EVALUATION OF CYTOTOXIC ACTIVITY OF ANDROGRAPHIS ECHIOIDES NEES.

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ABSTRACT

The present study evaluated the preliminary phytochemical and anticancer activity of the successive extracts (with the help of ethanol and ethyl acetate of the leaves of Andrographis Echioides by brine shrimp lethality bioassay, allium cepa root tip meristem model and 3-(4, 5-dimethylthiazol-2,5-diphenyltetrazolium bromide assay. the activity was compared with Cyclophosphamide as standard. The results revealed the presence of flavonoids in leaf. The anticancer activity was showed by brine shrimp lethality bioassay, allium cepa root tip meristem model and 3-(4, 5-dimethylthiazol-2,5-diphenyltetrazolium bromide assay.

KEYWORDS: MTT assay (3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay, Brine shrimp lethality bioassay (BSLB), cytotoxic activity, Flavonoids, Andrographis echioides.

INTRODUCTION

Andrographis echioides Nees, Family-Acanthaceae, echioides have been reported for their analgesic, anti-inflammatory and antipyretic activity, hepato-protective activity, anti-oxidant and anti-microbial activities. Flavones and flavones glycoside are the responsible active constituents for cytotoxic activity. The plant is richly constituted with a series of chemical constituents like Echiodinin, Flavone glucosides, Dihydroechioidin, Androechin flavanone, Andrographidene, Phytene, echiodinin-5-O-β-D-glucoside, 5,7,8-trimethoxy flavones, skullcapflavone I 2-O methyl ether and skullcapflavone I-2’-O-glucoside which makes the plant pharmacologically and therapeutically active. A perusal of literature revealed that cytotoxic effects remain to be studied. Here we reported the cytotoxic effect of the...
flavonoidal fraction extract of *Andrographis echioides* on Brine shrimp lethality bioassay (BSLB), Allium cepa root tip meristem model (acrtm) and 3-(4,5-dimethylthiazol-2,5-diphenyltetrazolium bromide assay (MTT).

**MATERIALS AND METHODS**

The plant *Andrographis echioides* Nees. was collected from Wardha (Mahatma Gandhi Hindi Vishwavidyalaya), in December 2013. The plant was botanically identified and authenticated by Prof. Dr. A. Chaturvedi, Department of Botany, Rashtrasant Tukadoji Maharaj (RTM) Nagpur University, Nagpur. The specimen Voucher No.9469 is deposited at the Department of Botany, Nagpur university for the future reference.

**Preparation of the Extract**

The shade dried and powdered leaves material of *Andrographis echioides* was subjected to refluxation in a round bottom flask with ethanol for 1 hr. The alcoholic extract was evaporated until dryness and suspended in 20% v/v ethanol in distilled water and then fractionated with the help of ethyl acetate using separating funnel. The combined ethyl acetate fractions separated and evaporated to dryness at 50º C. Flavonoids enrich fraction was then stored in desiccator till further use.

**Preliminary phytochemical screening**

The preliminary phytochemical analysis gives the information about phytoconstituents present in the crude drug. The chemical tests were performed on the flavonoidal fraction of *Andrographis echioides* leaves using different chemical tests in order to identify the classes of phytoconstituents. Table No. 1.

**EVALUATION OF CYTOTOXIC ACTIVITY BY IN VITRO ASSAYS**

**Model I: Brine Shrimp Lethality Bioassay (BSLB)**

*Artemia Salina L.* (Artemiidae), the brine shrimp, used in laboratory bioassay to determine toxicity through the estimation of median lethal concentration (LC$_{50}$ values) which has been reported for series of toxins and plant extracts. Several naturally extracted products which had LC$_{50}$ < 1000 μg/ml using BSLB were known to contain physiologically active principles.

The measuring the LC$_{50}$ of the several plants extract to the Artemia salina larvae that analogue with the cancer cells. The brine shrimp lethality assay consists of exposing larvae to test sample in saline solution and lethality is evaluated after 24 hrs BSLB has been used.
successfully to biomonitor the isolation of cytotoxic, antimalarial, insecticidal, and antifeedent compounds from plant extracts.

