Int. J. Drug Res. Tech. **2014, Vol. 4 (3), 46-54** **ISSN 2277 - 1506**

International Journal of Drug Research and Technology

Available online at http://www.ijdrt.com

Original Research Paper

PRELIMINARY PHYTOCHEMICAL SCREENING AND *IN VITRO* ANTIOXIDANT ACTIVITIES OF AQUEOUS EXTRACT OF *INDIGOFERA TINCTORIA* AND *INDIGOFERA ASTRAGALINA*

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ABSTRACT

The phytochemical screening of primary and secondary metabolites present in the plants use for various bioactive applications lead to the development of new novel plant based drugs. The present study aimed to address *in vitro* antioxidant activities of *Indigofera tinctoria* and *Indigofera astragalina* are commercially available and used in traditional medicine, because of its high nutritive value. The *in vitro* antioxidant study was carried out to study for its total reducing power, nitric oxide radicals scavenging, hydroxyl radicals scavenging, DPPH scavenging and total antioxidant activity. The results showed that the flavonoid and phenolic content of aqueous extracts of *I. tinctoria* and *I. astragalina* was 43.94 µg/mg, 55.05 µg/mg and 41.07 µg/mg, 36.55 µg/mg respectively. The IC_{50} value of *I. tinctoria* aqueous extract for DPPH, Nitric oxide radical scavenging and Hydroxyl radical scavenging was 512.66 ± 1.26 µg/mL, 585.28 ± 1.24 µg/mL and 483.78 ± 1.18 µg/mL. The IC_{50} value *I. astragalina* was 650.51 ± 1.20 µg/mL, 662.32 ± 1.26 µg/mL and 525.57 ± 1.28 µg/mL. The high absorbance of total reducing power and total antioxidant activity indicates a higher antioxidant activity of the extracts. In our knowledge aqueous extract of *I .tinctoria* and *I. astragalina* showed a potential antioxidant activity because of its high phenolic contents.

Keywords: *Indigofera tinctoria*, *Indigofera astragalina*, Antioxidant activity.

INTRODUCTION

India is the rich sources of medicinal plants. In traditionally, Indian peoples are using medicinal plants to treat variety of diseases. Plant contains substances that can be used for therapeutic purposes as precursors for the synthesis of useful drugs. Plants are involved significant role in allopathic medicine, herbal medicine, homeopathy and armatherapy. Pharmaceutical companies are involved in develop a plant based drugs, because of low toxicity, easily available, safe, efficient and rarely have side effects.[1] Over the years, the medicinally important plants have been studied and evaluated the various phytochemicals accumulated in the various morphological parts of plants. This has been leads to the isolation of organic compounds.[2] Plants are synthesized variety of chemical constituent's via primary and secondary metabolism. Preliminary phytochemical screening played a significant role to find out the chemical constituents, which is useful to develop a new drug for the pharmaceutical industry.[3] The phytochemical constituents like phenols, anthroquinons, alkaloids, glycosides, flavonoids and saponins showed a antibiotic role of plants,[4] flavonoids and phenols present in plants play a important role in prevention of human diseases.[5] The presence of these phytochemical constituents in the plant exhibits the antiashmatic, immunomodulatory, hepetoprotectivity, hyphocholestremic and antiinflamatory activities.[6] Free radicals like superoxide anions, hydrogen peroxide, hydroxyl radicals and nitric oxide radical leads to tissue injury. These free radicals play a vital role in

