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In-Vitro Anticancer Potential Of Eucalyptus Camuldulensis

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Abstract:

Cancer is the one of the most commonly occurring cancer. Intravenous chemotherapeutic drugs have endeavored side effects in several patients, therefore people turn to the administration of herbal drugs. Several natural compounds have been identified and isolated from medicinal plants for treatment of different human malignancies. The aim of this study was to investigate the anticancer potential of Eucalyptus camuldulensis leaf crude extract on kidney cancer HEK cell line The methanolic leaf extract of E. camuldulensis was prepared through soxhlet extraction. Phytochemical analysis revealed the presence of phytochemicals. The antioxidant activity of E. camuldulensis was performed by ABTS assay (1.56, 3.12, 6.25, 12.5, 25, 50, 100, 200, 400. 800, 1000µg/ml). The cytotoxic effects of E. camuldulensis leaf extract on HEK was analyzed by MTT Assay. The cytotoxic effect was more in HEK cell line in a concentration dependent manner.

Keywords: Eucalyptus camuldulensis, Phytochemicals, antioxidant activity, anticancer activity.

Introduction

Cancer is a malignant disease specified by an abnormal division and differentiation of cells further leading to uncontrolled growth and increase in number of cells which produce a mass of cells or tumor. Later this spreads to different parts of the body tissues via blood or lymph and resides there to cause different types of cancer. Cancer cells feed itself by a process called angiogenesis in which network of blood vessels grow resulting in the formation of angiogenesis activators and bring down the production of angiogenesis inhibitors (Musa et al ., 2011). Shrubs, herbs or plant derived compounds have proved to be a great natural source in treating various cancer cells. About 30 plant derived compounds have been isolated and are currently under clinical trials. Secondary metabolites of plants have proved to be a pool of new medical compounds.

The genus Eucalyptus belongs to family. They are Australian native tree and distributed worldwide. Eucalyptus plants are rich in antioxidants such as flavonoids, tannis and phoroglucinol derivatives (Kumar et al., 2011). Anti-cancer activity against a range of cancer cell lines both in vitro and in vivo studies have been done on the extract of several Eucalyptus species such as camuldulensis, globulis, citrodora, maiden and torquata (Voung, Quan et al., 2015). Eucalyptus camuldulensis, commonly known as the river red gum belongs to Myrtaceae family and Australian native tree widely used as timber for its brilliant red wood (Brooker et al., 2019). Some studies have been conducted on Eucalyptus camuldulensis as an antioxidant and anti-cancer agents (EL-Ghorab et al., 2003). In treatment the only used part of Eucalyptus is the leaves. Therefore, the aim of this study was to investigate the anti-cancer effect of Eucalyptus camuldulensis against cancer cell line.

Materials And Methods

2.1 COLLECTION AND IDENTIFICATION OF PLANT

Eucalyptus camuldulensis was collected from kollam city, Kerala, India and it was identified by M. S Kiranraj (Assistant Professor), Department of Botany, Sree Narayana College, kollam,

2.2 PREPARATION OF CRUDE EXTRACT OF Eucalyptus camuldulensis (EC) LEAVES.

Fresh Eucalyptus camuldulensis were washed and cleaned thoroughly under running water. The excess water was drained and the leaves were shade dried. Dried leaves were powdered to obtain a coarse powder. Dried leaf powder is placed inside a thimble, which is loaded into the main chamber of the Soxhlet extractor and were extracted by using solvents such as as ethanol in soxhlet apparatus for 18 hrs and the extractive was filtered through Whatman filter paper No. 4 then concentrated at 40°C in vacuum and stored at 4°C for this investigations (Vogal et al., 1978).

2.3 QUALITATIVE ANALYSIS OF EC

Following tests were performed qualitatively

2.3.1 Phenolic Compounds

2 ml of plant extract (EC) was taken in a test tube and add 1% lead acetate solution. Formation of white precipitate indicates the presence of phenolic compounds.(Trease and Evans 2002).

2.3.2 Tannin

Take 2 ml of plant extract (EC) and add few drops of 0.1% ferric chloride solution in it, formation of brownish green indicates the presence of tannins (Trease and Evans, 2002).

2.3.3 Flavonoids

2ml of plant extract (EC) was treated with 2ml of 10% Lead acetate solution. Appearance of yellowish green colour indicated the presence of flavonoid. (Trease and Evans, 2002).

2.3.4 Saponins

To about 1ml of extract (EC) was added to 2ml of distilled water in a test tube and shaken vigorously with few drops of olive oil. Foam which persisted was taken as an evidence for the presence of saponins.(Sofowora, 1993).

2.3.5 Terpenoid

2ml of extract of plant sample (EC) was mixed with 2ml of chloroform .Then allow to evaporate and add 2ml of concentrated sulfuric acid, then heat for 2 minutes. Greyish colour indicates the presence of terpenoids. (Trease,and Evans 2002).

2.3.6 Alkaloids

Take 2 ml of plant extract (EC) and add 2ml Wagner's reagent. Test tubes were observed for the appearance of reddish brown precipitate (Trease and Evans, 1989, Sofowora, 1993).

2.3.7 Glycoside

In 5ml plant extract (EC), 2ml glacial acetic acid, one drop of 5% FeCl3 and conc. H_2SO_4 were added. Brown ring appears, indicating the presence of glycosides (Trease and Evans.2002).

2.3.8 Quinone

2ml of extract of plant (EC) was mixed with 3 or 4 drops of concentrated HCl. A yellow colour precipitate indicates the presence of quinones.

2.3.9 Fatty Acids

0.5 ml of extract (EC) was added to 5ml of ether and allowed it to evaporate on filter paper. Then the filter paper was dried and the appearance of transparency on filter paper is the indication of presence of fatty acids (Sofowora, 1993,Trease and Evans, 2002)

2.3.10 Steroids
1ml of extract (EC) was dissolved in 10ml of chloroform and equal volume of concentrated sulphuric acid was added by the sides of the test tube. The upper layer turns red and sulphuric acid layer showed yellow with green fluorescence. This indicates the presence of steroids.

