.

Gamma glutamyl transferase (GGT)

GGT is a membrane-bound enzyme that participates in the metabolism of glutathione, cleaving its gamma-glutamyl peptide bond and transferring the glutamyl moiety to acceptor molecules. The enzyme is produced in many tissues, but most GGT in serum is derived from the liver. Serum GGT has been considered as surrogate marker of non alcoholic fatty liver disease(NAFLD) leading to oxidative stress and hepatocellular damage and it was found to be significant determinant in the pathogenesis of NAFLD (Hossain *et al*., 2016).

GGT activity was analysed in the serum of experimental group mice and the results are presented in Figure 34.

Figure 34 : Gamma glutamyl transferase



Values are mean ± SD(n=6)

a – GpII Vs GpIII, GpIV, GpV, GpVI

a,b - statistically significant ($p<0.05$)

Data presented in Figure 34 indicated that t*he serum of tumor bearing animals showed* more than three fold significant ($p<0.05$) increase in GGT activity. GGT, an enzyme involved in cellular glutathione homeostasis which is often increased in tumor conditions. This membrane bound enzyme GGT is expressed highly in embryo livers and decreases rapidly to lowest levels after birth. GGT is highly re-expressed during the development of (HCC) Hepatocellular carcinoma (Pompella *et al*., 2006; Lei *et al*., 2012). In the present study this elevated level was significantly ($p<0.05$) reduced after administration with DEE of C. senna leaves and the reduction rate was comparable with that of standard drug methotrexate.

Many researchers have reported GGT activity in cancer condition. The findings of Corti *et al*., (2009) revealed that GGT activity was able to promote iron-dependent DNA oxidative damage, thus potentially representing an important mechanism in initiation/progression of neoplastic transformation. Pro-oxidant activity of GGT can promote oxidative DNA damage, thus contributing to cancer genomic instability thereby suggesting a potential role for membrane-bound gamma-glutamyltransferase (GGT) in tumor progression.

Treatment with ethanolic leaf extract of *C. fistula* significantly reversed the alteration of GGT to normal levels, possibly by maintaining the hepatocellular membrane

integrity which is an indicator of possible hepatoprotective property (Pradeep *et al*., 2010). Usha *et al*., (2007) reported that the increased activity of GGT in experimental animals after liver damage with carbon tetrachloride was near to normal value when treated with aqueous extract of the root sample of *C. occidentalis* which proved the hepatoprotective effect. Hepatoprotective effect of *C. fistula* fruit extract against liver injury evoked by bromobenzene has been reported in mice in which gamma glutamyl transpeptidase (GGT) was found to be significantly and dose-dependently decreased (Kalantari *et al*., 2011).

The present study is in accordance with the above literatures and showed that DEE of *C. senna* leaves effectively decreased the activity of GGT to near normal which might be due to a reduction in the damage caused by ascites tumour.

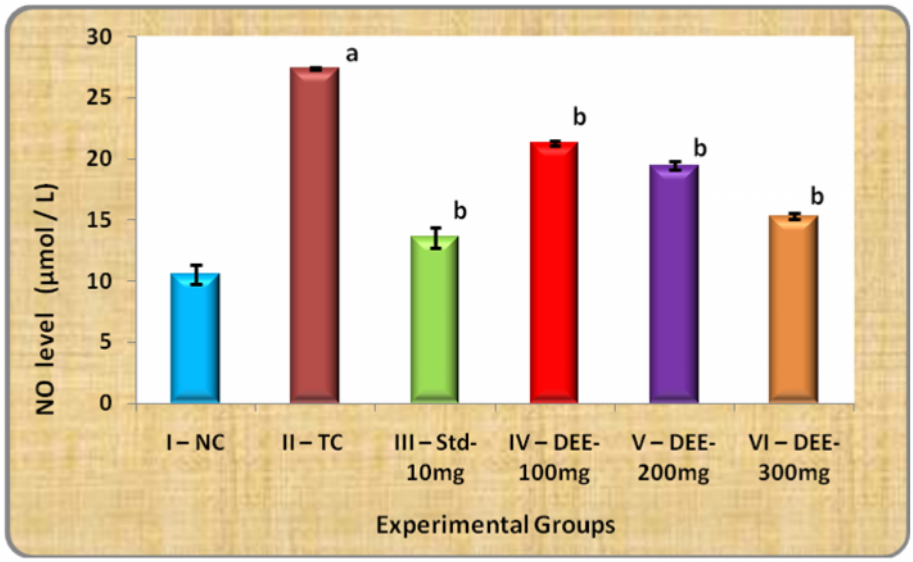
Nitric oxide (NO)

Nitric oxide is a multifunctional species that is implicated in a wide variety of physiological and pathological processes (Carla *et al*., 2000; Jenkins *et al*., 1995). It is an essential physiological signaling molecule mediating various cell functions but also induces cytotoxic and mutagenic effects when present in excess, thus pointing its dual role in biological system (Koh *et al*., 1999). Investigation of human cancers, including tumours of the central nervous system, stomach and cervix revealed high levels of expression of nitric oxide synthase(NOS) and nitric oxide in some tumours compared with normal tissue(Cobbs *et al*., 1995; Miles *et al*., 1996).

The NO level was measured in the serum of experimental group mice and the results are depicted in Figure 35.

Figure 35

Effect of DEE on NO level in serum of control and experimental animals



Values are mean ± SD(n=6)

a – GpII Vs GpIII, GpIV, GpV, GpVI

a,b - statistically significant ($p<0.05$)

The serum NO level of control animals was significantly($p<0.05$) elevated (27.36 ± 0.1µmol) after 14 days of tumour challenge whereas administration of DEE of C. senna leaves at high dosage level(300mg/kg b.wt.) significantly($p<0.05$) reduced the NO level to 15.32 ± 0.13µmol which was nearer to normal level (10.52 ± 0.39 µmol) and also comparable with that of methotrexate, standard drug (13.56±0.33) treated animals.

Angiogenesis, a crucial step in the growth and metastasis of cancers, is initiated with vasodilation that is mediated by nitric oxide(NO). To use antiangiogenesis approach successfully as an anticancer therapy, it is essential to identify the agents that can demote proangiogenic factors like NO. The results of Thejass and Kuttan, (2007) clearly demonstrated that allyl isothiocyanate (AITC) and phenyl isothiocyanate (PITC) which are two natural isothiocyanates occurring in *Brassica nigra*, *Lepidum sativum*, *Wasabia japonica*, *Raphanus sativus*, and *Synapis* spp (Boggards *et al*., 1990) inhibited tumour-specific angiogenesis at non-toxic concentrations in B16F-10 melanoma cell-induced C57BL/6 mice by downregulating NO and they also indicated the decreased tumour-directed capillary formation in treated mice. This is in accordance with our results where nitric oxide level was being downregulated by DEE that may indicates its antiangiogenic

effect and also suggested that DEE of *C. senna* leaves could be a novel anticancer therapy.

Cassigarol, a stilbene compound isolated from *Cassia* species has been reported for inhibition of tumor induced angiogenesis through *in vitro* and *in vivo* experiments which indicated antitumor and antimetastatic activities of that compound (Kimura, 2005). Treatment with methanolic extract of *Argyreia nervosa* leaves on Ehrlich ascites carcinoma induced mice significantly reduces the level of nitric oxide production in liver tissue, when compared with EAC control animals (Sharma *et al*., 2015).

The present findings are in accordance with the above literatures and showed that DEE of *C. senna* leaves effectively decreased the level of NO to normal which is probably due to its anticancer effect.

4.3.1.3. Effect of DEE of *C. senna* leaves on antioxidant status of liver tissue

After sacrifice, the liver of different experimental group animals were washed with ice cold saline, homogenized with phosphate buffer and used for the analysis of enzymic antioxidants namely superoxide dismutase(SOD), catalase(CAT) and glutathione peroxidase(GPx) activities and the levels of nonenzymic antioxidants namely Vitamin C, Vitamin E and reduced glutathione.

Determination of enzymic antioxidant activities

The primary antioxidant enzymes in mammalian tissues are superoxide dismutase SOD, CAT and GPx (Łukaszewicz-Hussain and Moniuszko-Jakoniuk, 2004).

Endogenous antioxidant system may counteract the ROS and reduce the oxidative stress with the enzymic antioxidants SOD, CAT and GPx. SOD accelerates the conversion of superoxide radical ($O_2^{\bullet -}$) to hydrogen peroxide while CAT or GPx converts H_2O_2 to H_2O. Depletion in the activity of these three antioxidant enzymes can be owed to an enhanced radical production (Kono and Fridovich, 1982).

Superoxide dismutase(SOD), a metallo protein is the most sensitive enzyme index in liver injury and one of the most important enzyme in the antioxidant defense system. It

scavenges the superoxide anion to form hydrogen peroxide and oxygen, hence diminishing the toxic effect caused by superoxide radical (Olaleye *et al*., 2010).

Catalase(CAT) is an enzymic antioxidant widely distributed in all tissues and the highest activity is found in liver. This enzyme catalyses the decomposition of H_2O_2 to water and oxygen, thus protecting the cell from oxidative damage by H_2O_2 and $OH^{\cdot}$ radical. The reduction in the activity of CAT may result in a number of deleterious effects due to accumulation of hydrogen peroxide (Curtis *et al*., 1972). The high concentration of H_2O_2 was accompanied by high activity of GPx and CAT. CAT and GPx play a significant role in the elimination of hydrogen peroxide. (Spolarics and Wu, 1997).

Glutathione peroxidase(GPx) has been suggested to be responsible for the detoxification of H_2O_2, when it is present in low concentration, whereas CAT plays its role when GPx pathway reaches saturation with substrate (De Bleser *et al*., 1999).

The results of enzymic antioxidant activities analysed in liver tissue of control and experimental animals are presented in Table 12.

