PHYTOCHEMICAL SCREENING OF OILS FROM *GOSSYPIUM BARBADENSE* AND *NELUMBO NUCIFERA* ON DISEASED HUMAN LUNG FIBROBLAST SHOWS FREE RADICAL SCAVENGING ACTIVITY

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Abstract

The search for natural antioxidants has gained considerable attention due to their potential health benefits and therapeutic applications. Phytochemical compounds derived from plants have gained significant attention due to their potential health benefits and therapeutic properties.

In this study, we conducted a comprehensive phytochemical screening of two plant species, *Gossypium barbadense* (commonly known as cotton) and *Nelumbo nucifera* (also known as lotus). The study revealed the presence of diverse bioactive compounds, particularly flavonoids, phenols, and tannins, which contribute to free radical scavenging activity.

Both the oils were evaluated for their total phenolic ccontent, total flavanoid content and free radical scavenging activity was carried out using the 2, 2-diphenyl-1-picrylhydrazyl (DPPH) method with ascorbic acid as reference standard and activity was extended for cell viability using human lung fibroblast (COPD), HCC7231 (TACC CCL-96).

The results showed that both *Gossypium barbadense* and *Nelumbo nucifera* are equally potent and showing promising free radical scavenging property. It was observed that *Nelumbo nucifera* potentiate the antioxidant activity of *Gossypium Barbadense*. In addition, both the oils showed non-toxic effects against a diseased human lung fibroblast (COPD), HCC7231 (TACC CCL-96) by protecting H_2O_2-induced apoptosis. This synergistic action of *Gossypium barbadense* and *Nelumbo nucifera* is an effective and alternative for the treatment of cancer due to their free radical scavenging activity.

Keywords: *Gossypium barbadense*, *Nelumbo nucifera*, phytochemical screening, free radical scavenging activity, DPPH method, synergistic action, cell viability.

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INTRODUCTION

Cotton is grown as annual crop in many countries for commercial purposes. Different commercially valuable *Gossypium hirsutum, Gossypium arboreum, Gossypium herbaceum, Gossypium barbadense,* and other cotton varieties developed conventionally to produce improved agronomic varieties of cotton for their application in textile, fibre, oil, and animal feed. Cottonseed oil is extracted from the seeds of cotton plants of various species, mainly *Gossypium barbadense and Gossypium herbaceum* [1]. They are grown primarily to produce cotton fiber and animal feed. Cottonseed oil (CSO) is the by-product of cotton manufacturer; extracted from the decorticated and delinted cottonseed. It is used as edible oil and industrial applications [2].

The active constituent of cottonseed oil is not a single compound but a combination of various components. CSO is primarily composed of triglycerides, which are esters formed from glycerol and fatty acids. It typically contains a mixture of saturated fatty acids, monounsaturated fatty acids, and polyunsaturated fatty acids. The most abundant fatty acid in cottonseed oil is linoleic acid (around 50-70%), which is an omega-6 polyunsaturated fatty acid which help to lower LDL (bad) cholesterol levels [3]. The vitamin E content in cottonseed oil can also contribute to skin health by providing antioxidant protection. The chemical composition of cottonseed oil can vary depending on factors such as the variety of cotton, processing methods, and cultivation conditions. Cottonseed oil offers several potential health benefits due to its composition of fatty acids and other nutrients. Some studies suggest that cottonseed oil may have anti-inflammatory effects. Chronic inflammation is associated with various health conditions, including heart disease, diabetes, and certain types of cancer [4].

Figure 1: Cotton plant and Cotton seed

Lotus is a perennial aquatic plant. It belongs to the small family of Nelumbonaceae, comprising of only one genus Nelumbo with two species: *Nelumbo nucifera Gaertn* and *Nelumbo lutea Pear*, which are popularly named as Asian lotus and American lotus, respectively [5].

