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Investigation of Antioxidant and Antiproliferative activity of *Inidigofera trifoliata* Linn. against Hep G2 cell line

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Abstract

The most important phyto-chemical constituents of plants are alkaloids, tannins, flavonoids and phenolic compounds which are capable of curing various human ailments and prevent disorders. Phenolic compounds and flavonoids are widely distributed in plants which have been reported to exert multiple biological effects, including antioxidant, free radical scavenging abilities, anti-carcinogenic activity etc. Antioxidants are compounds that protect cells against the damaging effects of free radicals such as singlet oxygen, superoxide, hydroxyl radicals and reactive oxygen species. Therefore, the present study is investigated on the effect of antioxidant and anti-proliferative capability of the medicinal plant *Indigofera trifoliata* Linn. by using leaf extracts with various solvents. Among the solvents like Methanol, Ethyl acetate and Hexane used for extraction, the methanol extract of *I. trifoliata* observed to be with significant antioxidant capacity by the formation of green phosphomolybdenum complex. The MTT assay was performed to study the anti-proliferative activity of *I. trifoliata* and found the plant posses potential in scavenging free radicals and in chelating metal ions. In addition, the plant extract proved to be an efficient in causing toxicity to liver cancer cells (Hep G2). Hence, this plant could be considered as an efficient source of therapeutic compounds against free radical induced cancer. However, further studies are required to isolate, purify and analyse the specific bioactive compounds for the antioxidant and anticancer activity.

Keywords: Phyto-chemical analysis, MTT assays, Antioxidant activity, Anti-proliferative activity, Hepatocellular carcinoma (Hep G2), *Indigofera trifoliata.*

Contents

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1. Introduction

Plants having potential phytochemical constituents like steroids, alkaloids, tannins, flavonoids and phenolic compounds which are capable of curing various human ailments and prevent disorders. Among the phytochemical constituents, steroids, flavonoids and phenolic compounds are playing a key role in society especially in health care

Industry. Plant and plant-derived products are part of the healthcare system since ancient human civilization (Khatun *et. al.,* 2011). For centuries, people using plants for their therapeutic values and today about 85,000 plants have been documented for therapeutic use globally (Sharma *et al.,* 2011). The use of herbs and alternative medicine has increased dramatically in the last two to three decades (Plengsuriyakarn *et al.,* 2012). The medicinal properties of plants have also been investigated worldwide, due to their potent pharmacological activities, low toxicity and economic viability (Zahin *et al.,* 2009). The traditional medicinal methods, especially the use of medicinal plants, still play a vital role to cover the basic health needs in the developing countries (Shihabudeen *et al.,* 2010). The most bioactive crude extracts in terms of high antioxidant activity and potent antiproliferative activity in plants were reported by Mohd Alfazari Mohd Ghazali *et al*., 2014). India has about 8000 species of medicinal plants among which about 1000 plants have been used in the traditional system of medicine like Ayurveda, Unani and Siddha. In a recent period, the development of more effective and safer drugs in stipulated and natural products from the plants and their synthetic derivatives are expected to play an important role (Sun *et al.,* 2011). The most important of these bioactive constituents are alkaloids, tannin, flavonoids and phenolic compounds (Shihabudeen *et al.,* 2010). Antiproliferative activities of isolated pure compounds from medicinal plants against HepG2 human liver cancer cells and MCF-7 human breast cancer cells were evaluated and reported (Xiangjiu He *et al.,* 2008).

In connection with the above scientific facts, the present study is planned to investigate the antioxidant and antiproliferative capability of the medicinal plant *Indigofera trifoliata* Linn. The study also involve in analysis of overall phytochemical properties of *I. trifoliata* after extraction with different solvent systems like chloroform, ethyl acetate and methanol.

2. Materials and Method

Plant collection

Fresh leaves of *Indigofera trifoliata* Linn.were collected from the fields located in Malachery forest, Villupuram District, Tamil Nadu and was identified and verified by a plant taxonomist Dr.S.Narasimman, Professor of Botany from Madras Christian College, Chennai, Tamil Nadu. The morphological appearance of *I. trifoliata* is shown in **fig. 1**.

Figure 1: Morphology of *Indigofera trifoliata* Linn.

