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PHYTOCHEMICAL SCREENING, *IN-VITRO* ANTIOXIDANT AND ANTI-CANCER ACTIVITIES OF *INDIGOFERA ASTRAGALINA*

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ABSTRACT

Natural products are core resources development of new medicines to treat current and emerging diseases. But there is lot of medicinal plant' biological activities are still unknown and are not documented. The current work was aimed to explore the phytochemical and biological activities of one such plant i.e. *Indigofera astragalina.* The phytochemical profile explore the presence of different bioactive compounds in its like amino acids, saponins, glycosides. The carbohydrate and protein concentrations were found to be 11.26%, 14.26% and 34.11%, 42.86% for the fresh and dry sample of *I. astragalina*. The oxalate and phytate contents of *I. astragalina* were found to be 225.52± 0.33 and 47.920 mg/100 gm dry matter. The plant extract posses antioxidant activity against DPPH free radicals. The methanolic extract of *I. astragalina* inhibited the growth of cancerous HepG2 and Huh-7 cells. The results of current study provide the evidence to the medicinal properties of *I. astragalina* and further studies are in progress to isolate effective compounds and different biological activities on *I. astragalina.*

INTRODUCTION

Nature has a treasure of medicines to treat all kinds of aliments. When our prehistoric ancestors first roamed the earth in search of food, they perhaps learned from bitter experience, which plants were edible and which was not. The importance of this information was vital to the health and to the tribes and was passed down from generation to generation (Ivorra *et al.*, 1989; Wang *et al.*, 2010). Not only have they discovered many source of food while foraying but also medicines and the medicinal plants. These medicinal plants are used in the production in herbal medicine preparation since ancient times and are main sources for pharmaceutical products (Mallikarjuna Rao *et al.*, 2018). A quarter of the entire plant species at one time or other has been used for the medicinal purpose. These

Plant species are spread across the world with more abundant in tropical countries. India is one of the origin place of traditional system of medicine such as UNANI and Ayurveda and with the presence of abundant species which are used for the traditional system of medicine (Talluri *et al.*, 2018). The medicinal plant contains many phytoconstituents, which are the main determinant of the medicinal activities. With the advent of modern synthetic drugs, about 100 years ago, herbal medicine is fringe in main stay of therapeutics. This process rendered medicinal herbalism almost extinct. But the emerging of new diseases, side effects and microbial resistance to currently synthetic drugs the world again looking back on herbal medicine because of their low cost, wide

spectral activities against different diseases and low side effects (Mallikarjuna Rao *et al.*, 2019). As mentioned earlier, many plants species which are using in traditional medicine are still unknown about their medicinal uses scientifically. The genus Indigofera belongs to the family Fabaceae, is one of the widely used most popular medicinal plants, consisting 700 species of flowering plants. The plants of Indigofera genus are known for a number of medicinal plants and is popular for the production of indigo dye form *Indigofera tinctoria and Indigofera suffruticosa* (Singh *et al.*, 2001; Narender *et al.*, 2006; Singh *et al.*, 2006). Some of the species have the medicinal value such as *I. articulate* use in the treatment of toothache, *I. oblongifolia, I. suffriticosa, I. aspalathoides, Indigofera erracta, Indigofera aspalathoids, Indigofera articulata, Indigofera oblongifolia, Indogofera suffruticoisa*. Phytochemical investigation of the Indigofera species reveals that they are reach in organic compounds, fatty acids, flavanoids (Wahyuningsih *et al.*, 2017; Taj Ur Rahman *et al.*, 2018). The cursory search of literature reveals that the scientific work has not been carried out on the particular species *Indigofera astragalina*. Therefore it is utmost important to investigate its phytochemical aspects and possible biological activities on this plant species. *Indigofera astragalina* is a wildly available plant with poor documentation. The plant is an herbaceous legume which is regarded as weed and is generally found in sandy location across Africa and Asia including all over India. This plant is mostly used in South Tropical Africa as a medicine of skin and mucosae but in India it is not so popular. Overall, the medicinal potential of the plant is totally unexplored hence taking view of the medicinal properties of other members of same genus, the current research was under taken to study the phytochemical properties as well as biological activities.