Lotus seed oil (LSO) contains various active constituents that contribute to its potential health benefits. LSO is a good source of polyunsaturated fatty acids, including linoleic acid and alpha-linolenic acid. LSO contains tocopherols, which are forms of vitamin E and serve as natural antioxidants. It contains phenolic compounds, such as gallic acid and ellagic acid. They are also known to contain various flavonoids, including quercetin, kaempferol, and myricetin. LSO contain various sterols, including beta-sitosterol and campesterol [6]. Lotus essential oil is used in skin care products such as massage oils, bath soaps, body lotions and aromatherapy. This product is a blend of floral and earthy scents. The relaxing effect of lotus essential oil increases feelings of relaxation and peace [7].

Figure 2: Lotus plant and Lotus seed

In Traditional Chinese Medicine (TCM), lotus seeds have been used for centuries to promote spleen and kidney health, as well as to treat digestive disorders and insomnia. In Ayurveda, an ancient Indian healing system, lotus seeds and oil are considered to have cooling properties and are used to reduce body heat and inflammation. It is also used in Ayurvedic massage to calm the mind and soothe the skin [8]. In Korean traditional medicine, lotus seeds are believed to have anti-aging properties and are used to improve skin elasticity and texture. Lotus seed oil is also used to moisturize the skin and reduce the appearance

of wrinkles[9].

Oxidative stress is a condition characterized by an imbalance between the production of reactive oxygen species (ROS) and the body's antioxidant defenses. Oxidative stress can contribute to the development and progression of cancer. ROS can cause DNA damage and mutations, disrupt cell signaling pathways, and promote inflammation, all of which can lead to the uncontrolled growth and proliferation of cancer cells. However, cancer cells also rely on ROS for their survival and growth, which has led to the exploration of targeting oxidative stress as a potential therapeutic strategy [10].

Excessive ROS can cause damage to cellular components such as DNA, proteins, and lipids, leading to various neurodegenerative diseases such as Alzheimer's disease, Parkinson's disease, and amyotrophic lateral sclerosis (ALS)[11]. Conditions such as rheumatoid arthritis, inflammatory bowel disease, and chronic obstructive pulmonary disease (COPD) are characterized by chronic inflammation, which can lead to oxidative stress [12].

DPPH (2, 2-diphenyl-1-picrylhydrazyl) assay is a popular method for evaluating the antioxidant activity of various substances, including natural products, food, and beverages. In DPPH assay, the DPPH free radical is used as a probe to measure the scavenging activity of antioxidants[1] using ascorbic acid as reference standard [13].

In the current investigation, the ability of CSO and LSO to scavenge 1, 1-diphenyl-2-picrylhydrazyl (DPPH) free radicals and safe-guard cell viability of diseased human lung fibroblast (COPD), HCC7231 (TACC CCL-96) by MTT assay was used to assess both compounds.

EXPERIMENTAL WORK

Materials and Methods

Chemicals:

1, 1-diphenyl-2-picryl-hydrazil (DPPH), ascorbic acid and methanol were procured from Sigma Aldrich Ltd and the chemicals used were of analytical grade.

Plant Materials

Cotton seeds and Lotus seeds were procured from Gandhi Krishi Vigyana Kendra (GKVK) Bengaluru. The seeds are dried under sunlight and they were thoroughly grinded in a domestic mixer.

Cell culture [14]

Human lung fibroblast (COPD), HCC7231 (TACC CCL-96) was obtained from National Centre for Cell Science, Pune, India, and was maintained in Duelbacco Minimum Eagle's Medium with 10% Fetal Bovine Serum at 37 ^{o}C, 5% CO_2 and 90% humidity throughout the study. The cell viability was assessed by the 3-(4, 5-dimethylthiazol-2- yl)-2, 5-diphynyltetrazolium bromide (MTT) assay.

Extraction Method [15]

Cold pressed method is one of the extraction methods to obtain natural essential oils. This mechanical process does not require any external heat to make the process proceed further because the temperature required for its functioning and the heat generated internally due to friction must not exceed 120^{O} C. This method is very remarkable organic extraction process to derive the essential oils from raw materials.