Procedure for BSLB
Brine shrimp (*Artemia salina*), eggs (150 mg) were hatched in a conical shaped vessel (1L) filled with sterile artificial sea water (NaCl, 38 g/ml) under constant aeration for 72 hrs to avoid risk of larvae death because of reduction in pH during incubation, pH was adjusted to 8.5. A 15 ml of yeast solution (0.06%) was added to vessel for every liter of salt water after 48 hours in order to feed larvae. It takes about 72 hrs for hatching active nauplii free from egg shells were collected and used for the assay.

For the Flavonoidal fraction, of *Andrographis echioides* seven concentrations were tested in order to determine dose response relationship and a control group was set with vehicle used for dilutions. Tested concentration of samples were 50, 100, 500, 1000, 1500, 2000, 3000 µg/ml. solution were prepared in distilled water. Ten nauplii were drawn through a glass capillary and placed in test tube containing sample, filled with 5 ml total volume of artificial sea water.

Experiment was conducted along with the control (vehicle treated) at above mentioned concentrations of test substances in a set of three test tubes per dose. In this study Cyclophosphamamide was used as a positive control. After 24 hrs, the test tubes were observed and the numbers of survived nauplii in each test tube were counted and the results were noted. The percentage of lethality of brine shrimp nauplii was calculated at each concentration for each sample. LC$_{50}$ values were estimated by Plotting of log of concentration (logC) versus percent mortality for all test samples by using Microsoft Excel 2000. LC$_{50}$ value lower than 1000 µg/ml is considered bioactive. Control shows no mortality.

Model II: *Allium cepa* root tip meristem model (ACRTM)
*Allium cepa* root tip meristems have been widely used for the evaluation of cytotoxicity, anti-mitotic, genotoxicity, antimutagenic and antioxidant activity by employing the growing roots of *Allium cepa*. The aim of this model was to evaluate the cytotoxic potential of flavonoidal fraction of *Andrographis echioides*, in *Allium cepa root tip* meristems in vitro that affect the proliferative kinetics.
1. Growing *Allium cepa* meristems
Locally available *Allium cepa* bulbs (50 ± 10 g) were grown in the dark over 100 ml tap water at ambient temperature until the roots have grown to approximately 2-3 cm. The water was changed daily.

2. Procedure
The flavonoidal fraction of *Andrographis echioides*, was dissolved in water to obtain 1 mg/ml and 10 mg/ml concentrations. Cyclophosphamide was used at 1 mg/ml and 10 mg/ml concentrations. The bulbs with root tips grown up to 2-3 cm were placed over drug solutions and incubation was carried out at ambient temperature. The length of roots grown in drug solution (newly appearing roots not included), root number were recorded at 0, 48 and 96 hrs and compared with that of control bulbs placed over tap water.

**Observation of root**

\[
\text{Number of roots} = n \\
\text{Average number of roots} = \frac{\text{Sum of all roots Length}}{\text{Number of roots (n)}}
\]

Observation was made for root length and root number attained following incubation with flavonoidal fraction of *Andrographis echioides* and cyclophosphamide in comparison to control. Statistical significance is given for comparison of root length and root number attained at 48 and 96 hrs with respect to the 0 hrs control.

**Model III: 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium Bromide (MTT) assay**
Cell Proliferation activity flavonoidal fraction of *Andrographis echioides* leaves was carried out by MTT Assay, which estimated the effect of drug on the growth of cells in vitro. The Flavonoidal fraction of leaves of *Andrographis echioides* was sent for ‘MTT’ evaluation at “Maratha Mandals Research Centre, Belgaum” for two cell lines Hela and MDA-MB at three different concentrations i,s. 10, 20 and 30 µg/ml.