MATERIALS AND METHODS

Collection and processing of plant material

The plant was identified, confirmed and authenticated by the Dr. P. Prayaga Murthy, Assistant Professor, Govt. Degree College, Yeleswaram, E.G. AP, India. These samples were washed with Tap water to get rid from dust and debris. The samples were dried by air dried (shade dried) method for 48 hours. Weight of samples was measured when they were fresh also after complete drying to determine the moisture content. There after the dried samples were made powder by mechanical grinder of lexus made. Subsequently the powder sample was subjected for extraction by methanol and it was stored for future use in an air tight container. The moisture content in the plant was evaluated by the following formula:

% Moisture content = wet weight of plant – dried weight of plant /Wet weight of plant x100

Fig 1. *Indigofera astragalina*

Phytochemical screening

The phytochemical test were carried to the extract using standard chemical test to explore its' chemical profile (Kokate *et al.*, 2001).

Estimation of carbohydrates

The carbohydrates content was estimated for fresh and dry sample of *Indigofera astragalina* using standard glucose.

Preparation of standard starch solution

30 mg of starch added with 3 ml of distilled water. The solution was used as standard for estimation of carbohydrate. An appropriate amount of starch (10mg/ml) such as 5µl, 10µl, 15µl , 20µl , 25µl, 30µl, 35µl, 40µl were taken in different test tubes and required volume of triple distilled water was added respectively to make up the final volume up to 100µl. Subsequently equal amount (5ml) of anthrone reagent was added to each test tube. The test tubes were heated at 90° C for 15 minutes and were cooled for half an hour. The OD (optical density) was measured by UV/V is spectrophotometer at 650 nm. The mean value of the samples (OD) were pointed on the Y axis and extrapolated towards the standard curve. The point at which it will touch the standard curve the point was extrapolated towards the X axis (concentration axis). The point at which joined to the X axis was considered as the

carbohydrate concentration for the respective OD. One OD value was calculated from the standard curve.

Estimation of protein

100mg of bovine serum albumin (BSA) was dissolved in 10ml of triple distilled water. 10µl, 20µl ,40µl, 60µl, 80µl, 100µl of BSA solution were dissolved with triple distilled water respectively to make up the final volume up to 100µl. Subsequently 4 ml of Biuret reagent was added to each test tube. The test tubes were then subjected to heat at 37^0 C for 30 minutes .There after 0.5 ml of folic reagent was added to each test tube. The mixture was taken kept aside for 30minutes at room temperature and OD were measured 690nm.

Determination of total oxalates

To 1 gm of the ground powder of *Indigofera astragalina*, 75ml of 1.5 M H_2SO_4 was added. The solution was carefully shaken in a mechanical shaker for one hour and then filtered by using Whattman no 1 filter paper. The filtrate was then collected and titrated against 1.0 M $KMnO_4$ solution till a faint pink colour appeared and that persist for 30 seconds [1 ml of 0.1 M $KMnO_4$ = 0.00450 g oxalic acid].

Determination of total phytates

4 gm of powered sample was soaked in 100 ml of 2% HCL for three hours and then filtered it through Whattmann no-1 filter paper. To the filtrate (25 ml), 5 ml of 3% NH_4SCN and 53.5ml of distilled water were added and dissolved. This solution was filtered $FeCl_3$ solution (containing 0.00195g Fe/Cl_3) until a brownish yellow colour which persisted for 5 minutes appeared. Phytine- phosphorus (1 ml=1019 mg phytine phosphorus) was determined. The photons content was calculated by multiplying the value of phytine phosphorus by 3.55.

Antioxidant activity:

To detect anti oxidant activity, qualitative 2,2 diphenyl-1- picrylhydrazyl (DPPH) assay was carried out (Prakash *et al.,* 2007). Chromatogram was developed by using various solvent systems as described below in commercially available TLC plates. The TLC plates were first air dried and then the chromatograms were sprayed with 0.2% 2,2, diphenyl -2- picryl- hydrrazyl in methanol as an indicator. The presence of antioxidant compounds were detected by yellow spots(band) against a purple background on the TLC plates.