oxidative damage to various biomolecules including proteins, lipids, DNA and pathogenesis of various diseases such as diabetes mellitus, cancer, atherosclerosis, arthritis, neurodegenerative diseases and ageing process.[7] Antioxidants are important to prevent the formation of free radical and avoid the cell damage, which is essential to prevent cancer and heart disease. A number of synthetic antioxidants were developed like butylated hydroxyl toluvene (BHT), butylated hydroxyl anisole (BHA) and tert-butyl-hydroquinone (TBHQ), but the usage of these agents was avoided due to their toxicity.[8] Various types of antioxidants were isolated from different kind of higher plants. Furthermore antioxidants from natural sources increase the shelf life of foods.[9] The plant *Indigofera tinctoria* belongs to the family of Fabaceae, its commonly known as "True Indigo'' and called as Neeli or Avuri in Tamil. *I. tinctoria* is a shrub, distributed throughout the India. The different parts of the plants are used for variety of diseases. The seeds of *I. tinctoria* containing galactomannon composed of galactose and mannose. Roots and leaves are used in epilepsy and hydrophophia.[10] The aerial parts of *I. tinctoria* used in treatment of antiproliferative activity in human lung cancer.[11] Different solvent extracts of *I. tinctoria* showed antibacterial activity.[12] Dry powder of *I. tinctoria* used to treatment of asthma.[13] The leaves of *I. tinctoria* used as antiinflamatory traditionally. Indirubin is the active compounds isolated from *I. tinctoria* leaves active as effective anticancer drug.[14] Indigotin is the active compounds isolated from this leaves posses hepatoprotective activity.[15] *Indigofera astragalina* belongs to the family of Fabaceae, commonly known as "Hairy Indigo'' in English. The flowers are small reddish purple in colour and produced racemes of 2-10 cm long. *I. astragalina* shows antibacterial activity against *S. aureus* and *E.coli* because of presence of rich amount of tannins, cardiac glycosides, saponins and alkaloids.[16] Presence of polyphenol lignin, phosphorous, nitrogen and potassium in *I. astragalina* shoots posses to rich nutritive value of this plant. The leaves contain nine essential and eight non essential aminoacids.[17] The present study focussed to study the phytochemical constituents and antioxidant activities of aqueous extracts of *I. tinctoria* and *I. astragalina*.

MATERIALS AND METHODS

Plant Material and Extraction

The plant materials *I. tinctoria* and *I .astragalina* were collected from tirunelveli district of Tamilnadu. The plant was identified by Dr. P. Jeyaraman, Director, Institute of herbal Botany plant anatomy research centre, West Tambaram, Chennai-45. The collected plant leaves were washed, and shade dried. Aqueous extract was prepared from the homogenized powder using magnetic stirrer. Briefly 20 g of homogenized powder mixed in 200 mL distilled water using magnetic stirrer for 24 h and filtered using Whatmann No.1 filter paper. The filtrate was further lyophilized and stored in refrigerator for further analysis.

Phytochemical Screening

Preliminary phytochemical screening of (Alkaloids, Aminoacids, Anthroquinons, Carotenoids, Flavonoids, Glycosides, Proteins, Reducing Sugar, Saponins, Steroids, Tannins and Terpenoids) aqueous extract of *I. tinctoria* and *I. astragalina* were carried out according to standard procedure.[18-20]

Determination of Total Phenolic Content

The total phenolic content of the aqueous extract of *I. tinctoria* and *I. astragalina* leaves were determined by Folin- Ciocalteu reagent method[21] using tannic acid as a standard with slight modifications. Briefly, 1 mL of different concentration of extract and standard (200 µg to 1000 µg) were taken in test tube and made up the volume to 2 mL using distilled water. Add 1 mL of 10% Na_2Co_3 solution and 1 mL of Folin-Ciocalteu reagent (1:10 dilution in distilled water). The mixture was incubated for 2 h at room temperature. The absorbance was read at 765 nm against Folin- Ciocalteu reagent as a blank. The amount of phenolic content was determined by tannic acid standard curve.

Determination of Total Flavonoid Content

The total flavonoid content of the aqueous extract *I. tinctoria* and *I .astragalina* leaves were determined by Hsu. C *et al.*[22] with slight modifications. Different concentration of extract and standard (200 µg-1000 µg/mL) were taken in 2 mL of distilled water and consequently add 0.15 mL of $NaNo_2$ solution and allowed to stand for 6 min followed by add 0.15 mL of 10% $AlCl_3$ in methanol and allowed to stand for 6 min at room temperature. To this reaction mixture add 2 mL of 4% NaOH solution and immediately add distilled water to make up the volume to 5 mL, and incubated for 15 min at room temperature. The absorbance of the reaction mixture was read at 510 nm against blank without plant extract. The amount of flavonoid content was determined by quercetin standard curve.

In Vitro Antioxidant Activity

Total reducing power

The total reducing power of aqueous extract was evaluated by Yan Zhao *et al.*[23] Briefly different concentration of extract (200µg-1000 µg/mL) was taken in 1mL 0.24 M phosphate buffer (pH 6.6), 5mL of reaction mixture containing 1% potassium ferricyanide, 0.2 M phosphate buffer (pH 6.6) was added to the extracts and incubated for 20 min at 50° C add 5mL of 10%. TCA (Trichloro Acetic acid) and centrifuge the content at 1000 rpm for 10 min. 2mL of supernatant mixed with 2mL of distilled water and then added 0.2mL of 0.1% $Fecl_3$. The absorbance was read at 700nm, using distilled water as a blank.

