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Exploring The Antiproliferative Mechanisms Of Cosmos Caudatus leaf Extracts In Lung Cancer Cell Lines

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Abstract: Objective: To determine the phytochemical constituents, antioxidant and anticancer activities of Cosmos caudatus leaf extracts on A549 and P 51 human lung cancer cell lines. **Methods:** Leaf sample was subjected to soxhlet extraction method and phytochemical screening was done using different biochemical tests. The antioxidant activity was tested using 2,2-diphenyl-1-picrylhydrazyl, ferric ion reducing power assay and 2,2-azino bis 3-ethyl benzothiazoline-b-sulfonic acid assay. In vitro anticancer activity on A549 and P 51 human lung cancer cell lines was evaluated by (3-(4,5-dimethyl thiazole-2yl)-2,5-diphenyl tetrazolium bromide) MTT assay. **Results:** Phytochemical screening confirmed the presence of phytoconstituents like tannins, flavanoids, alkaloids, saponins, glycosides and reducing sugar. The antioxidant assays exhibited significant inhibiting activity. MTT cell proliferation assay of methanolic extract of plant drug produced a cytotoxic effect on lung cancer cell lines A549 and P 51 with an IC50 value of 197.10 µg/ml, while it had no cytotoxic effect on normal cell lines. **Conclusion:** It has been reported the effect of flavanoid characteristics in reducing the cancer. C.Caudatus contains a wide variety of secondary metabolites that hold strong antioxidant capacity. Their leaf have found to be potential source of antioxidants and anticancer activity.

Keywords: Anticancer, Antioxidant, Phytochemical, Cosmoscaudatus, Lung cancer

1. INTRODUCTION

Lung cancer is one of the prominent causes of death to human being. At present it is one of the major reasons of fatality to both men and women worldwide [1]. It is the second most common type of cancer. The main risk factors of lung cancer include smoking, carbon inhalation, asbestos, environmental factors, lifestyle and diet. It was found that even after quitting smoking by the smokers the occurrence rate of lung cancer is not decelerate appropriately [2]. The diseased condition is detected only in the later stages after the

substantial progress of illness [3]. According to statistics from 2020 it is associated with distant metastasis in males and females. The development of malignant cells gradually declines the effective application of surgery, chemotherapy and radiotherapy [4]. The increased level of free radicals [5] has a great impact on the occurrence of cancer and serious effect on cellular protein, lipid and DNA instability at the genomic level. The role of oxidative stress in carcinogenesis has been reported [6]. The main purpose of the research using cancer cell lines is due to the substantial increase of lung cancer in recent years [7]. *Cosmos caudatus* is a commonly found weed of Rutaceae family, which widely distributed in India. It is also found in many other countries such as Ceylon, China, Malaysia, Australia, and countries in tropical Africa [1]. In Asian countries like India, medicine practitioners have been using *Cosmos caudatus* in homoeopathic systems of medicines [2-3]. Different parts of the plant possess valuable medicinal properties [4-6]. The plant is known for containing phenolics, flavonoids, and essential oils [7]. The plant has a strong odor of turpentine and is used as an antihelminthic and diuretic in indigenous medicine [8]. The plant is known for its several biological activities like astringent, stimulant, anthelmintic, antimicrobial, anti-inflammatory, and diuretic [9]. It exhibits a suppressive effect over the replication of HSV-1 and HSV-2 [10]. *Cosmos caudatus* is used in traditional medicine as an anticancer drug in combination with *Adhatodavasica* and *Achyranthus aspera* [11]. *C. Caudatus* demonstrates moderate to mild antileukemic activity against L1210, P3HR1, anti-K562, Raji, and U937 leukemia cells [10].

2. MATERIALS AND METHODS

2.1 Collection and authentication of plant part

C. Caudatus plant was collected from Medicinal and Aromatic Research Institute, Odakkali, Kerala. Voucher specimen was deposited in the Forest Research Institute, Peechi for botanical authentication. The dried, powdered leaf was extracted successively with different organic solvents such as Ethanol, Chloroform and Water in the increasing order of polarity using Soxhlet apparatus.