Preparation of chromatogram

The chromatography was carried out in the solvent system toluene: ethyl acetate: formic acid in the ratio (7:3:0.3) which was showed the anti oxidant activity. The plate was kept under UV lamp .The band which looks as yellow coloured band in case of anti oxidant activity testing was appeared as red fluorescent and the second band appeared as blue fluorescent under UV lamp. Both the bands were cut by the scalpel and were stored 2 eppendorf tube labelled as A and B. 2 ml of methanol was added to each eppendorf tube. Both the eppendorf were kept open for removal of the solvent. The dried residues were the probable compounds.

Cytotoxicity test using brine shrimp lethality test:

Brine shrimp (*Atemia salina*) eggs were incubated for 48 hrs in 8% saline water to get the desired growth of the larvae for biological evaluation (Wu, 2014). Stock solution of extract was prepared at a concentration of 10 mg/ml. Extract was evaluated at three different doses 50,100 and 200µg /ml. For each dose level three replicates were used. Positive control which contain same amount of ethanol as the doses of *Indigofera astragalina* and negative control which contain only brine shrimp were considered as two control samples. Mobility readings brine shrimp were taken every hour up to 4 hrs. and after 24 hrs and motility was graded as below. 4+ highly motile; 3+ motile; 2+ sluggish; 1+ slow, Nil- no activity at all. After 24 hrs the final reading was taken and the percentage of inhibition was calculated by comparing the treated samples with the controls. Standard deviation was also calculated.

Anticancer activity of Indigofera astragalina:

The anticancer activity of *Indigofera astragalina* (IA) following tests were carried out using four different types of cell lines such as Huh-7, Hep G2 (hepatocellular carcinoma cells) K562 and jurkat cells lines (leukemia cells) (Cotter 2009; Van Meerloo *et al.,* 2011). The Huh -7, Hep-G2 , K562 and jurkat cells were cultured in RPMI 1640 supplemented with 10% fatal bovine serum,100 unit /ml of penicillin and streptomycin and L- glutamine in

CO_2 incubator in a humidified atmosphere at 37° C. The cells at a density $1x10^4$ cells/ml were seeded in each well of flat bottom 96 wells plate containing 100µl of growth medium. The Huh-7 Hep-G2 cells were allowed to adhere and cultured for 24 hours. And subsequently treated with various concentration of methanolic extracts of IA at different concentration like 50µg/ml,100µg/ml and 200 µg/ml for 48 hours. The same concentrations of ethanol were used as controls since thick methanolic extract of IA was dissolved with ethanol were used as controls since thick methanolic extract of IA WAS DISSOLVED WITH EHANOL .Subsequently 20ul of 5mg/ml of MTT in PBS (phosphate buffer saline) was added to each well and the plate was incubated for 4 hours. Subsequently, 10µl of MTT solvent was added to dissolved the formazon for colorimetric assay. After incubating at 37° C for 1 hr the absorbance was taken at 570 nm on a micro plate ELISA reader. The optical density of the sample was compared to that of negative control to obtain the percentage of viability as follows: All the tests were run in triplicates.

[Cell viability (%0:= (OD of treated cells /OD of control cells)x 100]

Cell Cycle Analysis

Flow cytometric analysis was used to evaluate cell cycle distribution of Hep G2 using this method described previously. Exponentially increased cells were incubated at a density of $5x10^5$ cells/ml in 24 well plates. After 24 hours the cells were incubated in presence or absence of methanolic extract of IA for 24 hours and harvested by Tripsinization. The cells were centrifuged at 400 g for 10 minutes. The medium was removed, and the cells were rinsed with cold PBS before proceeding with cell cycle analysis. In brief the cells were re-suspended in 50µl of cold PBS. To it 70% ethanol was added and the cells were then incubated over the night at 20^0 c . After centrifugation the pellets were washed twice with cold PBS and suspended in 500µl PBS. The cells were kept on ice for 10 minutes and stained with propidium iodide at 37°C for 30 minutes. The cells cycle distribution was determined by using a BD Biosciences (San jose) LSR II analytical flow cytometer equipped with BD FACSDiva software (Decker and Lohmann-Matthes, 1988).

