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Research Article

Study on the Anti-Cancer Activity of *Tylophora* indica Leaf Extracts on Human Colorectal Cancer Cells

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ABSTRACT
The failure of existing therapeutic interventions and chemotherapy draws the necessity of new and efficient drugs in the treatment of cancer. Plant based drugs always hold pre-eminence owing to the factors like lesser side effects and decreased chances of development of drug resistance. *Tylophora indica* a plant belonging *to Asclepiadaceae* is widely used in traditional medicine for the treatment of different aliments as antibacterial, anti-inflammatory and antiviral agents. The lack of scientific evidences regarding the anticancer potential of this plant limits its validation as chemotherapeutic agents. In the current study the anti-proliferative effect of alcoholic extracts of *Tylophora indica* on HCT-15 colon cancer cell lines was studied in detail. Phytochemical analysis confirms the presence of alkaloids and flavaniods as the major secondary metabolites present in ethanolic extracts. Invitro free radical scavenging assays confirms significant antioxidant activity. Dose dependent invitro studies confirm an IC50 value of 40 mcg/ml with MTT and neutral red uptake assays. Increased LDH leakages shows increased membrane damage in cells treated with ethanol fractions of *Tylophora indica*. Apoptosis was determined by EtBr/Acridine orange staining methods which confirmed presence of apoptotic/ necrotic cells upon treatment with extracts. The results suggests therapeutic potential of Ethanolic extracts of *Tylophora indica* against colon cancer.

Key words: *Tylophora indica*, HCT-15, MTT, Neutral red uptake assay, LDH leakage assay, Apoptosis

INTRODUCTION

Colorectal cancer (CRC) is the third most common cancer in men (663,000 cases, 10.0% of the total cancers) and the second in women (570,000 cases, 9.4% of the total cases) worldwide. Incidence rates of CRC vary 10-fold in both sexes worldwide. The burden of CRC has risen rapidly in some economically developed Asian countries like Japan, South Korea and Singapore and Colorectal cancers accounts to the third major cause of mortality in these countries. The limitation of existing treatment regimens, increased economic burden and side effects raises concerns over treatment of colon cancer.

The research for complementary and alternative medicine for dealing with cancer management has lead intense research on plants prescribed in ayurvedic medicinal systems. Ayurveda, the traditional medicinal system of india is inturn recognized as a repository of valuable information regarding therapeutics and validating these in a scientific platform can offer potent information for discovery of new drugs with lesser side effects.

Tylophora indica (family: Asclepidaceae) is a perennial plant distributed throughout Southern and Eastern parts of India in plains, forests and hilly places. The plant is found growing normally in Uttar Pradesh, Bengal, Assam, Orissa, Himalayas, and Sub Himalayas in India (Wealth of India, CSIR publication 1995). Mainly the leaves are used as traditional medicine in asthma, anti-arthritisis and anti-cancer activity, but the lack of scientific evidences prompts more screening studies regarding the pharmacological values.

Tylophora alkaloids are common to various plants from Asclepidaceae family such as *Tylophora indica*. The anticancer activity of *Tylophora indica* is least studied and validation of activity can bring more light to the pharmacological activities of the same (Donald son et al., 1988, Gao W et al., 2004). Due to their diverse and potent pharmacological action, they confine to be target for synthesis modification and structural actively relationship. The present study is aimed to check invitro anticancer activity of alcoholic fractions of *Tylophora indica* in colon cell line HCT-15.

MATERIALS AND METHODS

Sample collection and extraction: *Tylophora indica* was collected from interior areas of Trivandrum and dried in

Table 1: Presence of Alkaloids and Flavanoids

Name of compound	Result
Alkaloids	+
Flavanoids	+

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Table 2: Determination of total anti - oxidant activity

Concentration(µg/ml)	*Tylophora indica* extract(OD at 690nm)	Ascorbic acid standard(OD at 690 nm)
Control	0.027±0.0018	0.027±0.0018
50	0.955±0.0049	0.580±0.023
100	0.725±0.027	0.438±0.016
200	0.570±0.093	0.382±0.049

Table 3: Hydroxyl radical scavenging assay

Concentration of sample(µg/ml)	Tylophoraindica extract(%inhibition)	Ascorbic acid standard(%inhibition)
50	35±2.13	51.76±3.91
100	49±3.68	55.29±2.68
200	54±1.76	63.52±5.31

shade, powdered and used for further studies.100g of sample was defatted using petroleum ether which subjected to soxhilation using 90% ethanol. The crude extract was dried and used for further studies. The crude extracts were further fractionated using and subjected to phytochemicals analysis.