2.2 Preparation of plant materials

Plant sample was washed thoroughly and shade dried at room temperature. The sample was crushed into powder with mechanical grinding machine, which promote the effective binding of solvent with plant materials. The weight of the plant material was measured before loading in the Soxhlet apparatus and solvent is heated so that the steam passes through the plant materials and vapourising the volatile compounds. The vapour flows through a coil and condenses back to liquid and can be collected in the receiving vessel. The dried and ground plant was extracted with different solvents by Soxhlet extraction. It was then concentrated and dried under reduced pressure and controlled temperature at 40-50 °C using rotary evaporator.

2.3 Phytochemical analysis

Very little phytochemical work has been done on the genus *Macranga*. The crude powder of *C. Caudatus* was extracted using aqueous and different organic solvents to ensure obtaining polar and nonpolar constituents. Qualitatively tested for different phytochemical constituents, namely alkaloids, flavanoids, saponins, tannins, phenols, glycosides, terpenoids and reducing sugar by following the standard procedures [14].

2.4 Determination of antioxidant activity by using in vitro methods

ABTS assay

The total antioxidant activity of the samples was measured by ABTS radical cation decolourization assay according to the method of [15]. ABTS was produced by reacting 7 mmol ABTS aqueous solution with 2.4 mM potassium persulfate in the dark for 12–16 h at room temperature. The solution was diluted with ethanol (about 1:89 v/v) and equilibrated at 25 °C. About 1 ml of diluted ABTS solution was added to about 30 µl sample solution and 10 µl of Trolox (final concentration 0–15 µM) in ethanol. A test tube containing 1 ml of diluted ABTS solution and 30 µl of ethanol served as the negative control. The test tubes were vortexed well and incubated exactly for 30 min at room temperature. After incubation, the absorbance of samples and standards were measured at 734 nm against the ethanol blank. The results were expressed as the concentration of Trolox having equivalent antioxidant activity expressed as µM/g sample extracts.

2.5 Ferric ion reducing antioxidant power assay

Freshly prepared working FRAP reagent was pipetted using 1-5 ml variable micropipette (3.995 ml) and mixed with 5 µl of the appropriately diluted plant sample and mixed thoroughly. An intense blue color complex was formed when the ferric tripyridyl triazine (Fe³⁺+TPTZ) complex was reduced to ferrous (Fe²⁺) form and the absorbance at 593 nm was recorded against a reagent blank after 30 min incubation at 37° [16]. All the determinations were performed in triplicates. The FRAP values were obtained by comparing the absorbance change in the test mixture with those obtained from increasing concentrations of Fe³⁺ and expressed as mg of Trolox equivalent per gram of sample.

2.6 Diphenyl-1-picrylhydrazil assay

To different volume of extract, 0.5 ml of methanolic solution of DPPH was added and made up to 2 ml using methanol. The mixture was allowed to react at room temperature for 30 min. Methanol served as the blank and a tube without the extracts served as the positive control [17]. After 30 min of incubation, the discoloration of the purple colour was measured at 518 nm in a spectrophotometer. The assay was calculated as:

$$\text{Radical scavenging activity} = \frac{\text{Control}-\text{Test}}{\text{Control}} \times 100$$

DPPH scavenging effect (%) or Percent inhibition = $A_0 - A_1 / A_0 \times 100$.

Where A₀ was the Absorbance of the control reaction and A₁ was the Absorbance in the presence of test or standard sample [18]. The results were analysed in triplicate [19]. The IC₅₀ value is the concentration of sample required to inhibit 50% of the DPPH free radical.