**Materials and equipment**
- 5 mg/ml MTT solution in PBS.(Sterilized by filtration and Stored in dark at 4°C)
- 96 well plates
- Multichannel pipettes
- Micro plate reader equipped with a 492 nm filter
Experimental procedure
1. A 100 µl of culture medium containing MTT solution (10: 1) is added to each well.
2. Treated with different concentrations of the flavonoidal fraction of *Andrographis echioides* appropriately diluted with dimethyl sulfoxide (DMSO). Control group contains only DMSO.
3. After 24 hrs incubation at 37°C in a humidified atmosphere of 5% CO₂, the medium was replaced with MTT solution for further 4 hr incubation.
4. The precipitated crystals of Formazan blue were solubilized by adding DMSO (200 µl) and waited until a homogenous color formed.

Reading of results and calculations
1. Absorbance is read at 492 nm of wells containing cells and blanks.
2. The mean of the absorbance of well is calculated with the same treatment after subtracting of blank.
3. The results are normalized considering control wells as 100% (maximum absorbance obtained), expressing the results as percentage of controls.
4. Using the appropriate method of calculation, IC₅₀ can be estimated.

RESULTS

Table 1: Preliminary phytochemical screening of *Andrographis echioides*

<table>
<thead>
<tr>
<th>Sr. No.</th>
<th>Plant Constituent</th>
<th>Test/ reagent</th>
<th>FF</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Lipid</td>
<td>Spot test</td>
<td>+</td>
</tr>
<tr>
<td>2.</td>
<td>Glycoside</td>
<td>Keller- killani test</td>
<td>+</td>
</tr>
<tr>
<td>3.</td>
<td>Tannins</td>
<td>feCl₃ test</td>
<td>+</td>
</tr>
<tr>
<td>4.</td>
<td>Flavonoids</td>
<td>Shinoda test</td>
<td>+</td>
</tr>
<tr>
<td>5.</td>
<td>Saponin</td>
<td>Foam test</td>
<td>+</td>
</tr>
</tbody>
</table>

+ indicates positive test results.

Brine Shrimp Lethality Bioassay (BSLB)

Table No. 2 Cytotoxic effect of Cyclophosphamide by BSLB

<table>
<thead>
<tr>
<th>Sr. No.</th>
<th>Concentration (µg/ml)</th>
<th>Log C</th>
<th>Average Mortality After 24 hrs</th>
<th>Percent Average mortality</th>
<th>LC₅₀ (µg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>50</td>
<td>1.698</td>
<td>1</td>
<td>10</td>
<td></td>
</tr>
<tr>
<td>2.</td>
<td>100</td>
<td>2</td>
<td>3</td>
<td>30</td>
<td></td>
</tr>
<tr>
<td>3.</td>
<td>500</td>
<td>2.698</td>
<td>6</td>
<td>60</td>
<td></td>
</tr>
<tr>
<td>4.</td>
<td>1000</td>
<td>3</td>
<td>8</td>
<td>80</td>
<td></td>
</tr>
<tr>
<td>5.</td>
<td>1500</td>
<td>3.176</td>
<td>9</td>
<td>90</td>
<td></td>
</tr>
<tr>
<td>6.</td>
<td>2000</td>
<td>3.301</td>
<td>10</td>
<td>100</td>
<td></td>
</tr>
<tr>
<td>7.</td>
<td>3000</td>
<td>3.602</td>
<td>10</td>
<td>100</td>
<td>194.98</td>
</tr>
</tbody>
</table>
In toxicity evaluation of plant extracts by BSLB, an LC$_{50}$ value lower than 1000 µg/ml is considered bioactive. The degree of lethality was found to be directly proportional to concentration of extract. LC$_{50}$ value for Cyclophosphamide is lesser than 1000 µg/ml. LC$_{50}$ value has been estimated (after 24 hrs live nauplii were counted and analyzed) by graphical method (Log concentration verses percent mortality) using Microsoft Excel and was found to be 194.98 µg/ml.