Phytochemical analysis

Alkaloids: Presence of alkaloids were determined by Mayer's test (Dipali, 2013). 0.36 g of mercuric chloride ($HgCl_2$) was dissolved in 60ml of distilled water and 5g of

KI in 10ml of water. The two solutions were mixed and diluted to 100ml with a few drops of reagent were added. Formation of white or pale precipitate shows the presence of alkaloids.

Flavanoids (Dipali, 2013).: In a test tube containing 0.5ml of alcoholic extract, 5-10 drops of dilute.HCl and a small piece of $ZnCl_2$ or $MgCl_2$ were added and the solution was boiled for few minutes. The presence of flavonoids resulted in production of a reddish pink, dirty brown colour.

Evaluation of invitro anti-oxidant activity

Determination of total anti-oxidant activity of simple: The anti-oxidant activity was evaluated by phosphomolybdenum method, as per the procedure described by Prieto. et al, 1999. 0.3ml extract was mixed with 3ml reagent (0.6ml H_2S0_4, 28mM sodium phosphate and 4mM Ammonium molybdate. The tubes were incubated at 950 C for 90 minutes. The absorbance was read at 695nm after cooling at room temperature. Blank containing 0.3ml metanol instead of extract was used.

Nitric oxide Radical Scavenging Activity (Marcocci L *et al* 1994): Weighed 10 mg of extract and made up to 1ml with methanol or Di-methyl sulphoxide. Different concentrations of (125, 250, 500 µg/ml). To that added 50µl of 10µm sodium nitroprusside and 50µl of the test solution and finally made-up the solution to 3ml with phosphate buffer(pH 7.4) mix properly.Placed that solution in the incubator and incubated it at 25 – 30 for two and a half hour under fluorescence light. After incubation, 125µl of Gris reagent was added and incubated it for 30 minutes at room temperature for colour development (light pink is developed). The absorbance is read at 546 nm.

Hydroxyl Radical scavenging Activity. (Elizabeth K *et al* 1990): Hydroxyl radical scavenging activity was carried out by measuring the competition between deoxyribose and the extracts for hydroxyl radicals generated from the Fe^{3}+/Ascorbate/ethylene diamine tetra acetic acid/H_2O_2system. Attacks of the Hydroxyl radicals on the de-oxy ribose led to TBARS (Thiobarbituric acid reactive substances) formation. Different concentrations of extracts such 50,100.200µg/ml were added to the reaction mixture containing 2.8mm L^{-1}deoxy ribose,100µm L^{-1} ferric chloride,104µmL^{-1} EDTA 100 µmL^{-1} ascorbic acid ,1mm$L^{-1}H_2O_2$ and 230mmL^{-1} phosphate buffer (pH 7.4) making a final volume of 1ml.1ml of TBA (1%) and 1ml of TCA(2.8) were added to the tube and kept at 100ºC for 20 minutes. After cooling, absorbance was measured at 532nm against a blank containing deoxy ribose and buffer. Reaction was carried out in a triplicates and mean average values were used for calculation.

Reducing power activity (Yen, Duh, 1993): The reducing power of extract was determined by the method of Yen and Duh (1993). Weighed different concentrations of the extract (50, 100, 200µl) and added 2.5ml of phosphate buffer (p^H 6.6) and 2.5ml of 1% potassium ferric cyanide and boiled for 20 minutes at 50℃. To it added 2.5ml of TCA and centrifuged for 10 minutes at 2000 rpm. The supernatant was collected and added 1ml of distilled water, 250µl of 0.1% ferric chloride, and the absorbance was read at 700 nm.

Invitro anti-proliferative effect of *Tylophora indica* on HCT-15: HCT-15 Human colon adeno carcinoma cell lines were purchased from NCCS, Pune. HCT-15 was sub cultured in DMEM supplemented with 10% FBS, antibiotics (Penicillin and Streptomycin) at 37^0C in 5% CO_2 incubator (Eppendorf Germany). 70-80% confluent cells were exposed with different concentration of crude extracts (10-100 µg) and incubated for 24 hours. The viability was assessed by standard methods.