Table 12 : Effect of DEE of *C. senna* leaves on activities of enzymic antioxidants in liver tissue of control and experimental animals

Groups	SOD (u/mg of protein)	CAT (u/mg of protein)	GPx (u/mg of protein)
Group I	29.57 ± 0.17	74.17 ± 0.07	13.52 ± 0.27
Group II	11.57 ± 0.06[a]	45.67 ± 0.06[a]	5.45 ± 0.32[a]
Group III	25.21 ± 0.11[b]	67.48 ± 0.07[b]	11.51 ± 0.12[b]

Group IV	**15.49 ± 0.06[b]**	**52.18 ± 0.13[b]**	**6.25 ± 0.27[b]**
Group V	**18.57 ± 0.30[b]**	**59.12 ± 0.05[b]**	**8.69 ± 0.31[b]**
Group VI	**22.58 ± 0.04[b]**	**64.62 ± 0.10[b]**	**10.69 ± 0.19[b]**
SEd CD (p<0.05)	**0.2736**	**0.1481**	**0.4571**

Values are mean ± SD(n=6)

a – GpI Vs GpII

b – GpII Vs GpIII, GpIV, GpV, GpVI

a, b - statistically significant (p<0.05)

Units:

a. Superoxide dismutase — Amount of enzyme that cause 50% reduction in NBT oxidation.

b. Catalase — Amount of enzyme required to decrease absorption at 240nm by 0.05 units/min.

c. Peroxidase — Amount of enzyme to cause change in absorption at 430nm/min

As oberved in Table 12, the activities of antioxidant enzymes such as SOD, CAT and GPx were found to be significantly(p<0.05) decreased in tumour bearing mice. A decrease in the activity of SOD, CAT and GPx in EAC bearing mice might be due to the mitochondrial injury. The decline in these enzyme activities could also be due to a reduction in their biosynthesis or their excessive utilization in trapping the free radicals generated. In our present study the administration of DEE significantly(p<0.05) increased the activities of those three antioxidant enzymes in a dose dependent manner. Moreover, flavonoids have a chemopreventive role in cancer through the induction of enzymes affecting carcinogen metabolism and inhibit various activities of tumor promoters, which are involved in the process of carcinogenesis (Hertog *et al*., 1992). The present observations which are in agreement with the above finding suggest that the high content

of flavonoids in DEE might be responsible for chemopreventive role of DEE in cancer through the induction of enzymes.

Enzymic antioxidants have been reported in earlier studies that support the present findings. Kannampalli *et al*., (2005) reported that the activities of SOD and CAT, which are mutually supportive antioxidant enzymes provide protection against reactive oxygen species and were found to be restored by phytochemicals of *C. fistula* leaves. Manonmani *et al.,* (2005) reported that the decreased activities of key antioxidant enzymes such as superoxide dismutase, catalase, glutathione peroxidase and the nonenzymic compound reduced glutathione were brought back to near normal range upon treatment with aqueous extract of *C. fistula* flowers(ACF). The results suggested that ACF has got promising antioxidative activity.

In the study reported by Shanmugasundaram *et al*., (2011), the ethanolic extract of *Senna auriculata* leaves have reversed the mitochondrial enzymatic antioxidant activity to near normal values in rats and suppressed lipid peroxidation. Veeraraghavan *et al*., (2015) reported that the decreased activities of SOD and CAT noted in hepatocellurar carcinoma induced rats were significantly increased with the administration of methanolic extract of *Garcinia mangostana* Linn pericarp and the drug had positive effects in inhibiting hepatic cancer.

The daily oral treatment of aqueous extract of *Terminalia chebula* to liver cancer bearing rats demonstrated a significant increase in enzymic antioxidants SOD, CAT and GPx (Srigopalram and Jayraaj, 2012). In the liver tumor bearing animals the decreased activities of SOD, CAT and GPx were significantly increased by the treatment with methanol extract of *Prosopis cineraria* which may extend its chemopreventive effect by modulating and augmenting antioxidant defense system. The antitumor properties of that extract may be due to the presence of flavonoids (Maideen *et al*., 2012).

The present findings are in tune with the earlier findings and suggest that DEE of *C. senna* leaves can improve the enzymic antioxidant status in the liver tissue.

Determination of nonenzymic antioxidant levels

Vitamin E, Vitamin C and GSH are well known non enzymic antioxidant defense system of cells. Among these vitamin E is a well recognized, important biological free radical scavenger in the cell membrane (Horwitt, 1976). These three nonenzymic antioxidants are inter related by recycling processes (Thirunavukkarasu *et al*., 2002).

Glutathione(GSH) is an important nonenzymic endogenous antioxidant. GSH, a non protein thiol is involved in many cellular processes including the detoxification of endogenous and exogenous compounds (Yu, 1994).

Vitamin C is synthesized in mitochondria and is transported to the other cell. Two functions of Vitamin C are: quenches O_2 in cytosol and recycles vitamin E after it captures free radicals and also it reacts not only with H_2O_2, but also with O_2, $OH^{\bullet}$ and lipid hydroperoxides (Wu *et al*., 2007b). Vitamin C is a good scavenger of most reactive oxygen species (Halliwell, 1990) thereby preventing degenerative diseases including cancer (Block and Mankes, 1989).

Vitamin E is a lipophilic antioxidant and interacts with the polyunsaturated acyl groups of lipids, stabilize membranes, and scavenge and quench various reactive oxygen species (ROS) and lipid soluble byproducts of oxidative stress. Vitamin E has been shown to provide protection against superoxides as well as H_2O_2 (Clemens and Walker, 1987).

The results of nonenzymic antioxidant levels analysed in liver tissue of control and experimental animals are presented in Table 13

Table 13 : Effect of DEE of *C. senna* leaves on levels of nonenzymic antioxidants in liver tissue of control and experimental animals

Groups	Vitamin C (mg/g)	Vitamin E (µg/g)	GSH (nm/g tissue)
Group I	1.41± 0.45	4.43 ± 0.12	7.39 ± 0.33
Group II	0.31 ± 0.03[a]	1.15 ± 0.17[a]	2.62 ± 0.30[a]
Group III	1.59 ± 0.06[b]	3.77 ± 0.29[b]	6.55 ± 0.33[b]
Group IV	0.52 ± 0.07[b ns]	2.18 ± 0.13[b]	3.47 ± 0.40[b]

Group V	**0.82 ± 0.05^{b}**	**2.85 ± 0.09^{b}**	**4.58 ± 0.37^{b}**
Group VI	**1.07 ± 0.05^{b}**	**3.36 ± 0.17^{b}**	**5.33 ± 0.31^{b}**
SEd CD ($p<0.05$)	**0.3350**	**0.3063**	**0.6099**

Values are mean ± SD(n=6)

a – GpI Vs GpII

b – GpII Vs GpIII, GpIV, GpV, GpVI

a, b - statistically significant ($p<0.05$)

b^{ns} – statistically not siginificant ($p<0.05$)

In the present study, the level of GSH, vitamin C and vitamin E was significantly ($p<0.05$) decreased (Table 13) in tumor bearing mice whereas mice fed with DEE showed a significant ($p<0.05$) improvement in those nonenzymic antioxidants which was comparable with that of standard drug. This may indicate that DEE of *C. senna* leaves was very effective in counteracting the damage caused by ascites tumour.

Many studies regarding nonenzymic antioxidants have been reported which are similar to present findings. The depleted level of GSH observed in hepatocellurar carcinoma induced rats were significantly increased on treatment with methanolic extract of pericarp of *Garcinia mangostana* thereby inhibiting hepatic cancer (Veeraraghavan *et al*., 2015). In a similar study by Maideen *et al*., (2012), the methanol extract of *Prosopis cineraria* significantly increased the levels of non enzymatic antioxidants namely GSH, Vitamin C and vitamin E in liver tumor bearing animals thereby effectively suppressing liver tumor. Thus our study is in agreement with the above research findings cited.

Administration of ethanolic leaf extract of *C. fistula* significantly protected the liver against damage caused by a fall in the level of vitamin C, vitamin E and GSH induced by ethanol + DEN treatment enhancing the glutathione production by providing more substrate for reactive intermediates that promote detoxification mechanisms. This

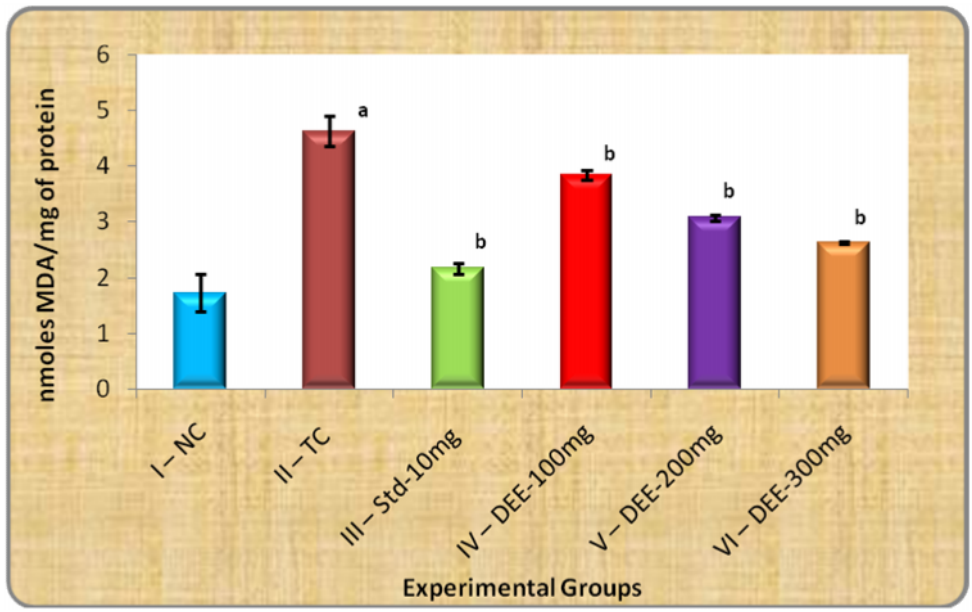
also might be the reason for the restoration of other antioxidant enzymes such as SOD, CAT and GPx by ethanolic leaf extract of *C. fistula* (Pradeep *et al*., 2010).

The remarkable($p<0.05$) decrease in the activities of enzymic antioxidants namely SOD, CAT and GPx and in the levels of non-enzymic antioxidants such as GSH, vitamin C and vitamin E in tumor control indicates the complete disruption of the antioxidant defense mechanism of the liver. This condition was reverted back to normal with the administration of DEE. This may indicate that DEE of *C. senna* leaves was very effective in counteracting the damage caused by ascites tumour.