2.4 QUANTITATIVE ANALYSIS OF EC
2.4.1 Estimation of phenol
The extract of sample (EC) was pipette out and the volume in the tube was made up to 3.0 ml with distilled water. Folin-Ciocalteu reagent (0.5ml) and 2mL 20% Na2CO3 were added and the tube was placed in a boiling water bath for exactly one minute. The tube was cooled and the absorbance was read at 750nm in a spectrophotometer against a reagent blank. Standard Gallic acid solutions (2.5-100µg/ml) were also treated as above. The amount of total phenols in the plant tissues was estimated by the method proposed by Mallick and Singh (1980).

2.4.2 Estimation of tannin
Content of tannins in sample was determined by Folin-Ciocalteu method. Colorimetric estimation of tannins is based on the measurement of blue colour formed by the reduction of phosphotungsto molybdic acid by tannin like compounds in alkaline medium. 1ml of extract (EC) and standard solution of tannic acid (20-100µg) was made up to 7.5mL with distilled water. Then 0.5mL of Folin-Ciocalteu reagent and 35% 1mL sodium carbonate solution were added. The volume was made upto 10mL with distilled water and the absorbance was measured at 700nm.

2.4.3 Estimation of total flavonoid
Total flavonoid content was measured by the aluminum chloride colorimetric assay. The reaction mixture consists of 1mg of extract (EC) and 4 ml of distilled water was taken in a 10 ml volumetric flask. To the flask, 0.30 ml of 5 % sodium nitrite was treated and after 5 minutes, 0.3 ml of 10 % aluminium chloride was mixed. After 5 minutes, 2 ml of 1M Sodium hydroxide was treated and diluted to 10 ml with distilled water. A set of reference standard solutions of Quercetin (20, 40, 60, 80 and 100µg/ml) were prepared in the same manner as described earlier. The absorbance for test and standard solutions were determined against the reagent blank at 510 nm with an UV/Visible spectrophotometer. The total flavonoid content was expressed as µg of QE/ mg of extract (Lee Wei Har et al 2012).

2.4.4 Estimation of glycosides
Cardiac glycosides develop an orange red colour complex with Baljet's reagent (Picric acid in alkaline medium). The intensity (absorbance) of colour produced is proportional to the concentration of glycosides .Cardiac glycosides were quantitatively determined according to Solich et al.by some modifications.

2.4.5 Estimation of steroid
0.5ml of extract was taken in a clean test tube; Cholesterol was used as standard and was taken at varying concentrations of (20-100µg/ml) in test tubes (1-5). To the standard and test samples, 5ml of ferric chloride reagent and 4ml of concentrated sulphuric acid were added. The reaction mixtures were incubated at RT for 30 min and OD was read at 540nm. A standard graph was plotted from which the unknown value of steroid in the test sample was determined (Zak et al 1954).

2.4.6 Estimation of terpenoid
Powdered sample of 10gm was soaked in 100ml of ethanol for about 24 hours and it was filtered and extracted using petroleum ether using separating funnel. The collected petroleum ether extract was allowed to dry and % of Terpenoid content was estimated using the following formula; % Terpenoid content = Weight of terpenoid extract/ weight of the sample x 100 (Ferguson., 1956).

2.4.7 Estimation of saponin
The vanillin-sulphuric acid assay for determining the total saponin content of plant materials is usually done by incubating 1mg/ml of plant sample extracts, standards or reagent blank with 0.25 mL of 0.8% (w/v) vanillin in ethanol and 2.5 mL of 72% (v/v) sulphuric acid in water for 15 min at 60°C in a shaking water bath, with the standard as diosgenin and the reagent blank made up with the solvent used for extracting the plant samples (extraction solvent). After cooling in water at the ambient temperature for 5 min, the absorbance of the standards and extract (EC) were measured at 544 nm using a UV–VIS spectrophotometer. (Chen et al 2010).

2.4.8 Estimation of alkaloids

The plant extract (1mg) was dissolved in 1 ml dimethyl sulphoxide (DMSO), added 1ml of 2N HCl and filtered. This solution was transferred to a separating funnel, 5 ml of bromocresol green solution and 5 ml of phosphate buffer were added. The mixture was shaken with 1, 2, 3 and 4 ml chloroform by vigorous shaking and collected in a 10-ml volumetric flask and diluted to the volume with chloroform. A set of reference standard solutions of atropine (20, 40, 60, 80 and 100 µg) were prepared in the same manner as described earlier. The absorbance for test and standard solutions were determined against the reagent blank at 470 nm with an UV/Visible spectrophotometer (Fazel Shamsa et al 2008).

2.5 ABTS -RADICAL SCAVENGING ASSAY

The reaction was initiated by the addition of 200 µl of diluted ABTS to 1.56 – 800 µg/ml of different concentrations of sample extract (EC) and in control 50 µl of methanol used instead of sample. Methanol is used as blank. The absorbance was read at 734 nm and the percentage inhibition was calculated. The inhibition was calculated according to the equation:

$$\%\ of\ inhibition = \frac{A0 - A1}{A0} * 100$$

Where, A0 is the absorbance of control, A1 is the absorbance of test compound (Re et al 1999)

2.6 CYTOTOXICITY AND ANTICANCER ACTIVITY SCREENING BY MTT ASSAY

The MTT assay is used to measure cellular metabolic activity as an indicator of cell viability, proliferation and cytotoxicity. This colorimetric assay is based on the reduction of a yellow tetrazolium salt (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide or MTT) to purple formazan crystals by metabolically active cells. The viable cells contain NAD(P)H-dependent oxidoreductase enzymes which reduce the MTT to formazan (Mosmann et al 1983). The insoluble formazan crystals are dissolved using a solubilizing solution (100% DMSO) and the resulting purple colored solution is quantified by measuring absorbance at 570 nm using an ELISA plate reader.