2.7 Culturing of cell lines

Human lung cancer cell line, A549 (Passage number 51), was procured from National Centre for Cell Sciences, Pune, India and maintained in RPMI-1640 media, supplemented with 10% fetal bovine serum, 100 U/ml penicillin and 100 µg/ml streptomycin and kept at 37 °C in an incubator with 5% CO₂. The cells were passaged at 80-90% confluency and medium was changed every third day. Test group: cells+Methanol extract.

2.8 MTT cell viability assays

Cytotoxicity of the drugs was estimated by MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide) assay [20]. Approximately 1x10⁵/ml cells were seeded in a 24 well plate, with complete growth medium (RPMI-1640) and allowed to attach. At 60% confluency, the medium was replaced with fresh media and different concentrations of drugs

(MO-I and MO-II) were added to each well (0-200 µg/ml). The cells were further incubated for 48h and at the end of the incubation period, the medium was again replaced with fresh medium. Then 50 µl of 0.5% MTT were added to each well and incubated for 4 h. The formazan crystals formed were dissolved in dimethyl sulfoxide and the absorbance was measured at 570 nm in UV/VIS spectrophotometer (Systronics, India) and percentage viability was calculated.

2.9 Statistical analysis

The result was expressed as mean standard deviation. Descriptive statistics was used to analyse the mean, standard deviation, variation and level of statistical significance between groups. When $p < 0.05$ and $p < 0.01$, it was considered statistically significant for analysis for analysis of percent inhibition of cell growth.

3. RESULTS

Total yield of crude extract

The total percentage yield of crude extract from *C. Caudatus* leaves by using solvents, namely ethanol, chloroform and water, were 8.32%, 7.25%, 8.91% (weight/weight)x100, respectively, with reference to the air-dried plant material.

Phytochemical analysis

Preliminary screening of *C. Caudatus* extracts (ethanol, chloroform and aqueous) showed the presence of a diversity of phytochemical constituents. Reducing sugar detected in ethanol, chloroform and aqueous extracts, presence of protein in ethanol and aqueous extract, presence of amino acid in ethanol, chloroform and aqueous extract, presence of phenol, tannin and flavanoids in all three solvents, the presence of alkaloids were detected in ethanol and chloroform, the presence of saponins in all the three solvents, the absence of phytosterols in ethanol, chloroform and aqueous extract, the presence of glycosides in ethanol and chloroform and similarly flavanoids and glycosides were present in ethanol and chloroform (table 1).

Table 1: Preliminary phytochemical screening of *Cosmoscaudatus* leaves extracts

S. No.	Phytochemical	Test	Ethanol	Chloroform	Water
1.	Carbohydrates	Molish's	++	++	++
2.	Proteins	Biuret	+		+
3.	Phenol	Ferric chloride test	++	++	+
4.	Tannins	Potassium Hydroxide test	++	++	+
5.	Flavanoids	Alkaline reagent	++	-	++
6.	Alkaloids	Hager's	-	-	-
7.	Saponins	Frothing	++	+	+
8.	Phytosterols	Liebermann and Burchard's	-	-	-
9.	Glycosides	Borntrager's test	++	+	+
10.	Flavanol glycosides	Magnesium and hydrochloride acid	+	+	+
11.	Cardiac glycosides	Keller Killiani	+	+	+

-, Absent; +, moderately present; ++, high presence.

Antioxidant assays

ABTS assay

In the present study chloroform, ethanol and water were subjected to ABTS assay along with standard trolox and ethanol extract showed the highest activity among chloroform and aqueous extract.

The ethanolic extract of *Cosmos caudatus* exhibited effective antioxidant activity and the inhibition was found to be concentration-dependent (fig. 1).

Sample	Absorbance (approx.)
Chloroform	1200
Ethanol	4200
Water	2200
Trolox	9200

Tab. 1: ABTS assay for extracts of *C.Caudatus*.

