Annals of R.S.C.B., ISSN:1583-6258, Vol. 25, Issue 4, 2021, Pages. 9410 - 9421
Received 05 March 2021; Accepted 01 April 2021.

Invitro Antioxidant and Antiproliferative Effect of Ethanolic Leaf Extract of *Trianthemadecandra* on Hep2 Cancer Cell Lines

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Abstract

To analyze the antioxidant and antiproliferative potential of *Trianthemadecandra*was evaluated. Free radical scavenging potential of the plant was studied by various *in vitro* assays such as DPPH, ßCarotene and Frap assay. The antiproliferative activity of *Trianthemadecandra*was analysed by MTT assay. The ethanol extract is more powerful in scavenging free radicals. The preliminary phytochemical screening of *Trianthemadecandra*revealed the presence of phenolics, carbohydrates alkaloids, flavonoids and tannins in high amount. The antiproliferative potential of the ethanol extract was studied on Hep2 cell lines by MTT assay. The extract had an IC50 value of100µg/mL which showed cell viability of 22.67%. From the result, it is clear that ethanol extract of *Trianthemadecandra*has cytotoxic effect on Hep2 cell lines. Thus, the study revealed that *Trianthemadecandra*could be considered as a significant source of antioxidant and antiproliferative agents.

Keywords: Antioxidant, antiproliferative, *Trianthemadecandra*, DPPH assay, Hep2 cell line.

Introduction

The human system creates reactive oxygen species (ROS), such as superoxide anion radical, hydroxyl radical, and hydrogen peroxide by many enzymatic systems through oxygen consumption (Dina *et al*., 2009). In small amounts, these ROS can be beneficial as signal transducers and growth regulators (Hancock*et al*., 2001). However, during oxidative stress, large amounts of these ROS may favor some human disease conditions such as cancer, hepatic diseases, cardiovascular diseases, ageing, and neurodegenerative diseases (Bagchi*et al*., 2000). Hence, certain amounts of exogenous antioxidants are constantly required to maintained adequate level of antioxidants in order to balance the ROS. Antioxidants are compounds that inhibit or delay oxidation of other molecules by terminating initiation or propagation of oxidizing chain reactions (Lindenschmidt*et al*., 1986).A great number of spices and aromatic herbs contain chemical compounds exhibiting antioxidant properties (Madsen and Bertelsen, 1995). These properties are attributed to a variety of active phytochemicals including vitamins,

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carotenoids,terpenoids,alkaloids, flavonoids, lignans, simple phenols and phenolic acids, etc(Lu, 2000).

Cancer is a complex multifactorial cell disease characterized by abnormal cellular proliferation. Cancer development and progression are dependent on the cellular accumulation of various genetic and epigenetic events (Giri*et al*., 2006 and Mbaveng*et al*., 2011), and is an aberrant net accumulation of typical cells arising from excess proliferation, insufficient apoptosis, or a combination thereof (Abdul *et al*., 2009). Cancer development is normally caused by oncogene, tumor suppressor gene, and microRNA gene alterations (Burstein and Schwartz, 2008). It imposes a serious burden on the public health system, and its treatment and cure are scientifically challenging. Cancer is expected to claim nine million lives worldwide by 2015 (Rajesh *et al*., 2011). Medicinal plants have a long history in both traditional and modern cancer treatments (Confort*iet al*., 2008). Hence, it is possible that traditional medicinal plants can serve as potential sources for developing new drugs and more effective anti-cancer agents for future therapy.

. It is used as a tonic and diuretic in hepatic and spleen enlargement. It is also used in catarrhal jaundice and for skin diseases (Dalal*et al*., 2010). It is commonly used in viral hepatitis to promote bile flow and protect the parenchyma (Thakur and Megni, 2005) and popularly used to enhance memory and learning (Jadav, 2009). In view of the reputed efficacies of this plant,this present study investigates its phytochemical constituents in an attempt to establish its most active form and antioxidant and anti proliferative potential of *Trianthemadecandra*was evaluated.