Total antioxidant activity

The total antioxidant activity of aqueous extracts was determined by Habib *et al.*[24] 1mL of different concentration of plant extracts (200 µg-1000 µg) were mixed with 3mL of reaction mixture containing Sulfuric acid (0.6 M), Sodium Phosphate (28 mM) and Ammonium Molypdate (4 mM). The above mixture was incubated for 90 min at 95 °C. After the incubation sample was kept for cooling and absorbance was read at 695 nm against reaction mixture as a blank.

Hydroxyl radical scavenging activity

The hydroxyl radical scavenging activity of aqueous extracts were evaluated by Yan Zhao *et al.*[23] 0.5 mL of extract (20 µg-1000 µg/mL) mixed with 0.5 mL of 9.1 mM Salicylic acid in ethanol and 0.5 mL of 9 mM $FeCl_2$ solution and then add 0.5 mL distilled water. Since, then add 0.5 mL of 88 mM H_2O_2 was added to start Fenton reaction. The absorbance (A_1) was measured at 510 nm. The absorbance (A_2) was measured with 0.5 mL of distilled water instead of $FeCl_2$. The absorbance (A_3) was measured with 0.5 mL of distilled water instead of plant extract. Without plant extract and $FeCl_2$ serve as a blank. The amount of hydroxyl radical scavenging activity was measured using the following formula,

% of scavenging activity- $[1-(A_1-A_2)/A_3] \times 100$

Nitric oxide radical scavenging assay

Nitric oxide radical scavenging activity of aqueous extracts was determined by senthilkumar *et al.*[25] The reaction mixture containing 2 mL of Sodium Nitroprusside (10 mM), 0.5 mL of phosphate buffer saline (pH-7.4) and 0.5 mL of different concentration of extracts (200 µg-1000 µg/mL) was incubated for 150 min at 25° C. After that 0.5 mL of Nitrite was pipette out and mixed with 1mL sulfonilic acid reagent (0.33% in 2% Glacial acetic acid) and kept for 5 min. Then add 1 mL of 1% Naphthyl ethylene diamine dihydrochloride was added (NEDD) and allowed to stand for 30 min at 25° C. The absorbance was read at 540 nm. The percentage of Nitric Oxide inhibition was calculated using the following formula,

% of Nitric oxide radical scavenging assay-$[A_0-A_1/A_0]\times 100$

A_0- control (without Plant Extract), A_1- Test Sample

DPPH radical scavenging assay

The DPPH radical scavenging activity of aqueous extracts was evaluated by senthilkumar *et al.*[25] About 2 mL of various concentrations of extracts and standard added to 2 mL of 0.1 mM Diphenyl picrylhydrazine solution in methanol (DPPH) and incubated 37° C in the dark for 30 min. The absorbance was read at 517 nm using methanol as a blank.The percentage of DPPH radical scavenging activity was determined by following formula,

% of DPPH radical scavenging- $[A_0-A_1/A_0]\times100$

A_0- Control (without plant Extract), A_1- Test Sample

RESULTS

Preliminary Phytochemical Screening

The preliminary phytochemical screening of aqueous extracts showed the presence of Aminoacids, Flavonoids, Glycosides, Protein, Reducing sugar, Saponins, in *I. tinctoria* and *I. astragalina* aqueous extracts. Alkaloids, Steroids and Tannins are present exclusively in *I. tinctoria* aqueous extracts. Both plants aqueous extracts are showed the absence of Anthroquinons, Carotenoids and Terpenoids (Table. 1).

Total Phenolic Content

Total phenolic content of aqueous extracts of *I. tinctoria* and *I. astragalina* calculated using the tannic acid standard curve. The phenolic content of *I. tinctoria*, *I. astragalina* showed 55.05 µg/mg and 36.55 µg/mg of aqueous extracts.

Total Flavonoid content

Total flavonoid content of aqueous extracts of *I.tinctoria* and *I.astragalina* calculated using the quecertin standard curve. The amount of total flavonoid content present in aqueous extracts of *I.tinctoria*, *I.astragalina* showed 43.94 µg/mg and 41.07 µg/mg espectively.