Preparation of *Indigofera trifoliata* plant extract

The leaves were carefully washed with tap water, rinsed with distilled water and air-dried for 1 hour at room temperature. Then the leaves were separated and shadow dried in room temperature for few days till the leaves were completely dried and able to crush into powder. Then the air dried leaves were ground into powder and subjected to direct extraction with chloroform, ethyl acetate and methanol. The coarsely ground plant material was extracted with chloroform, ethyl acetate and methanol in the ratio of 1:10 (w/v) by repeated extraction. The extract was filtered through the Whatmann No. 1 filter paper and the excess solvent was removed by condensation by steam batch (Eloff, 1998).

Antioxidant activity assays

DPPH assay: **(2, 2-diphenyl-1-picrylhydrazyl)**

The Radical Scavenging Activity of different extracts was determined by using DPPH assay according to Chang *et al.,* (2008) with little modification. The decrease of the absorption at 517nm of the DPPH solution after the addition of the antioxidant was measured in a cuvette containing 2.96ml of ethanolic DPPH (0.1 mM) solution and 20 to 200 µg/ml of plant extract. The setup was left at dark in room temperature and the absorption was monitored after 20 minutes. Ascorbic acid was used as standard. The ability of the plant extract to scavenge DPPH radical was calculated by the following equation:

$$\% \text{ of DPPH Radical Scavenging Activity (\% RSA)} = \frac{\text{Abs. control} - \text{Abs. sample} * 100}{\text{Abs. control}}$$

Abs. control is the absorbance of DPPH radical + ethanol Abs. sample is the absorbance of DPPH radical + plant extract

Phosphomolymbdenum assay

The antioxidant activity of samples was evaluated by the green phosphomolybdenum complex formation according to the method of Prieto *et al.,* (1999). An aliquot of 100µl of sample solution was combined with 1ml of reagent solution (0.6M H_2SO_4, 28 mM Sodium phosphate and 4 mM Ammonium molybdate) and incubated in a water bath at 95°C for 90 min. After the samples had cooled to room temperature, the absorbance of the mixture was measured at 695 nm against a blank. The result was expressed as percentage of inhibition.

Super oxide anion radical scavenging activity

About 1 ml of NBT solution (156µM NBT in 100mM PO_4 buffer, pH 8.0) mixed with 1ml of NADH solution (468µM in 100mM PO_4 buffer, pH8) and 0.1ml of test sample (10mg/m). The reaction was started by adding 100µl of PMS solution (60µM PMS in 10mM PO_4 buffer, PH 8.0). The mixture was incubated at 25ºC for 5 minutes and the absorbance was measured spectrophotometrically at 560 nm (Nishikimi *et al*., 1992).

Hydroxyl radical scavenging activity

The scavenging activity of methanol extract of *I. trifoliata* on hydroxyl radical was measured according to the method of Klein *et al.,* (1992). Various concentrations (50, 100, 150 and 200 µg/mL) of extracts were added with 1.0 ml of iron-EDTA solution (0.13% ferrous ammonium sulfate and 0.26% EDTA), 0.5 ml of EDTA solution (0.018%) and 1.0 mL of dimethyl sulphoxide (DMSO) (0.85% v/v in 0.1M phosphate buffer, pH 7.4). The reaction was initiated by adding 0.5 ml of ascorbic acid (0.22%) and incubated at 80–90°C for 15 min in a water bath. After incubation, the reaction was terminated by the addition of 1.0 mL of ice-cold TCA (17.5% w/v). About 3 ml of Nash reagent (75.0g of ammonium acetate, 3.0 mL of glacial acetic acid, and 2 mL of acetyl acetone were mixed and raised to 1000 mL with distilled water) was added and left at room temperature for 15 min. The reaction mixture without sample was used as control. The intensity of the color formed was measured spectrophotometrically at 412 nm against reagent blank. The % hydroxyl radical scavenging activity is calculated by the following formula:

$$\% \text{ HRSA} = \text{from } [(A0 - A1)/A0] \text{ X } 100$$

Where A0 is the absorbance of the control and A1 is the absorbance of the extract/standard.