Hence, Cyclophosphamide is bioactive by using BSLB with LC$_{50} = 194.98$ µg/ml.

Table No. 3. Cytotoxic effect of flavonoidal fraction by BSLB

<table>
<thead>
<tr>
<th>Sr. No.</th>
<th>Concentration (µg/ml)</th>
<th>Log C</th>
<th>Average Mortality After 24 hrs</th>
<th>Percent Average mortality</th>
<th>LC$_{50}$ (µg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>50</td>
<td>1.698</td>
<td>1</td>
<td>10</td>
<td></td>
</tr>
<tr>
<td>2.</td>
<td>100</td>
<td>2</td>
<td>3</td>
<td>30</td>
<td></td>
</tr>
<tr>
<td>3.</td>
<td>500</td>
<td>2.698</td>
<td>5</td>
<td>50</td>
<td></td>
</tr>
<tr>
<td>4.</td>
<td>1000</td>
<td>3</td>
<td>6</td>
<td>60</td>
<td></td>
</tr>
<tr>
<td>5.</td>
<td>1500</td>
<td>3.176</td>
<td>7</td>
<td>70</td>
<td></td>
</tr>
<tr>
<td>6.</td>
<td>2000</td>
<td>3.301</td>
<td>8</td>
<td>80</td>
<td></td>
</tr>
<tr>
<td>7.</td>
<td>3000</td>
<td>3.602</td>
<td>10</td>
<td>100</td>
<td>498.8</td>
</tr>
</tbody>
</table>

LC$_{50}$ value has been estimated (after 24 hrs live nauplii were counted and analyzed) by graphical method (Log concentration verses percent mortality) using Microsoft Excel and was found to be 498.8 µg/ml. In this study LC$_{50}$ value for the flavonoidal fraction was less than 1000 µg/ml.

Hence, flavonoidal fraction is bioactive by using BSLB with LC$_{50} = 498.8$ µg/ml.
Allium cepa root tip meristem model (ACRTM)

Table No. 4. Cytotoxic effect of flavonoidal fraction of Andrographis echioides by ACRTM

<table>
<thead>
<tr>
<th>Sr. no</th>
<th>Groups</th>
<th>Concentration (mg/ml)</th>
<th>Root length in cm</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Control</td>
<td>-</td>
<td>0 hrs</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>48 hrs</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>96 hrs</td>
</tr>
<tr>
<td>2</td>
<td>Flavonoidal fraction(FF)</td>
<td>1</td>
<td>2.89+0.04 (n =14)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>3.02+0.27 (n =26)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>3.25+0.33 (n =35)</td>
</tr>
<tr>
<td>3</td>
<td>Flavonoidal fraction(FF)</td>
<td>10</td>
<td>2.55+0.26 (n =12)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>2.43+0.22 (n =23)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>2.31+0.16* (n =16)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>2.31+0.16* (n =16)</td>
</tr>
<tr>
<td>4</td>
<td>Cyclophosphamide</td>
<td>1</td>
<td>2.43+0.14 (n =18)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>2.20+0.17* (n =16)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>1.95+0.1** (n =11)</td>
</tr>
</tbody>
</table>

Values expressed as mean ± SEM (n=10-35) Two way ANOVA followed by Bonferroni post test. Statistical significance (root length & root number attained at 48 and 96 hr. compared with to 0 hr.)

* p<0.05; ** p<0.01; ***P<0.001.
3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium Bromide (MTT) assay

Regents of cytotoxic activity of flavonoidal fraction of *Andrographis echoides* by MTT assay.

Results for MDA- MB cell line

Control treated MDA-MB

FF treated MDA-MB
Table No.5. Cell line- MDA-MB - Human adenocarcinoma, mammary gland.