RESULTS

Moisture content

Moisture content (MC) of any material is very important to, this affects the shelf life, quality and usability of the products. The accurate MC value is crucial in pharmaceutical product. So, the MC of dry plant material was calculated as above mentioned formula and was found to be as 77.13%.

Photochemical screening

The pH of IA methanolic extract calculated and observed it as slightly acidic (pH 6.0). Then the extract was used for further phytochemical screening to know its chemical profile using standard methods. The extracts gave the positive results for the presence of amino acids, proteins, starch, glycosides, saponins, tannins. The extract gave negative results for flavanoids, alkaloids, anthraquinones. The results were showed in Table no 1.

Estimation of carbohydrate

The carbohydrates content of samples were calculated as per the standard curve plotted by glucose absorbance as showed in table no 2 and figure 2 and the concentration was found to be 11.26% and 14.26% for the fresh sample and dry sample *I. astragalina* (IA) respectively.

Estimation of protein in mathanolic extract of *I. astragalina*

The standard curve was plotted by considering the mean values OD of different concentrations of BSA solution. The protein concentration of samples was calculated from the standard curve. On the basis of above standard curve one OD corresponded to 31µg of protein. Thus the values of samples were calculated as per the standard curve. Protein concentration was calculated as per one OD value. According to the calculation the protein concentration in fresh and dried powered sample were found to be 34.11% and 42.86% respectively.

Oxalate content

Oxalate present in the food is insoluble, which generally bind with calcium obtained from diet and result in the formation of calcium oxalate. The calcium oxalate is insoluble and get precipitate around the soft tissues such as kidney, causing kidney stones. So, the measuring of oxalate contents in medicinal products is very important and the oxalate

content of IA was found to 225.52± 0.33 mg/100 gm dry matter (Table no 4).

Phytate content

Phytic acids can bind to mineral such as Ca , Zn , Mn, Fe and Mg to form complexes that are indigestible, by decrease the bioavailability of these elements for absorption . The low phytate contents in the leaf indicate that the consumption of the leaves will not affect the bioavailability of the minerals. The phytate content of the IA extract was found to be 47.920mg/100 gm of dried power (Table no 5).

Antioxidant activity

Antioxidant assay was carried out by DPPH assay method. Two bands were found with the Rf values of 0.33 and 0.50 in the solvent toluene: ethyl acetate:formic acid (7:3:0:3) . In the solvent chloroform: methanol one yellow streak was found but there were no yellow bands found in case of other solvents (Table no 6) . The oxidation activity of isolated compounds from methanolic extracts showed one yellow band for compounds 1 and no yellow bands in case of compounds 2 (obtained from preparative TLC).

TLC Analysis

The TLC analysis shows no spots in the solvent hexane, toluene, toluene: ethyl acetate (9:1) & chloroform : ethyl acetate (9:1). This indicates absence of such constituents which are soluble in these respective solvents (Table no 7), Whereas in the solvent toluene: ethyl acetate (97:3), three spots were found in the naked eye but after spraying of the reagent (vanillin sulphuric acid) nine spots were found in the naked eye and subsequently four spots each were observed after spraying the reagent. In the solvent Toluene: ethyl acetate :formic acid (7:3:0:3) 1 streak was found In the naked eye but after spraying the reagent 2 blue colours spots were observed . In chloroform : methanol (9:1) no spots were found in naked eye but in UV chamber blue fluorescent spot was found . After spraying vanillin sulphuric acid four spots were found.