Lipid peroxidation(LPO)

Lipid peroxidation, initiated in the presence of hydroxyl radicals leads to the formation of malondialdehyde (MDA) directly which further results in oxidative stress (Surapaneni and Priya, 2008). Membrane lipids are easily susceptible to deleterious actions of reactive oxygen species. Measurement of lipid peroxidation is considered to be a convenient method to monitor oxidative membrane damage (Surapaneni and Priya, 2010)

Figure 36 : Effect of DEE of *C. senna* leaves on extent of lipid peroxidation in liver tissue of control and experimental animals



Values are mean ± SD(n=6)

a – GpII Vs GpIII, GpIV, GpV, GpVI

a, b - statistically significant ($p<0.05$)

Figure 36 represents the extent of lipid peroxidation in the form of malondialdehyde (MDA). The tumour bearing mice showed significantly($p<0.05$) elevated level of MDA than that of normal control. The lipid peroxidation(Malondialdehyde level) was found to be higher in cancerous tissue than normal tissue. The activities of some intracellular antioxidant enzymes such as SOD, CAT and GPx have been reported to be decreased with the increase in lipid peroxidation and this fact is concomitant with the results of the present study. The present findings indicate that the level of MDA was found to be significantly($p<0.05$) decreased with the administration of DEE in a dose dependent manner.

Many researchers have reported the effect of plant extracts in reducing lipid peroxidation. Treatment with ethanolic leaf extract(ELE) of *C. fistula* completely prevented the 3 fold increase in LPO induced by ethanol + DEN in the liver tissue of rats (Pradeep *et al*., 2010). Ilavarasan *et al.,* (2005) reported that antioxidant activities of the aqueous (CFA) and methanolic extracts (CFM) of the *C. fistula* bark assayed in Wistar albino rats showed significant radical scavenging by inhibiting lipid peroxidation initiated

by CCl_4 and $FeSO_4$ in rat liver and kidney homogenates. Both extracts showed dose-dependent protective effect against lipid peroxidation and free radical generation in liver and kidney homogenates.

Vasudevan *et al*., (2008) investigated the chemopreventive efficacy of *C. fistula* bark extracts in 7, 12-dimethyl benz(a)anthracene (DMBA) induced hamster buccal pouch carcinogenesis and found that oral administration of *C. fistula* bark extract completely prevented the formation of oral squamous cell carcinoma and also restored the status of lipid peroxidation by-products, antioxidants and detoxification enzymes in DMBA induced animals which is probably due to the presence of one or more potent anticarcinogenic principles and their synergistic effect.

In a similar study by Srigopalram and Jayraaj, (2012), the administration of aqueous extract of *Terminalia chebula* in diethylnitrosamine (DEN) induced liver cancer bearing rats significantly decreased the lipid peroxidation which may be due to the free radical scavenging activity of that extract. In the study by Maideen *et al*., (2012), an increase in MDA was presumably associated with increased free radicals, confirming the fact that these free radicals inhibited the activities of SOD, CAT and GPx. The present findings are in accordance with the above reports.

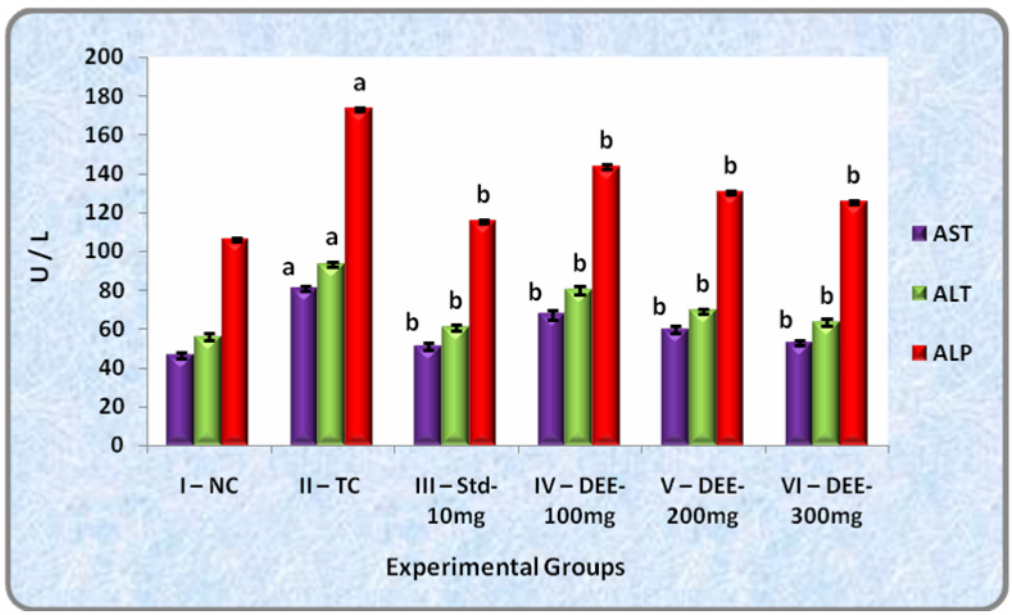
Thus, the present results with the support of the above cited literatures showed that DEE of *C. senna* leaves decreased the extent of lipid peroxidation which shows that the extract can protect the normal cells from oxidative injuries.

4.3.1.5. Liver marker enzymes in serum

Abu Sienna *et al*., (2003) suggested that, the consumption of free amino acid for building the proteins of rapidly dividing tumor cells might result in the disturbance of the enzyme activity in the liver. Serum AST, ALT and ALP are the most sensitive markers employed in the diagnosis of hepatic damage (Sallie *et al*., 1991).

Liver marker enzymes such as aspatate tranaminase (AST), alanine tranaminase (ALT) and phosphatase(ALP) were analysed in serum of experimental group mice and the results are shown in Figure 37.

Figure 37 : Effect of DEE on AST, ALT and ALP activities in serum of control and experimental animals



Values are mean ± SD(n=6)

a – GpII Vs GpIII, GpIV, GpV, GpVI

a, b - statistically significant ($p<0.05$)

From the Figure 37, it is evident that the activities of liver marker enzymes such as AST, ALT and ALP in serum were significantly($p<0.05$) increased in EAC group as compared to those of normal group. The increased activities of AST, ALT and ALP in serum are indicative of cellular leakage and loss of functional integrity of liver cell membrane (Drotman and Lowhorn, 1978).

Many researchers have reported elevated activities of liver marker enzymes in cancer condition. Tissue damage is the sensitive feature in the cancerous conditions so any deterioration or destruction of the membrane can lead to the leakage of the enzymes from the tissues. Hence elevation of these liver specific enzymes observed in breast cancer condition may be due to the progression of tumor growth (El-Beshbishy, 2005). Serum AST and ALT levels increased as a result of metabolic changes in the liver such as liver cancer, cirrhosis and hepatitis (Chalasani *et al*., 2004). As a marker for liver metastases in breast cancer patients and also as a marker for hepatotoxicity, AST and ALT were found to be increased (Lox *et al*., 1998). This is in agreement with our present

findings where the elevated activities of liver specific enzymes might be due to the progression of tumor growth and metastasis of ascites tumour to liver.

In the present findings, with the administration of DEE to the EAC induced mice, the activities of the above enzymes were significantly($p<0.05$) reduced as compared to EAC group and were also found to be on par with methotrexate(standard drug) in 14 days treatment period. The significant ($p<0.05$) recovery of the elevated hapato-specific enzyme activities confirmed the preventive role of the extract on liver damage (Kannampalli *et al*., 2007).

In a previous study reported by Shanmugasundaram *et al*., (2011), it was found that the ethanol extract of *S. auriculata* leaf regulated the activity of AST, ALP and and ALP in liver of rats intoxicated with alloxan. The substantially elevated serum enzymatic levels of AST, ALT and ALP were significantly restored towards normalization by ethanolic extract of *C. sophera* leaves which was clear indication of the improvement in the functional status of the liver cells (Mondal *et al*., 2012).

A significant 2 fold increase in AST, 3 fold increase in ALP and 4 fold increase in ALT observed in serum of experimental rats with hepatocellular damage were reversed after treatment with ethanolic leaf extract of *C. Fistula* (Pradeep *et al*., 2010). Bhakta *et al*., (2001) showed that the n-heptane extract of *C. fistula* leaves lowered the serum levels of AST, ALT and ALP which was indicative of its hepato protective activity.

The reports given by Asirvatham and Christina, (2012) demonstrated that altered levels of liver enzymes namely ALT, AST and ALP of DAL control group were restored as that of the normal group on treatment with ethanol and aqueous extracts of *Drosera indica* and the cancer induced metabolic changes were also normalized. The daily oral treatment of aqueous extract of *Terminalia chebula* to liver cancer bearing rats demonstrated a significant decline in AST, ALT, ALP and GGT and those results have confirmed the efficacy of that extract as an effective chemotherapeutic agent (Srigopalram and Jayraaj, 2012). Vennila *et al*., (2010) stated that chemopreventive effect of taxol derived from an endophytic fungus, significantly reduced the elevated activities of AST and ALT in cancer bearing rats.

The present study has been supported by the above findings cited. In line with these supportive findings, DEE of *C. senna* leaves was found to be effective in normalization of activities of liver marker enzymes.

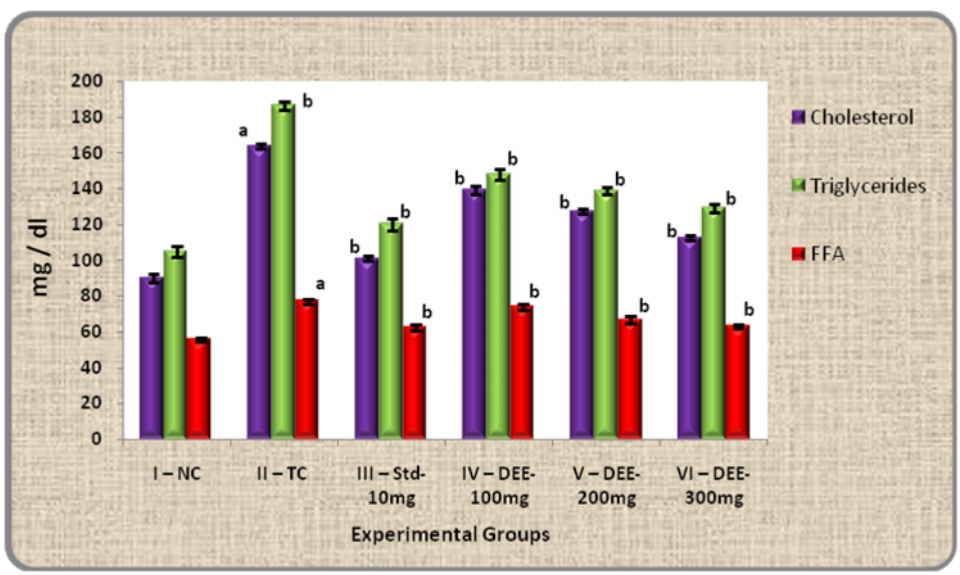
4.3.1.6. Effect of DEE on lipid content of tumour induced mice

Fundamentally the development of a malignancy requires the uncontrolled and excessive proliferation of cells (Gupta *et al*., 2011). These rapidly forming cells need many basic components well above the normal limits which are used in physiological process. One such component which forms major cell membrane components essential for various biological functions including cell division and growth of normal and malignant tissues is lipids. Lipid stores are diminished due to increased use of lipids by this rapidly dividing cells (Neville and Day, 2002; Nayak *et al*., 2010).

