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RESEARCH ARTICLE

ANTIOXIDANT AND *INVITRO* CYTOTOXICITY OF *TODDALIA ASIATICA* L

***Gopi Krishna, S., Sudarsanam, G. and Penchala Pratap, G.**

Department of Botany, S.V.University, Tirupati, India

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ABSTRACT

Whole plant of *Toddalia asiatica* were selected to evaluate antioxidant activities and invitro cytotoxic activity using commonly accepted assays. They were extracted with chloroform, methanol and petroleum ether respectively and selected for the best antioxidant and cytotoxic results. Flavonoids, such as quercetin, rutin, and antioxidant BHA (Butylated Hydroxyanisole) were included and used as standards in this study. For all the assays concentrations of samples are maintained from 62.5 to 1000 µg/ml. Each sample under assay condition showed a dose-dependent antioxidant effect of DPPH (1,1-diphenyl-2-picryl hydrazyl radical), FRAP (Ferric reducing ability of plasma) and LPO (Lipid peroxidation inhibitory activity). Among the crude plant extracts, Methanol extract showed stronger IC_{50} values in antioxidant studies in DPPH and LPO methods. IC_{50} of Methanolic extract in DPPH is 500 ±0.00 followed by Pet.ether and Chloroform, IC_{50} of Pet.ether extract in LPO is 300±0.00 followed by Chloroform and Methanol. Overall FRAP absorbance range is 0.051 – 0.472, all the extracts showed moderate reducing power. The extracts also showed dose dependent cytotoxic effect on normal (Vero) and cancer cell lines (HaCaT,A549,HeLa,HT-29) when analyzed by MTT assay. Of the different crude extracts, extracts demonstrated maximum cytotoxicity to cancer cell lines, CTC_{50} values ranging from 116.67± 5.8 to 710 ± 10, and all the extracts showed high toxicity toward normal cell line Vero. Nearly all cancer cells could be killed by extracts, where as small fractions of cells from cancer cell lines showed resistance even at much higher concentrations.

INTRODUCTION

Nature has been a source of medicinal agents for 1000's of years and an impressive number of modern drugs have been isolated from natural sources. Many of these isolations were based on the uses of the agents in traditional medicine. The plants continues to plays a main role in traditional medicine system for health care (Owolobi *et al*., 2007). Phytochemicals, the non-nutritive plant chemicals that have protective or disease preventive properties. There is a growing interest in correlating phytochemical constituents of a plant with its pharmacological activity (Gupta, 1994). Collection of information and documentation of traditional knowledge plays an important role in scientific research on drug development (Ragupathy *et al*., 2008). Free radicals produced in our body due to aerobic respiration and substrate oxidation, can cause oxidative stress which may contribute to the development of several diseases including cancer, Alzheimer's disease, aging, diabetes, Parkinson disease and atherosclerosis (Moure *et al.,* 2001; Madsen and Bertelsen, 1995; Hazra *et al.,* 2008; Ani and Naidu, 2011; Rohman *et al.,* 2010; Kleinsmith, 2006; Herceg and Hainaut, 2007). Overproduction of free radicals in our bodies may be increasing due to pollution and other external factors, and their removal by our antioxidant systems may be lower than before due to a number of factors related to our lifestyle among others. Oxidative stress causes serious damage to important cellular macromolecules such as protein and DNA. However, the production of free radicals can be balanced by antioxidant actions of endogenous enzymes as well as natural and synthetic antioxidants (Reuter *et al*., 2010; Rahman, 2007). Antioxidants exert its action through several mechanisms including prevention of chain initiation, chelating of transition metal ion catalysts, decomposition of peroxidases, prevention of continued hydrogen abstraction and radical scavenging (Volka *et al*., 2006). These deleterious effects of free radicals have drawn the attentions of scientists to the importance of antioxidants in prevention and treatment of diseases (Niki, 2010). Thus, there has been increasing interest in finding natural diet derived antioxidant to prevent oxidative damage (Moure *et al*., 2001; Madsen and Bertelsen, 1995). Thus many studies have been carried out on natural sources to unravel the components which possess antioxidant properties and with low cytotoxicities (Ani and Naidu, 2011).