Metal chelating activity

The chelating of ferrous ions by methanol extract of *I. trifoliata* was estimated by the method of Dinis *et al.,* (1994). Briefly the extract samples (250 µl) were added to a solution of 2 mmol/L $FeCl_2$ (0.05 mL). The reaction was initiated by the addition of 5 mmol/L ferrozine (0.2 mL) and the mixture was shaken vigorously and left standing at room temperature for 10 min. Absorbance of the solution was then measured spectrophotometrically at 562 nm. The result was expressed as percentage of inhibition.

Qualitative phytochemical analysis

The different qualitative chemical tests were performed for establishing the profile of given extract for its chemical composition. The tests were performed according to the methods of Harborne *et al*., (1989).

Quantitative analysis of phytochemicals

The total phenols and total flavonoids were estimated using standard techniques as established by McDonald *et al*, (2001). A linear correlation has been obtained by comparing the antioxidant activity and polyphenols and flavonoid content of the extracts.(SakshimaThusoo et al., 2014)

Thin Layer Chromatography

The plant extract was loaded on pre-coated silica plates which were then developed using the solvents methanol, chloroform in the ratio of 0.75:9.25. The spots were identified both in the UV light, far light and in the iodine chamber. Then R_f value was calculated as the ratio of distance travelled by the solute to the distance travelled by the solvent. The extract which showed DPPH inhibition of more than 90% was examined by Thin Layer Chromatography (TLC). The plant extract was applied on pre - coated TLC sheets and developed in solvent mixture then allow it for dry. After drying the plates, dipped in a 0.2% DPPH reagent in methanol or ethanol and were left for 30 minutes at room temperature. Antioxidant activity was confirmed when the DPPH purple color changed to yellow under white light.

Evaluation of anti-proliferative potential

Chemicals and reagents: MTT (3-[4,5-dimethylthiazol-2-dimethyl]-2,5-diphenyl tetrazolium bromide) invitrogen, USA. Acridine orange were obtained from Sigma, USA. All other fine chemicals were obtained from Sigma–Aldrich, St. Louis.

Cell culture:

Hep G2 cells obtained from NCCS (National Centre For Cell Science, Pune) were cultured in Rose well Park Memorial Institute medium (RPMI), supplemented with 10% fetal bovine serum, penicillin/streptomycin (250 U/mL), gentamycin (100ug/mL) and amphotericin B (1mg/mL) were obtained from Sigma Chemicals, USA. All cell

cultures were maintained at 37◦C in a humidified atmosphere of 5% CO_2. Cells were allowed to grow to confluence over 24 h before use.

Cell growth inhibition studies by MTT assay:

The methanolic extracts of the plants were prepared and their cytotoxic effects on four human cancer cell lines (A549, human lung adenocarcinoma; MCF7, human breast adenocarcinoma; HepG2, hepatocellular carcinoma and HT-29, human colon carcinoma) and one normal cell line (MDBK, bovine kidney) were examined using the MTT assay.(Sahar Behzad *et al.*, 2014) .Cell viability was measured with the conventional MTT reduction assay, as described previously with slight modification. Briefly, Hep G2 cells were seeded at a density of 5×103 cells/well in 96-well plates for 24 h, in 200ul of RPMI with 10% FBS. Then culture supernatant was removed and RPMI containing various concentrations (1–1000µg/ml) of methanol extract of *I. trifoliata* was added and incubated for 48 h. After treatment cells were incubated with MTT (10 µl, 5 mg/mL) at 37 ◦ C for 4 hour and then with DMSO at room temperature for 1 hour. The plates were read at 595 nm on a scanning multi-well spectrophotometer. Data represented the mean values for six independent experiments.

3. Results

Radical scavenging activity (RSA) *Indigofera trifoliata* extracts (DPPH assay)

From the dose dependent response curve of DPPH radical scavenging activity of different leaf extracts of, the methanol extract of *I. trifoliata* was observed to have higher scavenging activity than ethyl acetate and chloroform (**Table 1 and Fig.2**). At a concentration of 200 µg/mL, the scavenging activity of Methanol extract reached 67.24%. The Methanol leaf extract of *I. trifoliata* showed excellent antioxidant and free radical scavenging activity. In considering this, the Methanol leaf extract was chosen for further study.