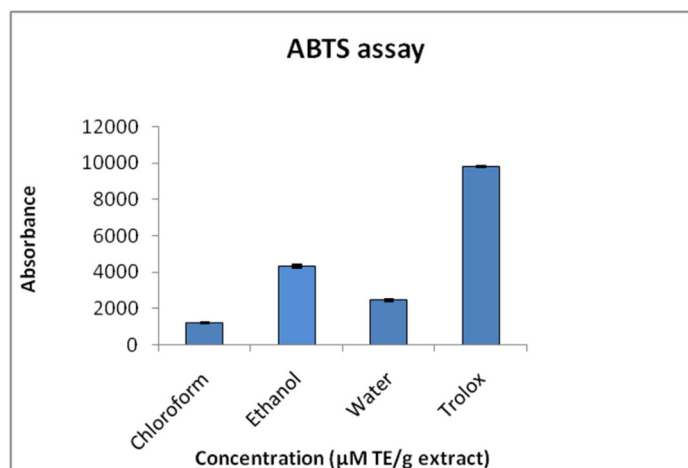


Fig. 1: ABTS assay for extracts of *C.Caudatus*. Data is present as mean standard error of the mean (n=3). Statistical significance was assessed using one-way as compared with standard.*p<0.05. (Standard Used-Trolox Equivalents (TE))

FRAP assay

In this assay with leaf extract, the presence of antioxidants in the sample would results in the reduction of ferricyanide Fe^{3+} to ferrocyanide Fe^{2+} by donating electron. Methanol extract were subjected to FRAP assay along with standard Trolox. In the result obtained, ethanol extract showed higher activity than chloroform and aqueous, which was comparable to standard Trolox (fig. 2).

Sample	Absorbance (approx.)
Chloroform	15
Ethanol	38
Water	27
Trolox	70

Tab. 2: FRAP assay for extracts of *C.Caudatus*.

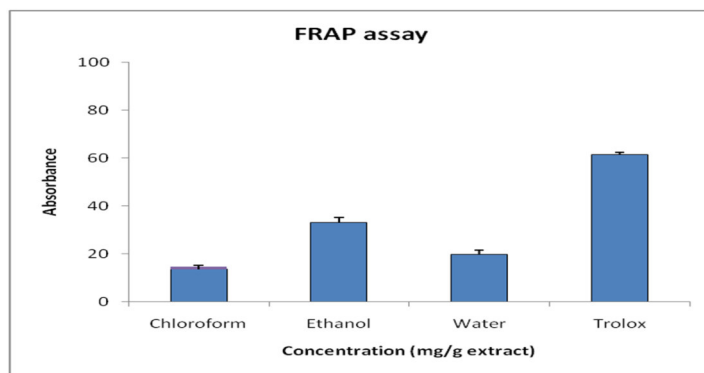


Fig. 2: FRAP assay for extracts of C.Caudatus. Data is present as mean standard error of the mean (n=3). Statistical significance was assessed using one-way as compared with standard.*p<0.05

DPPH assay

In the present study, different concentration of methanol of leaves of *C.Caudatus* were subjected to DPPH free radical scavenging assay.

The antioxidant capacity of the extract was compared with ascorbic acid as the standard antioxidant. It was detected as the concentration of the leaf extracts increases the percentage of inhibition also increases (fig. 3).

Concentration (µl)	% Inhibition (approx.)
50	70
100	80
150	90

Tab. 3: DPPH assay for extracts of C.Caudatus.