Cancer is a genetic disease, which is mainly driven by genetic instability, including changes in oncogenes and tumor suppressor genes which leads to the expression of abnormal proteins involved in the stimulation of cell proliferation and

Corresponding author: Gopi Krishna, S.
Department of Botany, S.V.University, Tirupati, India.

survival (Kleinsmith, 2006; Herceg and Hainaut, 2007). A large body of evidences have shown that free radicals have been implicated in the development of cancer in humans (Reuter *et al*., 2010; Rahman 2007). One example of the free radicals, is the hydroxyl radical which can cause genetic mutation by forming adduct with guanine to form hydroxylated bases of DNA (8 hydroxyl-2′-deoxyguanosine) causing transversions of GC (guanine-cytosine) to TA (thymine-adenine) (Lombardi *et al*., 1998; Lunec *et al*., 2002). Epidemiologic studies have also shown that cancer may be due to several factors such as exposure to environmental carcinogenic agents, lifestyle (tobacco and alcohol consumption), nutritional habit and infectious agents. These factors can initiate and promote carcinogenesis which may progress to cancer.

T. asiatica belongs to family Rutaceae. It grows in forested riparian habitat with high rain fall. It is commonly named as Orange climber. The bioactive compounds like alkaloids, flavonoids, tannins and phenolic compounds are identified in this plant. It is used medicinally by many herbalists from ancient times. The fruit is used as a cough remedy and the roots in the treatment of indigestion and influenza. The leaves are used for lung diseases and rheumatism. In some places the root and its bark have been used as a remedy for fever, malaria, cholera, diarrhoea and rheumatism. Usher 1974 reports that in India a yellow dye is extracted from the roots (called Lopez Root) and the root bark is used medicinally as a tonic and for stomach ailements. To the best of our knowledge, there is no antioxidant and cytotoxic investigation on whole plant extracts of this plants. Thus, this paper reports the antioxidant and cytotoxic activities of the crude chloroform, methanol and petroleum ether extracts.

MATERIALS AND METHODS

Plant collection and Extraction

The Plant materials were collected from different locations of southern India during the month april 2014. Tirupathi, Andhra Pradesh. India. The plants were confirmed by comparing with the housed authenticated specimens. The collected plant materials were shade dried and powdered sample was extracted with methanol, chloroform and petroleum ether successively with soxhlet apparatus, the extracted materials were dried under reduced pressure.

Media and Chemicals

2,2-Diphenyl 1- picryl solution (DPPH), Dimethyl sulfoxide (DMSO), Potassium ferricyanide, 10% trichloroacetic acid, TBA,3-(4,5–dimethyl thiazol–2–yl)–5–diphenyl tetrazolium bromide (MTT), Trypan blue, were obtained from Sigma Aldrich Co, St Louis, USA. EDTA, glucose and antibiotics from Hi-Media Laboratories Ltd Mumbai. Dimethyl Sulfoxide (DMSO) and Propanol from Merck Ltd, Mumbai, India.

Antioxidant activity test

DPPH free radical scavenging activity

The assay was carried out in a 96 well microtitre plate. To 200 µl of DPPH solution, 10 µl of each of the test sample or the standard solution was added separately in wells of the microtitre plate. The final concentration of the test and standard solutions used were 1000, 500, 250, 125, 62.5, 31.25 and 15.625, 7.812 µg/ml. The plates were incubated at 37^{o} C for 30 min and the absorbance of each solution was measured at 490 nm, using a microplate reader.

Ferric Reducing antioxidant Power Assay

A method developed by Oyaizu, 1986 for reducing power test was used. The above samples were spiked with 2.5 mL of phosphate buffer (0.2 M, pH 6.6) and 2.5 mL of 1% potassium ferricyanide. The mixture was then kept in a 50°C water-bath for 20 min. The resulting solution was then cooled rapidly, spiked with 2.5 mL of 10% trichloroacetic acid, and centrifuged at 3000 rpm for 10 min. The supernatant (5 mL) was then mixed with 5 mL of distilled water and 1 mL of 0.1% ferric chloride. The absorbance at 700 nm was then detected after reaction for 10 min. The higher the absorbance represents the stronger the reducing power.

Lipid peroxidation inhibitory activity

The reaction mixture containing egg lecithin (1ml), ferric chloride (0.02ml), ascorbic acid (0.02ml) and extract or standard (0.1ml) in DMSO at various concentrations was kept for incubation for 1 hour at 37ºC. After incubation 2 ml of 15% TCA and 2ml of 0.37% TBA were added. Then the reaction mixture was boiled for 15 min, cooled, centrifuged and absorbance of the supernatant was measured at 532 nm.

Invitro Cytotoxicity by MTT

Cell lines and Culture medium

HeLa (Human Cervical Carcinoma), HT-29 (Human Colon carcinoma), A549 (Human Lung carcinoma), HaCaT (Human Keratinocyte Carcinoma), VERO (African Green monkey Kidney) cell lines were procured from National Centre for Cell Sciences (NCCS), Pune, India. Stock cells were cultured in DMEM supplemented with 10% inactivated Fetal Bovine Serum (FBS), penicillin (100 IU/ml), streptomycin (100 µg/ml) and amphotericin B (5 µg/ml) in an humidified atmosphere of 5% CO_2 at 37°C until confluent. The cells were dissociated with TPVG solution (0.2% trypsin, 0.02% EDTA, 0.05% glucose in PBS). The stock cultures were grown in 25 cm^2 culture flasks and all experiments were carried out in 96 microtitre plates (Tarsons India Pvt. Ltd., Kolkata, India).