HEK (Human embryonic kidney) and L6 cell lines were seeded on 96 well plates and allowed to acclimatize to the culture conditions such as 37 °C and 5% CO2 environment in the incubator for 24 h. The test sample EC was prepared in DMEM media (100 mg/mL) and filter sterilized using 0.2 µm Millipore syringe filter. The sample EC were further diluted in DMEM media and added to the wells containing cultured cells at final concentrations of 6.25, 12.5, 25, 50, 100 µg/mL respectively. Untreated wells were kept as control. All the experiments were done in triplicate and average values were taken in order to minimize errors. After treatment with the test sample the plates were further incubated for 24 h. After incubation period, the media from the wells were aspirated and discarded. 100 µL of 0.5 mg/mL MTT solution in PBS was added to the wells. The plates were further incubated for 2 h for the development of formazan crystals. The supernatant was removed and 100 µL DMSO (100%) were added per well. The absorbance at 570 nm was measured with micro plate reader. Two wells per plate without cells served as blank. (Joseph et al 2012)

All experiments were done in triplicates. The cell viability was expressed using the following formula:
Percentage of cell viability= Average absorbance of treated *100
Average absorbance of control

2.6.1 IC 50 value

The IC50 value is the half maximal inhibitory concentration of the sample. The IC50 values were calculated using the equation for slope (y = mx + C) obtained by plotting the average absorbance of the different concentrations of the test sample (6.25-100 µg/mL) in Microsoft Excel.

Results And Discussion

3.1 CRUDE EXTRACT OF Eucalyptus camuldulensis

EC-MeOH- 40.173g sample with 500ml extraction solvent methanol

Sl.No.	Solvents	Weight of extract (g)
1	Methanol	10.959

Table 1 . Weight of extract obtained from soxhlet extraction

3.2 Physical properties of sample

The physical properties of sample, like consistency of extract, colour of extract and percentage yield of extract where recorded in (Table5.2).

Sl.No:	Solvent Extract	Sample Code	Consistency	Extract colour	Yield(%)
1	Methanol	EC-MeOH	Sticky	Dark Green	28%

Table 2: .Physical properties of crude extract .

The percentage yield obtained through the soxhlet methanol extraction for the Eucalyptus camuldulensis leaves was 28%

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3.3 QUALITATIVE ANALYSIS OF EC

The phytochemical content of Eucalyptus camuldulensis (EC) leaves was analyzed by soxhlet extraction of the dried leaves using methanol. Maximum amount of phytochemicals present in EC leaves were retained in methanolic extract (Table 3).. The qualitative analysis of extract revealed the presence of bioactive compounds such as phenol, tannin, flavonoid, saponin, terpenoid, alkaloid, glycoside, quinones, fatty acid and steroid.

SL. NO:	Name of test	Sample code
		EC
1	Phenol	+++
2	Tannin	+++
3	Flavonoid	+++
4	Saponin	+
5	Terpenoid	++
6	Alkaloid	+
7	Glycoside	+++
8	Quinones	+++
9	Fatty acid	+
10	Steroid	+++

Table 3: .Phytochemical components of the leaf extracts of E. camuldulensis

In a study reported by Mohammed El Hassan et al (2015) the alcoholic extract of E. camuldulensis obtained by soxhlet extraction revealed only the presence of tannins, sterol, terpenoid, saponin, flavonoids and phenolic

compounds and in another study by Shubhreet Kaur et al (2019) reported the presence of quinones, saponins, carbohydrates, tannins, phenols, flavonoids and fat in the methanolic extract of Eucalyptus leaves

3.4 QUANTITATIVE ANALYSIS OF EC

In this present study, the quantitative analysis of E. camuldulensis methanolic leaf extract, the phenolic content was found to be 63µg/mg, tannin (35.6µg/mg), flavonoids (55 µg/mg), glycosides (13 µg/mg), steroid (14.2 µg/mg), terpenoid (0.014%), saponin (168.13 µg/mg), alkaloid (21.25 µg/mg). In a previous study by Shubhreet Kaur et al (2019), the phenolic content was found to be 11.41% and total flavonoid content was 35.88%.