MTT assay (Arung *et al.*, 2009): MTT is a colorimetric assay that measures the reduction of yellow 3-(4, 5dimethythiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT) by mitochondrial succinate dehydrogenase. The MTT enters the cells and passes into the mitochondria where it is reduced to an insoluble, coloured (dark purple) formazan product. The cells are then solubilised with DMSO and the released, solubilized formazan reagent was read spectrophotometrically. Since reduction of MTT can only occur in metabolically active cells the level of activity is inturn a measure of the viability of the cells.

The cells were washed with 1x PBS and then added with 50µl MTT (5mg/ml in PBS) and incubated at 37^0C for 3

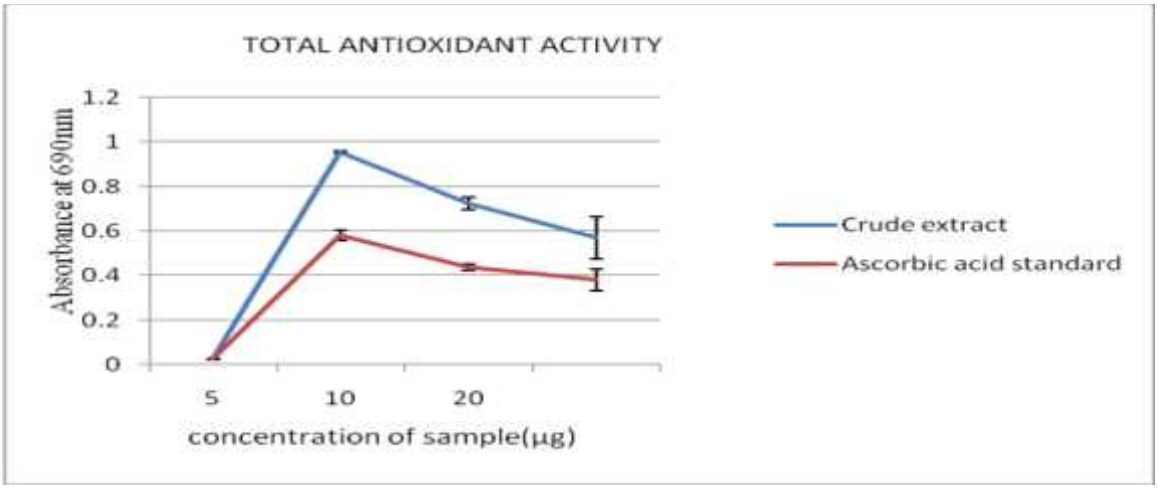

Fig. 1: total antioxidant activity of Tylophora extracts. Along X axis concentration in µg/ml and Y axis percentage inhibition

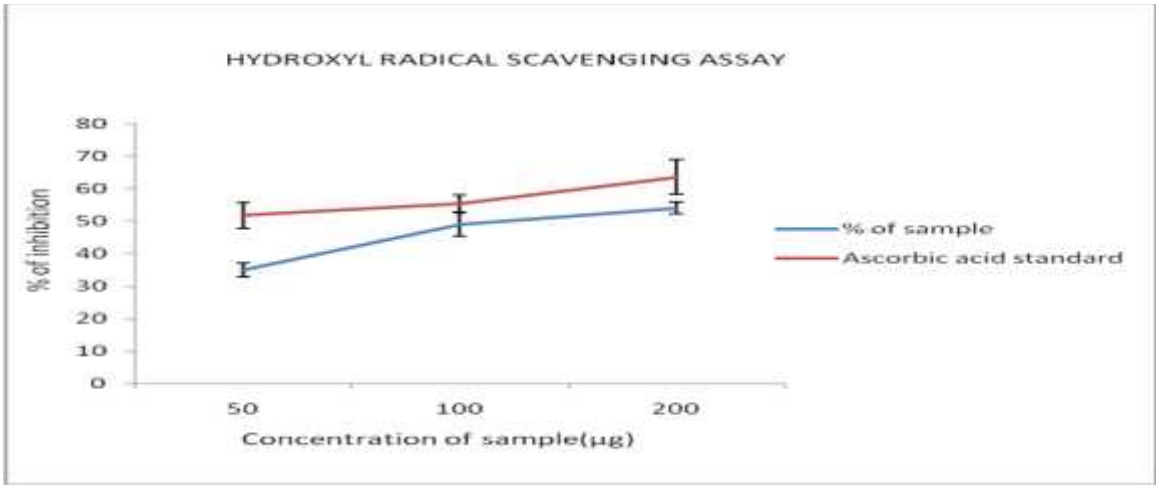

Fig. 2: Hydroxyl radical scavenging assay of Tylophora extracts. Along X axis concentration in µg/ml and Y axis percentage inhibition

hours, followed by addition of 300µl of DMSO.