Preparation of Test Solutions

For Cytotoxicity studies, each weighed test drugs were separately dissolved in distilled DMSO and volume was made up with DMEM supplemented with 2% inactivated FBS to obtain a stock solution of 1 mg/ml concentration and sterilized by filtration. Serial two fold dilutions were prepared from this for carrying out cytotoxic studies.

Procedure

The monolayer cell culture was trypsinized and the cell count was adjusted to 1.0 x 10^5 cells/ml using DMEM containing

10% FBS. To each well of the 96 well microtitre plate, 0.1 ml of the diluted cell suspension (approximately 10,000 cells) was added. After 24 h, when a partial monolayer was formed, the supernatant was flicked off, washed the monolayer once with medium and 100 µl of different test concentrations of test drugs were added on to the partial monolayer in microtitre plates. The plates were then incubated at 37° C for 3 days in 5% CO_2 atmosphere, and microscopic examination was carried out and observations were noted every 24 h interval. After 72 h, the drug solutions in the wells were discarded and 50 µl of MTT in PBS was added to each well. The plates were gently shaken and incubated for 3 h at 37° C in 5% CO_2 atmosphere. The supernatant was removed and 100 µl of propanol was added and the plates were gently shaken to solubilize the formed formazan. The absorbance was measured using a microplate reader at a wavelength of 540 nm. The percentage growth inhibition was calculated using the following formula and concentration of test drug needed to inhibit cell growth by 50% (CTC_{50}) values is generated from the dose-response curves in graph pad prism for each cell line.

Statistical analysis: Data representative of three independent experiments with similar results were presented as mean ± SD.

RESULTS

DPPH radical scavenging activity

The results of DPPH free radical scavenging activity on the three crude extracts are shown in Table 1. The highest radical scavenging activity (IC_{50} value 500±0.00 µg/ml) was shown by methanolic extract, pet.ether showed moderate antioxidant activity with IC_{50} value of 850±0.00µg/ml and chloroform extract showed lowest antioxidant activity with IC_{50} values of 1000µg/ml.

Lipid proxidation inhibitory activity

The results of Lipid peroxidation inhibitory activity on the three crude extracts are shown in Table 1. The highest inhibition activity (IC_{50} value 300 ± 0.00µg/ml) was shown by Petroleum ether extract, chloroform extract showed good inhibition with IC_{50} 600±0.00µg/ml. Whereas methanol extract showed lowest inhibition with IC_{50} value >1000µg/ml.

Table 1. Antioxidant studies of *T. asiatica*

DPPH Method:

Pet-ether					
Concentration (µg/ml)	% of Inhibition				
	1	2	3	Avg	Std Dev
1000	56.52	56.27	55.88	56.22	0.32
500	34.53	31.47	31.55	32.51	1.74
250	11.00	14.13	14.71	13.28	2.00
125	8.18	2.40	4.81	5.13	2.91
62.5	1.79	0.00	0.00	0.60	1.03
IC50	850	850	850	850.00	0.00

Methanol					
Concentration (µg/ml)	% of Inhibition				
	1	2	3	Avg	Std Dev
1000	56.08	58.26	55.96	56.77	1.30
500	50.83	50.42	50.97	50.74	0.29
250	44.75	48.74	41.83	45.11	3.47
125	45.58	39.50	39.61	41.56	3.48
62.5	35.08	32.49	33.52	33.70	1.30
IC50	500	500	500	500.00	0.00

Chloroform					
Concentration (µg/ml)	% of Inhibition				
	1	2	3	Avg	Std Dev
1000	41.50	39.44	42.78	41.24	1.69
500	31.75	30.42	31.67	31.28	0.75
250	23.68	19.15	18.06	20.30	2.98
125	10.58	10.14	8.61	9.78	1.04
62.5	8.08	7.04	5.00	6.71	1.57
IC50	>1000	>1000	>1000		

FRAP Method:

Pet-ether				
Concentration (µg/ml)	Absorbance			
	1	2	3	Avg
1000	0.253	0.258	0.254	0.255
500	0.11	0.137	0.12	0.122
250	0.079	0.076	0.076	0.077
125	0.049	0.099	0.053	0.067
62.5	0.025	0.075	0.052	0.051