<table>
<thead>
<tr>
<th>Sr. No.</th>
<th>Compound</th>
<th>Concentration (µg)</th>
<th>O.D. at 492nm</th>
<th>% of cell lysis</th>
<th>IC50</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>FF</td>
<td>10</td>
<td>0.445</td>
<td>No lysis</td>
<td>20 µg</td>
</tr>
<tr>
<td>2.</td>
<td>FF</td>
<td>20</td>
<td>0.894</td>
<td>50%</td>
<td></td>
</tr>
<tr>
<td>3.</td>
<td>FF</td>
<td>30</td>
<td>1.003</td>
<td>75%</td>
<td></td>
</tr>
<tr>
<td>4.</td>
<td>Control</td>
<td>-</td>
<td>0.240</td>
<td>No lysis</td>
<td>-</td>
</tr>
</tbody>
</table>

The effect of flavonoidal fraction (10, 20 & 30 µg) of *Andrographis echioides* leaves on cell line-MDA-MB-human adenocarcinoma, mammary gland. Control group concentration Optical density (O. D.) at 492 nm shows no lysis of human adenocarcinoma, mammary gland cell. Treatment with (20µg) of *Andrographis echioides* leaves O. D. at 492 nm shows 50% of cell lysis of human adenocarcinoma, mammary gland.

Inhibitory effect against MDA-MB was observed with IC_{50} value 20 µg/ml. Suggesting that the flavonoidal fraction of *Andrographis echioides* leaves possess cytotoxic activity.

Results for Hela cell line

![Control treated Hela cells](image1)

![FF treated Hela cells](image2)

Table No. 6. Cell line- Hela - Human cervix

<table>
<thead>
<tr>
<th>Sr. No.</th>
<th>Compound</th>
<th>Concentration (µg)</th>
<th>O.D. at 492nm</th>
<th>% of cell lysis</th>
<th>IC50</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>FF</td>
<td>10</td>
<td>0.538</td>
<td>No lysis</td>
<td>20 µg</td>
</tr>
<tr>
<td>2.</td>
<td>FF</td>
<td>20</td>
<td>0.938</td>
<td>&gt;50%</td>
<td></td>
</tr>
<tr>
<td>3.</td>
<td>FF</td>
<td>30</td>
<td>1.288</td>
<td>&gt;75%</td>
<td></td>
</tr>
<tr>
<td>4.</td>
<td>Control</td>
<td>-</td>
<td>0.221</td>
<td>No lysis</td>
<td>-</td>
</tr>
</tbody>
</table>

The effect of flavonoidal fraction (10, 20 and 30 µg) of *Andrographis echioides* leaves on cell line Hela- Human cervix. Control group concentration O. D. at 492 nm showed no lysis of Hela- Human cervix. Treatment with flavonoidal fraction (20µg) of *Andrographis echioides* leaves O. D. at 492 nm shows 50% of cell lysis of Hela- Human cervix. Inhibitory
effect against Hela was observed with IC$_{50}$ value 20µg/ml. Suggesting that the flavonoidal fraction of *Andrographis echioides* leaves possess cytotoxic activity.

**DISCUSSION**

In the present study BSLB had been used. It is a simple bioassay useful for screening large number of extracts in the drug discovery process and has been used in a number of previous studies. The technique has been used to identify over 300 novel antitumor and pesticidal natural products. BSLB found to have a positive correlation with human nasopharyngeal carcinoma cytotoxicity. BSLB also has been reported to be useful in predicting other biological activities such as phototoxicity, trypanocidal, enzyme inhibition, ion regulation activities and hepatotoxicity. The brine shrimp lethality activity of flavonoidal fraction of leaves of *Andrographis echioides* showed the linear dose-effect relationship between flavonoidal fraction concentrations and LC$_{50}$ value. The degree of lethality was found to be directly proportional to the concentration of the Flavonoidal fraction. The flavonoidal fraction of *Andrographis echioides* leaves has shown the LC$_{50}$ value of 498.88 µg/ml while LC50 value of Cyclophosphamide was found to be 194.98 µg/ml. Results showed that flavonoidal fraction of *Andrographis echioides* leaves has potent cytotoxic effect by using BSLB which is comparable to that of standard anticancer drug Cyclophosphamide. LC$_{50}$ lower than1000 µg/ml in the BSLB is considered to biologically active. The LC$_{50}$ value for flavonoidal fraction of *Andrographis echioides* leaves in study was found to be lower than 1000 µg/ml. Hence, flavonoidal fraction of leaves were bioactive by BSLB method with LC$_{50}$ values 498.88µg/ml. Several naturally extracted products which had LC50 < 1000 µg/ml using BSLB were known to contain physiologically active principles.[53]