Table 1: DPPH Radical Scavenging effect of extracts of *I. trifoliata*

S.No	Concentration (µg)	% inhibition		
		Methanol	Ethyl acetate	Hexane
1	20	**36.86**	28.16	16.42
2	40	**39.47**	31.45	19.3
3	60	**42.51**	35.87	25.82
4	80	**44.81**	37.94	27.94
5	100	**49.00**	39.1	31.75
6	120	**53.64**	43.51	32.93
7	140	**57.19**	47.68	36.24
8	160	**59.99**	50.75	39.88
9	180	**63.48**	53.49	41.4
10	200	**67.24**	55.77	41.82

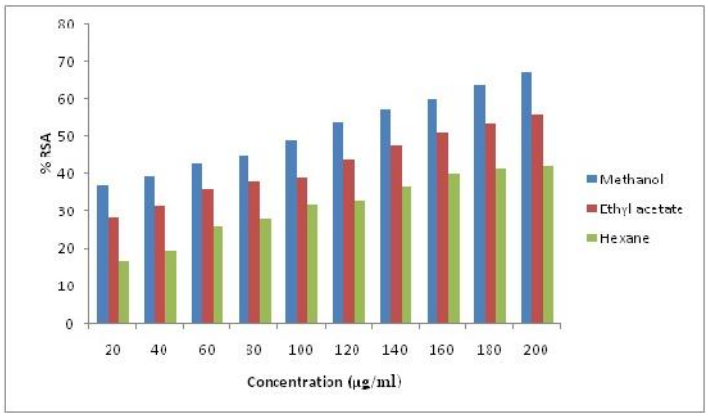

Figure 2: DPPH Radical Scavenging effect of extracts of *I. trifoliata*

Phosphomolybdenum assay:

The assay is based on the reduction of Mo (VI) to Mo (V) by the extract and subsequent formation of a green phosphate/Mo (V) complex at acid pH. The phosphomolybdenum method is quantitative since the antioxidant activity is expressed as the number of equivalents of ascorbic acid. The methanolic extract of *I. trifoliata* was used to determine their antioxidant capacities by the formation of green phosphompolybdenum complex (**Table 2**). The

results indicate that the test has 33.22% against the standard 90.98% which shows the methanol extract is having moderate antioxidant in the reduction of phosphomolybdenum complex.

In vitro superoxide anion radical scavenging of *I. trifoliata* leaf extracts

In this system, superoxide anion derived from dissolved oxygen by PMS-NADH coupling reaction reduces NBT. The decrease of absorbance at 560nm with antioxidants indicates the consumption of superoxide anion in the reaction mixture. The methanol extract have strong superoxide radical scavenging activity and exhibited higher superoxide radical scavenging activity than control (**Table - 2**) as 1.387 in control, 44.84 in test sample and 96.46 in standard sample.

Metal chelating activity:

Presence of transition metal ions in a biological system could catalyse the Haber–Weiss and Fenton-type reactions, resulting in generation of hydroxyl radicals (OH). However, these transition metal ions could form chelates with the antioxidants, which result in the suppression of OH generation, and inhibit ion of peroxidation processes of biological molecules. In this assay, the presence of chelating agents in the extract of *I. trifoliata* disrupts the ferrozine - Fe^{2+} complex formation, thus decreasing the red colour. The data presented in this study indicated that *I. trifoliata* ability for iron binding and could reduce the generation of hydroxyl radicals as the value of 0.074 in control, 35.13 in test sample and 67.56 in standard sample (**Table 2**).

Table 2: Comparative analysis showing Phosphomolybdenum assay, Superoxide radical scavenging assay and Metal chelating activity of *Indigofera trifoliata*

Name of the test	***Activity (OD values) in different species***
Phosphomolybdenum assay in methanol extract	
Control	0.695
Test	33.22
Standard	90.98
Superoxide radical scavenging activity	
Control	1.387
Test	44.84
Standard	96.46
Metal chelating activity	
Control	0.074
Test	35.13
Standard	67.56

Hydroxyl radical scavenging activity

The hydroxyl radical is one of the representative reactive oxygen species generated in the body. These radicals are produced through various biological reactions; one of the common reactions is the Iron (II)-based Fenton reaction. Hydroxyl radical scavenging activity of methanol extract and standard is presented in the **Table 3 and Figure 3**.The increased concentration of sample showed increasing effect of activity as from 14.88% in 25 µg to 65.04% in 100 µg concentration. The radical scavenging capacity may be attributed to phenolic compounds in methanol extract with the ability to accept electrons, which can combine with free radical competitively to decrease hydroxyl radical.

