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Summary and Conclusion

Cancer is a global challenge as this disease remains the second largest cause of death around the world. It is the most deadly disease that causes serious health problems, physical disabilities, mortalities and morbidities. Cancer is also the leading cause of death in developing countries like India. Every year in India about 8,50,000 new cancer cases being diagnosed resulting in 5,80,000 cancer related.

Cancer is an abnormal type of tissue growth and is commonly defined as an uncontrolled growth of cells with loss of differentiation commonly with metastasis, the spread of cancer to other tissues and organs. The uncontrolled growth and spreading of cancer cells make them dangerous.

The available synthetic chemotherapeutic drugs for cancer are reported to exhibit toxicity towards normal tissues and possess undesirable side effects. The non-discriminatory use of these drugs leads to severe side effects in normal cells with the high proliferative index, leading to drug resistance and limiting the effective dose of anticancer drug that can be administered. The limited success of clinical therapies including radiation, chemotherapy, immunomodulation and surgery in treatment of cancer indicates that there is an imperative need of alternative strategies in cancer management.

It is necessary to develop new anticancer agents with antitumor and antimetastatic activities but without side effects and adverse reactions. Overcoming the side-effects of synthetic anticancer drugs with natural anticancer agents from medicinal plants may provide a strategy for overcoming the adverse effects of chemotherapy as they are less toxic.

Oxidative stress is excess formation or incomplete removal of highly reactive molecules such as ROS including free radicals as well as nonradical species such as hydrogen peroxide. Oxidative stress, a key player in several diseases such as cancer, diabetes mellitus, atheroscleorosis, cardiovascular diseases, ageing and inflammatory diseases results from an imbalance between formation and neutralization of prooxidants.

Oxidative stress is initiated by free radicals, which seek stability through electron pairing with biological macromolecules such as proteins, lipids and DNA in healthy human cells and causes protein and DNA damage along with lipid peroxidation.

There are restrictions on the use of synthetic antioxidants, as they are suspected to be carcinogenic. Therefore development of a potential natural antioxidant molecule is gaining importance in the recent years as it plays an important role in preventing or delaying the onset of certain pathological consequences such as cancer, heart diseases and hepatotoxicity.

Medicinal plants are the essential parts of the traditional health care systems. Today about 80% of the world's population relies mainly on plants and plant extracts for healthcare. There are more than 8,000 plant species in South Asia with known medicinal uses. In recent years, researches on medicinal plants are useful for developing new products and medicines to treat diseases. Medicines derived from plant are potentially safer and more reliable compared to synthetically produced drugs.

Medicinal plants have always been the principal source of medicine in India. The medicinal plant wealth is our national heritage and it seems to be the first and foremost line of defence for the treatment of more diseases almost in various communities. In Indian medicine systems, Ayurveda, Siddha and Unani entirely and Homeopathy partially depend either on plant materials or their derivatives for treating human ailments. Generally, herbal drugs are easily available, safe, less expensive, efficient and rarely have side effects.

Plants are the effective source of anticancer agents and over 60% anticancer agents are derived from plants. Still there are number of plants with anticancer potential have not yet been fully investigated. The use of plant-derived products in the treatment of cancer may reduce adverse and toxic side effects. Worldwide efforts are ongoing to identify new anticancer compounds from plants.

Antioxidants derived from plants are presumed to be safe since they are natural origin and have capability to counteract the damaging effect of ROS. Plants have developed a complex enzymic anti-oxidative defence system such as SOD, CAT, POD,

GPx and GST to combat oxidative damage caused by ROS and they also produce a very impressive array of nonenzymic antioxidants such as ascorbic acid, α-tocopherol, flavonoids, polyphenols, reduced glutathione and carotenoids to prevent oxidation of the susceptible substrates.

Phytochemicals are naturally occurring chemicals present in medicinal plants and have their own defence mechanisms and protection against various diseases. Secondary phytochemical compounds namely alkaloids, flavonoids, phenols, tannins and terpenoids possess some therapeutic properties. These secondary metabolites contribute significantly towards the biological activities of medicinal plants such as antioxidant, anticarcinogenic, hypoglycemic, antidiabetic, antimicrobial, antiinflammatory, antimalarial, anticholinergic and antileprosy activities.

Cassia species have been of keen interest in phytochemical and pharmacological research due to their excellent medicinal values. All *Cassia* species are rich source of secondary metabolites and they are used extensively in various parts of the world against a wide range of ailments, the synergistic action of its metabolite being probably responsible for the plants beneficial effects. The clinical research on *Cassia* species indicated it as a source of effective liver tonic, antibiotic, antiinflammatory and antifungal agents. Due to their medicinal, agricultural and economic value, *Cassia* species have more important attention worldwide.