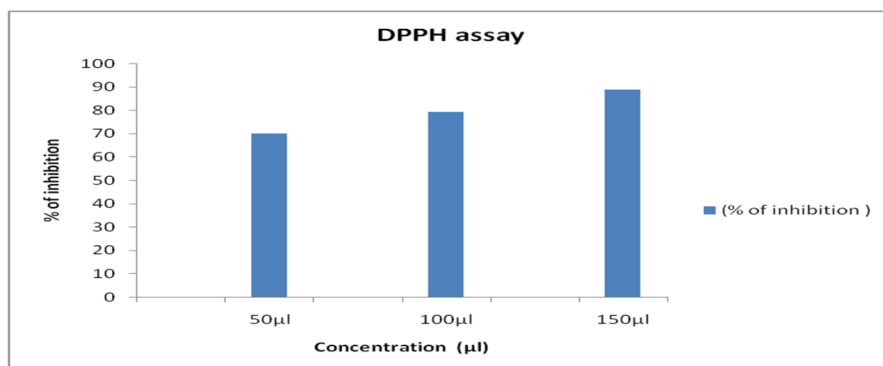


Fig. 3: DPPH assay for extracts of C.Caudatus. Data is present as % of inhibition at 3 different concentration in µl Effect of C.Caudatus methanol leaf extract on A549 and passage no. 51 lung cancer cell lines

The result of MTT assays revealed that the methanol extract of *C.Caudatus* leaves induce cytotoxicity towards cancer cell lines A549 and passage no. 51. It was found that as the concentration of extract increases the percentage of cell death also increases. The IC 50 values of extract at different intervals are represented. The anticancer activity of the plant is evaluated for safe treatment. The MTT assay of the methanolic extracts of the leaves are carried out using A549 and passage no. 51. The present study demonstrated the cytotoxic action of the extract of *C.Caudatus*, percentage of inhibition at 734 nm using cell lines. Concentration required to produce inhibition of viability of 50% cell were calculated. Among these five concentrations, 200 µg/ml of methanol extract was most effective in producing

percentage growth inhibition (fig. 4: A). The correlation graph of real-time measurement of IC 50 values at various intervals is shown in (fig. 4: B, C, D). Morphology of the cell exposed to drug at different concentrations and effective anticancer activity of the extract is determined (fig. 4: E).

Concentration (X)	% Cell Death (Y)
0	0
20	8
40	15
80	25
120	35
200	55

Tab. 4: A) Cytotoxicity and % of cell death on *C.Caudatus* leaf extract

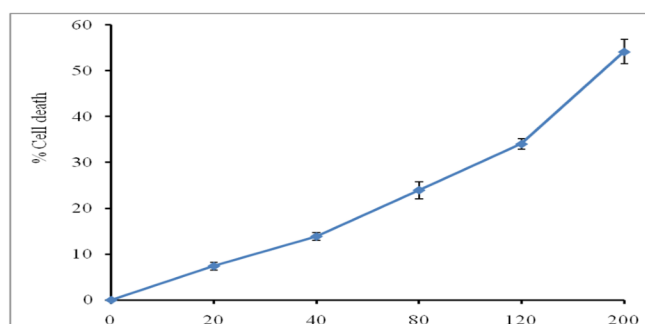


Fig. 4: A) Cytotoxicity and % of cell death on *C.Caudatus* leaf extract

Concentration of extract (µg/ml)	% Cell Death
0	0
25	8
50	14
80	25
120	33
200	53

Tab. 4: B) Cytotoxicity and % of cell death on *C.Caudatus* leaf extract

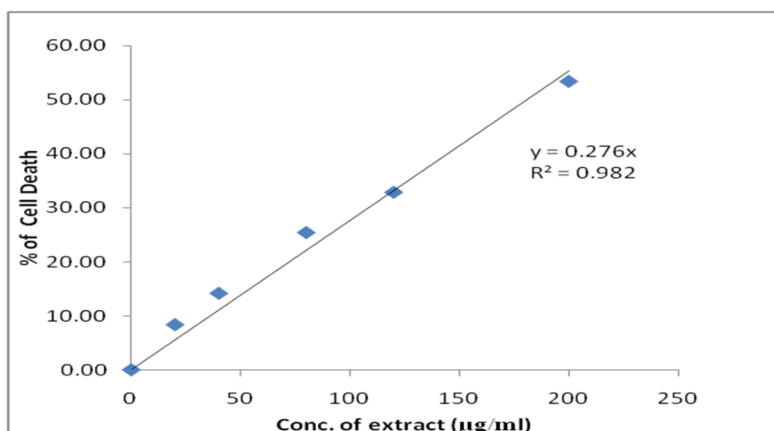


Fig. 4: B) Correlation between real-time measurements of IC 50 values using the RTCA system. The average IC 50 values of lung cancer cell lines A549 and passage no. 51 at 24h, after treatment with *C.Caudatus* leaf extract. RTCA–real-time analysis