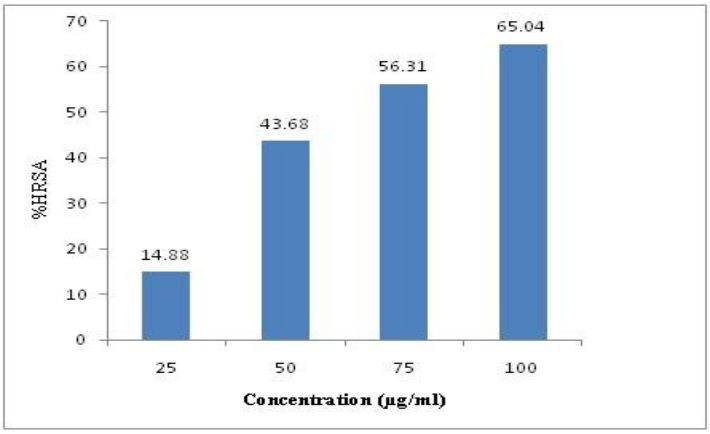

Figure 3: Hydroxyl radical scavenging activity of Methanol extract of *Indigofera trifoliata*

Table 3: Hydroxyl radical scavenging activity of Methanol extract of *Indigofera trifoliata*

Concentration (µg)	HRSA (%)
25	14.88
50	43.68
75	56.31
100	65.04

Qualitative Phytochemical screening of methanol extract of *I. trifoliata*

The preliminary phytochemical screening of *I. trifoliata* has revealed the presence of phenolics and flavonoids in high amounts, whereas, terpenoids, reducing sugars, saponins, proteins and tannins were present in moderate amount. Alkaloids were found to absent (**Tab 4).**

Quantitative analysis of phytochemicals

Determination of total phenols and Flavonoids by spectrophotometric method

Based upon the preliminary phytochemical test, Quantitative determination of phytoconstituents was carried out for the extracts of *I. trifoliata.* From the standard methods used and found that total phenolic compound 189.81mg/ml and flavonoids 783.59mg/ml was present in the plant extract (**Table 4).**

Table 4: Qualitative and Quantitative phytochemical analysis of Methanol extract from *Indigofera trifoliata.*

S.No	phytochemicals	Test	Result
1	Detection of alkaloids	Mayer's test	-
2	Detection of phenolic compound	Ferric chloride test	+++ 189.81 GA E/g
3	Detection of glycosides	Borntrager's test	++
4	Detection of terpenoids	Salkowski test	++
5	Detection of flavonoids	---	+++ 783.59 Q E/g
6	Detection of tannins	---	++
7	Detection of reducing sugars	Fehling's test	++
8	Detection of saponins	Foam test	++
9	Detection of proteins	Millon's test	++

Thin Layer Chromatography

The chromatogram developed with 10% methanol in chloroform revealed the presence of 7 major compounds at R_f value of 0.81, 0.71, 0.61, 0.55, 0.47, 0.42 and 0.21 as visualized UV illumination and 4 more compounds with R_f values of 0.68, 0.33, 0.24 and 0.18 (**Fig 4**).

Autobiography

The specific compound (band) which has anti oxidative properties was found out as the compound with R_f value of 0.81 (**Fig. 4**). The antioxidant activity was confirmed when the DPPH purple color changed to yellow.