Total Reducing Power

Total reducing power of aquous extracts of *I.tinctoria* and *I. astragalina* were evaluated by potassium ferricyanide reduction method. The aqueous extracts showed the possible of iron reducing power, when increasing the quantity of aqueous extracts. A higher absorbance indicates the higher antioxidant activity of aqueous extracts (Fig. 1). The higher absorbance of total reducing power of *I. tinctoria* and *I. astragalina* was 0.611/1 mg, and 0.465/1 mg against potassium Ferricyanide indicates the antioxidant activity of the extracts.

Total Antioxidant Activity

Total antioxidant activity used to determine the antioxidant level of the any compounds. The increasing absorbance indicates the high antioxidant activity of the compounds. Total antioxidant activity of aqueous extracts of *I. tinctoria* and *I. astragalina* were increased, when increasing the quantity of aqueous extracts. The higher absorbance of total antioxidant activity of *I. tinctoria* and *I. astragalina* was 0.481/1 mg and 0.315/1 mg indicates a strong antioxidant activity of the extracts (Figure 2).

Hydroxyl radical scavenging activity

The hydroxyl radical scavenging activity of aqueous extracts of *I. tinctoria* and *I. astragalina* was measured by Fenton reaction. The IC_{50} value of *I. tinctoria* and *I. astragalina* were 483.78 ± 1.18 µg/mL and 525.57 ± 1.28 µg/mL, which was compared to the standard ascorbic acid IC_{50} value was 372.01 ± 1.24 µg/mL (Figure 3).

Nitric oxide radical scavenging activity

The nitric oxide radical scavenging activity of aqueous extracts of *I. tinctoria* and *I. astragalina* were measured by percentage of nitric oxide inhibition using the sodium nitroprusside as a reaction mixture. The IC_{50} value of *I. tinctoria* and *I. astragalina* was 585.28 ± 1.24 µg/mL and 662.30 ± 1.26 µg/mL. Which was compared to the standard ascorbic acid showed 598.02 ± 1.20 µg/mL. However the IC_{50} value of *I. tinctoria* was higher than the ascorbic acid (Figure 4).

DPPH radical scavenging activity

The DPPH free radical scavenging is a widely accepted technique to evaluate the free radical scavenging activity of antioxidants. The IC_{50} value of aqueous extract of of *I. tinctoria* and *I. astragalina* showed 512.66 ± 1.26 µg/mL and 650.51 ± 1.20 µg/mL compared to the standard ascorbic acid IC_{50} value was 345.57 ± 1.10 µg/mL (Figure 5).

DISCUSSION

Preliminary phytochemical analysis known to find out their pharmological active compounds. Analysis of total phenolic and flavonoid content is possible to identify the antioxidant properties of the plant extracts.[26] The phytochemicals such as polyphenols, flavonoids found in the medicinal plants leads to the higher antioxidant activity. The phenolic compound plays a vital role in absorbing and neutralizing free radicals. The presence of phenolic content was noticed both aqueous

extracts of *I. tinctoria* and *I. astragalina* was 55.05 µg/mg and 36.55 µg/mg respectively. According to the Senthil kumar *et al.*[25] methanolic extract of *Indigofera cassiodes* phenolic content was 38.70 µg/mg and it was posses to be a potential antioxidant activity. Similarly the flavonoid compounds present in the plants exhibit potent antioxidant properties in the role of scavenging of free radicals, chelating metal ions and inhibition of enzyme responsible for free radical formation.[27] According to the Shreya *et al.*[18] *Terminalia arjuna* bark methanol extract showed a better antioxidant activity because of large amount of flavonoid content (200 mg/g). In the contrast our study the flavonoid content of aqueous extract of *I. tinctoria* and *I. astragalina* showed 43.94 µg/mg and 41.07µg/mg indicates the strong antioxidant activities of *I. tinctoria* and *I. astragalina.*

The measurement of total reducing power indicates the antioxidant activity of the plant extracts. The iron reducing indicates the electron donating mechanism. The antioxidants donate one electron to free radicals and neutralize it. The aqueous extracts of *I. tinctoria* and *I. astragalina* showed the higher reducing power. The total antioxidant activity of aqueous extracts of *I. tinctoria* and *I. astragalina* showed the high activity. These results were indicated an elevated activity with dose response relationship.