Lipid content in serum

Lipids such as total cholesterol(TC), triglycerides(TGL) and free fatty acids(FFA) were analyzed in the serum of experimental group mice and the results are shown in Figure 38.

Figure 38. Lipid content in serum



Values are mean ± SD(n=6)
a – GpII Vs GpIII, GpIV, GpV, GpVI
a, b - statistically significant ($p<0.05$)

As observed in Figure 38, the levels of TC, TGL and FFA in serum were significantly($p<0.05$) increased in tumour induced mice as compared to those of normal group. Our results are supported by a number of research findings, where hyperlipidemia was reported in many types of cancer condition. Increased proliferation and progression of carcinogenesis may be correlated with increased total cholesterol and triglycerides. Cholesterol content is responsible for the fluidity of lipid membrane. Carcinoma increases level of cholesterol that may contribute to the further progression of cancer (Damen *et al*., 1984). Increased level of total cholesterol and triglycerides in untreated cancer bearing animals was recorded due to reduced activities of fat-splitting enzymes namely lecithin: Cholesterol acetyltransferase (LCAT) and Lipoprotein lipase(LL) (Pattanayak *et al*., 2014). LCAT and LL control the clearing of free cholesterol and triglycerides from the plasma by esterification and cleavage respectively (Rouser *et al*., 1970).

Serum total cholesterol was increased in ascites hepatoma cell line AH109Abearing rats (Kawasaki *et al*., 2004). Another study showed that in Lewis lung carcinoma implanted mice, the serum total cholesterol and free cholesterol concentrations were increased (Kawasaki, 2006). In a previous study of tumor-bearing rats, an increase in the plasma triglyceride concentration and a suppression of the activities of tissue lipoprotein lipase were seen with increased tumor burden and tumor removal completely reversed these changes (Noguchi *et al*., 1991).

Triglyceride pool was increased by 50 % in tumor bearing rat compared to controls (Nagarajan and Sankaran, 1973). The accumulation of triglycerides in fibrosarcoma condition suggests that there should be some diversion of fatty acids from the oxidative sequences to the esterfication pathway (Jayamathi, 2010).

It is reported that plasma FFA are increased in tumor bearing animals and increased plasma FFA is attributed to the hypermetabolic state in cancers (Jiang 2006). Excessive rates of lipid peroxidation might result in hyperlipidemia, found in many cancer patients (Bast *et al*., 1991) which is in accordance with our results.

In the present study the levels of serum TC, TGL and FFA were significantly($p<0.05$) lowered in Group VI animals which received high dose of

DEE(300mg/kg body wt.) than EAC control *a*nd it was also found to be on par with methotrexate(standard drug) in 14 days treatment period. The effect of DEE in minimizing the elevated free fatty acid level to normal can be attributed to a reduction in the hypermetabolic rate which is a characteristic of cancer cells. The recovery of normalcy level of all the above lipids could be due to the hypolipidemic effect of DEE via anticancer mechanism.

The present results are also supported by earlier findings, where plant extracts have exhibited hypolipidemic effect through antitumor activity. Naitik *et al*., (2012) reported the effect of antitumor activity of ethanolic extract of leaves of *Terminalia catappa* on lipid lowering activity in transplanted fibrosarcoma(malignant tumor) in Wistar albino rats in which the elevated levels of serum total cholesterol and triglycerides of tumor bearing rats were restored to normal levels.

Ethanol and aqueous extracts of *Drosera indica* were effective in inhibiting the tumor growth of DLA-bearing mice by reducing the elevated serum triglycerides to normal level and it was found that ethanol extract was comparatively better than aqueous extract (Asirvatham and Christina, 2012).

A significant raise in the level of plasma lipids such as TC, TGL and FFA in mammary carcinoma induced rats was potentially reverted back to near normal by bioflavonoid, Naringenin indicating anticancer potential of the same (Kumar *et al*., 2016). Pari and Latha, (2002) reported that the elevated serum level of TC, TGL and PL were reduced to closer to normal level upon treatment with the aqueous extract of *C. auriculata* in diabetic rats.

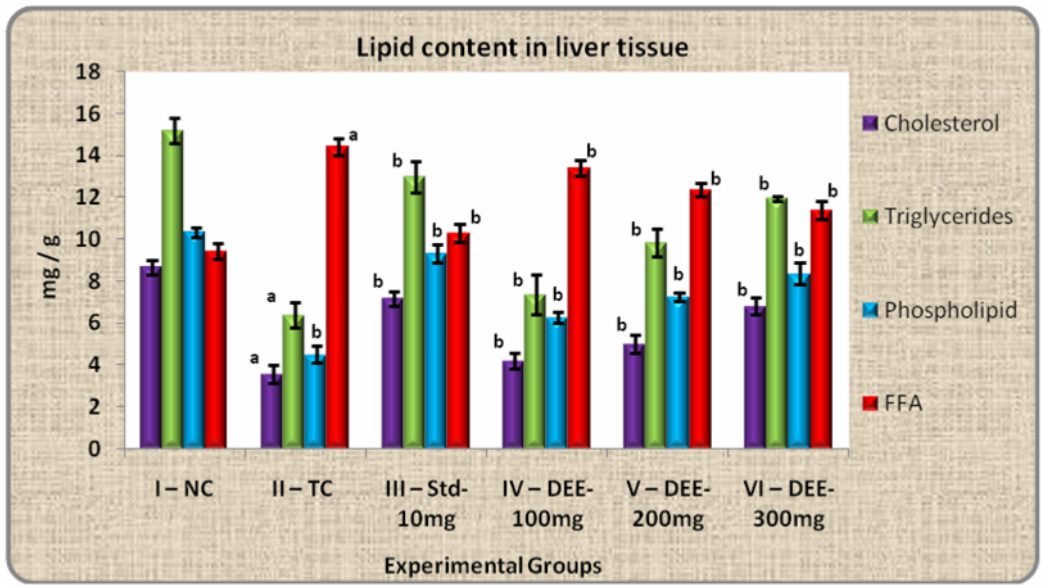
The present results are concordant with the reports obtained in the above cited literatures and DEE of *C. senna* leaves was effectively regulate the plasma lipids to near normal which may be probably due to hypolipidemic effect through antitumor activity.

Lipid content in liver tissue

Liver is the major organ involved in lipid metabolism and distribution. The alteration in the level of lipids in plasma as well as liver during carcinogenesis and tumour progression has been observed previously (Dessi *et al*., 1992). The changes that occur during cancer progression in liver mediated metabolism leads to an alteration in the plasma lipid level.

Lipids such as total cholesterol(TC), triglycerides(TGL), phopholipid(PL) and free fatty acids(FFA) were analyzed in the liver tissue of experimental group mice and the results are presented in Figure 39.

Figure 39 : Effect of DEE of *C. senna* leaves on lipid content in liver tissue of control and experimental mice



Values are mean ± SD(n=6)

a – GpII Vs GpIII, GpIV, GpV, GpVI

a - statistically significant ($p<0.05$)

Figure 39 represents the level of lipids in liver tissue. In contrast to elevated level of serum lipids, a significant ($p<0.05$) depletion of cholesterol, triglyceride and phospholipids in liver tissue of the EAC induced animals was observed as 4.3mg/g, 7.12mg/g and 4.9mg/ g of tissue respectively indicating an increased lipolytic activity in tumor–bearing animals. The present findings have been supported by earlier findings in which lipolysis and hyperlipidemia were found to be correlated in cancer condition.

In the tumor–bearing animals, increased lipolytic activity was reported, associated with hyperlipidemia (Devereux and Hollander, 1987). Hyperlipidemia could reflect the increased activity of total lipase(TL-enzyme catalyzing lipolysis) during progressive carcinogenesis (Pattanayak *et al*., 2014). The depletion of fat stores associated with tumor

growth indicates high energy requirement and hence an increased lipolytic activity(Ekman *et al*., 1982). Lipid stores are diminished due to increased use of lipids by the rapidly dividing cells (cancer cells) (Nayak *et al*., 2010). DMBA-induced mammary carcinoma bearing animals bear a significant increase in total lipase activity in liver samples (Kumar *et al*., 2016). The decreased concentrations of phospholipids indicate accelerated phospholipid degradation by phospholipase enzyme that hydrolyzes membrane phospholipids into fatty acids and lysophopholipids (Basavarajappa *et al*., 1998).

In the present findings, as observed in serum, in *liver tissue* also there was an increase in the FFA(14.4mg/g of tissue) in tumour induced mice as compared to that of normal group(9.41mg/g of tissue). Free radicals and reactive oxygen species generated by carcinogens are responsible for high rate of oxidation / per oxidation of polyunsaturated fatty acids. It results in greater utilization of lipids including total cholesterol and triglycerides for new membrane biogenesis (McCarthy *et al*., 1980). Increased lipogenesis in cancer-bearing animals was mediated by excess production of lipid peroxides (Pattanayak *et al*., 2014).

Significant increase in the concentration of free fatty acids in liver tissue might be due to free radical mediated membrane phospholipid breakdown induced by the carcinogen in addition to increased activity of phospholipase. Free radicals oxidize polyunsaturated fatty acids directly and a single hydroxyl molecule can result in peroxidation of many polyunsaturated fatty acids because of a series of cyclic reactions leading to formation of short chain alkanes and lipid acid aldehydes totally destroying lipid structures.