*Allium cepa* root tip meristem model, a standardized test for cytoxicity monitoring has been used by various researchers. In the present study, flavonoidal fraction of *Andrographis echioides* leaves produced dose and time dependent growth inhibition. Incubation of bulbs in different concentrations of flavonoidal fraction of *Andrographis echioides* leaves produced a growth retarding effect that was associated with a decrease in the root number. *Allium cepa* root tip meristem growth inhibition was highest with significance of (p < 0.001) at 10 mg/ml concentration (96 hrs) for flavonoidal fraction. Flavonoidal fraction of *Andrographis echioides* leaves and cyclophosphamide arrested the root growth. Cytotoxic effects were also evident in the form of shortening and decaying of roots. However, the root number did not increase any further at 10 mg/ml concentration. Cytotoxic effect of flavonoidal fraction of
Andrographis echioides leaves by using Allium cepa root tip meristem model was comparable to that of Cyclophosphamide.[63]

Furthermore in the present study, cell proliferation activity of flavonoidal fraction of Andrographis echioides leaves was carried out by 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium Bromide (MTT) assay using human cell lines Hela and MDA-MB in three concentrations (10, 20 and 30 μg/ml). MTT assay still remains an adequate method for in vitro study of the cell viability and proliferative activity of the cell. Measurement of cell viability and proliferation forms is used as basis for this in vitro assay. The MTT bioassay is a rapid, versatile, quantitative and highly reproducible colorimetric assay. It also has been used for mycotoxins screening, cytotoxic activities of natural killer cells and neurotoxicity studies of Aβ peptides and in vitro drug interactions studies. The MTT assay has been used as an alternative to the radioactive techniques.

Present study estimated the effect of extract on the growth of cell in vitro. Measurement of cell viability and proliferation forms is used as basis for this in vitro assay. The flavonoidal fraction of leaves of Andrographis echioides was sent for ‘MTT’ evaluation at “Maratha Mandals Research Centre, Belgaum” The analogous to the results obtained in previous models, flavonoidal fraction was found to be active with IC$_{50}$ value 20μg/ml. The MTT assay generally shows a good correlation with other viability tests and in vivo results.

In the present study, using three reported models (BSLB, ACRTM & MTT assay) for evaluation of cytotoxic activity which have different sensitivity confirms the cytotoxic activity of flavonoidal fraction Andrographis echioides leaves, data showed consistant results and confirmed the cytotoxic activity of flavonoidal fraction. Thus, present study for the first time demonstrated that flavonoidal fraction of Andrographis echioides leaves exhibits potent cytotoxic property by all three methods used which comparable to standard anticancer drugs.

CONCLUSION

From the experimental results of the present study it is concluded that the flavonoidal fraction of Andrographis echioides leaves shows cytotoxic activity using in vitro models (Brine Shrimp Lethality Bioassay, Allium cepa root model and MTT assay). The Andrographis echioides flavonoidal fraction may have potential therapeutic value for the management of cancer. The results of the study revealed that the flavonoidal fraction of Andrographis echioides has a potential cytotoxic and antitumor activity. Further study is required to
establish the antitumor activity of flavonoidal fraction *Andrographis echioides* leaves in vivo and in vitro with different human cell lines.

**ACKNOWLEDGEMENT**

We would like to thank Maratha Mandals Research Centre, Belgaum (India), for experimental analysis (MTT Assay).

**REFERENCES**