3.4.1 Estimation of Phenol

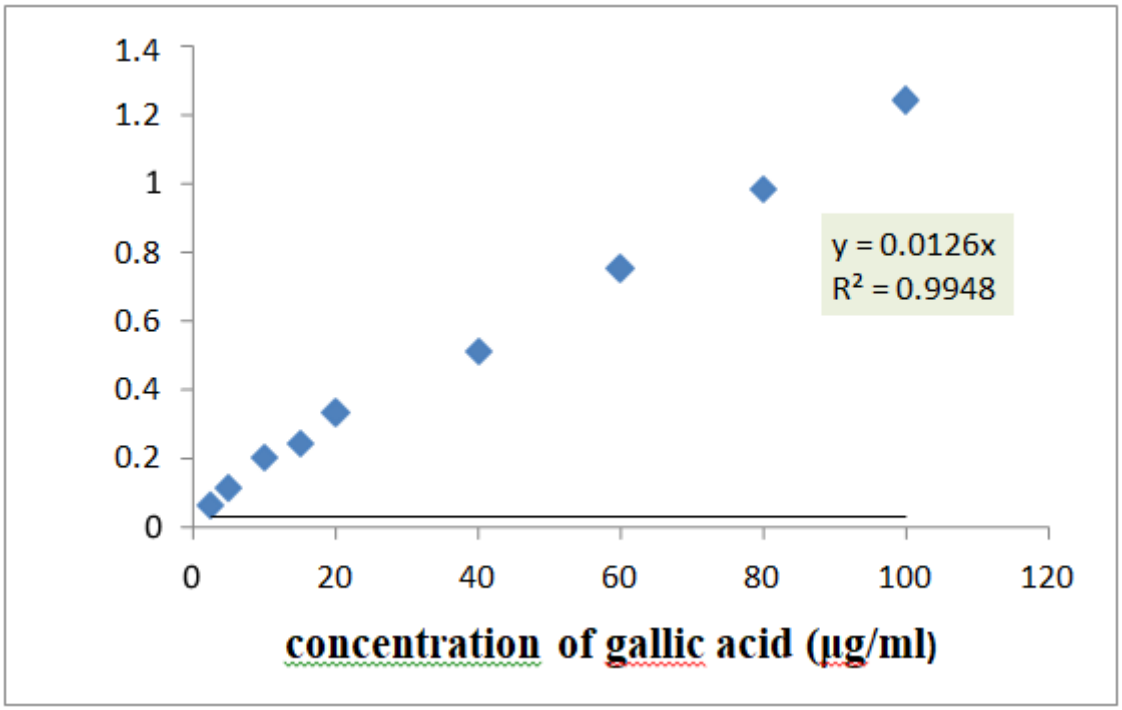

Fig.1: Standard curve for total phenolic content

3.4.2 Estimation of Tannin

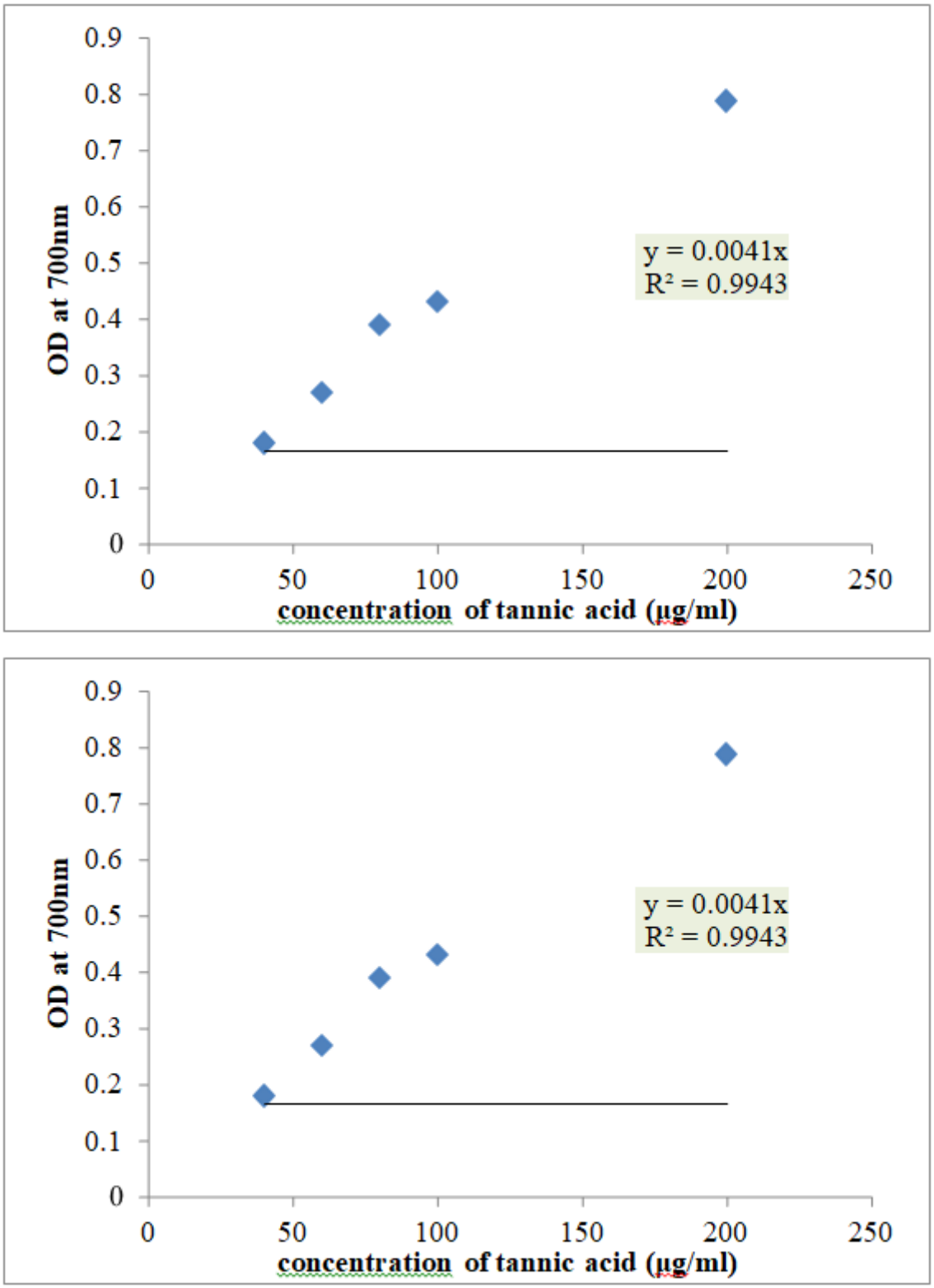

Fig.2: Standard curve for total tannin content

3.4.3 Estimation of total Flavonoid

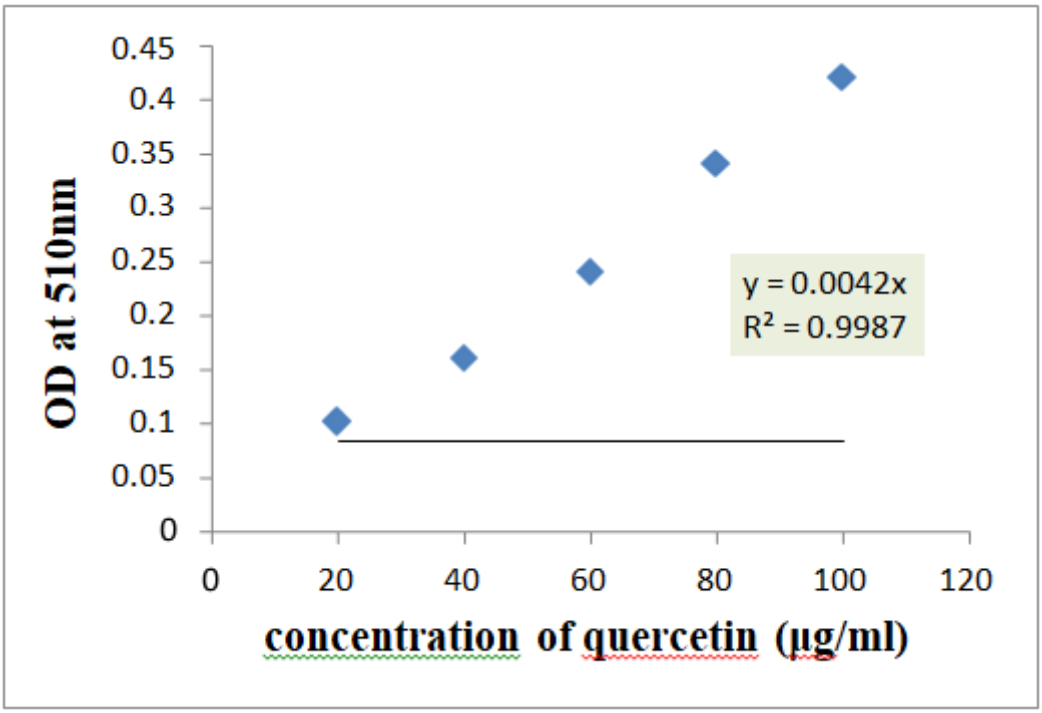

Fig.3: Standard curve for total flavonoid content

3.4.4 Estimation of Glycoside

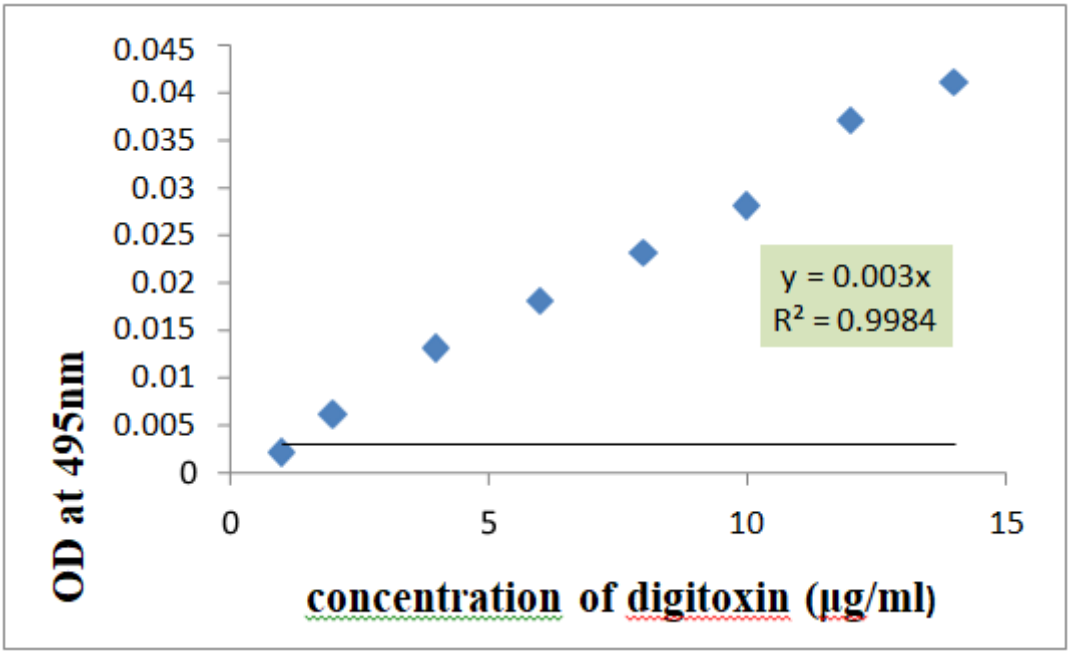

Fig.4: Standard curve for total glycoside

3.4.5 Estimation of Steroid

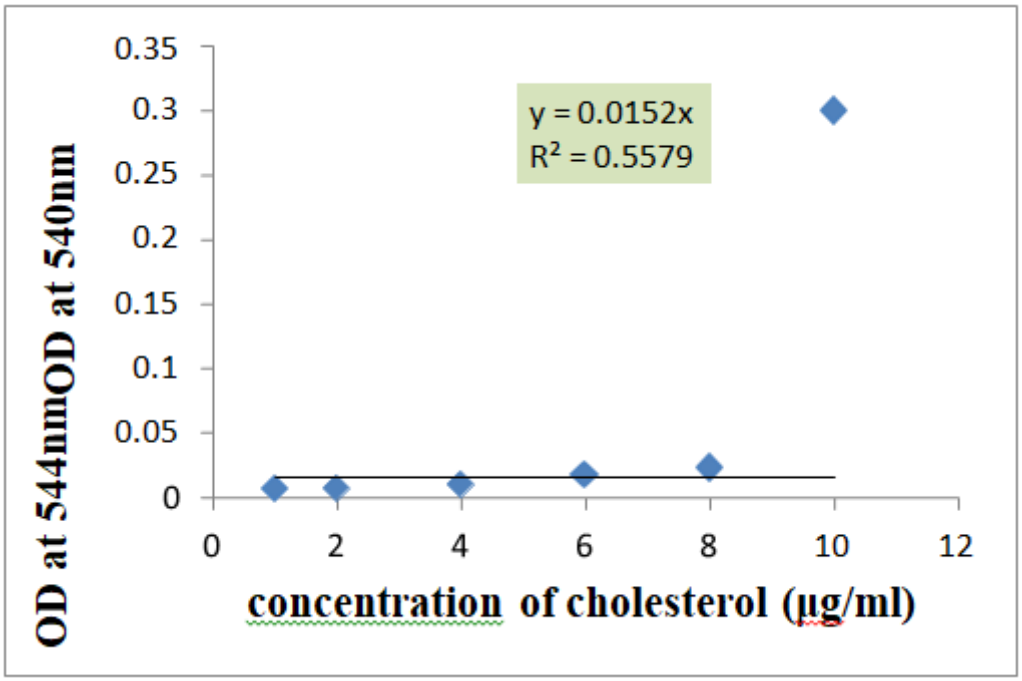

Fig.5 : Standard curve for total steroid content

3.4.6 Estimation of Terpenoid

Sl.No.	Sample Code	% terpenoids content