Many studies have been carried out on the various species of *Cassia* belonging to the family Fabaceae with respect to their various biological activities. Although *C. senna* leaves have been investigated for the presence of secondary metabolites and evaluated for the various biological activities such as cytotoxic, thrombolytic and antimicrobial activities, not much work has been done on the anticancer activiy of *C. senna.*

With this background, the present study entitled "Evaluation of antioxidant and anticancer potential of *Cassia senna* L. using *in vitro* and *in vivo* methods" was designed to determine the antioxidant potential and the phytochemical constituents of leaf and pod of *C. senna* and to screen the effective extract of *C. senna* by radical scavenging effect, chromatographic analysis and *in vitro* cytotoxic activity. *In vivo* anticancer activity of DEE of *C. senna* leaves was performed on cancer induced mice and spectral analyses were carried out to characterize the active principles of DEE.

The present research work was carried out in three phases.

In Phase I, the fresh plant of *C. senna* was collected from Madurai District. The leaf and pod parts of the plant were shade dried and coarsely powdered. Known weight of leaf and pod samples of *C. senna* were subjected to extraction with appropriate buffers and the extracts were analyzed for the enzymic antioxidants such as superoxide dismutase(SOD), catalase(CAT), peroxidase(POD), glutathione s-transferase(GST) and polyphenol oxidase(PPO) and the nonenzymic antioxidants namely ascorbic acid, α-tocopherol, polyphenols, flavonoids and reduced glutathione. Total antioxidant activity was also measured in leaf and pod samples of *C. senna.* The different solvent extracts of leaf and pod of *C. senna* such as petroleum ether, benzene, chloroform, ethyl acetate, successive ethanolic(SEE), direct ethanolic(DEE) and aqueous extracts were prepared and analysed for qualitative phytochemical analysis and quantitative estimation of alkaloids, flavonoids, phenols, tannins and steroids.

In Phase II the free radical scavenging effect of various solvent extracts of *C. senna* leaves on DPPH, superoxide, nitric oxide, hydroxyl, ABTS radicals and the non radical, hydrogen peroxide was evaluated. HPTLC profile for flavonoids and alkaloids was carried out in the leaf extracts of *C. senna* along with the respective standard marker compounds. HPLC analysis was performed for the two ethanolic leaf extracts namely DEE and SEE with the standard markers such as caffeine for alkaloids and quercetin and kaempferol for flavonoids to further screen and determine the bioactive constituents. *In vitro* cytotoxic effect of DEE and SEE of *C. senna* leaves was determined on selected cancer cell lines namely EAC, HCT116 and MCF7 and noncancer cell line, L929 by MTT assay.

In Phase III *in vivo* study was carried out to ensure the promising antioxidant and anticancer potential of DEE of *C. senna* leaves and compared with that of standard anticancer drug methotrexate. Animal ethical clearance was obtained(Reg.no AUW.IAEC.2013-14.BC:07) before starting the experiment. Adult male swiss albino mice weighing approximately 25-30g were used as experimental model. The mice were divided into six groups of six each. EAC cells were obtained from the AMALA cancer research center, Trichur, Kerala and the cell lines were injected into all groups of mice

except Group I(normal control) animals by intraperitoneal inoculation of 10^6 cells/mouse. After 24 hrs of the tumor cell induction, treatment was started. Group I was treated as normal (negative) control and Group II animals were treated as tumour(positive) control. Group III animals were treated with the standard anticancer drug namely methotrexate at dose level of 10mg/kg b.wt. and Group IV, V and VI were treated with various concentrations of DEE ranging from 100, 200 and 300mg/kg b.wt. respectively for 14 days.

After the experimental period, the animals were sacrificed by cervical decapitation. Serum and liver samples were collected and used for the various biochemical parameters. In serum, tumour markers namely GGT and NO and liver marker enzymes such as ALT, AST and ALP were analysed. The levels of cholesterol, triglycerides and free fatty acids were also determined in serum. The enzymic and nonenzymic antioxidants and the level of various lipids namely cholesterol, triglycerides, phospholipids and free fatty acids were analysed in the homogenate of liver tissue. Samples of liver tissues from each group were subjected to histopathological observation. In order to identify the nature of the components and to analyze the functional groups present in DEE of *C. senna* leaves GC-MS analysis and FT-IR spectroscopy were performed.

Salient findings of the present study

Phase I

- From the enzymic and nonenzymic antioxidant assays of leaf and pod samples of *C. senna*, the leaf sample was found to contain *significantly*($p<0.05$) higher activity of enzymic antioxidants and elevated levels of nonenzymic antioxidants and higher total antioxidant activity than pods which might be due to the nature of antioxidants that are present in photosynthetic cells other than reproductive part.