In the present study, after the treatment with DEE of C. senna leaves at various concentrations(100-300mg/kg body wt), the depleted level of TC, TGL and PL and the elevated level of FFA were significantly($p<0.05$) reverted back to near normal when compared to tumour bearing animals. The animals which received the higher dose(300mg/kg body wt) of DEE exhibited the lipid levels similar to those of standard drug(methotrexate) treated ones.

Similar observations were reported in many research findings. The reversal of depleted levels of total cholesterol and triglycerides observed in the liver of tumor-bearing rats into normal levels showed antitumor activity of ethanolic extract of *Terminalia catappa* and this normalization of lipid level may be due to an enhanced lipogenesis or due to decrease in lipolysis, or both (Naitik *et al*., 2012) which is in accordance with our results. A significant raise in the level of FFA in liver tissue of carcinoma induced rats was potentially reverted back to near normal on treatment with a bioflavonoid, Naringenin thereby exhibiting its anticancer potential of against carcinogenesis (Kumar *et al*., 2016). Aqueous extract of *C. auriculata* flowers has been reported for reducing elevated FFA level in diabetic rats into near normal (Pari and Latha, 2002).

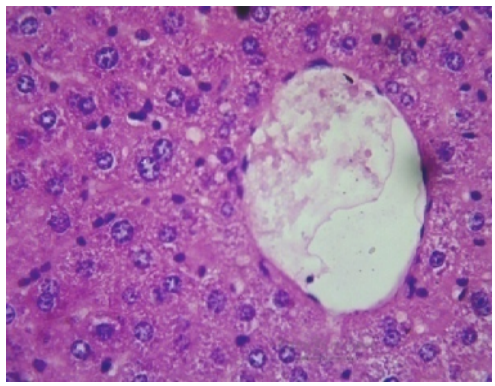
The present observations are supported by the above findings and indicate that DEE of *C. senna* leaves was found to modulate the lipid content of liver tissue to near normal which could be attributed to the antitumor activity.

From the results obtained by assessment of lipid content in serum and liver tissue, it is clearly evident that the treatment of DEE of *C. senna* leaves significantly($p<0.05$) attenuated those alterations of lipid levels in serum as well as in liver tissue, which might be indicating the efficiency of DEE to regulate lipid metabolism via its anticancer effect.

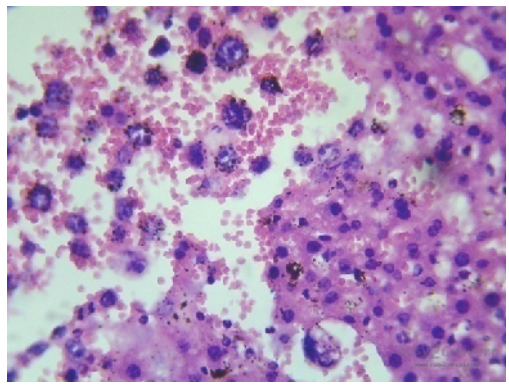
4.3.1.7. Effect of DEE on histological status

A small portion of liver and kidney of the control and experimental mice *was* taken and fixed in to 5% formaldehyde. *A*fter microtomy, the sections of both *liver and kidney* were stained with hematoxylin-eosin and were observed for histological status. The microscopic observations of l*iver* are illustrated in Plate 7.

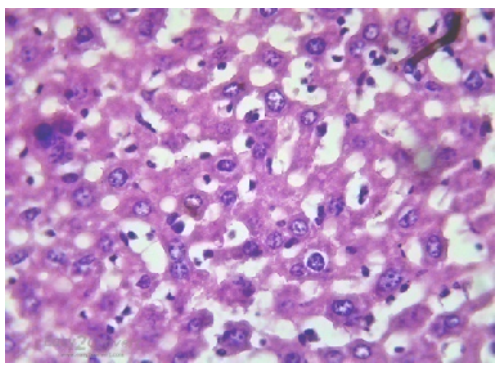
Plate 7 : Histopathological liver sections of mice



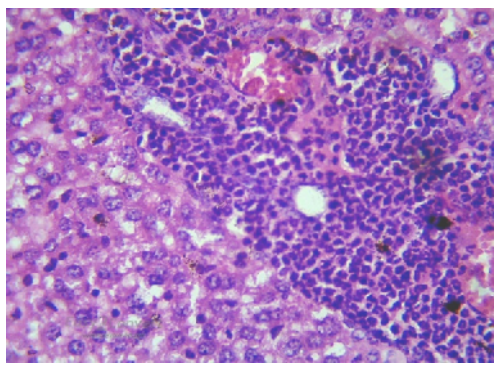
a) I-Normal control



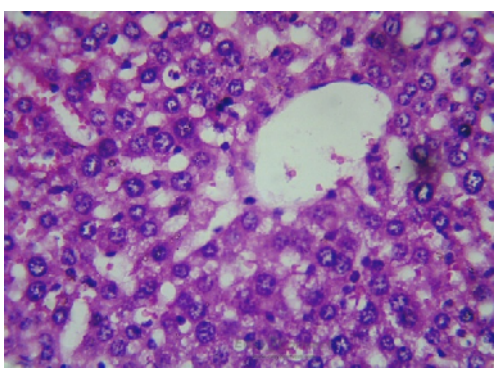
b. II-Tumor control



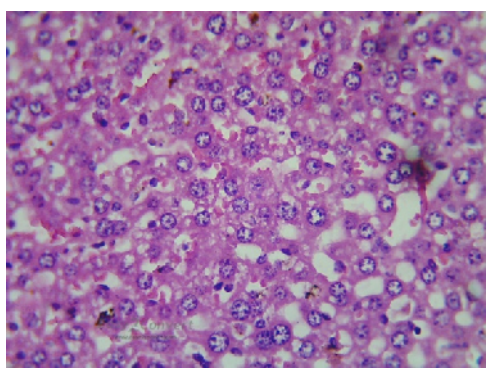
c) III-Std drug 10mg/kg b.wt



d) IV-100mg/kg b.wt.



e) IV-200mg/kg b.wt.



f) IV-300mg/kg b.wt.

The histopathological observations of liver sections of experimental mice are shown in Plate 7. Group I(normal control) showed normal lobular architecture and the portal tracts as shown in Plate 7a. Sinusoids and central veins were normal.

Histopathological view of liver sections of EAC induced mice(Plate 7b) showed increased size of hepatocytes, enlarged hyper chromatic nuclei, cellular infiltration(inflammation), dilated sinusoidal and central vein congestion suggesting severe hepatotoxicity. A marked changes in the lobular architecture is due to severe necrosis and inflammation.

Liver sections of experimental animals treated with standard drug(methotrexate) (Plate 7c) showed less cellular infiltration, reduced inflammation and almost normal lobular architecture.

Histological observations of Group IV, V and VI treated with DEE at increasing concentrations of 100, 200, 300 mg/kg body wt. respectively showed gradual reduction in cellular infiltration, hyper chromatic nuclei and inflammation. Dysplastic and premalignant state of hepatocytes were not observed in these groups. A pattern of recovery similar to that of standard drug(methotrexate) and almost normal hepatocellular architecture were observed in this DEE treated groups. This may be due to the diminution of oxidative stress and prevention of necrosis. These histopathological observations further authenticate our results of the biochemical findings of serum and liver tissue of mice.

Similar observations have been reported in earlier findings. Kumar *et al*., (2010) reported that histopathological examination showed significant changes like hyperplastic prostatic acini and malignant proliferation of ductal epithelial cells in the prostate and seminal vesicle of carcinogen induced rats, while after treatment with ethanolic extract of *C. senna*, normal and dilated ducts and acini with regular epithelial lining were observed in prostate and partially hyperplastic and partially flattened epithelium in seminal vesicle were observed.

Pradeep *et al*., (2010) showed that the histopathological patterns of liver injury observed in rats showed improved hepatocellular architecture and signs of recovery after

treatment with ethanolic leaf extract of *C. fistula* indicating the protective effect. Jafri *et al*., (1999) studied hepatoprotective activity of aqueous–ethanolic (50% v/v) extract of leaves of *C. occidentalis* and observed that the plant produced significant hepatoprotection on rat liver damage induced by paracetamol and ethyl alcohol by monitoring histopathological alterations.

Histological observations of the liver tissue of CCl_4 treated animals showed fatty degeneration with severe necrosis of the parenchyma cells in the central lobular region of the liver whereas the animals treated with ethanolic extract of *C. sophera* restored the altered histopathological changes that reavealed the effective hepatoprotective activity (Mondal *et al*., 2012). All these observations are in accordance with our results.

The present findings in agreement with the above literatures cited for histopathology of liver tissues support that DEE of *C. senna* leaves showed remarkable improvement in the hepatocellular architecture which may be due to the diminution of oxidative stress and prevention of necrosis that is induced by ascites tumour.

4.3.2.1. GC-MS

GC-MS analysis is used to identify and analyse the components present in the extracts and also provides insight into further development of research (Ze-kun and Chen-Haixia, 2012). The GC-MS analysis of DEE(Direct Ethanolic Extract) of *C. senna* leaves was carried out to identify the nature of the components present.

The Gas chromatogram of DEE is shown in Figure 35, in which a total of 8 major peaks were obtained at retention times 6.53, 15.82, 17.90, 22.69, 23.35, 26.03, 26.02 and 35.81 minutes. The mass spectra of easch of major peaks of chromatogram is represented(Figure 40 to 49) with individual retention times(RT).