Materials and Methods

Collection of plant material

The leaves of *Trianthemadecandra*were collected from wild and grown in controlled environment in the herbal garden. Leaves were collected from these plants. The collected plant parts were cleaned and shade-dried for 7 d and powdered. Twenty gram of each powdered samples were extracted with 200 mL of methanol for 1 min using ultra turax mixer(13, 000rpm) and soakedovernight at room temperature. The sample was then filtered through Whatman No. 1 paper in a Buchner funnel. The filtered solution was evaporated under vaccum in a rota-vator at 40ºC to a constant weight and dissolved in methanol solution. The solution was stored at 18ºC until use.

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Phytochemical evaluation

Phytochemical screening is to identify the presence of phyto constituents such as alkaloids, flavonoids, saponins, tannins, phenols, glycosides and steroids .

Estimation of total free phenolics

Total phenolic constituents of plant extracts were estimated by Folin-Ciocalteau's method using Folin-Ciocalteu reagent. The estimation was done spectrometrically at 760 nm and the results were expressed as gallic acid equivalents (GAE).

Estimation of total flavonoids

Aluminium chloride method was employed to quantify the total flavonoid content in the plant extracts. The results were expressed as quercetin equivalents (QE).

Estimation of total alkaloids

Total alkaloid content of the plant extracts was determined. Five gram of the sample was filtered and concentrated to one quarter of the original volume on a water bath after treatment with 200 mL of 10% acetic acid in ethanol. Concentrated NH_4OH was added drop wise to the extract until theprecipitation was complete. The whole solution was allowed to settle and the precipitate was collected and washed with dilute NH_4OH, filtered and weighed.

Estimation of total saponins

Powdered sample (20 g) was treated with 100 mL of 20% aqueous ethanol, heated over a hot water bath for 4 h at about 55°C with continuous stirring. The mixture was filtered and the residue re extracted. The combined extracts were reduced to 40 mL over water bath at about 90°C and the concentrate was transferred into a separating funnel and 20 mL of diethyl ether was added and shaken vigorously. The aqueous layer was recovered while the ether layer was discarded. The purification process was repeated and 60 mL of n-butanol was added to the combined extracts and washed twice with 10 mL of 5% aqueous NaCl. The remaining solution was heated in a water bath, dried in an oven to a constant weight and the saponin content was calculated as percentage.

Thin layer chromatography

Preliminary identification of phytochemicals was made by thin layer chromatography (TLC) using silica gel plates (5gm of silica gel dissolved with 90ml of water). The extracts were eluted with chloroform: methanol: water (30:20:4) and the chromatogram was developed by

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spraying with methanol: sulphuric acid (1:1) and heating to 110ºC. Then Rf value was calculated as the ratio of distance travelled by the solute to the distance travelled by the solvent.

Free radical scavenging activity on DPPH

Antioxidant activity of the extracts was determined in terms of hydrogen donating or radical scavenging ability using the stable radical DPPH according to (Eloff *et al*.,1998). Sample extracts at various concentrations was taken and the volumewas adjusted to 100 µL with ethanol. Ethanolic solution (5 mL) of DPPH (0.1 mM) was added and shaken vigorously. The tubes were allowed to stand for 20 min at 27ºC. The absorbance of the sample was measured at 517 nm. Radical scavenging activity was expressed as the inhibition percentage of free radical by the sample and was calculated using the formula:

% DPPH activity = (Control OD–Sample OD/Control OD) × 100.

Determination of antioxidant activity - β-carotene assay

The antioxidant capacity was estimated by thermally induced β-carotene bleaching assay was evaluated according to (Chellaram*et al*., 2011). A solution of β - carotene was prepared by dissolving 2 mg of β - carotene in 10 ml of chloroform. 2 ml of this solution was pipetted into 100mlconical flask after chloroform was removed under vacuum. 40 mg ofpurified linoleic acid, 400 mg of tween-40 emulsifier and 100 ml of aerated distilled water were added and shaken vigorously. 4.8 mlofthis emulsion were added to test tubes containing 200µl of ethanolic leaf extract of BHT was used a control for comparison. As soon as the emulsion was added to each tube, zero time absorbance was measured on UV-VIS spectrophotometer at 470nm. The tubes were then placed in water bath at 50ºC and the measurement of absorbance was continued until the colour of β- carotene disappeared in the control tube which was devoid of the sample. A blank of β- carotene was also prepared for background correction.