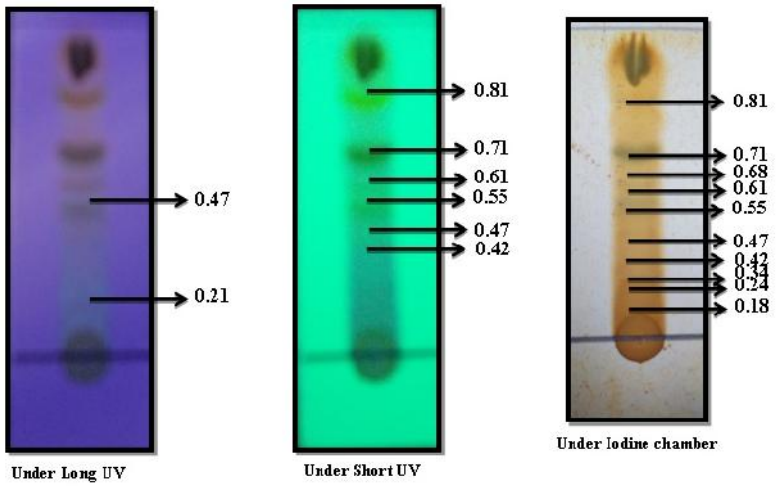

Figure 4: Thin layer chromatography

Antiproliferative effect of methanolic extract on HepG2 cell line

The result for MTT assay is shown in **Fig. 5** which shows the decreasing cell viability in increasing concentration of sample extract. In this assay cell viability was found to be reduced to 43% by the methanol extract of *I. trifoliata* (at a concentration of 100µg/ml) It is thus derived from the results that the plant has significant cytotoxic effect on HepG2 cell lines (**Fig. 6**) and hence it can be considered as a promising source of antiproliferative bioactive compounds.

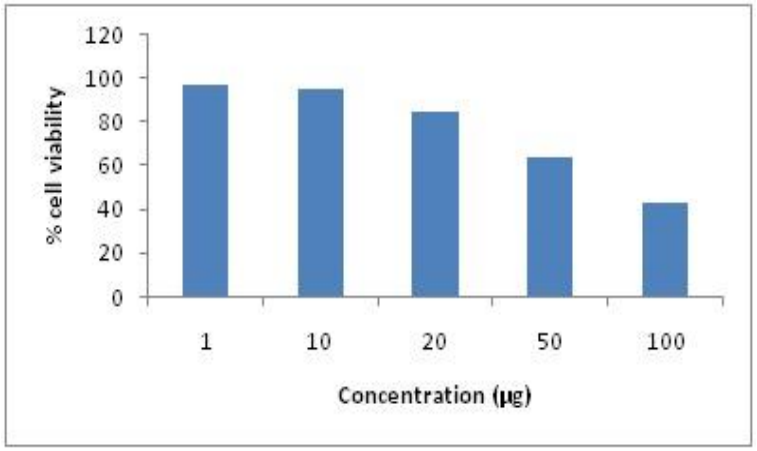

Figure 5: Effect of Methanol extract of *I. trifoliata* on viability of HepG2 cells