Figure 40 : Gas chromatogram of DEE of *C. senna* leaves

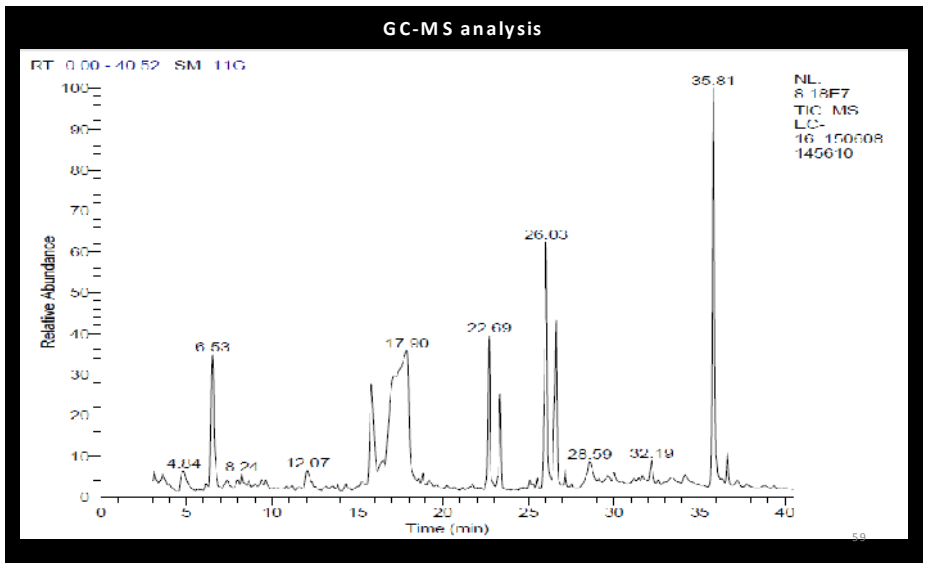
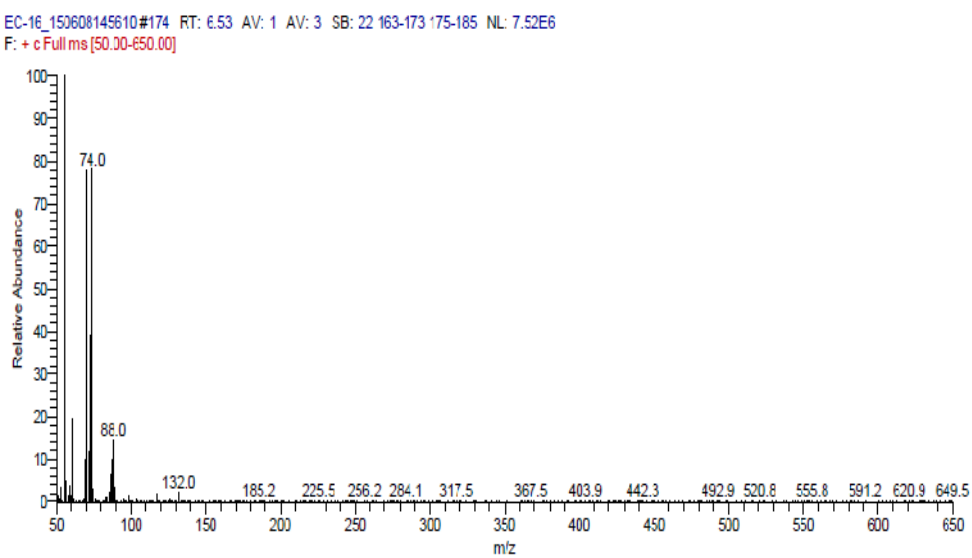
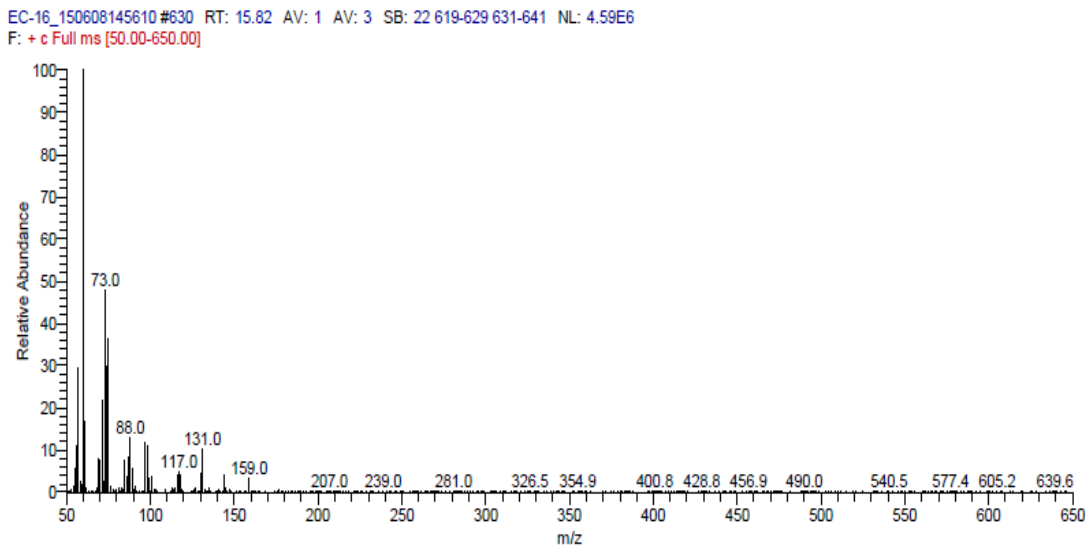


Figure 41 : Peak fragmentation of GC-MS spectrum at RT 6.53



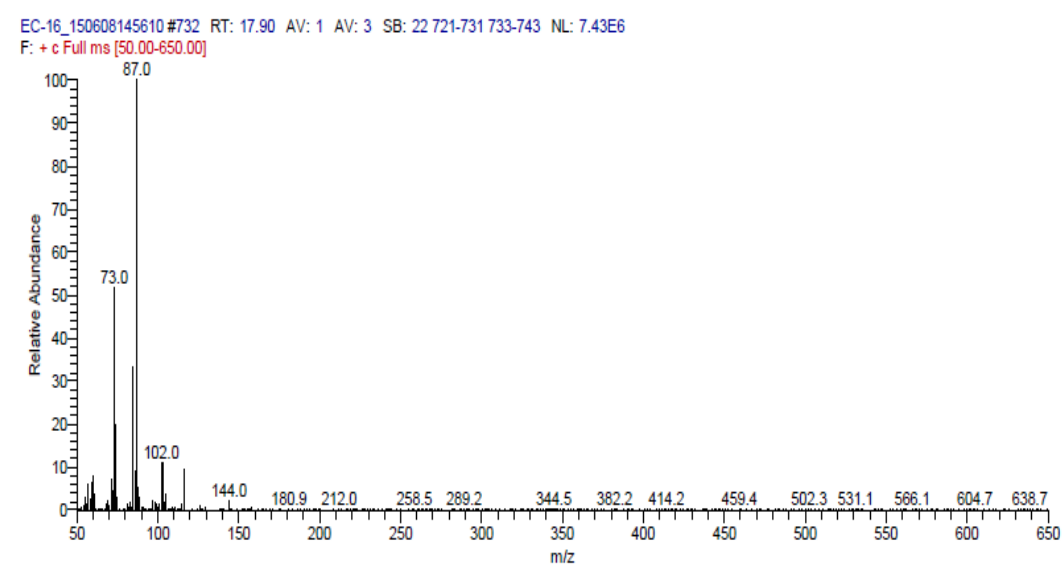
The mass spectrum(Figure 41) of the peak at retention time 6.53 showed a characteristic M-14 peak at m/e 74 and indicated the presence of $–CH_2$ group. Also M-44 peak at m/e 88 indicated the presence of carboxyl group and M-27 peak observed at m/e 256.2 is due to the loss of CH_2=CH group. M-53 peak at m/e is due to the presence of fatty acid esters.

Figure 42 : Peak fragmentation of GC-MS spectrum at RT 15.82



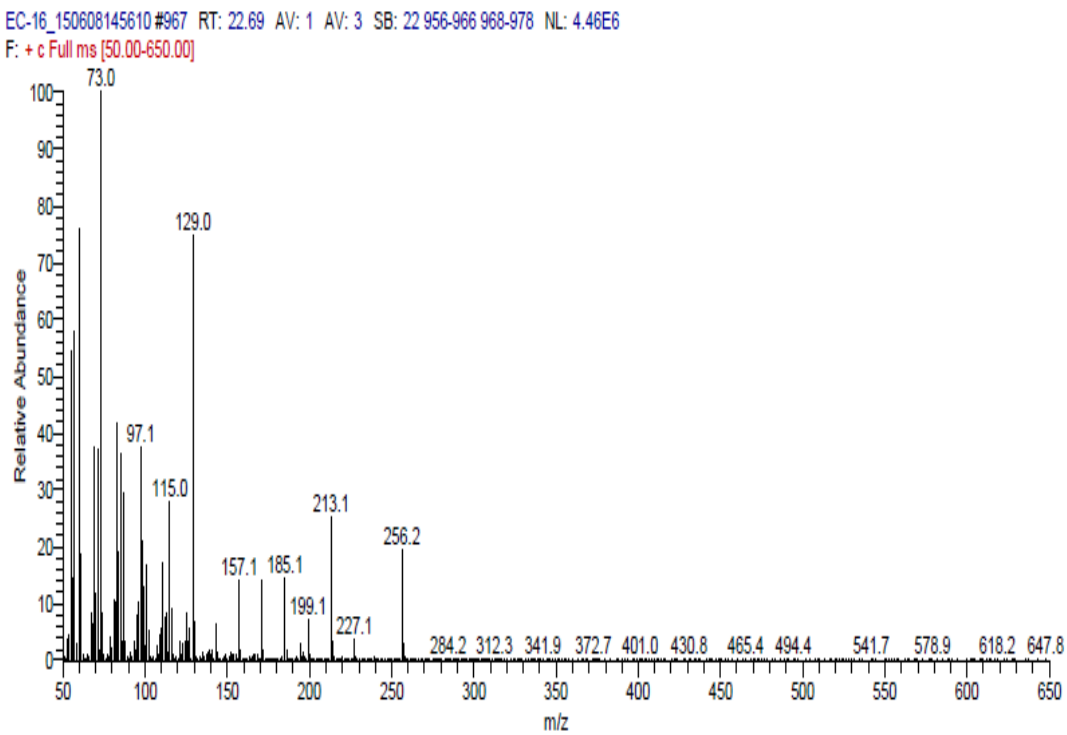
The mass spectrum(Figure 42) of the peak at retention time 15.82 showed characteristic two M-28 peaks at m/e 131 and at m/e 326.5 indicating the presence of –CO and CH_2=CH_2 groups. The M-29, M-14 and M-15 peaks were observed at m/e 88, m/e 117 and m/e 73 respectively. This may be due to the loss of –CHO, –CH_2 and –CH_3 groups respectively. These functional groups may indicate the possible presence of flavonoid.