1- (Initial absorbance of the sample - Final absorbance of the sample)X100

Ferric reducing antioxidant power (FRAP Assay)

The total antioxidant potential of the sample determined by means of ferric reducing ability (FRAP) assay. FRAP solution (3.6 mL) add to distilled water (0.4 mL) and incubated at 37˚C for 5 min. Then this solution mixed with certain concentration of the leaf extract (0.5mL) and incubated at 37˚C for 10 min. The absorbance of the reaction mixture was measured at 593 nm. For construction of the calibrationcurve, five concentrations of FeSO4, 7H2O (0.4, 0.8, 1.2,

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1.6, 2.0 mM) were used and the absorbance values were measured as for sample solutions. Qualitative and quantitative phytochemical screening*:* The extractswere subjected to preliminary

Anticancer activity

In oncology research and clinical practice many anti-proliferative assays are used in the assessment of cancer types of individual patients. Methylthiazolyldiphenyl-tetrazolium bromide (MTT) assay has been described as rapid, simple and reproducible method,widely used in the screening anticancer drugs and to measure the tumor cell proliferation.

Result and Discussion

Qualitative phytochemical analysis

The preliminary phytochemical screening of *Trianthemadecandra*revealed the presence of phenolics, carbohydrates, alkaloids,flavonoids, sopanins, tannins and steroids, in high amounts followedby glycosides, amino acids and proteins. (Table 1)

Table 1: Phytochemical estimation.

Compound	**Result**
Alkaloids	+
Carbohydrates	+
Glycosides	+
Saponins	+
Proteins and amino acids	+
Phenolic compounds	+
Steroids	+
Flavonoids	+
Tannins	+

Quantitative phytochemical analysis*:*

The major phytochemicals present in the selected plant extracts were phenols, flavonoids, alkaloids and tannins were quantified. According to the results, suggested with the antioxidant activity of each extract tested (Harborne 1984). In general, the ethanolic extracts showed higher content of phenols and flavonoids, and likewise higher antioxidant activity. The results of total phenol content, alkaloids, saponins and flavonoids are given in (Table 2).

Table2: Quantitative phytochemical estimation.

Bioactive compound	**Result**
Total phenols	259.63 µg GAE/g sample
Alkaloids	1.44 mg/g sample
Saponins	0.116 mg/g sample
Tanins	1.42mg/g dry weight
Flavonoids	64.2µg Quercetin equivalent/g

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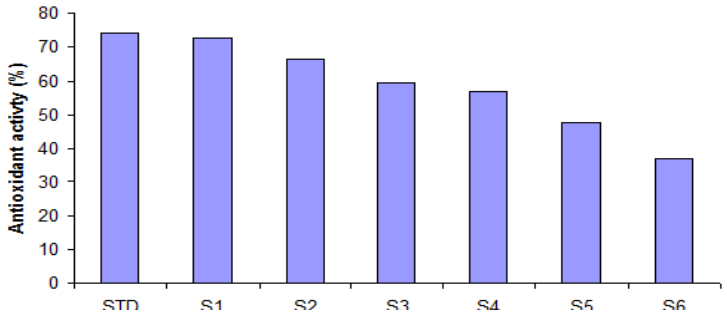
Thin layer chromatography

The chromatogram developed with 10% methanol in chloroform revealed the presence of five major compounds at Rf value of 0.34; 0.48; 0.57; 0.78; 0.87 as visualized under iodine vapour and UV illumination.

Radical scavenging activity of leaf extracts

The DPPH radical is widely used to evaluate the free-radical scavenging capacity of antioxidants. From the dose dependent response curve, DPPH radical scavenging activity of different plant extracts of *Trianthemadecandra*was observed. The scavenging activity of ethanol extract reached 98%. The ethanol extract of *Trianthemadecandra*showed good antioxidant and free radical scavenging activity (Figure. 1). Being a stable free radical, the DPPH assay is a simple and rapid method frequently used to evaluate the ability of antioxidants to scavenge free radicals. It gives reliable information concerning the antioxidant ability of the tested compounds to act as free radical scavengers or hydrogen donors. The old electron in DPPH free radicals gives a strong absorption maximum at 517nm. When DPPH becomes paired with hydrogen from a free radical scavenging antioxidant, its purple color fades rapidly to yellow to form reduced DPPH-H (Kolev*aet al*., 2002). The resulting decolorization is stoichiometric with respect to number of electrons captured.