Incubated at room temperature for 30 minutes until the cell get lysed and colour was obtained. The OD was read at 540nm using a microplate reader (Erba) and the percentage viability were calculated against untreated control samples.

Lactate dehydrogenase assay (Erich Gnaiger 2006): LDH leakage assay was performed with cell free supernatant as per methods described by Erich and Andry, collected from tissue culture plates. Briefly 100µl of supernatant were mixed with 2.7 ml potassium phosphate buffer, 0.1 ml 6 mM NADH solution, 0.1ml sodium pyruvate solution, into a cuvette and mixed well. The decrease of OD was recorded at 340nm in a plate reader. The blank solution was prepared by adding enzyme dilution buffer instead of sample. Activity of lactate dehydrogenase was calculated using formula.

Volume of activity (U/ml) = Δ [(OD at 5 min– OD at 0 min) x 3(ml) x DF] ÷ [6.2 x 0.1]

Neutral red assay (Repetto et al, 2008): The neutral red assay is based on the protocol that determines the accumulation of the neutral red dye in the lysosomes of viable, uninjured cells. The HCT-15 cells were incubated for 3 hours with 10µl of neutral red dye, followed by 200µl of fixation solution Fixing solution: 1% Formalin containing 1% w/v $CaCl_2$), 100µl extraction buffer (Extraction buffer: 50% ethanol containing 1%w/v Glacial acetic acid) were added and OD was read in a microplate reader.

% viability was calculated using formula

% viability= (Average OD of Test- Average OD of blank) / (OD of control – Average OD of blank) x 100

Determination of Apoptosis using Acridine orange (AO) and ethidium bromide (EB) double staining method.*:* DNA-binding dyes AO and EB were used for the morphological detection of apoptotic and necrotic cells (Zhang et al, 1998). HCT 15 cells were treated with different concentration of extracts such as 10, 50 and 100µg/ml respectively. Untreated cells were used as control. Following incubation the cells were washed by cold PBS and then stained with a mixture of AO (100 µg/ml) and EB (100 µg/ml) at room temperature for 10min. The stained cells were washed twice with 1X PBS and observed by blue filter (exicitation) of fluorescent microscope (Olympus CKX41 with Optika Pro5 camera). . The cells were divided into four categories as follows: living cells (normal green nucleus), early apoptotic (bright green nucleus with condensed or fragmented

Table 4: Reducing power activity

Concentration of sample(µg/ml)	Tylophora indica extract(OD a 700nm)	Ascorbic acid standard(OD at 700nm)
20	0.26±0.03	0.52±0.1
40	0.29±0.05	0.58±0.2
80	0.36±0.02	0.66±0.05

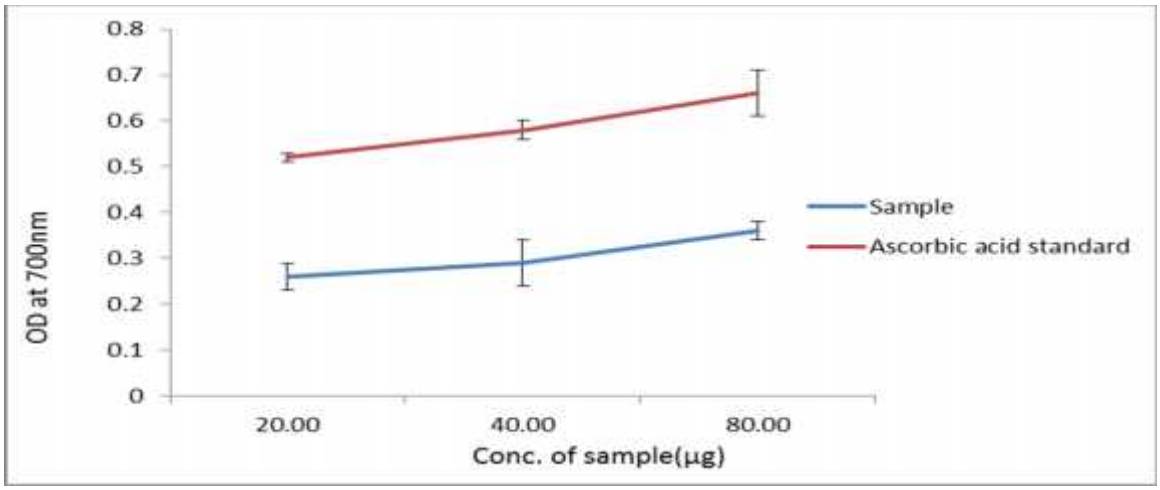

Fig. 3: Reducing activity of Tylophora extracts. Along X axis concentration in µg/ml and Y axis percentage inhibition