Figure 43 : Peak fragmentation of GC-MS spectrum at RT 17.90



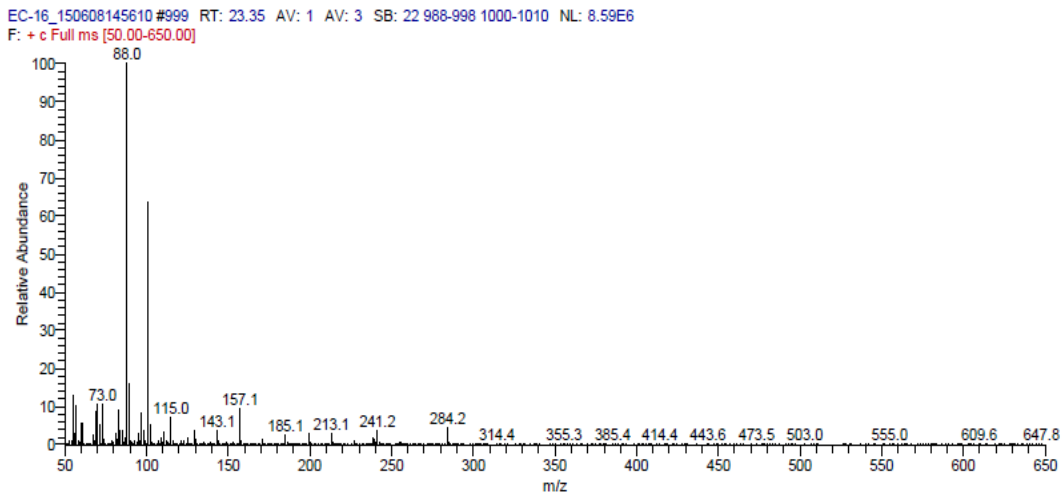
The mass spectrum(Figure 43) of the peak at retention time 17.90 showed characteristic fragmentation peaks M-55, M-42, M-31 and M-30 at m/e 289.2, m/e 102, m/e 344.5, m/e180.9 and at m/e 258.5. This may be due to the loss of CH_2=CH_2, CH_3=CHC=O, C_3H_6, –OCH_3 and –CH_2NH_2 groups. The M-14 and M-15 peaks were observed at m/e 73 and m/e 87 respectively indicating the presence of –CH_2 and –CH_3 groups.

Figure 44 : Peak fragmentation of GC-MS spectrum at RT 22.69



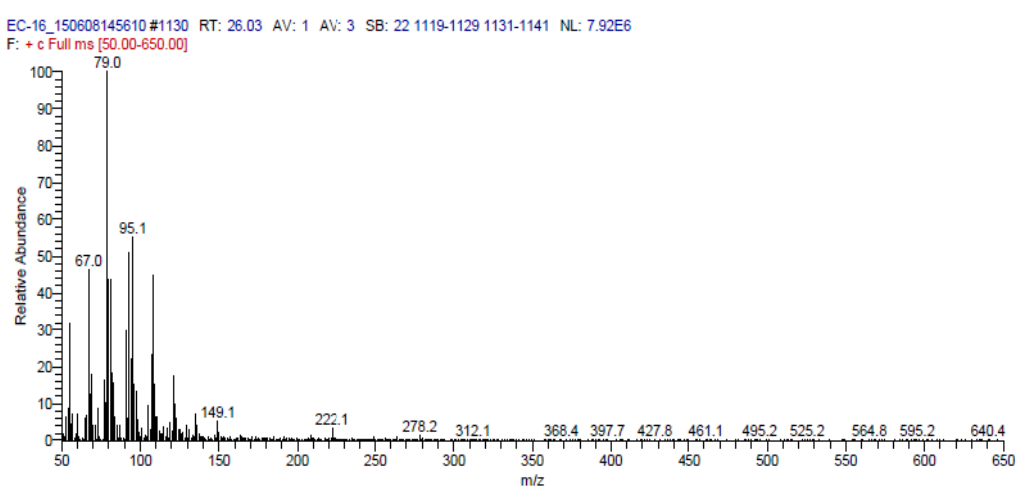
The mass spectrum(Figure 44) of the peak at retention time 22.69 showed characteristic five M-28 peaks at m/e 372.7, 284.2, 256.2, 157.1 and at m/e 129 and four M-14 peaks at m/e 115, 185.1, 199.1 and 213.1. This may be due to the loss of –CO, CH_2=CH_2 and –CH_2 groups respectively. Two M-29 peaks at m/e 227.1 and at m/e 312.3 were observed due to the loss of –C_2H_5 and –CHO groups and also M-17 peak was observed at 97.1 indicating the presence of OH group. These functional groups may indicate the possible presence of flavonoid.

Figure 45 : Peak fragmentation of GC-MS spectrum at RT 23.35



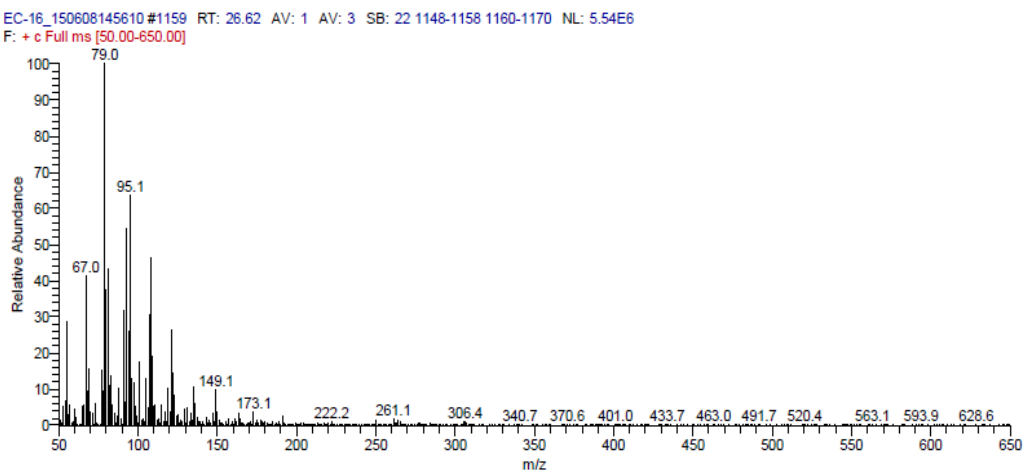
The mass spectrum(Figure 45) of the peak at retention time 23.35 showed characteristic four M-28 peaks at m/e 213.1, 185.1, 157.1 and at m/e 115. This may be due to the loss of –CO, CH_2=CH_2 and CH=NH groups. Two M-14 peaks at m/e 143.1 and 101 and one M-15 peak at m/e 73 may be due to loss of –CH_2 and –CH_3 groups respectively. M-40 and M-43 peaks at m/e 314.4 and at m/e 241.2 were observed that indicating the presence of –CH_2C=N and OH groups respectively. And also two M-30 peaks observed at m/e 355.3 and at m/e 284.2 indicating the presence of –CH_2NH_2 groups The existence of the above functional groups may indicate the possible presence of alkaloid.

Figure 46 : Peak fragmentation of GC-MS spectrum at RT 26.03



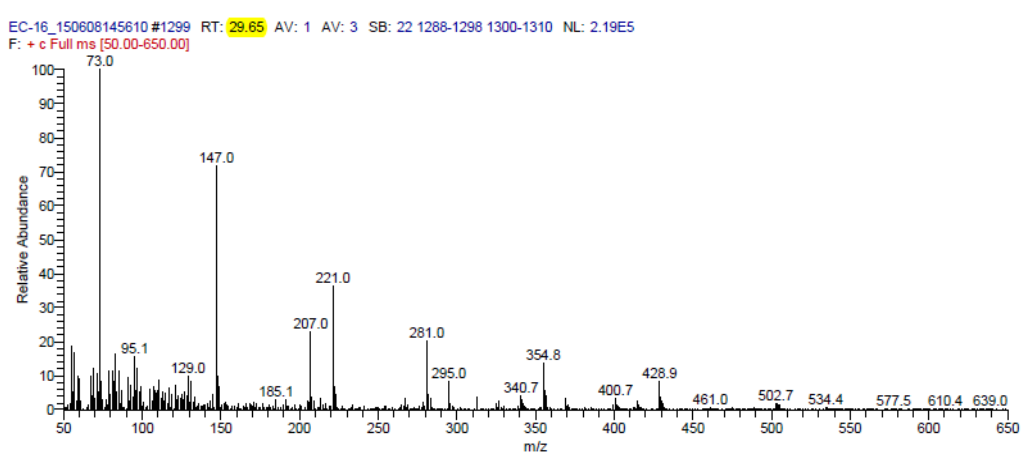
The mass spectrum(Figure 46) of the peak at retention time 26.03 showed characteristic fragmentation peaks M-28, M-29 and M-73 at m/e 121, at m/e 368.4 and at m/e 149.1 which may be due to the loss of –CO, CH_3–CH_2, CHO and $(CH_3)_2COH$ groups respectively. Two M-56 peaks at m/e 312.1 and at m/e 222.1 were observed indicating the presence of C_4H_8 group. These functional groups may indicate the possible presence of flavonoid.

Figure 47 : Peak fragmentation of GC-MS spectrum at RT 26.62



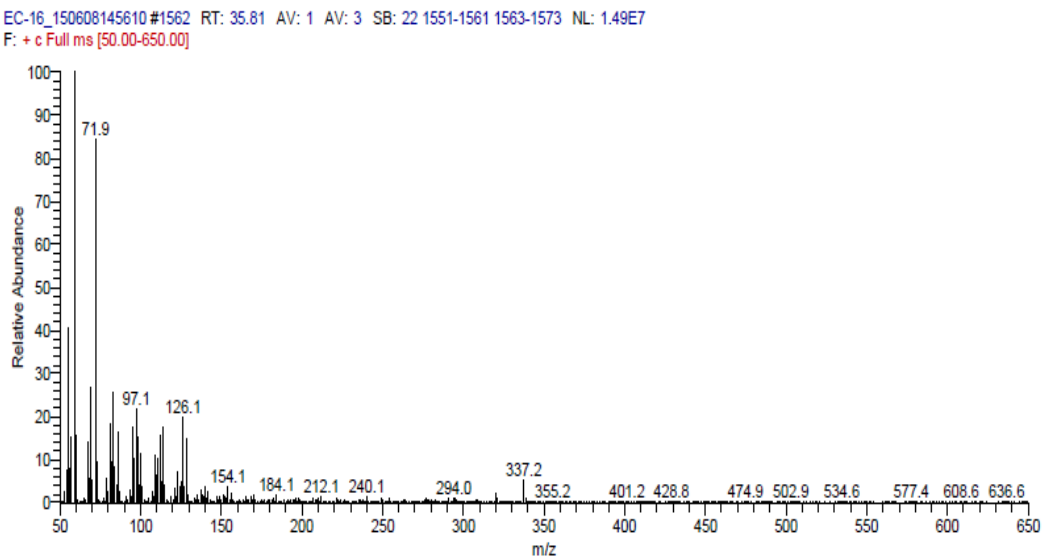
The mass spectrum(Figure 47) of the peak at retention time 26.62 showed characteristic fragmentation peaks M-14, M-27, M-29 and M-30 at m/e 108, 122, 340.7 and at m/e 370.6 indicating the presence of CH_2, C_2H_3, –CO, CH_3–CH_2, CHO and –OCH_3 groups respectively.