Methanol				
Concentration (µg/ml)	Absorbance			
	1	2	3	Avg
1000	0.41	0.412	0.416	0.413
500	0.4	0.408	0.408	0.405
250	0.34	0.351	0.356	0.349
125	0.253	0.247	0.246	0.249
62.5	0.151	0.149	0.151	0.150

Chloroform				
Concentration (µg/ml)	Absorbance			
	1	2	3	Avg
1000	0.4627	0.4747	0.4777	0.472
500	0.227	0.227	0.231	0.228
250	0.16	0.16	0.154	0.158
125	0.107	0.109	0.111	0.109
62.5	0.07	0.069	0.069	0.069

LPO Method:

Pet-ether					
Concentration (µg/ml)	% of Inhibition				
	1	2	3	Avg	Std Dev
1000	98.33	95.24	95.45	96.34	1.73
500	88.33	87.30	89.39	88.34	1.05
250	40.00	41.27	43.94	41.74	2.01
125	33.33	34.92	39.39	35.88	3.14
62.5	26.67	28.57	31.82	29.02	2.60
IC50	300	300	300	300.00	0.00

Methanol					
Concentration (µg/ml)	% of Inhibition				
	1	2	3	Avg	Std Dev
1000	41.46	42.17	38.27	40.63	2.08
500	12.20	13.25	12.35	12.60	0.57
250	4.88	7.23	6.17	6.09	1.18
125	1.22	2.41	2.47	2.03	0.70
62.5	0.00	0.00	0.00	0.00	0.00
IC50	>1000	>1000	>1000		

Chloroform					
Concentration (µg/ml)	% of Inhibition				
	1	2	3	Avg	Std Dev
1000	98.65	98.65	97.30	98.20	0.78
500	33.78	36.49	37.84	36.04	2.06
250	21.62	18.92	17.57	19.37	2.06
125	9.46	9.46	12.16	10.36	1.56
62.5	8.11	6.76	5.41	6.76	1.35
IC50	600	600	600	600.00	0.00

IC_{50} VALUES of DPPH,LPO and Absorbance range of FRAP

SAMPLES	IC_{50} VALUES µg/ml		Absorbance Range (1000-62.5)
	DPPH	LPO	FRAP
Pet. Ether	800 ± 0.00	300 ± 0.00	0.255 – 0.015
Methanol	500 ± 0.00	> 1000	0.413 – 0.150
Chloroform	> 1000	600 ± 0.00	0.472 – 0.069
Standard	Rutin 15.77 ± 0.12	BHA 27 ± 1.00	Quercetin 0.674 – 0.382

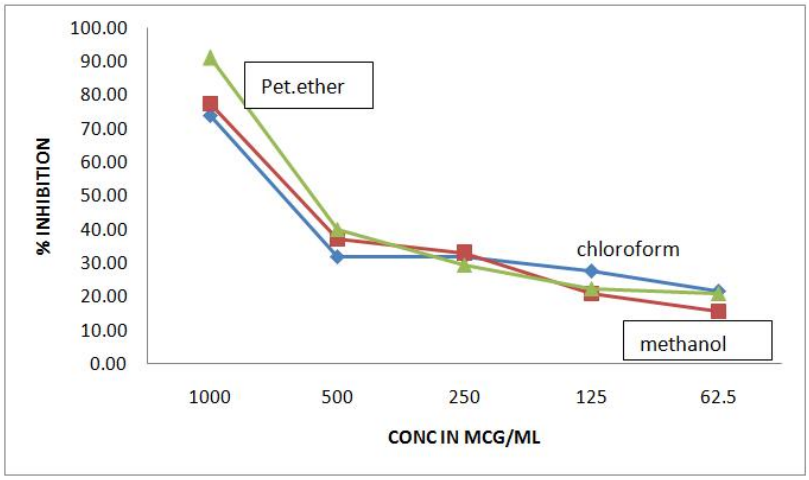
Table 2. Cytotoxicity of Toddalia asiatica extracts against Cancer and Normal cell lines by MTT assay

S.No	EXTRACT	CELL LINE (CTC_{50} µg/ml ± SD) Average of 3 replicates				
		HaCaT	A549	HeLa	HT-29	Vero
1.	Pet.ether	236.67±5.8	593.33±11.5	116.67±5.8	693±5.8	121.67±5.8
2.	Methanol	156.67±11.5	660±10.00	283.33±5.8	546.67±5.8	110.00±10
3.	Chloroform	193.33±5.8	710±10	183.33±11.5	693.33±5.8	196.67±5.8