Concentration of extract (µg/ml)	% Cell Death
0	0
20	7
40	13
80	22
120	36
200	59

Tab. 4: C) Cytotoxicity and % of cell death on *C.Caudatus* leaf extract

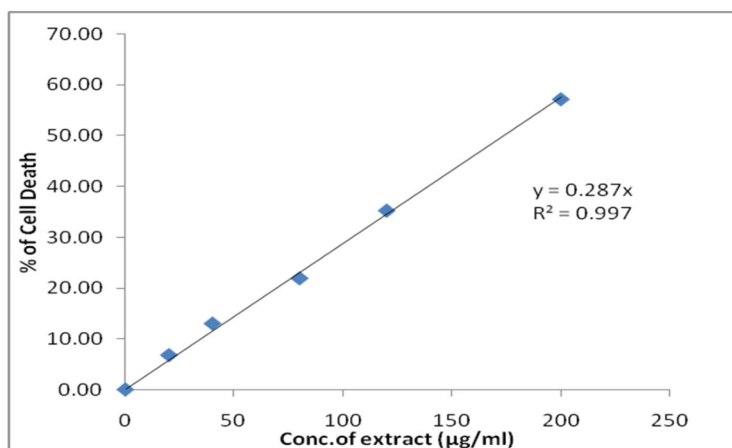


Fig. 4: C) Correlation between real-time measurements of IC 50 values using the RTCA system. The average IC 50 values of lung cancer cell lines A549 and passage no. 51 at 48h, after treatment with *C.Caudatus* leaf extract. RTCA–real-time analysis

Concentration of extract (µg/ml)	% Cell Death
0	0
20	7
40	15
80	25
120	35
200	54

Tab. 4: D) Cytotoxicity and % of cell death on *C.Caudatus* leaf extract

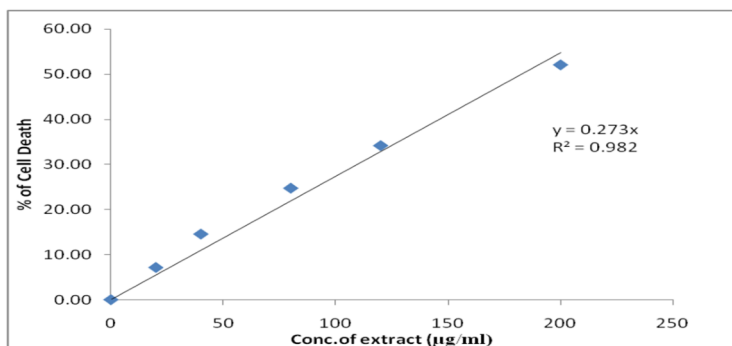


Fig. 4: D) Correlation between real time measurements of IC 50 values using the RTCA system. The average IC 50 values of lung cancer cell lines A549 and passage no. 51 at 72h after treatment with *C.Caudatus* leaf extract. RTCA–real time analysis

Morphology of Cells exposed to drug at different concentrations

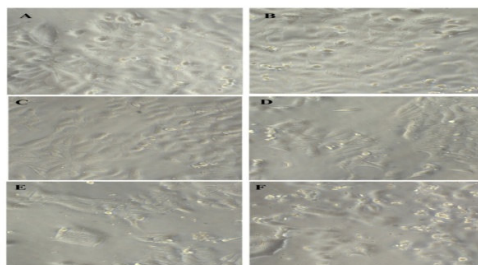


Fig. 4: E). A-control, B-F low to high doses (20, 40, 80, 120, and 200 ug/ml)