1	EC	0.014

Table 4 : Terpenoid content present in the crude extract EC

3.4.7 Estimation of Saponin

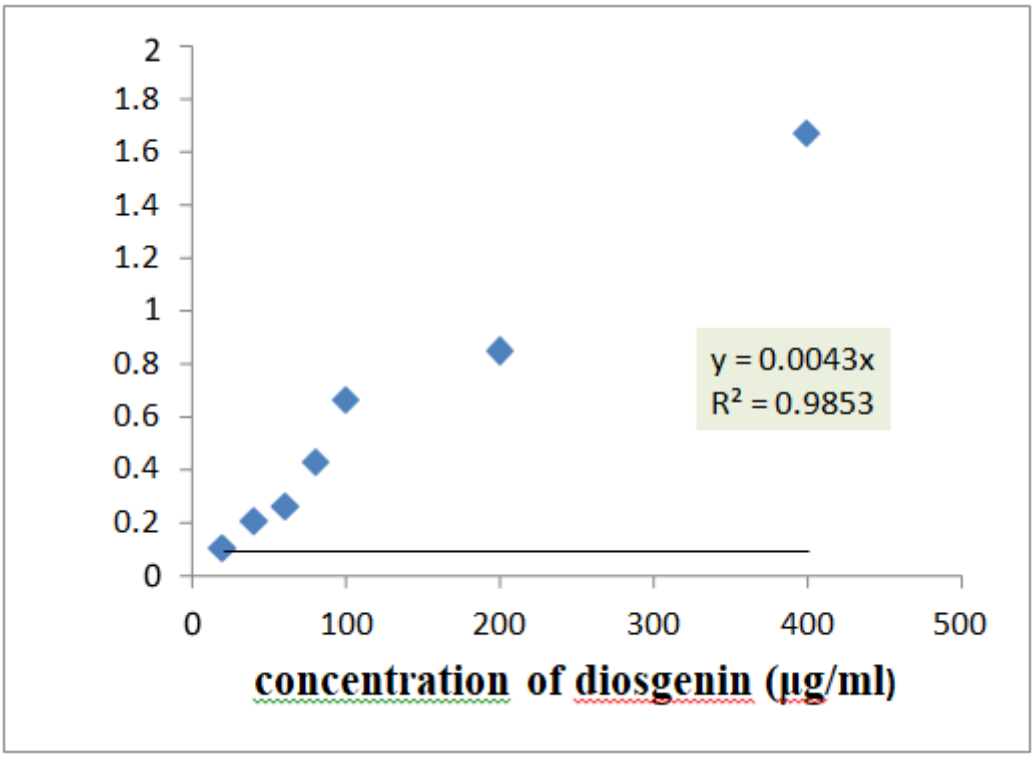

Fig.6 .Standard curve for total saponin content

3.4.8 Estimation of Alkaloids

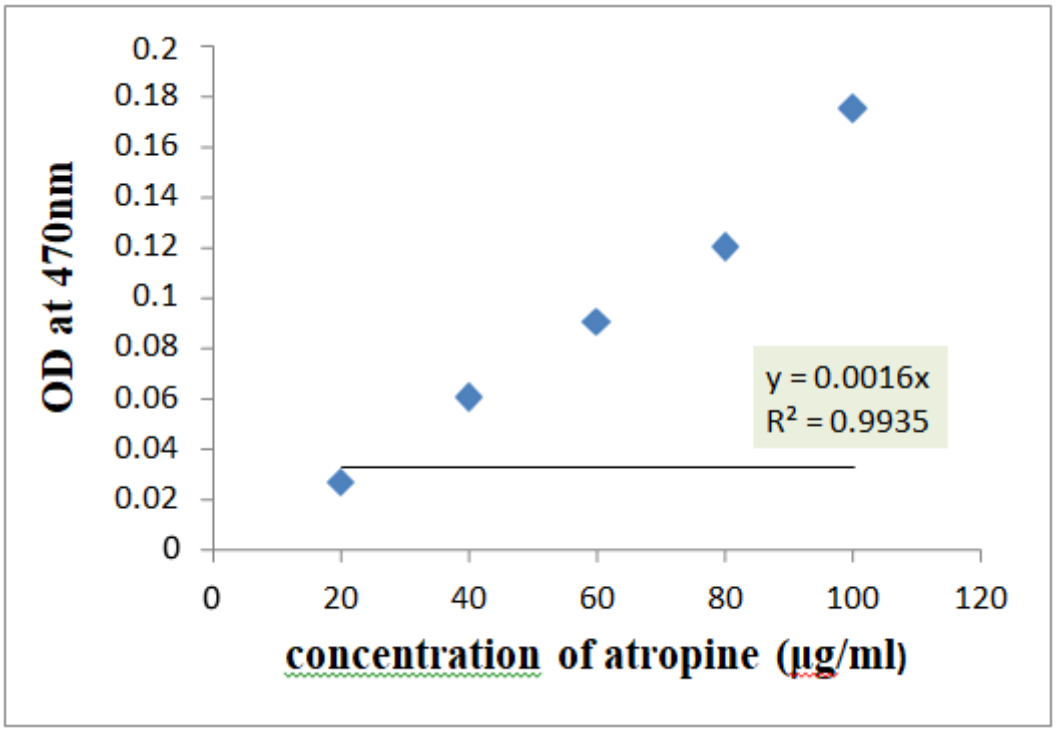

Fig.7: Standard curve for total alkaloid content

3.5 ABTS- RADICAL SCAVENGING ASSAY

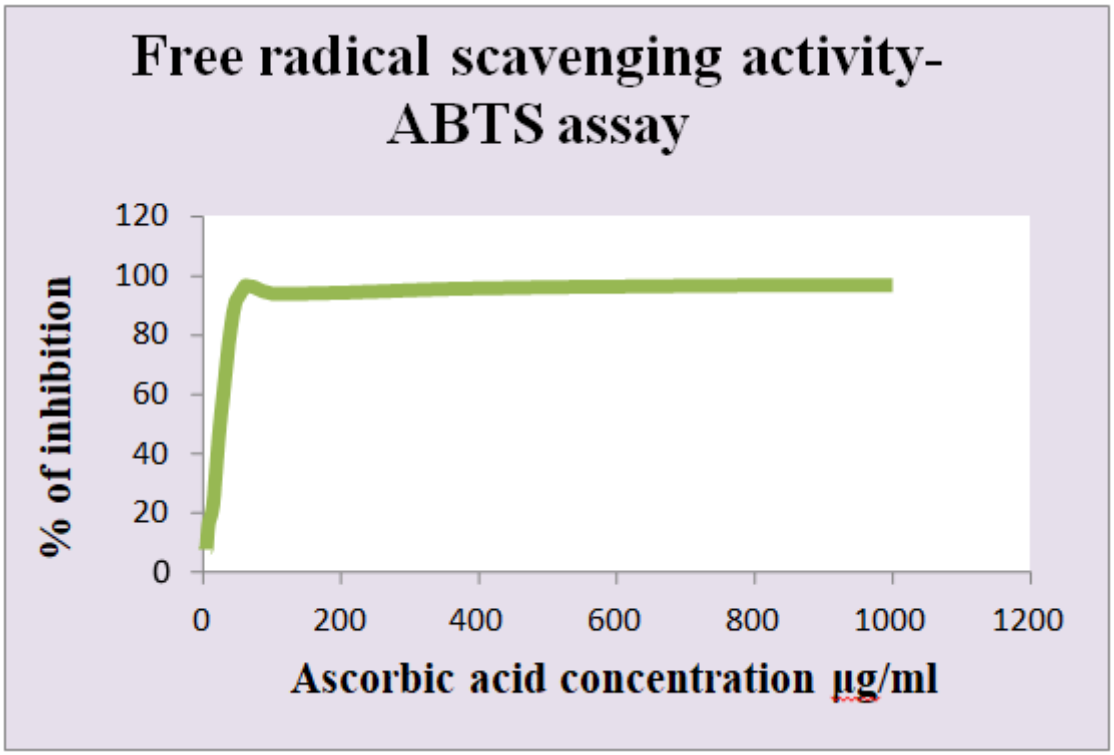

Fig 8. Standard curve for antioxidant activity of standard ascorbic acid

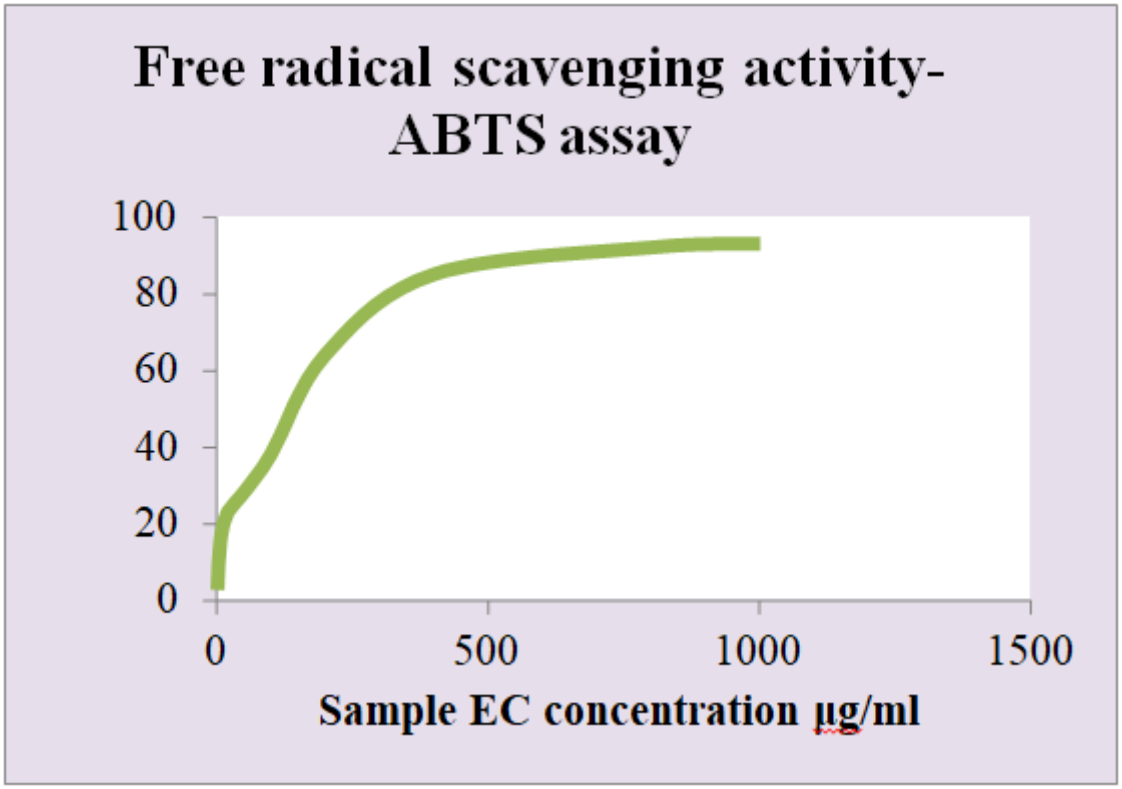

Fig.9: Standard curve for antioxidant activity of sample EC