- Preliminary phytochemical screening of different solvent extracts of leaf and pod of *C. senna* showed the presence of various phytochemicals. Among the different solvent extracts of leaf and pod analysed, leaf extracts of *C. senna* were found to be positive for most of the phytoconstituents such as alkaloids, flavonoids, phenols, tannins, steroids, terpenoids, phytosterols, saponins, carbohydrates,

glycosides and proteins when compared to pod extracts indicating that leaf may be a rich source of secondary metabolites than pod.

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- Quantitative estimation of the selected phytochemicals in the various extracts of leaf and pod of *C. senna* showed that leaf samples recorded significantly($p<0.05$) higher content of alkaloids, flavonoids, phenols, tannins and steroids when compared to that of pod and it is clear that the two ethanolic extracts namely DEE and SEE of *C. senna* leaves are rich sources of the above phytochemicals quantified than all other extracts including pod extracts.

- Since leaf of *C. senna* was found to be better than pod with respect to antioxidant assays and phytochemical analysis, only leaf extracts were selected for Phase II studies.

Phase II

- From the evaluation of free radical scavenging effect of different solvent extracts of leaf of *C. senna* it was observed that both the ethanolic extracts namely DEE and SEE exhibited significantly*(*$p<0.05$) higher percentage of scavenging activity against the free radicals namely DPPH, ABTS, OH, $O_2^{\bullet -}$, and NO and non radical, H_2O_2 than other extracts studied, thereby confirming the stronger scavenging ability of the polar extracts than the nonpolar ones. Between the two ethanolic extracts, DEE was found to be more powerful scavenger than SEE.

- From the HPTLC profile for alkaloids and flavonoids of leaf extracts of *C. senna*, it is clear that both the ethanolic extracts namely DEE and SEE were found to possess more number of components, as polar solvents have higher ability to extract the maximum phytoconstituents than nonpolar solvents. It could also 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

- Since DEE and SEE were found to be more effective than the other solvent extracts on the basis of results obtained from free radical scavenging effect and

HPTLC profile, only these two ethanolic extracts(DEE and SEE) were selected for further Phase II analyses.

- From the results of HPLC analysis of DEE and SEE with standard markers namely caffeine, quercetin and kaempferol, DEE was found to separate more number of peaks for alkaloids and flavonoids than SEE at respective wavelength. The RT of DEE was found to exactly match with that of kaempferol while in the case of caffeine and quercetin the RT of DEE was found to be closer to that of standards than SEE. Hence HPLC study confirmed that DEE was an effective extract containing more essential active principles than SEE. The lower ability to separate peaks of active principles by SEE when compared to DEE might be due to the sequential extraction procedure, where ethanol was used after petroleum ether, benzene, chloroform and ethyl acetate.

- The evaluation of *in vitro* cytotoxicity by MTT assay revealed that DEE and SEE mediated a concentration dependent increase in cytotoxicity towards cancer cell lines namely EAC, HCT116 and MCF7 cells lines. Out of the two ethanolic extracts tested, DEE was found to be significantly($p<0.05$) more cytotoxic to all the selected cancer cell lines than SEE. This might be due to the extraction of more number of essential phytoconstituents namely alkaloids and flavonoids in DEE whereas SEE being a product of sequential extraction procedure might contain lesser concentration of phytoconstituents than DEE. In case of noncancer cell line L929, both DEE and SEE were found to be less toxic. These observations indicated a differential effect of DEE of *C. senna* leaves which was selectively more toxic to the cancer cells and less toxic to the non-cancerous cells, which may validate DEE as a successful anti-cancer agent.

- Based on the results of HPLC analysis and cytotoxic activity, DEE of *C. senna* leaves was found to be more effective than SEE and hence DEE was selected for Phase III studies.

Phase III

- In *in vivo* studies, the effect of DEE of *C. senna* leaves on tumour growth response revealed that the administration of DEE to cancer induced mice resulted

in maintenance of body weight to near normal and also remarkable($p<0.05$) improvement in the Mean survival time(MST) and Increased life span(ILS%). These observations could be attributed to the efficiency of DEE in preventing ascites fluid accumulation in the peritoneal cavity of cancer induced mice thereby reducing tumour burden.

- In case of tumour markers, the GGT activity and the NO level were significantly($p<0.05$) increased in the serum of tumor bearing mice as compared with the normal control and the same were significantly($p<0.05$) reduced with the treatment of DEE. This reduction rate was comparable with that of standard drug methotrexate. The reversion of GGT activity into near normal may possibly due to the ability of DEE in reducing the hepatic damage caused by ascites tumour. The downregulation of nitric oxide level by DEE may indicate its antiangiogenic effect and hence DEE of *C. senna* leaves could be a novel source of anticancer therapy.