Fig.1: DPPH Radical Scavenging Assay of TrianthemadecandraM**ethanol extract.**



Note: S1-Methanol, S2-Ethanol, S3-Ethylacetate, S4-Aqueous, S5- Hexane, S6-Dichloromethane

The comparable beta-carotene bleaching rates of control, BHT (Standard) and ethanolic extracts of leaf. The highest antioxidant activity of leaf was found to be 48.87%. The beta carotene bleaching method is one of the most frequently applied methods for determining the total antioxidant property of the extract. In the beta carotene bleaching assay linoleic acid produces hydroperoxides as free radical during incubation at 50ºC and attacks the beta carotene molecules that cause reduction in absorbance at 470nm. Beta carotene in the systems undergoes

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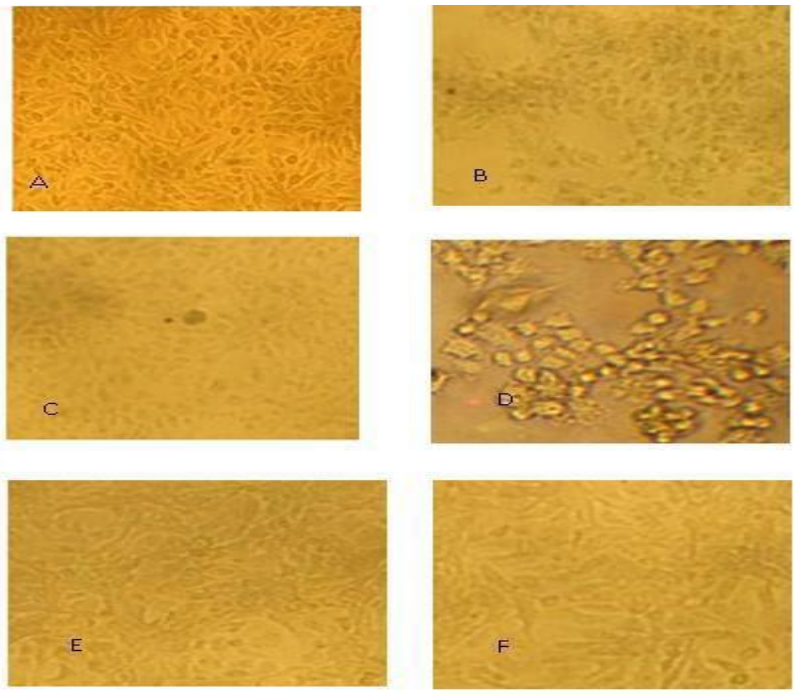
rapid discoloration in the absence of antioxidant and vice versa in it presence (Yamaguchi *et al*., 1998). The presence of different antioxidants can delay the extent of beta carotene bleaching by neutralizing the linoleate free radical and other free radical system. Thus, degradation rate of beta carotene-linoleatedepends on the antioxidant activity of the extracts. The result showed the control had substantial and rapid oxidation of beta carotene (Fattouch et al., 2007).

Accordingly, the absorbance decreased rapidly in the sample without antioxidant, while the sample extract with the presence of antioxidant retained their colour and also absorbance for a longer time. In the present study, free radical scavenging activity was determined by the FRAP (Ferric reducing ability of plasma) method, which depends upon the reduction of ferric tripyridyltriazine (FE(III)-TPTZ) complex to the ferrous tripyridyltriazine. Fe(II)-(TPTZ) by a reductant at low PH. Fe(II)-TPTZ has an intensive blue color and can be monitored at 593nm. FRAP values are obtained by comparing the absorbance change at 593nm in test reaction mixtures with thosecontaining range with antioxidant mixtures (Repetto *et al*., 2002). The free radical scavenging power of leaf extract of *Trianthemadecandra*increased with increased amount of extract. There is a clear difference between the control and the sample containing theextract (Singh *et al*.,1995). Among all the extracts the ethanol extract was showing maximum antioxidant power (13.7mmol of feso4/100g dry plant equivalents in *Trianthemadecandra*.



Note: A: Control cells (Untreated), B: Ethanol extract 25 µg/ml, C: Ethanol extract 50 µg/ml, D: Ethanol extract 100 µg/ml, E: Ethanol extract 150 µg/ml, F: Cyclophasphamide (Positive control) 180 µg/ml.