Cytotoxicity

For cytotoxicity testing of the methanolic extract of IA the motility of brine shrimps was observed. We did not observe any significant difference in the motility of the brine shrimps after hours among the different doses of extract as compared to the controls. Nevertheless, a dose dependant activity was noted after 24 hours (Table no 8). As the dose increases the activity of the larvae decrease in case of the methanolic extract as indicated by the percentage of inhibition. The percentage of inhibition in different doses was calculated by the formula mentioned in the material method section. % of inhibition ={mean of a lived larvae (control) – a lived larvae after doses/mean (control) } X100

There is a significant difference between control and test sample in the Percentage of inhibition of the brine shrimp larvae after 24 hours for all the doses i.e. 50 µg/µl, 100 µg/µl and 200µg/µl . It indicates that in dose 50µg/µl and 100 µg/µl the cytotoxic activity is relatively same but for 200 µg/µl the cytotoxic activity is nearly two fold increase.

Cytotoxicity activity on cancer cell lines

The methanolic extract of IA inhibited the growth of HepG2 and Huh-7 cells in a dose dependent manner. The cell viability decrease with the increasing concentration of the extract. In case of the cell line K562 and jurkat cell, the cell viability decrease is less pronounced even with the 200µg/ml dose of extract. Hence, it is clearly pronounce that the effect of the methanolic extract of *Indigofera astragalina* has cytotoxic activity for the hepatocelluar cells but not as pronounced in case of Leukaemia cell lines even after treatment with the dose 200 µg/ml (Table no 9) .

Cell cycle analysis

The anticancer activity prompted observed in the cell lines promoted us to perform cell cycle analysis. HepG2 cell were labelled by propidium iodide, which binds to double stranded nucleic acid i.e. DNA and subsequently sorted on a flow cytometer . Cell in different stages of cell cycle were sorted according to their DNA content in the cell. A diploid cell in the G1 phase content 2n DNA and the cells in the S phase contains between 2-4n DNA and the cell in G2/M phase posses 4n DNA. There fraction of the cell present in each phase was taken as readout. The IA extract at dose 50 µg/ml and 100 µg/ml shows significant accumulation of the cells at G2/M phases as compared to the no extracts control. The cells in both the doses accumulate to 21-23 percents as compared to 11% in control cells. This indicates a cell cycle arrest in G2/M phase with IA extract.

Table no 1: Photochemical screening of *I. astragalina*

Sl. No	Parameter	Result
1	Nature of compound	Slightly acidic
2	Test for flobatannin	-ve
3	Test for amino acid	+ve
4	Test for flavonoid	-ve
5	Test for presence of aleurone	+ve
6	Test for starch	+ve
7	Test for anthraquinone	-ve
8	Test for acidity	+ve
9	Test for protein	+ve
10	Test for saponin	+ve
11	Test for glycoside	+ve
12	Test for steroid	-ve
13	Test for tannin	+ve
14	Test for alkaloid	-ve

+ve =present and –ve = absent

Table no 2: Absorbance of Starch Solution

Concentration	1st reading	2nd reading	3rd reading	Mean
50µg	0.4863	0.4863	0.4864	0.4864
100µg	0.9505	0.9505	0.9505	0.9505
150µg	1.2451	1.2449	1.2449	1.2449
200µg	1.6888	1.6888	1.6888	1.6888
250µg	2.0763	2.0763	2.0673	2.0763
300µg	2.3059	2.3059	2.3059	2.3059
350µg	2.6930	2.6930	2.6931	2.6931
400µg	3.2937	3.2937	2.2938	2.2938

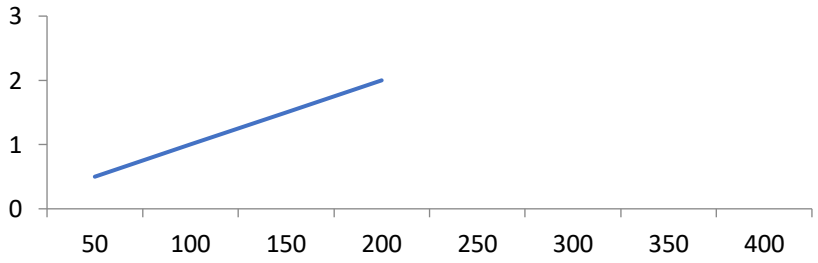

Fig 2. Standard cure of glucose

Table no 3: Absorbance (OD) of BSA

Concentration (µg)	1st reading	2nd reading	3rd reading	Mean
100	0.1419	0.1420	0.1419	0.1419
200	0.33557	0.3557	0.3557	0.3557
400	0.0.5486	0.5487	0.5486	0.5486
600	0.8321	0.8322	0.8321	0.8321
800	0.9979	0.9978	0.9979	0.9979
1000	1.2600	1.2601	1.2600	1.2600