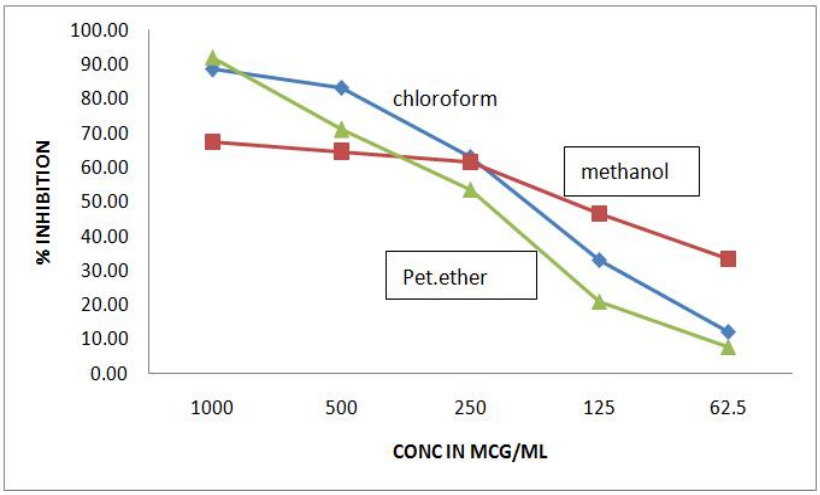
*Average of three independent determinations, 3 replicates, values are mean±SEM.
+CTC50 = concentration of the sample tolerated by 50% of the cultures exposed.

Graphical representation:

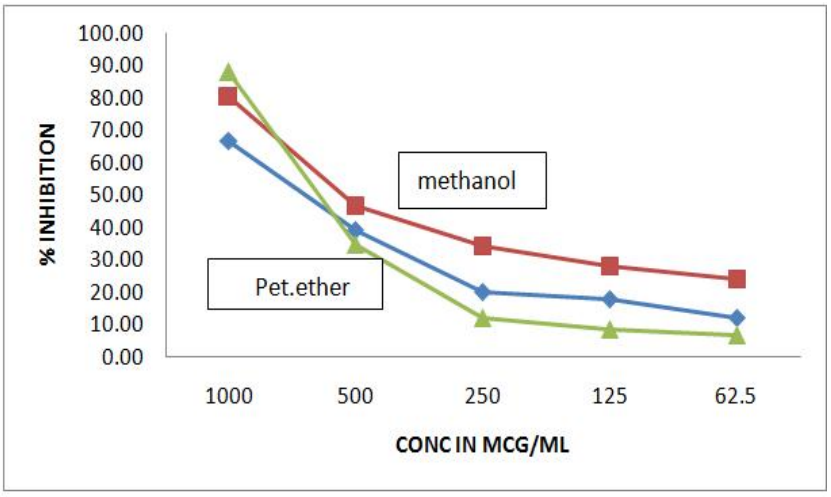
Cytotoxicity of extracts on A 549 Cell line



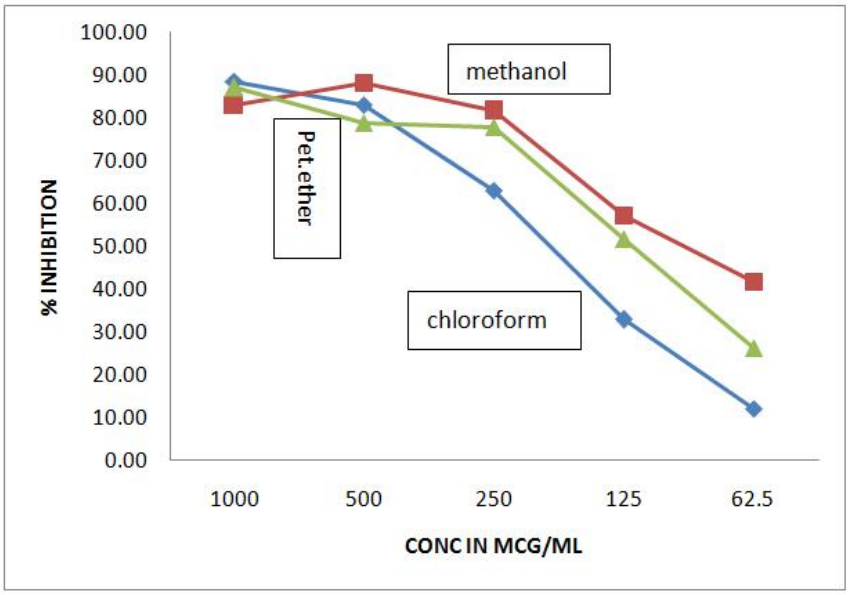
Cytotoxicity of extracts on HaCaT Cell line