In this present study the Free radical scavenging capacity of the standard ascorbic acid and extract EC was determined by ABTS assay. The % of inhibition was higher in dose dependent manner. The antioxidant

capacity of standard ascorbic acid was 97% at the highest concentration 1000 µg/ml (table5.19) and the antioxidant capacity of EC extract was 93% at the highest concentration 1000 µg/ml (fig 8 and 9).

In a study by Mahdavi et al (2017) reported high antioxidant activity in E. camuldulensis ethanolic extraction. Another study by Elham Taheri et al (2020) also reported the antioxidant activity of E. camuldulensis oil at different concentrations and was highest at 1000 µg/ml.

3.6 CYTOTOXICITY AND ANTICANCER SCREENING BY MTT ASSAY

The extract EC reduced the cell viability of HEK cell lines at different concentration for 24hr exposure. The result is summarized in table 6 and fig 10. Maximum reduction in cell viability was observed in HEK cancer cells administered with different concentrations of the sample EC .Therefore IC50 value was calculated for HEK cell line. The IC50 value was obtained as 87µg/mL of the sample. The effect of extract EC on L6 cells viability was determined to check the toxicity of EC on normal cells at various concentrations incubated for 24hrs by MTT assay (table 5). There is no much decrease in cell viability of L6 at different concentrations.