This process requires the grinding of seeds, while grinding the seeds the temperature should be kept minimal, so that the excessive heat won't spoil the natural essence of oil. The fatty portion of these raw materials which contain oil glands is pricked and then it is pressed mechanically to obtain the complete juice. In the last step, centrifugation process is used to part the essential oil from the juice obtained. Moreover, these oils are interesting for consumers due to their natural and safe as well as prevent certain diseases and improve human health due to including a higher level of lipophilic phytochemicals such as antioxidants. These oils have better nutritive properties than refined oils. However, they have a lot of advantages; one of the disadvantages of this technique is low productivity. Another disadvantage of this technique is hard to extract same quality product.

Then the oil obtained from *Gossypium Barbadense* (GB) and *Nelumbo Nucifera* (NN) is than subjected to total phenolic content, total flavanoid content, DPPH assay method for testing Free radical scavenging activity using ascorbic acid as reference standard and cell viability on diseased human lung fibroblast (COPD), HCC7231 (TACC CCL-96).

Phytochemical screening [16] of CSO and LSO

Phytochemical screening of CSO & LSO for the presence of Carbohydrates, proteins, alkaloids, glycosides, flavonoids, phenols & tannins and terpenoids was carried out.

Test for alkaloids

0.4 g of CSO & LSO was stirred with 8 ml of 1%

HCl and the mixture was warmed and filtere. 2ml of filtrate was treated separately with (a) with few drops of potassium mercuric iodide (Mayer's reagent) and (b) potassium bismuth (Dragend-roff's reagent). Turbidity or precipitation with either of these reagents was taken as evidence for existence of alkaloids.

Test for terpenoids

Presence of terpenoids in CSO & LSO was carried out by taking 5 ml (1 mg/ml) of CSO & LSO and mixed with 2 ml of chloroform, followed by 3 ml of concentrated H_2SO_4. A reddish brown colouration of the interface confirmed the presence of terpenoids.

Test for glycosides

200 mg of CSO & LSO was boiled with 6 ml of 1% HCl and filtered. The filtrate was shaken with 5 ml of benzene, filtered and 2 ml of 10% ammonia solution was added to the filtrate. The mixture was shaken and the presence of a pink, violet or red colour in the ammoniacal phase indicated the presence of glycosides.

Test for flavonoids

50 mg of CSO & LSO was suspended in 100 ml of distilled water to get the filtrate. 5 ml of dilute ammonia solution was added to 10 ml of filtrate followed by few drops of concentrated H_2SO_4. Presence of flavonoids was confirmed by yellow coloration.

Test for phenols & tannins

50 mg of CSO & LSO was boiled in 20 ml of distilled water and filtered. A few drops of 0.1% $FeCl_3$ was added in filtrate and observed for color change; brownish green or a blue-black coloration was taken as evidence for the presence of tannins.

Test for carbohydrates

A few drops of Molisch's solution was added to 2 mL of both CSO and LSO, thereafter a small volume of concentrated sulphuric acid was allowed to run down the side of the test tube to form a layer without shaking. The interface was observed for a purple color as indicative of positive for carbohydrates.

Test for proteins

To 1ml of CSO & LSO and few drops of Ninhydrin reagent was added and heated in a boiling water bath. A purple blue color indicates the presence of proteins.

Total phenolic content [17]

Phenolic compounds are important plant constituents with redox properties responsible for antioxidant activity. The hydroxyl groups in plant extracts are responsible for facilitating free radical scavenging. As a basis, phenolic content was measured using the Folin–Ciocalteu reagent in each extract. The results were derived from a calibration curve ($y = 9.53x - 0.13$, $R^2 = 0.996$) of gallic acid (0–250 µg/mL) and expressed in gallic acid equivalents (GAE) per gram dry extract weight. Total phenolic contents were expressed as milligram of gallic acid equivalent (GAE)/gThe aliquots (200 lL) of both the individual were analysed or standard solution of gallic acid (0.02, 0.04, 0.06, 0.08 and 0.1 mg/mL), used, were added to 500 lL of Folin–Ciocalteu reagent (10 %). The reagents were thoroughly mixed by shaking. The mixture was incubated at room temperature for 5 min, before 1500 lL of Na2CO3 (7.5 %) were added. All the reaction mixtures were then shaken and incubated for 30 min. The absorbance of 0.1 mg/mL was recorded at 765 nm using a double beam UV–Visible spectrophotometer (Shimadzu UV-1601, Japan).