Table 5: Nitric oxide Radical scavenging assay

Conc. of sample(µg/ml)	*Tylophora indica* extract(% inhibition)	Ascorbic acid standard(% inhibition)
125	30.769±2.12	31.5±2.18
250	38.46±3.48	39±3.61
500	53.84±4.18	53.9±4.23

Table 6: MTT cell viability assay in HCT-15

Concentration of sample	% viability
C	100
10µg	65±4.18
50µg	42±5.16
100µg	28±8.13

Table 7: Neutral Red Uptake assay in HCT-15

Concentration of sample	% viability
C	100
10µg	72±3.14
50µg	63±6.12
100µg	38±2.13

chromatin), late apoptotic (orange-stained nuclei with chromatin condensation or fragmentation) and necrotic cells (uniformly orange-stained cell nuclei).

RESULTS AND DISCUSSION

Preparation of sample: The powdered leaves were extracted with 75% ethanol which was then dried in vacuum and mixed with DMSO. The extract was re-suspended and used for further studies (Alirezasarkaki*et al*, 2007) in a final concentration of 1000mg/ml.

Phytochemical analysis: As per the studies done by Kim *et al.*, *Tylophora indica* contains many substances which exhibit antioxidant, anti-inflammatory (Amritpal Singh et al 2008), anti-asthmatic and anti-carcinogenic properties. These compounds were collectively termed as phytochemical and were detected by qualitative analysis and our result clearly depicits the presence of alkaloids and flavaniods.

From the observation of the colors it was found that the *Tylophora indica* contains mainly alkaloids when compared with other secondary metabolites which are in accordance with previous findings (R Dhananjayan et al 1975).

Determination of total anti-oxidant activity: The antioxidant activity of crude samples of *Tylophora indica* was determined and was compared with ascorbic acid standard. Crude sample of Tylophora shows comparable antioxidant activity with ascorbic acid standard.

Hydroxyl radical scavenging assay: Hydroxyl radical scavenging activity directly measures the efficiency of extracts to scavenge OH radicals which can offer protection against oxidative damage and development of cancer. From the results it can be observed that Alcholic extracts of *Tylophora indica* shown an IC50 value of approximately 100µg/ml which is comparable with pure standards of ascorbic acid. Results clearly depict the potential of extracts to scavenge OH radicals.

Reducing power activity: Reducing power shows reducing efficiency of extracts. The increasing concentration shows reducing power.

Nitric oxide Radical scavenging assay: In addition to reactive oxygen species, nitric oxide is also implicated in inflammation, cancer and other pathological condition, the plant or plant products may have the property to counteract the formation of nitric oxide radicals and in turn may be of considerable interest in preventing the ill effect of excessive nitric oxide generation in human body. In our study *Tylophora indica* shows potent nitric oxide radical activity in different concentrations (125µg, 250µg, 500µg) a lethal concentration of value is 2.6mg