Hydrogen peroxide in the cells is not cause cell damage, but its rise to the hydroxyl radical which is toxic to cells lead to cell damage. Phenolic compounds are potentially involved in inhibition of hydroxyl radical formation. The IC_{50} value of aqueous extract of *I. tinctoria* and *I. astragalina* at 483.78 ± 1.18 µg/mL and 525.57 ± 1.28 µg/mL inhibited the formation of hydroxyl radicals. According to the Senthilkumar *et al.*[25] the methanolic extract of *I. cassiodes* IC_{50} value 126.13 ± 5.83 µg/mL was potentially inhibit the formation of hydroxyl radicals.

Nitric oxide was formed during the reduction with oxygen. It is lead to alter the structure and functional behaviour of many cellular components. Oxygen reacts with nitric oxide and inhibits the formation of free radicals.[28] In the contrast of our study, the aqueous extract of *I. tinctoria* and *I. astragalina* IC_{50} value 585.28 ± 1.24 µg/mL and 662.30 ± 1.26 µg/mL inhibited the formation of nitric oxide radicals.The IC_{50} value of methanolic extract of *Imperalla cylindrical* was 400.14 ± 1.93 µg/mL inhibit the nitric oxide radical formation potentially[29]. The IC_{50} value of *I. tinctoria* was higher than the standard ascorbic acid was 598.02 ± 1.20 µg/mL, because high amount of Phytochemicals constituents.

DPPH is a free radical and it accepted electron and destroy it.[30] Nagarajan and Sellamuthu[31] quated that, IC_{50} value- 218 ± 1.73 µg/mg of petroleum ether extract of *I. tinctoria* showed potential effect against DPPH radical scavenging activity. Similarly aqueous extract of of *I. tinctoria* and *I. astragalina* showed the IC_{50} value 512.66 ± 1.26 µg/mL and 650.51 ± 1.20 µg/mL were potentially inhibit the formation of DPPH radicals.

CONCLUSION

The present study concluded that aqueous extract of *I. tinctoria* and *I. astragalina* has the strong antioxidant activity. It potentially inhibited the formation of free radicals like nitric oxide radicals, hydroxyl radicals and DPPH radical formation, because of high phenolic contents. Up to our knowledge this is the first report in *in vitro* antioxidant activities of aqueous extracts of *I. tinctoria* and *I. astragalina*.

ACKNOWLEDGEMENT

We acknowledge to UGC- UPE – PHASE II New Delhi (No. 2013/PFEP/C3/280) for financial support.

Table 1. Phytochemicals presence in aqueous extracts of *I.tinctoria* and *I.astragalina*

S. No	Phytochemicals	*I. tinctoria*	*I. astragalina*
1	Alkaloids	+	-
2	Aminoacids	+	+
3	Anthroquinons	-	-
4	Carotenoids	-	-
5	Flavonoids	+	+
6	Glycosides	+	+
7	Protein	+	+
8	Reducing Sugar	+	+
9	Saponins	+	+
10	Steroids	+	-
11	Tannins	+	-
12	Terpenoids	-	-