Table no 4: Determination of oxalate

No	Req. Amount	Oxalate (g)	Oxalate (mg)	Mean &S.D
1	50.2	0.2259	225.9	225.52±0.33
2	50.1	0.22545	225.45	
3	50.1	0.22545	225.45	
4	50.1	0.225	225	

Table 5: Determination of phytates

NO	Req. amount (ml)	Phytine phosphorus (mg)	Phytate (mg)	Mean& S.D
1	13.4	15.946	47.838	47.92±0.17
2	13.5	16.065	48.195	
3	13.4	15.946	47.838	
4	13.4	15.946	47.838	

Table no 6: Antioxidant activity of *Indigofera astragalina* extract

Benzene	Yellow band found	Nil
Chloroform: methanol (9:1)	Streak found	Nil
Toluene: ethyl acetate (97:3)	No band	Nil
Toluene: ethyl acetate: formic acid(7:3:0.3)	2 bands found	Rf=0.33 Rf=0.50

Table no 7: Phytochemical tests

Solvent	No. of spots colour and Rf values	No. of spots colour and Rf under uv	No. of spots colour and Rf after spraying reagent
Hexane	Nil	Nil	Nil
Toluene	Nil	Nil	Nil
Toluene :ethyl acetate(9:1)	Nil	Nil	Nil
Chlroform: ethyl acetate(9:1)	Nil	Nil	Nil
Benzene	1-grey(0.02)	1-red flourscent	1-grey (0.02) 2-grey(0.07) 3-purple(0.17) 4-blue(0.35)
Toluene :ethyl acetate(98:2)	1-green(0.08)	1-red fluorescent(0.08)	1-brown(0.06) 2-green(0.12) 3-purple(0.22) 4-blue (0.34)
Toluene :ethyl acetate: formic acid	Streak –green	Red fluorescent	1-blue(0.67) 2-blue(0.83)
Toluene :ethyl acetate(97:3)	1-light grey(0.10) 2-grey(0.12) 3-brown(0.80)	1-bluefluroscent (0.10) 2-red flourscent(0.12) 3-flourscent (0.80)	1-blue(0.07) 2-green(0.10) 3-grey (0.12) 4-yellow(0.15) 5-bluish grey(0.23)7 6-grey(0.17) 7-blue(0.39) 8-Blue (0.71) 9-green (0.80)
Choloroform:methanol (9:1)	Nil	Streak 1 (Brown flourscent)	

CONCLUSION

Several species of genus indigofera has been documented to be having medicinal activities. In this study we have put an effort to screen the phytoconstituents and biological activity of a less well documented species *Indigofera astragalina*. The extracts of IA found to be presence of different secondary metabolites indicates possible medicinal values. The methanolic extract show anti oxidant activity by DPPH assay. Upon checking the cytotoxicity by the brine shrimp motility assay the IA extract showed a significant cytotoxicity at 24 hours in a dose dependant manner. Based on this, we further checked the anti tumor activity of this IA extract in different human cancer cell lines such as heapatocellular carcinoma and leukemic cell lines. The extract is effective in inhibiting proliferation or killing the hepatoma cells by apoptosis. But this is not effective against the leukemia cell lines. This may be due to the heterogenecity or differential nature of the tumors. Furthermore, we checked how the anti cancer activity relates to the cell cycle distribution of the HepG-2cells. The HepG-2 cell upon administration of IA extracts show G2/M checkpoint arrest. The cells are generally proliferate in an uncontrolled manner in cancerous situation. Anticancer drugs or agent put a break on it and inhibits its cell division. The IA extracts is arresting the cells atG2/M subsequently slowing down the cells division and work like an anticancer agent.