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Anticancer activity

Since, IC50 value for Hep2 (liver) cell line (100 µg/ml) ethanol leaf extract was found to be effective, the reduction percentage of MTT at 48Hrs also estimated for Hep2 (liver) cells. When incubated with the extract, it induced Cytotoxicity in a significant manner which implicit the damage to the member integrity of the cell when contributed with control (Sutharsingh*et al* 2011).

The cytotoxicity was minimized in the extract treated cells and near normal level was attained at various concentrations (25, 50, 100 and 150µg/ ml) and maximum effect was found when treated at 100 µg/ml. From the above results, it was confirmed that *Trianthemadecandra*methanol leaf extract at 100µg/ml seems to offer significant protection and maintain the structural integrity of the hepatocellular membrane and this active concentration was followed for further studies. Tryphan blue is one of the several stains recommended for use in dye exclusionprocedure for viable cell counting. This assay is based on the principle that live cells do not take up blue, where as dead cells do and appear as blue under microscope - depicts the viability of cells by Tryphan Blue assay. The viability is measured in terms of percentage was found to decreased 98% in drug treated hepatic cell line. The cell treated with *Trianthemadecandra*ethanol extract at various concentrations (25, 50, 100 and 150µg/ml) showed protective nature of the extract act against the deleterious effects and the maximum effect was observed at 100 µg/ml. (figure 3). The extract had an IC50 value of100µg/mL which showed cell viability of 22.67%. (Table 3)From the result, it is clear that ethanol extract of *Trianthemadecandra*has cytotoxic effect on Hep2 cell lines. Cell cycle arrest is a common feature of cells that are undergoing terminal differentiation and defective proliferation (Edmondson *et al*.,1988).

Table: 3% MTT reduction on Hep2 cell line:

S. No	Concentration(µg)	MTT reduction (%)
1	Control	98.17
2	25	84.12
3	50	71.47
4	100	46.3
5	150	22.67
6	PC	11.13

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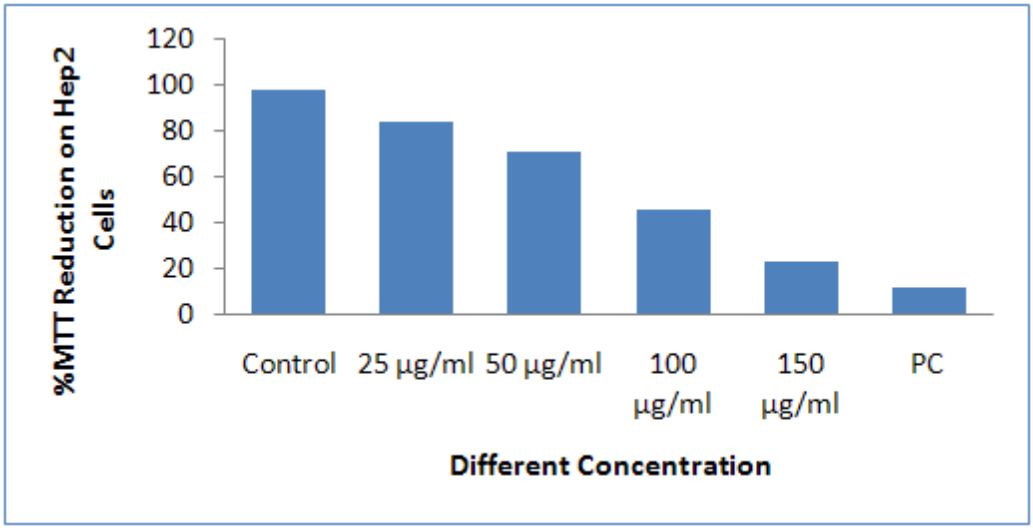
Fig. 3: MTT reduction on Hep2 cell line:



Note: A: Control cells (Untreated), B: Ethanol extract 25 µg/ml,C: Ethanol extract 50 µg/ml, D: Ethanol extract 100 µg/ml, E:Ethanol extract 150 µg/ml, F: Cyclophasphamide (Positivecontrol) 180 µg/ml.

Conclusion

The present investigation suggests that *Trianthemadecandra*possesses significant antioxidant and anti-proliferative potential.Hence, we can conclude that with further mechanistic studies, theplant can be considered as an efficient source of antioxidant andanti-proliferative agents.

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