Cytotoxicity of extracts on HT- 29 Cell line

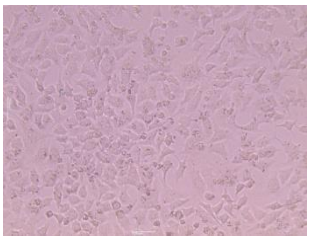


Cytotoxicity of extracts on Vero Cell line



Photos:

A549 Cell line:



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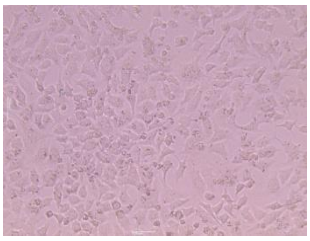
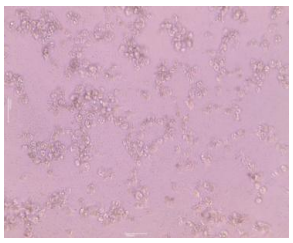


Chloroform – 1000



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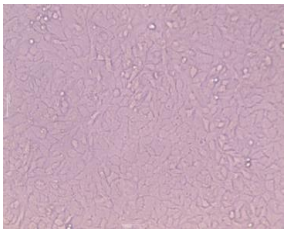
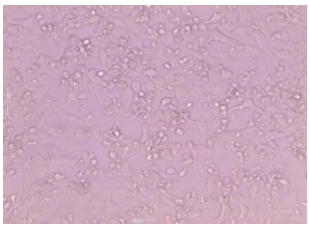


Methanol -1000



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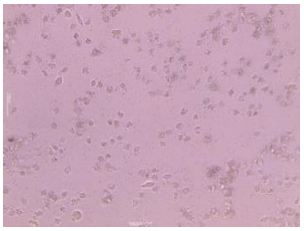
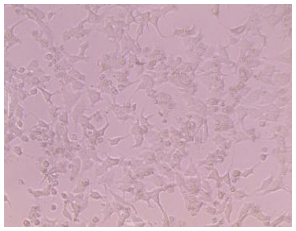
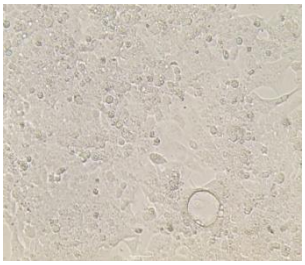


Pet. ether – 1000



Pet. Ether - 500

HaCaT Cell line:



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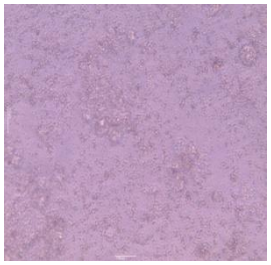
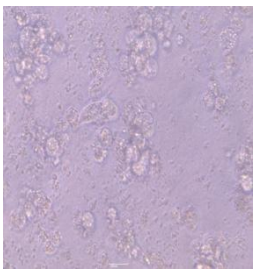


Chloroform -1000



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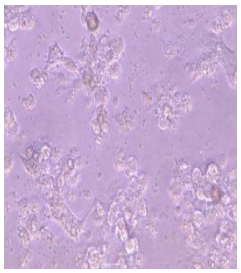

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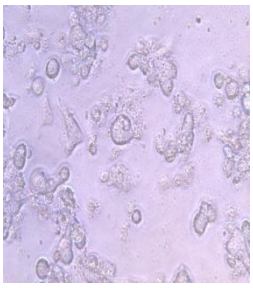
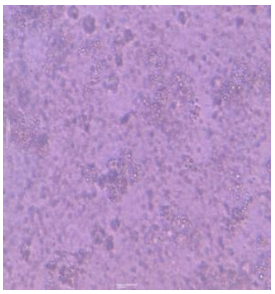
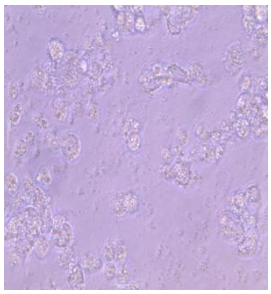
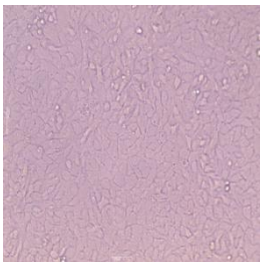
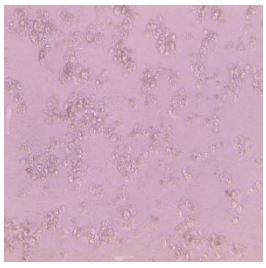
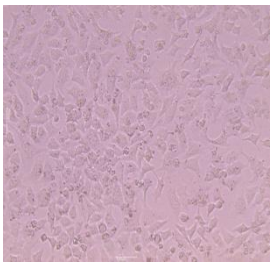
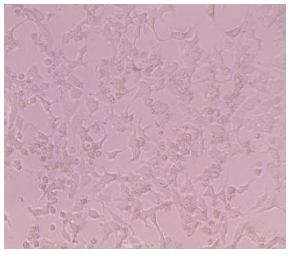
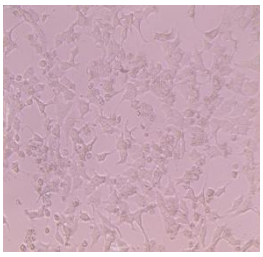
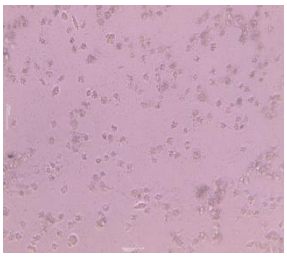
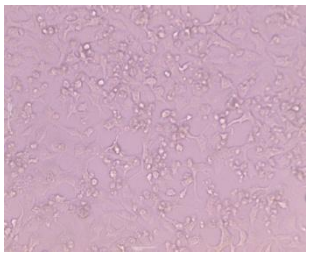