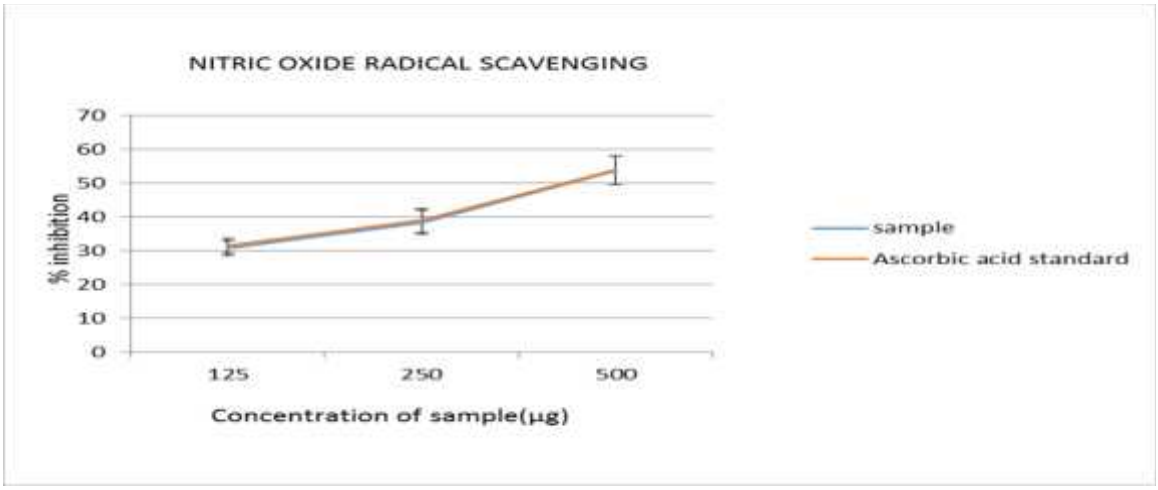

Fig. 4: Nitric oxide radical scavenging assay of Tylophora extracts. Along X axis concentration in µg/ml and Y axis percentage inhibition

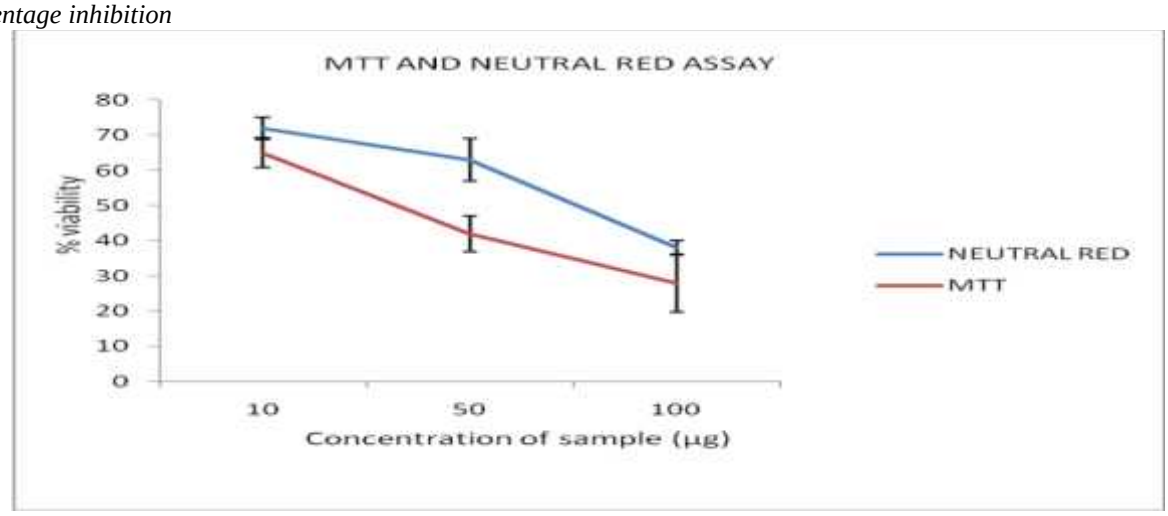

Fig. 5: Viability assessment of HCT 15 cells treated with extracts of Tylophora indica. Along X axis percentage cell viability and X axis concentration of treated extracts in µg/ml

Table: 8. LDH leakage assay in HCT-15

Concentration of sample	Enzyme unit/ml
C	0.12±0.008
10µg	0.23±0.012
50µg	0.45±0.017
100µg	0.76±0.004

Cytotoxity assay: Previous studies confirmed anti-asthamatic, anti-allergic, cytotoxic and also anti-cancer activity of *Tylophora indica* . The main aim of the study was to check the effect of alkaloids in modulating the biological properties of *Tylophora indica* of which anti-proliferative effect was considered significiant. MTT assay was done to determine the proliferative effect of *Tylophora indica*.

MTT cell viability assay in HCT-15: The MTT assay is based on the conversion of yellow tetrazolium salt MTT to purple Formazan crystals by metabolically active cells. The amount of formazan produced is proportional to the number of viable cells. In the study the MTT assay in HCT-15 showed highly reduced cell viability. 10µg crude *Tylophora indica* showed 65% of cell viability. 50µg crude sample showed 42% cell viability and 100µg sample showed 28% viability.

Neutral Red Uptake assay in HCT-15: Neutral red assay is based on the initial protocol described by Boren Jreunel and Puerner (1984) and determines the accumulation of the neutral red dye in the lysosomes of viable, injured cells.

In this study it showed highly reduced cell viability. 10µg crude *Tylophora indica* showed 72% cell viability. 50µg crude showed 63% cell viability and 100µg sample showed 38% viability.