Total flavonoid assay [18]

The total flavonoid contents were measured by colorimetric assay, using quercetin for preparing the calibration curve. One mL of all individual and combined extracts or standard solution of quercetin (0.005, 0.01, 0.02, 0.03 and 0.04 mg/mL) was reacted with 1 mL of aluminum chloride (2 %). After incubation at room temperature for 1 h, the absorbance of 0.04 mg/mL mixture was measured at 420 nm. Total flavonoid contents were calculated as milligram of quercetin equivalent (QE)/g from the calibration curve: y = 6.406 x 0.012 (R2 = 0.993).

***In vitro* screening for antioxidant activity**

Free radical scavenging activity by DPPH assays method[19]

DPPH (1, 1-diphenyl-2-picryl-hydrazil) is stable free radical. Methanol solution of DPPH is usedto appraise the antioxidant activity of numerous synthetic compounds. Antioxidant on interface with DPPH, transfer electron or hydrogen atom to DPPH, thus neutralizing its free radical character and convert it to1, 1-diphenyl-2-picryl hydrazine. The extent of discoloration indicates the scavenging action of the drug. The change in absorbance produced at 517 nm has been used as measure of its antioxidant activity.

Chemicals used

1,1-diphenyl-2-picryl-hydrazil (DPPH)-Sigma Ltd., Ascorbic Acid-Qualigens, Methanol-Qualigens.

Preparation of DPPH solution: It was prepared by dissolving 33 mg of DPPH in 1 lit. Of methanol just before use and kept in dark amber colored bottle to protect from sunlight.

Sample preparation

Preparation of stock solution of derivatives

It was prepared by dissolving 50 mg of TZD derivatives in 100 ml of methanol.

Standard preparation

Preparation of Ascorbic Acid solution

It was prepared by dissolving 50 mg of ascorbic acid in 100 ml of methanol.

Procedure

A 20, 40, 60, 80 µg/ml concentrations of derivatives and ascorbic acid were prepared. From this stock solution 1ml has been pipette out and 5ml methanol solution of DPPH was added, traumatized well and the mixture was incubated at $37^{O}C$ for 30 minute and absorbance of all samples was measured against blank at 517 nm. The absorbance of DPPH reagent alone was taken as control.

Percentage DPPH radical scavenging activity was calculated by the following equation:

% DPPH radical scavenging activity= {(A0− A1)/A0} × 100%

Where A_0 is the absorbance of the control, and A_1 is the absorbance of the extractives/standard. Then % of inhibition was plotted against concentration, and from the graph IC_{50} was calculated.

MTT Assay/ Cell viability [20]

The cells were preincubated at a concentration of 1 × 106 cells/ml in culture medium for 3 hrs at 37 °C and 6.5 % CO_2. Then, the cells were seeded at a concentration of 5 × 104 cells/well in 100 µl culture medium and at various concentrations (0.005-100 µM/ml) of standard doxorubicin and synthesized compounds (dissolved in 2 % DMSO (dimethylsulphoxide) solution) into microplates (tissue culture grade, 96 wells, flat bottom) and incubated for 24 hrs at 37 °C and 6.5 % CO_2. The cell proliferation is based on the ability of the mitochondrial succinate-terazolium reductase system to convert 3-(4, 5- dimethylthiazol-2-yl)-2,5- diphenyltetrazolium bromide(MTT) to a blue colored formazan. The test denotes the survival cells after toxic exposure. Then, 10 µl MTT labelling mixture was added and incubated for 4 hrs at 37 °C and 6.5 % CO_2. Each experiment was done in triplicates. Then 100 µl of solubilization solution was added into each well and incubated for overnight. The spectro-photometric absorbance of the samples was measured using a microplate (ELISA) reader. The wavelength to measure absorbance of the formazan product in between 550 and 600 nm according to the filters available for the ELISA reader was used. The reference wavelength should be more than 650 nm.