Figure 48 : Peak fragmentation of GC-MS spectrum at RT 29.65



The mass spectrum(Figure 48) of the peak at retention time 29.65 showed three characteristic M-14 peaks at m/e 207, 281 and at 380.7 and also two M-45 peaks at m/e 295 and at m/e 354.8. This may be due to the loss of CH_2 and CH_3CHOH groups respectively. The M-18 and M-60 peaks at m/e 129 and at m/e 221 indicating the presence of H_2O and CH_2COOH groups respectively. These functional groups may indicate the possible presence of fatty acid esters.

Figure 49 : Peak fragmentation of GC-MS spectrum at RT 35.81



The mass spectrum(Figure 49) of the peak at retention time 35.81 showed three characteristic M-28 peaks at m/e 126.1, 184.1 and at m/e 212.1 which may be due to the loss of –CO, CH_2=CH_2 groups. The M-43, M-30, M-29 and M-18 peaks were observed at m/e 294, 154.1, 97.1 and at m/e 337.2 indicating the presence of CH_3C=O, OH, CH_2NH_2, CHO and H_2O groups respectively. These functional groups may indicate the possible presence of flavonoid.

GC-MS analysis of DEE of *C. senna* leaves showed the possible presence of major secondary metabolites such as flavonoids, alkaloids and fatty acid esters thereby supporting the results of HPTLC and HPLC.

Silva *et al*., (2016) reported that the GC-MS analyses of methanolic, diethyl ether and *n*-hexane extracts of leaves of four *Senna* species namely *S. gardneri, S. macranthera, S. splendida* and *S. trachypus* identified 34 compounds in different

groups that included mainly flavonoids (chrysin and quercetin), carboxylic acids, fatty acids, fatty alcohols, steroids, diterpenes and triterpenes.

The compounds such as rhein, emodin, chrysophanol and physcione were isolated identified in *Senna podocarpa* (Malmir *et al*., 2015). A compound namely 2,4-(1*H*,3*H*)-pyrimidinedione was quantified in the leaf extract of *C. spectabilis* using GC-MS analysis. In GCMS analysis A new anthraquinone aglycone called anthraquinone 1-carboxylic acid was identified in *C. laevigata* leaves as a key molecule executing antifungal activity (Panigrahi *et al*., 2013).

According to Sermakkani and Thangapandian (2012), the methanolic extract of *C. italica* leaf subjected to GC-MS analysis showed the presence of 17 components including phytol, squalene and n-hexadeconoic acid. Anthraquinone glycosides and a flavonoid, quercetin-3-O-a-L-rhamnopyranoside were isolated from the methanolic extract of the leaves of *Cassia roxburghii* DC., and it was reported that both the methanolic extract and quercetin-3-O-a-L-rhamnopyranoside showed strong antioxidant activity(El-Toumy *et al*., 2012).

A new xanthone, 1,8-dihydroxy-3-methoxy-6-methylxantone and a new polyketide derivative, (4R*,5S*,6E,8Z)-ethyl-4-((E)-but-1-enyl)-5-hydroxypentdeca-6,8-dienoate, together with 20 known secondary metabolites, including 2 steroids, 4 xanthones, 10 anthraquinones, 2 triterpenoids, 1 fatty ester, and (E)-eicos-14-enoic acid, were isolated from the leaves of *Cassia obtusifolia* (Sob *et al*., (2008).

Fatty acid esters, fatty acid amide, triterpene, diterpene alcohols and phytol were identified as the major chemical groups in the methanol fractions of *C. auriculata* leaf extracts (Anandan *et al*., 2011).

Aqueous acetone leaf extract showed the presence of benzocoumarin glycoside, avaraoside I, avaraol I with protective effect on liver disorder. Other constitutents include luteolin, kaempferol, quercetin, myricetin,3-methoxyluteolin, kaempferol 3-O- _-d-glucopyranoside, epigallocatechin and emodin which contains functional groups such as hydroxyl, ester, aromatic ring, and ether functionalities which shows antipyretic, antiulcer, antihelmintic and hepatoprotective activities (Nakamura *et al*., 2014).

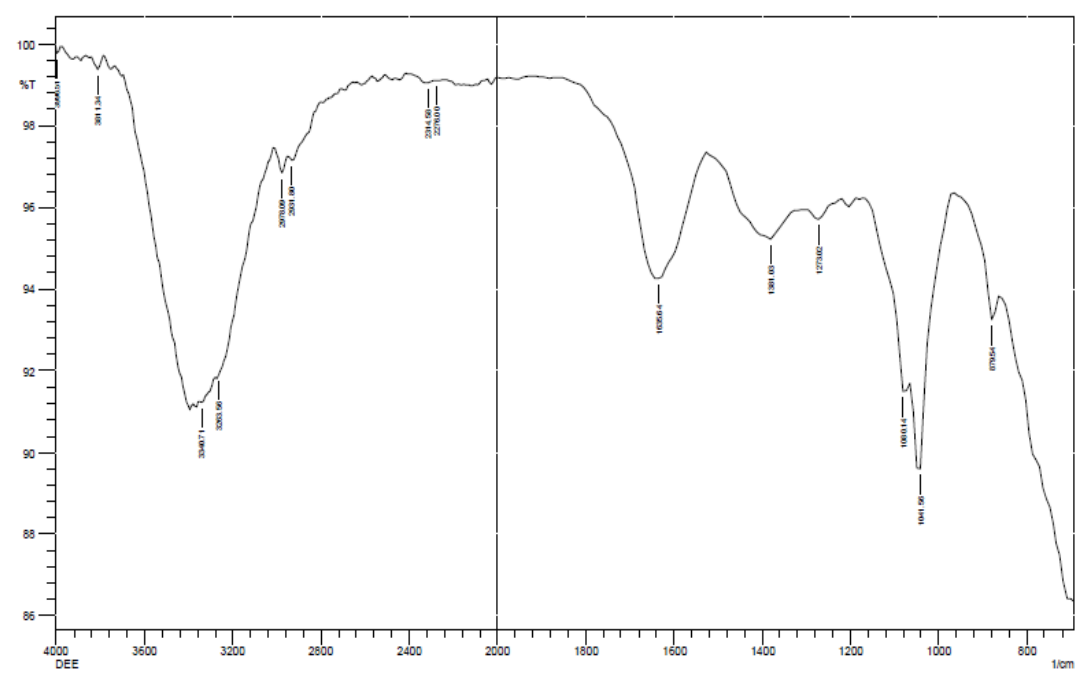
The present findings of GC-MS analysis of DEE are in accordance with the above literatures cited and confirmed the presence of CHO, C= O, OH, C–N, NH, C-H and COOH groups which indicating the presence of alkaloid and flavonoid type of compounds.

4.3.2.2. FTIR

FT-IR technique is used in the identification of functional groups of active components based on the peak value in the region of IR (Singh *et al*., 2011). Every compound exhibits a characteristic absorption or emission pattern in the IR spectral region that can be analyzed both quantitatively and qualitatively by FT-IR absorption spectroscopy. The FT-IR absorption spectroscopy is more sensitive and selective than colorimetric methods that can be performed both on pure compounds and complex mixtures, without separation into individual components (Janakiraman *et al.*, 2011).

The IR spectrum of the DEE of *C. senna* leaves were done by FT-IR spectrophotometer using KBr pellet method (Figure 50) in order to identify the functional groups present in the same.

Figure 50 : FT-IR spectrum of DEE of *C. senna* leaves



The result of the FT-IR spectrum of the DEE is presented in Figure. The characteristic intense broad bands at 3340.71cm^{-1} and 3263.56cm^{-1} reveal O–H, N–H and C stretching vibrations of alcohols, phenols, primary amines and secondary amines. The absorption bands range from 2978.09cm^{-1} - 2931.8cm^{-1}reveal C-H stretching vibrations of alkanes and a characteristic broad medium band at 1635.64cm^{-1} is the indicative of N=H stretching vibrations of primary amines. The band at 1273.02cm^{-1} shows C–N stretch and C–O stretch groups due to presence of aromatic amines, alcohols, carboxylic acids, esters and ethers. The band at 1080.14cm^{-1} reveals C–N functional group which might be due to the presence of aromatic and aliphatic amines. The FT-IR spectrum also showed an intense sharp absorption band at 1041.56cm^{-1} indicating the presence of C–N stretching vibrations of aromatic and aliphatic amines and showed a band at 879.54cm^{-1} indicating the presence of =C–H bending vibrations of alkanes

In earlier studies bioactive compounds have been identified in some of *Cassia* species by FTIR. FTIR spectroscopy of *C. auriculata* leaf extract indicated that the compounds attached with silver NPs could be polyphenols with aromatic ring and bound amide (Parveen *et al*., 2012). Parveen *et al*., (2016) isolated a novel isoflavone along with a known pyranocoumarin from the ethanolic extract of the leaves of *C. siamea* and also elucidated their structures on the basis of elemental analysis, UV, FT-IR, NMR and mass spectral analysis. The FT-IR spectrum showed absorption bands at 1640, 1610 and 1060 cm−1, indicating the presence of α,β-unsaturated carbonyl (C=O), C=C and C\\O functional groups in the molecule.

The present findings of FT-IR analysis confirmed the presence of compounds with functional groups such as CHO, C= O, OH, C–N, NH, C-H. Those functional groups indicates the presence of alkaloid and flavonoid type of compounds in DEE of *C. senna* leaves thereby supporting the results of HPTLC, HPLC and GC-MS.

From the spectroscopical characterization of DEE of *C. senna* leaves using GCMS and FTIR analysis, it can be deduced that DEE contains flavonoid and alkaloid type of compounds that may be the major active principles responsible for its antioxidant and anticancer potential.