(+) – Present, (-) –Absent

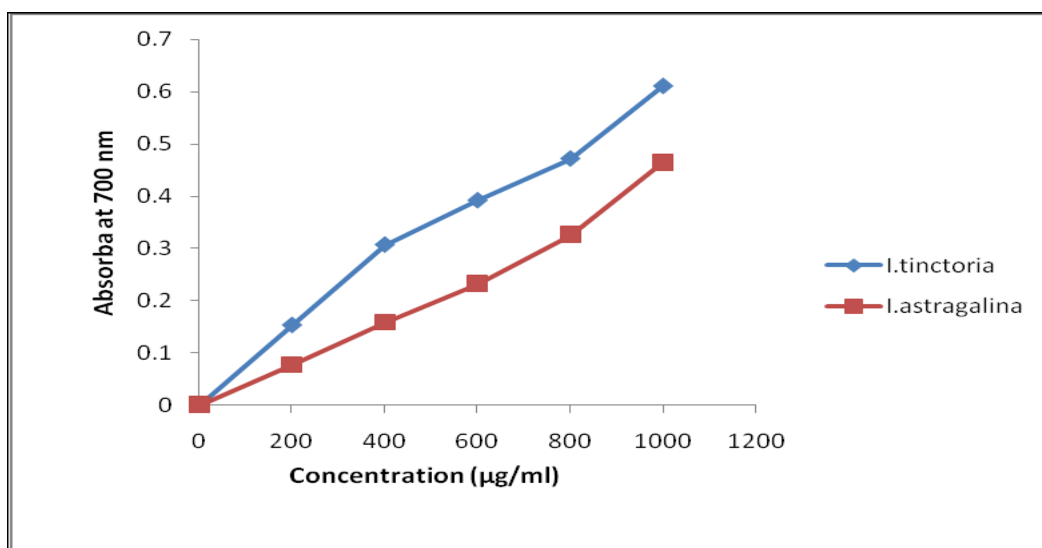

Figure 1: Total reducing power

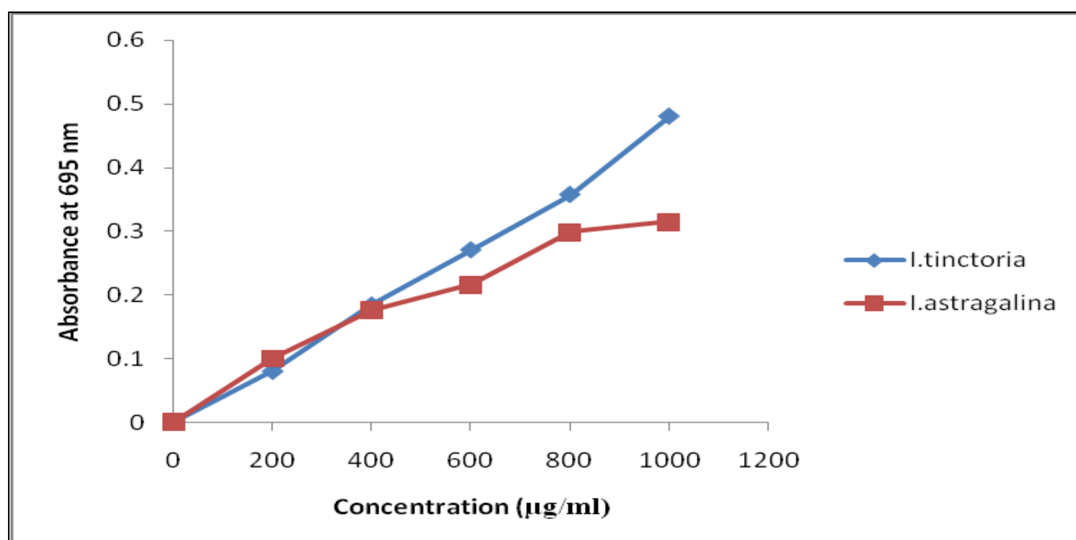

Figure 2: Total antioxidant activity

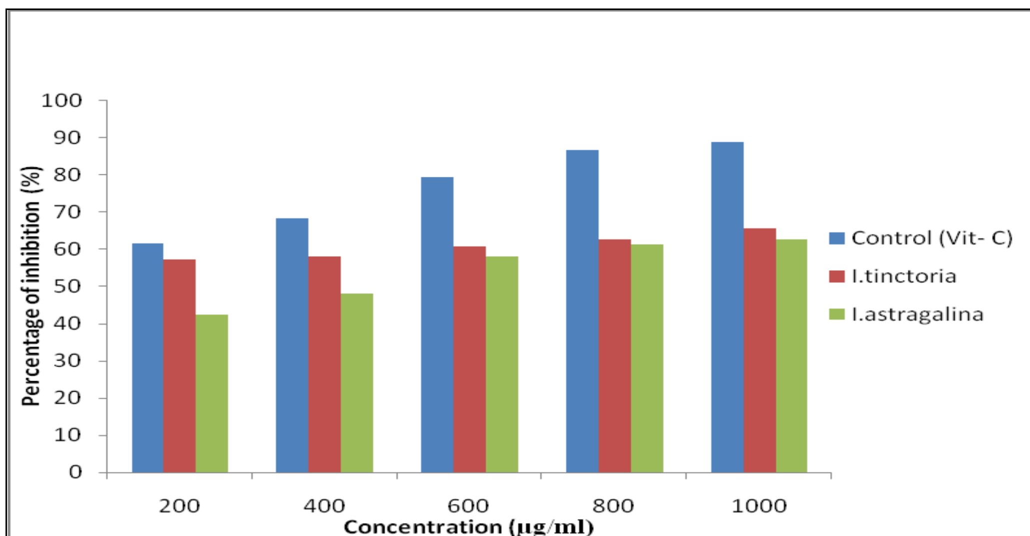

Figure 3: Hydroxyl radical scavenging activity

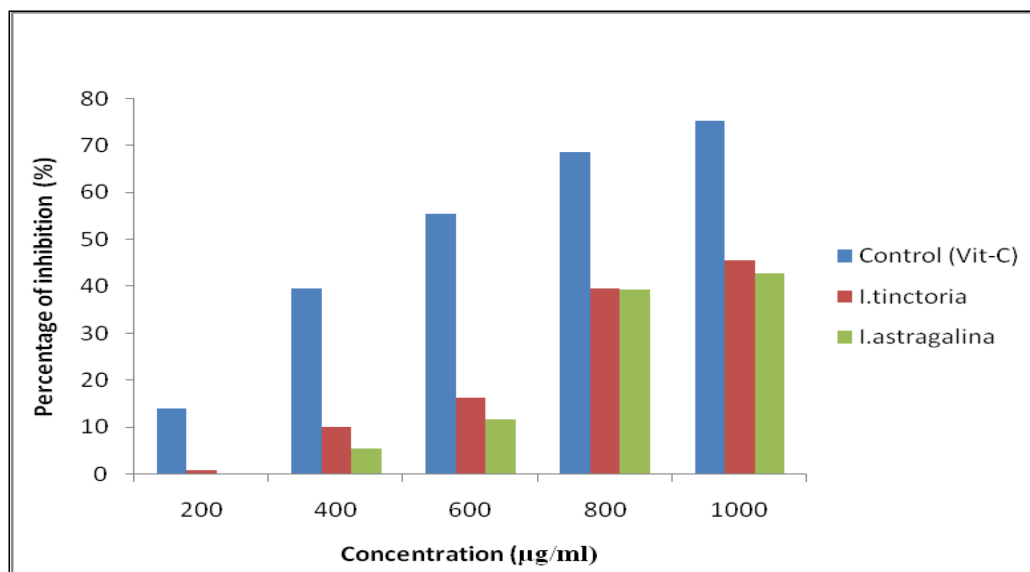

Figure 4: Nitric oxide radical scavenging activity

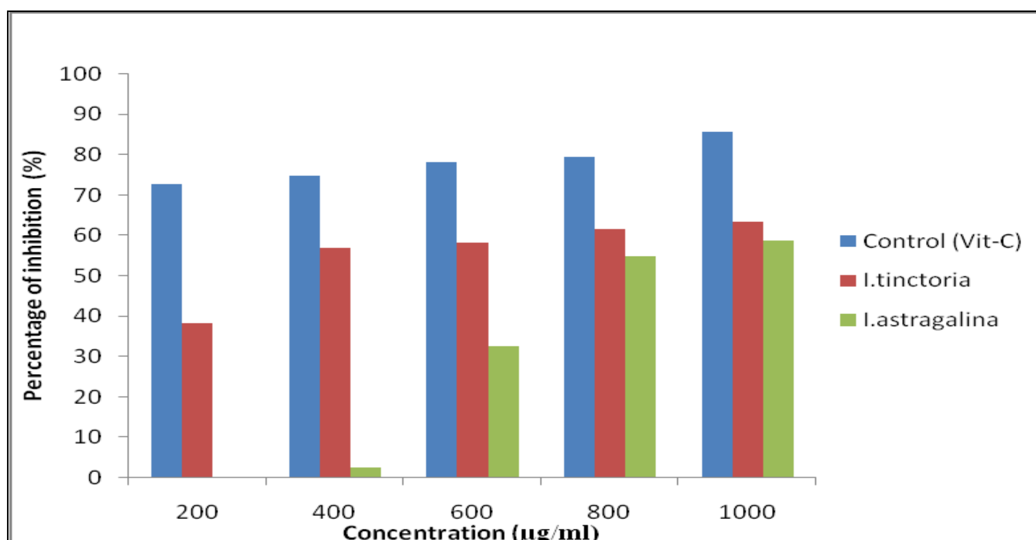

Figure 5: DPPH radical scavenging activity

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Cite This Article: R, Ramani Bai; M, Boothapandi and A, Madhavarani (2014), "Preliminary phytochemical screening and in vitro antioxidant activities of aqueous extract of *indigofera tinctoria* and *indigofera astragalina*", ***International Journal of Drug Research and Technology***, Vol. 4 (3), 46-54.

INTERNATIONAL JOURNAL OF DRUG RESEARCH AND TECHNOLOGY