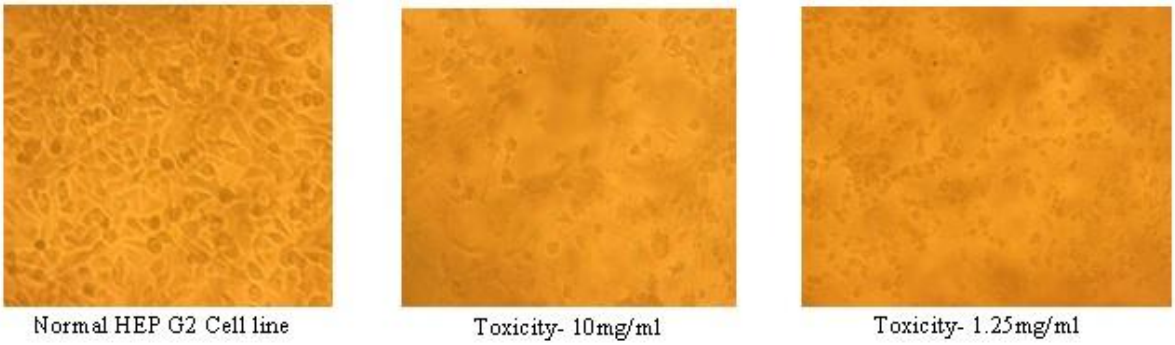

Figure 6: Cytotoxicity of Methanol extract of *I. trifoliate* on HepG2 cells

4. Discussion and Conclusion

The important advantages for therapeutic uses of medicinal plants in various ailments are their safety besides being economical, effective and easy availability (Rajamurugan et al., 2013). Several techniques have been used to determine the antioxidant activity *in vitro* in order to allow rapid screening of substances since substances that have low antioxidant activity *in vitro*, will probably show little activity *in vivo*. Free radicals are known to play a definite role in a wide variety of pathological manifestations. Antioxidants fight against free radicals and protect us from various diseases. They exert their action either by scavenging the reactive oxygen species or protecting the antioxidant defence mechanisms (**Saeed *et al*., 2012).**In oxidative stress, reactive oxygen species (ROS) such as superoxide (O2 -, OOH), hydroxyl (OH) and peroxyl (ROO) radicals are generated. The ROS play an important role in several human diseases such as cancer, neurodegenerative disorders, cardiovascular diseases, atherosclerosis, cataracts and inflammation Dinesh *et al*., 2014).

The electron donation ability of natural products can be measured by 2,2′-diphenyl-1- picryl hydrazyl radical (DPPH) purple-coloured solution bleaching. The method is based on scavenging of DPPH through the addition of a radical species or antioxidant that decolourizes the DPPH solution. The degree of colour change is proportional to the concentration and potency of the antioxidants. A large decrease in the absorbance of the reaction mixture indicates significant free radical scavenging activity of the compound under test. In the present study among all the fractions tested, methanol extract showed significantly higher inhibition percentage and positively correlated with total phenolic content and total flavonoid content. Results of this study suggest that the plant extract contain phytochemical constituents that are capable of donating hydrogen to a free radical to scavenge the potential damage (**Saeed *et al*., 2012).**

The antioxidant capacity of the fractions was measured spectrophotometrically through phosphomolybdenum method, based on the reduction of Mo (VI) to Mo (V) by the test sample and the subsequent formation of green phosphate/Mo (V) compounds with a maximum absorption at 765 nm (**Saeed *et al*., 2012).** The present study showed that methanol extract was capable of reducing the phosphomolybedenum complex which was recorded as reduction in OD of the sample when compared with control.The •OH radical is an extremely reactive in biological systems and has been implicated as highly damaging species in free radical pathology, capable of damaging biomolecules of the living cells. These radical combines with nucleotides in DNA and cause strand breakage leading to carcinogenesis, mutagenesis and cytotoxicity. Hydroxyl radical (•OH) scavenging capacity of an extract is directly related to its antioxidant activity (Khan *et al*., 2012).

The chelating of Fe2+ by extracts was estimated by the method of Dinis *et al*. (1994). Ferrozine can quantitatively form complexes with Fe2+. However, in the presence of chelating agents, the complex formation is disrupted with the result that the red colour of the complex is decreased. Measurement of colour reduction, therefore, allows the estimation of the chelating activity of the coexisting chelator. The transition metal ion, Fe2+ possess the ability to move single electrons by virtue of which it can allow the formation and propagation of many radical reactions, even starting with relatively non-reactive radicals (Aboul- Enein et al., 2003). *I. trifoliata* has the most active extract interfered with the formation of ferrous and ferrozine complex, suggesting that it has chelating activity and captures ferrous ion before ferrozine. Methanol extract of *I. trifoliata* was used to determine their antioxidant capacity by the formation of green phosphomolybdenum complex. The result indicates that the methanol extract is more powerful antioxidant in the reduction of phosphomolybdenum complex. The MTT assay performed to study the antiproliferative activity of *I. trifoliata* depicts that the extract possed significant inhibits activity on the proliferative of Hep G2 cell lines. This denotes that *I. trifoliata* could be considered as an effective source of antiproliferative bioactive compounds.

Since the result of the present study revealed the presence of phenols and flavonoids in major amounts, it can be derived that these phytochemicals might be respresent for the potential of *I. trifoliata*. Further mechanistic studies are required to isolate, purify and analyse the specific bioactive compound respectively for the antioxidant activity. To conclude, the plant *Indigofera trifoliata* was studied and found a potential in scavenging free radicals and in chelating metal ions. In addition, the plant extract proved to be efficient in causing toxicity to liver cancer cells (Hep G2). Hence this plant could be considered as an efficient source of therapeutic drugs against free radical induced cancer.

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