Methanol- 500 **Pet. ether - 1000** **Pet.ether – 500**

HeLa Cell line:

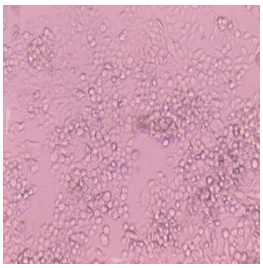
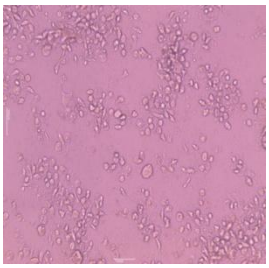
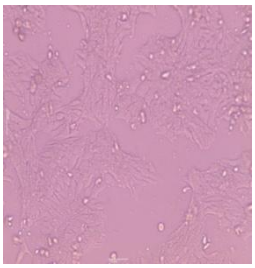
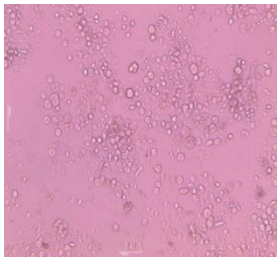





Control **Chloroform – 1000** **Chloroform – 500** **Methanol – 1000**

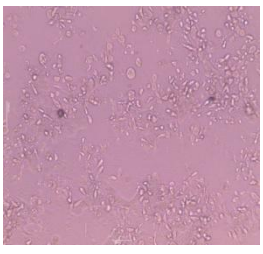
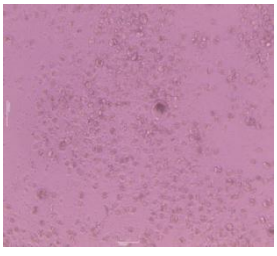
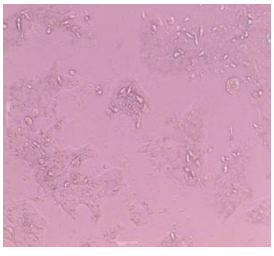