REFERENCES

1. Ivorra MD, Paya M, Villar A. A review of natural products and plants as potential antidiabetic drugs. J Ethnopharmacol. 1989;27(3), 243-75.
2. Wang YF, Ni ZY, Dong M, Cong B, Shi QW, Gu YC, Kiyota H. Secondary metabolites of plants from the genus Saussurea: chemistry and biological activity. Chem Biodivers. 2010; 7(11), 2623-59.
3. Mallikarjuna Rao T, Glucose Lowering Effect of *Zornia gibbosa* extracts in Diabetic Rats. Turkish Journal of Pharmaceutical Sciences 2018;15(3):339-346.
4. Talluri MR, Ketha A, Battu GR, Tadi RS, Tatipamula VB. Protective effect of *Aurelia aurita* against free radicals and streptozotocin-induced diabetes. Bangladesh J Pharmacol. 2018; 13.
5. Mallikarjuna Rao T, Veda Priya G, Ganga Rao B. Evaluation of Hepatoprotective Activity of *Zanthoxylum armatum* on Paracetamol-induced Liver Toxicity in Rats. Indian J Pharm Sci. 2019; 81(1):138-145.
6. Narender T, Khaliq T, Puri A, Chander R. Antidyslipidemic activity of furano-flavonoids isolated from *Indigofera tinctoria*. Bioorg Med Chem Lett. 2006; 16 (13), 3411-4.
7. Singh B, Chandan BK, Sharma N, Bhardwaj V, Satti NK, Gupta VN, Gupta BD, Suri KA, Suri OP. Isolation, structure elucidation and in vivo hepatoprotective potentioal of trans-tetracos-15-enoic acid from *Indigofera tinctoria* Linn. Phytoher Res. 2006; 20(10), 831-9.
8. Singh B, Saxena AK, Chandan BK, Bhardwaj V, Gupta VN, Suri OP, Handa SS. Hepatoprotective activity of indigtone- a bioactive fraction form *Indigofera tinctoria* Linn. Phytother Res. 2001; 15(4), 294-7.
9. Wahyuningsih S, A H Ramelan, D K Wardani, F N Aini, P L Sari, B P N Tamtama, Y R Kristiawan. Indigo Dye Derived from *Indigofera Tinctoria* as Natural Food Colorant. IOP Conf Ser: Mater Sci Eng. 2017; 193, 012048.
10. Taj Ur Rahman, Muhammad Aurang Zeb, Wajiha Liaqat, Muhammad Sajid, Sajjad Hussain, M. Iqbal Choudhary. Phytochemistry and Pharmacology of Genus Indigofera: A Review. Rec Nat Prod. 2018; 12(1), 1-13.
11. Kokate CK, Purohit AP, Gokhale SB. Carbohydrate and derived Products, drugs containing glycosides, drugs containing tannins, lipids and protein alkaloids. Text book of pharmacognosy 2001; 7, edition: 133-166, 167- 254, 255-2 69, 272-310, 428-52 3.
12. Prakash D, Suri S, Upadhyay G, Singh BN. Total phenol, antioxidant and free radical scavenging activities of some medicinal plants. Int J food Sci Nutr. 2007; 58(1), 18-28.
13. Wu C. An important player in brine shrimp lethality bioassay: The solvent. *J Adv Pharm Technol Res*. 2014; 5(1), 57–58.

14. Van Meerloo J, Kaspers GJ, Cloos J. Cell sensitivity assays: The MTT assay. Methods Mol Biol. 2011; 731, 237-45.
15. Cotter TG. Apoptosis and cancer: the genesis of a research field. Nat Rev Cancer. 2009; 9(7), 501-7.
16. Decker T and Lohmann-Matthes ML. A quick and simple method for the quantitation of lactate dehydrogenase release in measurements pf cellular cytotoxicity and tumor necrosis factor (TNF) activity. J Immunol Methods. 1988; 115(1), 61-9