LDH leakage assay in HCT-15: Lactate dehydrogenase is used as a quantitative marker enzyme for the intact cell, its activity providing information on cellular glycolytic capacity. Measurement of LDH leakage is an important and frequently applied test for severe irreversible cell damage.

The study showed increased lactate dehydrogenase. Increased LDH leakage shows loss of membrane integrity. 10µg crude *Tylophora indica* showed 0.23Enzyme unit/ml. 50µg crude sample showed 0.45 Enzyme unit/ml leakage and 100µg sample showed 0.76

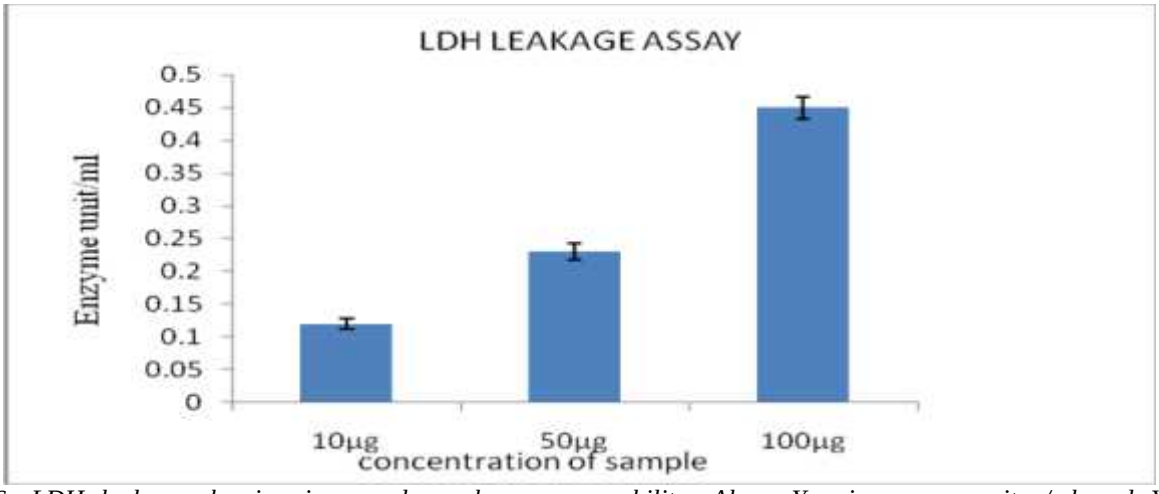

Fig. 6: LDH leakage showing increased membrane permeability: Along Y axis enzyme units /ml and X axis concentration in µg/ml

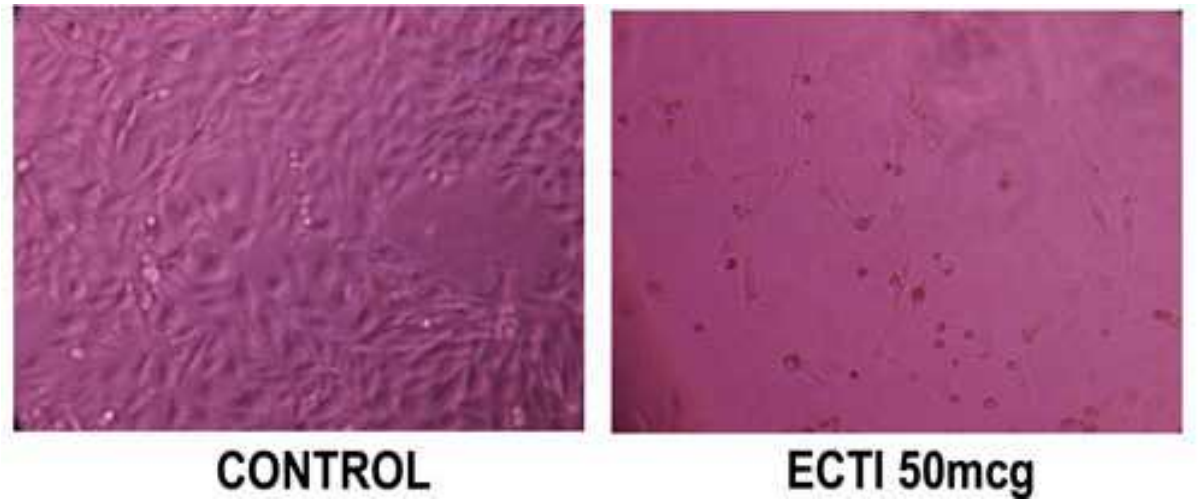

Fig. 7: Phase contrast analysis of HCT 15 cells treated with Tylophora indica.Treatment with samples decline cell morphology and nuclear blebbing observed (Olympus CKX 41- 20X)