Methanol – 500 **Pet. ether - 1000** **Pet. ether – 500**

HT -29 Cell line

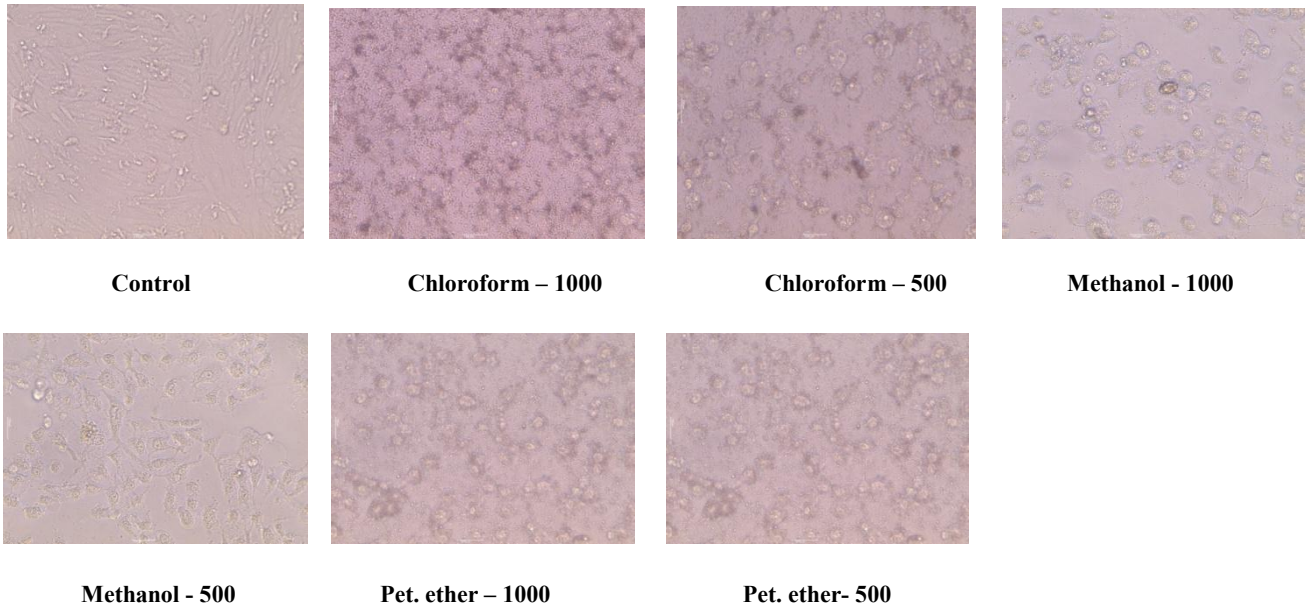





Control **Chloroform - 1000** **Chloroform – 500** **Methanol - 1000**

Methanol – 500 **Pet. Ether - 1000** **Pet. Ether - 500**

Vero Cell line



Control **Chloroform – 1000** **Chloroform – 500** **Methanol - 1000**

Methanol - 500 **Pet. ether – 1000** **Pet. ether- 500**

Ferric Reducing antioxidant Power Assay

The free radical scavenging power of different extracts increased with increase in concentration of extract. As can be seen in Table 1, there is a clear difference in absorbance in highest and lowest concentrations. Chloroform extract showed stronger reducing power at high concentration, absorbance ranging from 0.472 – 0.069. Followed by methanol and pet. ether with absorbance ranges of 0.413 – 0.150 and 0.255 – 0.051.

Invitro cytotoxicity by MTT

In the present study, cytotoxicity was evaluated on four different cancer cell lines and one normal cell line. All the extracts showed dose dependent activity for the concentrations ranging from 62.5 to 1000µg/ml. The cytotoxicity of plant extracts were classified in to three groups- highly toxic (100 – 400), moderately toxic (400 – 800), low toxic (800 – 1000). *T. asiatica* showed 50 % highly toxic and 50 % moderately toxic. HeLa and HaCaT cell lines showed higher affinity towards cytotoxicity in all the extracts. Overall cytotoxicty were in the range of 116.67 ± 5.8 to 710 ± 10 as shown in Table 2. The cytotoxicity of extracts are in increasing order pet.ether > methanol > chloroform. All the extracts exhibited high toxicity towards normal cell line Vero, with CTC_{50} values ranging from 110±10 to 196 ± 5.8.

DISCUSSION

Antioxidants have been widely used in the food industry to prolong shelf life. However, there is a widespread agreement that some synthetic antioxidants such as butylhydroxyanisole and butylhydroxytoluene (BHA and BHT, respectively) need to be replaced with natural antioxidants because of their potential health risks and toxicity. Thus, the search for antioxidants from natural resources has received much attention, and efforts have been made to identify new natural resources for active antioxidant compounds (Dudonne *et al*., 2009). Phenolic natural products such as flavonoids are of particular interest because of their antioxidant activity through scavenging oxygen radicals and inhibiting peroxidation. Antioxidants that scavenge free radicals play an important role in cardiovascular disease, aging, cancer, and inflammatory disorders (Cioffi *et al*., 2002). In addition, these naturally occurring antioxidants can be formulated to give nutraceuticals, which can help to prevent oxidative damage from occurring in the body. One way of estimating antioxidant activity is by the use of the stable free radical DPPH (Molyneux, 2004; Brand- Williams *et al*., 1995; Dudonne *et al*., 2009; Moon and Shibamoto, 2009). Lipid peroxidation assay is based on inhibition of lipid peroxidation. FRAP principle is higher the absorbance represents the stronger the reducing power. The cell viability was tested by MTT assay. It is one of the gold standard protocols to assess cytotoxicity due to its rapidity and precision (Mosmann, 1983). The IC50 and CTC values of antioxidant and cytototoxic activity of the present study showed the best results, however, a detailed study is required to understand the molecular mechanism of its activity.

Conclusion

From the present findings, it can be concluded that the studied extracts Toddalia asiatica showed toxicity against both cells (cancer and normal) irrespective of their origin. The results showed both the plants had nearly 50% of higher toxicity and 50% of moderate toxicity. Hence the extracts need to be thoroughly studied using animal models.

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