IC_{50}, the concentration of compound required to inhibit 50 % cell growth, was determined by plotting a graph of Log (concentration of compound) vs % cell inhibition. A line drawn from 50 % value on the Y axis meets the curve and interpolate to the X axis. The X axis value gives the Log (concentration of compound). The antilog of that value gives the IC_{50} value.

Percentage inhibition of novel compounds against all cell lines was calculated using the following formula:

$$\% \text{ cell survival} = \frac{(At - Ab)}{(Ac - Ab)} \times 100$$

Where,

At = Absorbance of Test

Ab= Absorbance of Blank (Media)

Ac= Absorbance of control (cells)

% cell inhibition = 100 − % cell survival

RESULTS AND DISCUSSION

According to the modern theory of free radical biology, reactive oxygen species are involved in several disorders. The main free radicals are superoxide radicals (SOR), hydroxyl radical (OHR), hydroperoxyl radical (HPR), alkoxyl radical (AR), peroxyl radical (PR) and nitric oxide radical (NOR). These free radicals are associated with DNA damage, attack lipids, causes carcinogenesis, cardiovascular and neurodegenerative diseases. These harmful actions of free radicals can be blocked by antioxidant substances which scavenge the free radicals and detoxify the organism.

The present research was done to assess the potential of *Gossypium Barbadense* (GB) and *Nelumbo Nucifera* (NN) oils through phyto-chemical screening, using the total phenolic content, total flavanoid content and DPPH (2,2-

diphenyl-1-picrylhydrazyl) assay method.
Phytochemical screening of CSO & LSO demonstrated the presence of carbohydrates, proteins, phenols & tannins, flavonoids, glycosides, terpenoids and alkaloids. Plants possess high amounts of polyphenols and flavonoids and potent antioxidant activity leading therefore to various defensive and disease fighting properties.
In this study we aimed to determine which plant oil exhibited a more potent antioxidant activity. Phytochemical analysis was conducted to identify the presence of various bioactive compounds. The results obtained from this study contribute to the understanding of the potential applications of CSO and LSO in developing natural antioxidant-based therapeutic interventions.

The seed were dried in sunlight and the oil was extracted from cold press method. This method ensured the preservation of delicate compounds and maintained the integrity of the extracted samples. The resulting extracts were used for the subsequent evaluation of free radical scavenging activity. The phytochemical screening revealed the presence of phenols, flavonoids, and tannins in both CSO and LSO, indicating their potential as sources of natural antioxidants.

The contents of total phenolic and flavonoid have been proved to be positively correlated with antioxidant activity. Therefore, the variation in the contents of phenolic and flavonoid compounds in individual oils was investigated. It was found that the phenolic contents of CSO with 35.47 ± 0.08 and LSO 32.03 ± 0.47 were significantly high. As well, the total flavonoid contents were significantly higher in both CSO and LSO with 25.5 ± 0.06 and 27.49 ± 0.32 respectively. (**Table 2**)