The anticancer and anti-proliferation effects of Eucalyptus essential oil on the Breast and Hepatocyte cancer cells was described in a study by Ashour H. M (2008) in which the oils extracted from both the Eucalyptus sideroxylon and Eucalyptus torquata does not exerted cytotoxic effects on HepG2 cells but showed cytotoxicity on Breast cancer cell line. The extraction of E. citriodora causes cancer cell death in colon, prostate, lung, cervix, ovary, neuroblastoma and liver cancer cell lines reported by Bhagat et al (2012). In another study by Elham Taheri et al (2020) reported an appropriate cytotoxic activity of E. camuldulensis on Colorectal cancer cell line Caco2 in a time and concentration dependent manner.

Table5: Percentage of viability exerted by the extract EC on the L6 cell line following 24hr exposure

Samples	**Triplicate1**	**Triplicate 2**	**Triplicate 3**	**Average**
Control	0.789	0.795	0.784	0.789
6.25	0.775	0.776	0.769	0.773
12.5	0.772	0.770	0.765	0.769
25	0.769	0.766	0.760	0.765
50	0.763	0.761	0.758	0.760
100	0.760	0.756	0.753	0.756
Concentration (µg/ml)	**Percentage of viability**			
6.25	97			
12.5	97			
25	96			
50	96			
100	95			

Table 6 .Half maximal inhibitory concentrations (IC50) of the extract EC in HEK cell line following 24hr exposure

Samples	**Triplicate1**	**Triplicate2**	**Triplicate3**	**Average**
Control	0.692	0.688	0.674	0.684
6.25	0.646	0.641	0.633	0.640
12.5	0.591	0.587	0.581	0.589
25	0.511	0.502	0.494	0.502
50	0.411	0.432	0.424	0.422

100	0.342	0.333	0.329	0.334
Concentration (µg/ml)	**Percentage of viability**			**IC 50**
6.25	93			
12.5	86			
25	73			
50	61			
100	48			**87**

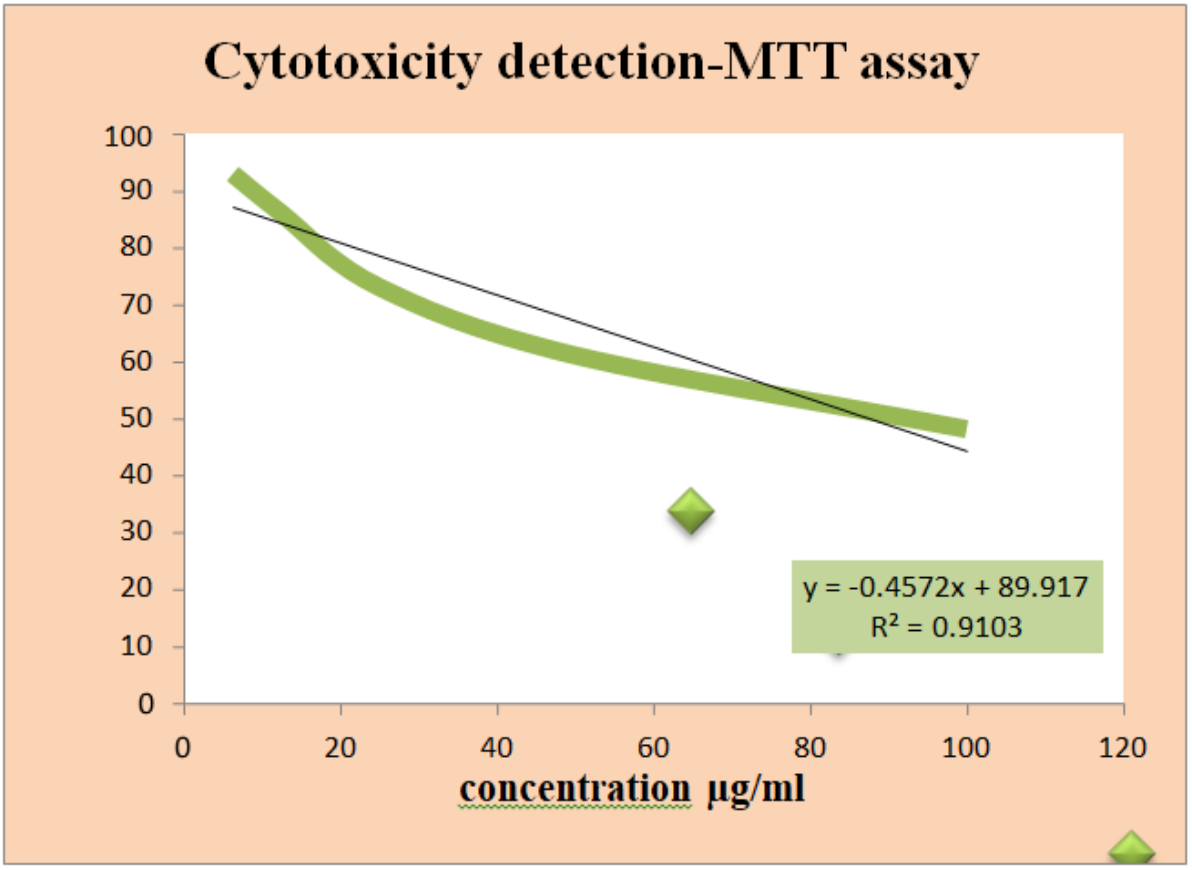

Fig. 10. Standard curve for cytotoxicity of EC on HEK cell line

Conclusion And Future Enhancement

Eucalyptus species have been used traditionally due to their antimicrobial, antifungal and anti-inflammatory properties. In the present study, it was observed that E. camuldulensis methanolic leaf extract has various phytochemicals and the extract exhibited antioxidant activity which indicates that the E. camuldulensis are rich in antioxidants.. Also this study has indicated the anticancer potential of E. camuldulensis leaf crude extract against Human Embryonic Kidney cancer cell line (HEK) which might be due to scavenge the free radicals in cancer cell lines. Kidney cancer is one of the most common cancers worldwide. Targeted therapies are proving to be important in treating kidney cancer and at the same time it causes many serious side effects to the patients. Therefore, the identification and isolation of the bioactive compounds with anticancer potential in Eucalyptus camuldulensis might prove a novel anticancer drug in future for the treatment of cancer such as kidney cancer.

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