4. DISCUSSION

In the recent years herbal medicine has been used widely in cancer treatment due to their varied phytometabolic contents and multiple biological characteristics [21]. The plant collected from Odakkali Research Institute was identified according to their taxonomical characters as *C.Caudatus* and analysed for the presence of secondary metabolites in selected extracts of the plant. The phytochemical constituents of the leaf greatly contribute to the medicinal value of plants [22]. The diverse and broad range of phytochemical with potent antioxidant activity is a promising source for drug synthesis [23]. Phytochemical constituents were detected by different biochemical tests. Phenol, tannin and flavanoids present in high level in ethanol, chloroform and aqueous (table 1). The antioxidant property of the extract is an important factor to the plants for the management and treatment of various diseases. The oxidative stress caused by free radical's damage cells and biomolecules. The medicinally important antioxidant-inducing plant trigger the free radicals and inhibit their oxidation reactions [24]. Many secondary metabolites like phenols, polyphenols and flavanoids are sources of antioxidants and have scavenging activity [25]. Reactive oxygen species have fast reactions and oxidize biomolecules like carbohydrates, lipids, proteins and instability of DNA causes the damage of cells tissues and organs leading to progression of cancer [26]. In the present work, three methods were used to evaluate the total antioxidant capacity of methanol extracts (ABTS, FRAP and DPPH). The antioxidant activity of *C.Caudatus* was measured by method of ABTS radical cation decolourisation assay. Total antioxidant activity was assessed by measuring the reduction of ABTS radical cation. The effect of various concentrations of extract on ABTS-free radical is shown in fig. 1. The ethanolic extract of *C.Caudatus* exhibited effective antioxidant activity and the inhibition was found to be concentration dependent. The ferric ion reducing antioxidant power assay of extract may serve as a significant indicator of its potential antioxidant activity. The antioxidants break the free radical chain reaction by donating hydrogen molecules [27]. The presence of antioxidants in the extract would result in the reduction of ferricyanide Fe^{3+} to ferrocyanide Fe^{2+} by donating an electron which was measured spectrophotometrically at 700 nm. In the assay the yellow colour of the test solution gradually changes to other shades of green and blue depending upon the reducing power of the plant extract. It serves as a significant indicator of effective antioxidant activity. Hence the result indicates the methanolic extract of *C.Caudatus* may have a high amount of antioxidant properties which was comparable to the standard used (fig. 2). The decrease in the absorbance of DPPH as a result of reaction between antioxidant molecules and radicals that leads to the scavenging of the radicals by hydrogen donation. Similar results are reported by authors. In the present study the antioxidant activity of *C.Caudatus* was at three different concentration evaluated using methanol extract of the

plant and was compared with standard ascorbic acid. The experimental data revealed the properties of scavenging of free radicals with methanol extracts showing antioxidant capacity.

The anticancer activity of the plant is evaluated for the safe treatment. The MTT assay of the methanolic extracts of the leaves are carried out using A549 and passage no. 51. The present study demonstrated the cytotoxic action of the extract of *C.Caudatus* percentage of inhibition at 734 nm using lung cancer cell lines. Concentration required to produce inhibition of viability of 50% cell were calculated. Among these five concentrations 200 µg/ml of methanol extract was most effective in producing percentage growth inhibition.

5. CONCLUSION

In conclusion, the result from the study support the view that *C.Caudatus* are promising source of natural antioxidants. The specific phytochemicals of *C.Caudatus* has a great influence on reducing the risk of cancer. It has been reported the effect of flavanoid characteristics in reducing the cancer. *C.Caudatus* contains a wide variety of secondary metabolites that hold strong antioxidant capacity. Their leaf has found to be a potential source of antioxidants and anticancer activity.

5.1 AUTHORS CONTRIBUTIONS

All the authors have contributed equally.

CONFLICT OF INETRESTS

The authors declare that there are no conflicts of interest.

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