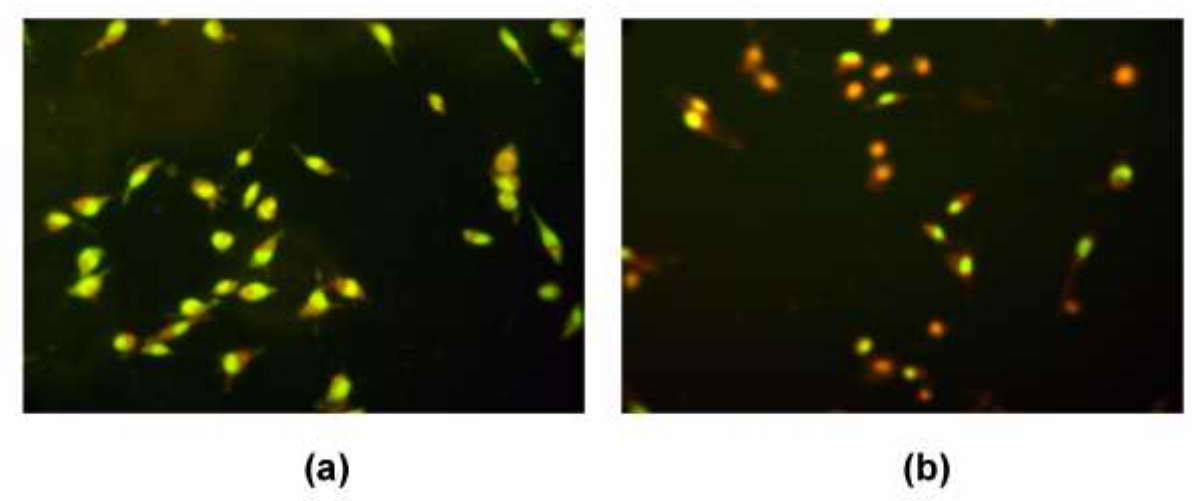

Fig. 8: Determination of apoptosis (a) Untreated control cells showing intact cells with Acridine orange stained green nucleus (b) Cells treated with 50µg/ml Tylophora indica extracts showing increased apoptotic cells (red stained nuclei).

Enzyme unit/ml leakage which confirms dose dependent membrane damage induced by Tylophora extracts in cells.

The increased incidence of cancer across the globe alarms the existing therapeutics, the need for a more safer and promising drug. Plant helds a unique position as a

greater repository for medicines. The present study discuss the pharmacological effects of Tylophora indica as candidate for anticancer activity. Phytochemical studies revealed the presence of compounds especially alkaloids and flavanoids. The significance of phytochemicals alkaloids and flavaniods for treatment of different cancer cells are previosuly described by workers like * Abhishek et al, 2011. the treatment of cancer and reactive oxygen species generation is one area of extreme controversy (Lopez-Lazaro, 2007; Shetab-Boushehri and Abdollahi,2012). The generation of free radicals by chemotherapeutic agents can harm normal cells and pose serious threat to the efficacy of the treatment. Hence we determined whether antioxidant effect of Tylophora extracts can mask the oxidative damage. The different antioxidant scavenging assays performed in our study clearly depicts the antioxidant property of extracts.

Being the third most common cancer in men and second most in women colo rectal cancer (CRC) prevails an increased rate of mortality and morbidity among a larger population. Decades of expensive and replicating research studies have had little impact on primary prevention of CRC and treatment regimens and here the unexplored repository of ayurveda holds a prime position. Tylophora indica is least studied for its anticancer potential apart from few screening studies reported by Gau et al, 2004; Chia et al, 2009, but apart from that no relevant data is avaliable regarding the anticancer activity in cancer of larger incidence namely colon or lung cancer. Our results is clearly showing increased cell death and membrane damage in HCT 15 cells treated with crude extracts of Tylophora. LDH leakage was increased with increasing concentration which confirms the membrane damage induced by Tylophora.

The overall results confirms potent anti oxidant activity of Tylophora indica and effective radical scavenging potential can find significant application in therapeutic applications.

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