- The antioxidant assays performed in liver homogenate revealed the remarkable($p<0.05$) decrease in the activities of enzymic antioxidants namely SOD, CAT and GPx and the non-enzymic antioxidants such as GSH, vitamin C and vitamin E in tumor control group which may indicate the complete disruption of the antioxidant defense system. This condition was reverted back to near normal with the administration of DEE. These results indicate the efficiency of DEE of *C. senna* leaves in counteracting the damage caused by ascites tumour and suggest that DEE has got promising antioxidant activity.

- The activities of liver marker enzymes, namely AST, ALT and ALP in the serum of EAC induced mice were significantly increased when compared to those of normal group. While in DEE treated mice, the activities of these enzymes were significantly($p<0.05$) reduced as compared with the tumor control group. The standard drug methotrexate also produced similar effect when compared to that of DEE treated groups. The significant($p<0.05$) recovery of the elevated hapato-specific enzyme activities into near normal may indicate the preventive role of DEE of *C. senna* leaves on liver damage caused by ascites tumour.

- From the investigation of lipid levels, it is revealed that tumour bearing mice showed a significant($p<0.05$) increase in the level of CL,TGL and free FFA in serum while in DEE treated mice, there was a regulation in the plasma lipids to near normal which is probably due to hypolipidemic effect exerted through antitumor activity.

- In case of liver tissue, a significant($p<0.05$) depletion of cholesterol, triglyceride and phospholipids observed in EAC induced mice may indicate the fast rate of liopolysis in the liver. There was a significant($p<0.05$) increase in FFA concentration in the liver of tumour control mice which might be due to the free radical mediated breakdown of membrane phospholipid into FFA for energy demand of cancer cells. 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.

- Assessment of lipid content in serum and liver tissue clearly indicates that administration of DEE of *C. senna* leaves to tumour bearing mice significantly($p<0.05$) modulated the abnormal lipid levels in serum as well as in liver tissue and this could be due to the efficiency of DEE in regulating the lipid metabolism through its anticancer effect.

- In histopathological studies, it was observed that liver sections of EAC induced mice(Group II) showed invasion of tumour cells with enlarged hyper chromatic nuclei, cellular infiltration(inflammation) and also showed marked changes in the lobular architecture due to severe necrosis and inflammation whereas Group IV, V and VI treated with DEE at an increasing concentrations of 100, 200, 300 mg/kg body wt. respectively showed gradual reduction in hyperchromatic nuclei, cellular infiltration and inflammation. A pattern of recovery similar to that of standard drug(methotrexate) and almost normal hepatocellular architecture were observed in DEE treated groups which may be due to the diminution of oxidative stress and prevention of invasion of tumour cells. These histopathological observations further authenticate the results obtained for the associated biochemical parameters

of serum and liver tissue of mice indicating the anticancer activity of DEE of *C. senna* leaves.

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

- From the spectroscopical characterization of DEE of *C. senna* leaves using GCMS and FTIR analyses, 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.

Conclusion

- The research outcome of the present study revealed that DEE of *C. senna* leaves appear to be a good source of antioxidants and essential phytoconstituents which could have novel therapeutic value in the mechanism of free radical scavenging activity. The present findings of *in vitro* studies indicated a differential effect of DEE of *C. senna* leaves which was selectively more toxic to the cancer cells and less toxic to the non-cancerous cells, which may validate DEE as a successful anticancer source. From the *in vivo* studies, it could be stated that DEE of *C. senna* leaves exhibited significant antioxidant and anticancer activity in cancer induced mice. Based on the results of chromatographical and spectral analysis it can be deduced that DEE of *C. senna* leaves is a rich source of flavonoid and alkaloid type of compounds that may be the active principles responsible for the antioxidant and anticancer potential of *Cassia senna*.

Suggestions for future research

The outcome of the present study has opened several promising propositions for future research. Some of them that can be adopted for an active research are suggested below.

- The anticancer properties of DEE of *C. senna* leaves can be tested in various types of cancer cell lines to determine whether a tissue specific response exists.

- The exact mechanism of anticancer activity such as apoptotic and antiangiogenic effect of DEE of *C. senna* leaves can be predicted through various *in vitro* and *in vivo* studies and by evaluating the expression of various genes/proteins involved in the apoptotic and antiangiogenic pathways.

- The biologically active flavonoid and alkaloid type of compounds present in DEE of *C. senna* leaves can be isolated, purified and their structures can be elucidated.

- *In silico* analyses can be done to characterize the interaction of the active components present in DEE of *C. senna* leaves with the target proteins to predict the mode of action, potential, adverse drug reactions and the absorption, distribution, metabolism, and excretion(ADME) profiles of the active compounds.