The comparable antioxidant activity of CSO and LSO suggests that both the plants possess equally potential natural antioxidants. While CSO exhibited slight higher levels of phenols, flavonoids, and tannins, it is important to note that the antioxidant activity cannot be solely attributed to these compounds. Other factors, such as the presence of other bioactive constituents or synergistic interactions between different compounds, may contribute to the observed equal potential of CSO and LSO extracts in scavenging free radicals.
In DPPH assay, both CSO and LSO displayed the free radical scavenging activity against DPPH radicals. The percentage inhibition of DPPH radicals was measured at various concentrations of the extracts at 20, 40, 60, 80µg/ml. Surprisingly, the results demonstrated by both CSO and LSO extracts exhibited similar levels of free radical scavenging activity as that of reference standard ascorbic acid with 0.8 absorbance, showing comparable percentages of inhibition at the tested concentrations **(Figure 3).** The absorbance value for the combination of CSO and LSO was comparatively same has that of absorbance of ascorbic acid. The IC_{50} values based on the DPPH assay revealed that, CSO (0.65±0.021), LSO (0.65±0.011) and CSO+LSO (0.81±0.021) and ascorbic acid (0.89±0.05) **(Figure 4).**

The values of phenolic content in this current study varied slightly compared to those in the literature. This may be due to the presence of different amounts of sugars, carotenoids or ascorbic acid, or the duration, geographical variation or methods of extraction, which may alter the amount of phenolics. The results showed that individual extracts contained important quantity of phenolics and flavonoids and their combination was found to produce best antioxidant activity and promotes cell viability.

The CSO, LSO and CSO+LSO and doxorubicin (standard) on the growth of Human lung fibroblast (COPD), HCC7231 (TACC CCL-96) cell lines were examined by the MTT assay. Dose response curves constructed between the range 0.005 – 100 µg/ml and 0.005 – 100 µM for compound aliquots and doxorubicin (control) respectively **(Table 3).** Comparable cytotoxicity was found against lung cancer cell line in CSO and LSO. For CSO IC_{50} was found to be 50.48 µg/ml and LSO it was 51.45 µg/ml and for combination it was found to be 51.45 µg/ml but no activity found against normal cell line. However, all compounds were found to be devoid of any activity against HEK-293T (normal) cell line but doxorubicin (standard) was found active against lung cancer cell lines with lower IC_{50}.

These findings may afford useful basis for the alleged synergistic effects of natural food and facilitate their application in combination as functional foods and dietary supplements.

Table 1: Phytochemical tests for CSO and LSO

PHYTO CONSTITUTES	COTTON SEED OIL	LOTUS SEED OIL
Carbohydrates	+	+
Proteins	-	+
Phenols and Tannins	+	+
Flavonoids	+	+
Glycosides	+	+
Terpenoids	+	+
Alkaloids	+	+

Table 2: Total phenolic and flavonoid contents of CSO and LSO and CSO+LSOcombination

Sample	Phenolic content (mg GAE/g)	Flavonoid content (mg QE/g)
CSO	35.47 ± 0.08	25.5 ± 0.06
LSO	32.03 ± 0.47	27.49 ± 0.32
CSO+LSO	37.27 ± 0.26	28.58 ± 0.64

Values are expressed as mean ± standard deviation (n = 3). Values are statistically different at $p<0.05$

Figure 3: Free radical scavenging activity of CSO, LSO and CSO+LSO by DPPH method

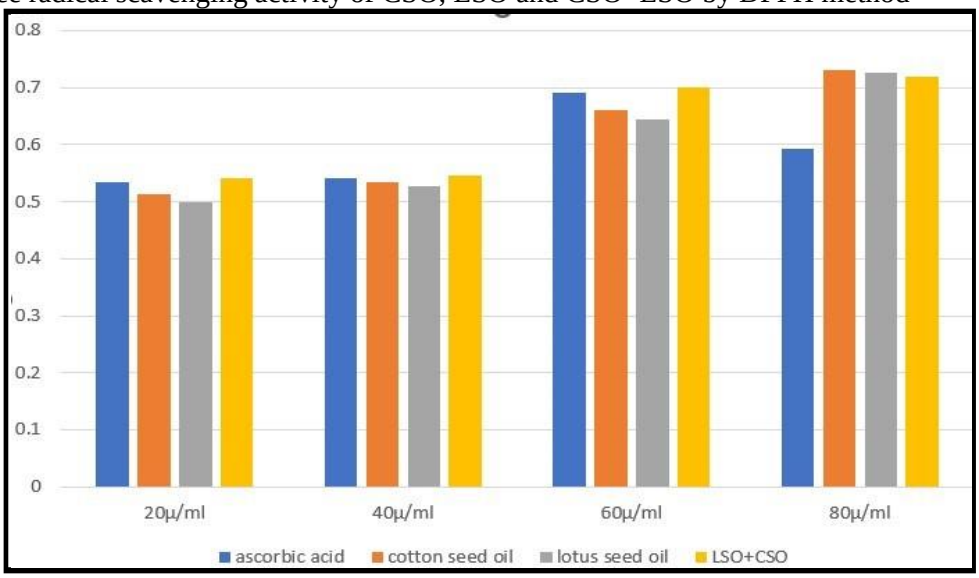
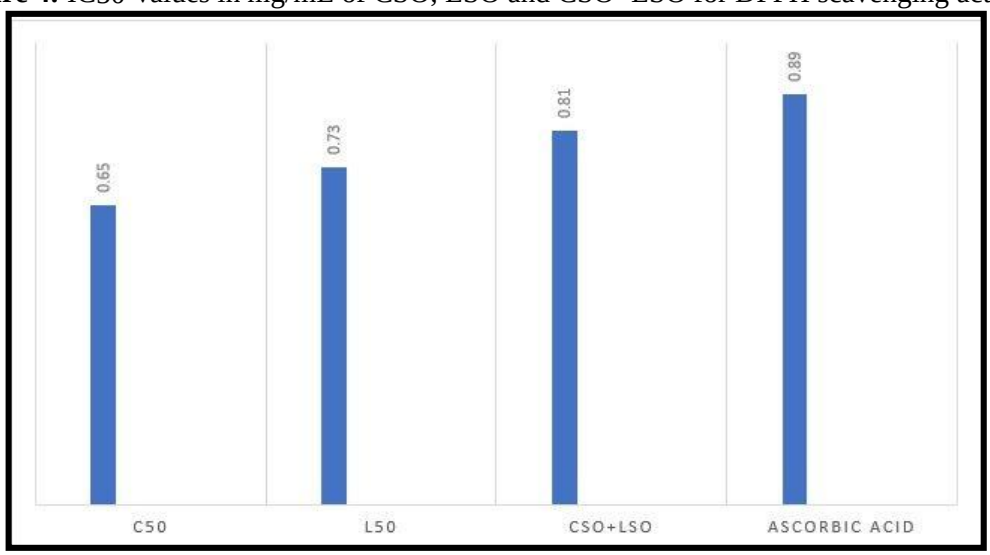


Figure 4: IC50 values in mg/mL of CSO, LSO and CSO+LSO for DPPH scavenging activity



X-axis-Concentration of oils in mg/mL Y-axis-DPPH radical scavenging

Table 3: IC50 values of CSO, LSO, CSO+LSO and Doxorubicin on cell lines by MTT Assay

Sample	IC50 (µg/ml) at 48h	
	Lung Cancer Cell Line (HCC7231 (TACC CCL-96)	**Normal Cell Line (HEK-293T)**
CSO	**50.48**	**> 100**
LSO	**47.41**	**> 100**
CSO+LSO	**51.45**	**> 100**
Doxorubicin (Standard)	**25.92**	**70.23**

CONCLUSION

Different herbs have different bioactive constituents with different antioxidant activities. When taken together, the mixtures shows higher or lower antioxidant capacity than their individual extracts. It may be due to synergistic, additive or antagonistic interaction among different compounds. In the present work we observed the synergistic action of CSO and LSO that leads to enhanced therapeutic potential and its remarkable free radical scavenging activity provides a valuable link for cancer studies by protecting cell viability. Further explorations of both the oils make its potential as an anticancer agent. Both hold great promise in advancing cancer research and therapeutic interventions in preventing or slowing the oxidative stress associated with degenerative diseases such as cancer and various other human ailments.

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