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Biochemical Profiling and Evaluation of Antioxidant and Cytotoxic Effect of Different Extracts of *Strychnos potatorum*

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A B S T R A C T

Emergence of different life style diseases increases the scope for developing natural remedies. *Strychnos potatorum* is a traditional medicinal plant used in the treatment of Asthma, diabetes, urinary infection etc. In the present analysis it was found that it is potential antioxidant agent. HPLC analysis showed that it contains flavanoids like Kaempherol and quercetin.

Introduction

The seeds of the plant *Strychnos potatorum* are widely used in the treatment of different ailments like bronchitis, diarroea, dysentery, urinary tract infections (Kavitha *et al.*, 2014). Curative properties of a plant product are due to the presence of secondary metabltes lke flavanoids, alkaloids, terpenods either alone or as synergistic action. Flavonoids are very important in preventing different diseases like cancer, inflammation, diabetes etc. as it is a strong antioxidant agent (Almedia et al., 2008).Evidences suggest that cellular damage or oxidative injury arising from production of free radicals or reactive oxygen species (ROS) are critical causative factors in the pathogenesis of many neurodegenerative disorders, inflammatory conditions, auto-immune diseases, atherosclerosis, aging diabetes, cancer and gastrointestinal disorders. Results from biological and phytochemical studies indicate that medicinal plants have profound antioxidant potential that can be exploited further in the prevention and treatment of these devastating disorders (Mazumdar *et al.*, 2008).

Hepatoprotective activity of this plant was also reported. The Ayurvedic preparations contain 75 to 80% of natural products (Aiyer and Komalammal, 1963). It is believed that Ayurvedic products have the capacity to purify the cells and tissues and also to prevent

or remove the toxic products from the live cells. These are an important constituent in some ayurvedic preparations (Ani *et al.,* 2011).

Strychnos potatorum (Linn.) belongs to the family *Loganiaceae*. Vernacular names of the plant are 'Thettambaral', 'Kathka', 'Nirmala', 'Kath', 'Chethaneeya', 'Chakshushya'. This plant is a medium sized deciduous tree having height upto 12 meters. Seeds and roots are used in medicinal preparations. It is popularly used to purify water in India. Seeds of this plant cure head diseases and urinary discharges. The roots are useful to treat Leucoderma and fruits for curing eye diseases, poisoning etc.

A study was carried out to evaluate the antioxidant potential and elucidate the active principles in it.

Materials and Methods

Extract preparation

Different plant materials were collected from local areas and other parts of the State. Plant materials were washed, rinsed with distilled water and dried in air. About 10 to 20 grams of the dried materials were taken and powdered using a warring blender. This powder was then taken for extraction with different solvents (Harborne, 1989, Cseke, 2004, Omer Ertuk 2006., Green, 2004., Nostro *et al.,* 2000).

As solvents, methanol, ethanol, sterile water, hexane, chloroform, ethyl acetate, benzene, diethyl ether and acetone were used initially, on trial and error basis, and according to the yield, solvents were selected for final use of extraction. All these solvents were purchased from M/s Merck, Mumbai. Plant extracts were prepared using different extraction methods depending on the nature of the plant material and yield, as mentioned in the table 4.1. In general, the following methods were used for the extraction.

Cold method (Harbone method, 1989) (Thara *et al.,* 2016)

This method was used with slight modification. In this method, 10 gm powder of the dried plant material (weight varies depending on the plant material) was taken and mixed with 100 ml of AR grade methanol. It was mixed well and kept on an orbitory shaker at 200 rpm for 15 to 20 hrs in room temperature. The extract was then filtered using Whatman no. 1 filter paper. The procedure was repeated for three times or until clear supernatant solvent was formed. The supernatant was collected and evaporated to dryness. The residue thus obtained was weighed and used for further analysis. The percentage yield is calculated (Handa *et al.,* 2006) .This method was employed for different plant materials using different solvents. Solvent having maximum yield was selected.

Test for phenolic compounds (Harborne, 1989, Mc Donald *et al.,* 2001)

The presence of phenolic compounds was tested using Folin's - Ciocalteu (FC) reagent. In this method 1ml of the extract (1mg/ml) was taken in a clean dry test tube and 5ml of FC reagent was added to it. 4 ml of 1.5% sodium carbonate was added and then kept for 30**',** at room temperature for incubation. Development of blue colour confirmed the presence of phenolic compounds. The intensity of blue colour was estimated using a colorimeter and the same was graded as + for low, ++ for medium and +++ for high intensities. The gradation of intensity represents the quantity of the phenolic compounds present in it.

Tests for determining the presence of flavonoids

Ferric chloride method (Harborne, 1989.Parekh *et al.,* 2006)

The presence of flavanoid compounds in the extract was determined by ferric chloride method. In this method, 1 ml of the extract was taken in a test tube and a few drops of ferric chloride (0.1 % in distilled water) were added to the test tube. It was mixed well and kept at room temperature for few minutes. The presence of flavanoid compounds was indicated by the development of green colour. The intensity of the colour was noted using a colorimeter.

Alkaline test for flavanoids (Harborne, 1989)

This test was used to identify the presence of flavanoids. In this method, 1ml of the extract was taken in a clean dry test tube and 0.2 ml of 0.1 N NaOH was added to it. Increase in intensity of the yellow colour was an indication of the presence of flavanoids. Few drops of dil. HCl were then added. The solution became colorless, showing the presence of flavanoids.

Test for alkaloids (Chopra *et al.,* 2005)

The presence of alkaloids was determined using the following method. 5 ml of the extract (0.2%) was taken in a test tube and 1 ml of 2mM HCl and 1 ml of the Nessler's reagent were added to the tube and mixed well. Presence of orange precipitate was an indication of the presence of alkaloids.

Test for terpenoids (Obeyedi *et al.,* 1990)

1.0 ml of the extract (0.2 %) was taken in a clean dry test tube and a few drops of concentrated H_2SO_4 were added to the test tube. It was kept in room temperature for 30 min. Lower layer of the above mixture was turned yellow to indicate the presence of terpenoids.

Test for saponins (Vogel, 1958)

5ml of the extract was taken in a clean dry test tube and a few drops of Na_2CO_3 were added to it and mixed well. Presence of saponins was indicated by the formation of froth.

Test for glycosides (Parekh *et al.,* 2007)

2ml of the extract was taken in clean dry test tube and a few drops of 0.1% of $FeCl_3$ and con. H_2SO_4 were added to it. It was then kept for few minutes. Formation of reddish lower layer and bluish green upper layer was an indication of the presence of glycosides.

The quantity of the compounds present in the mixture were graded and recorded as + for low level, ++ medium level, +++ high level and – for complete absence.

Biochemical estimation of total phenolic and flavanoid compounds

Biochemical estimations were done to determine the quantity of various secondary metabolites present in each extract.

Estimation of total phenolic content

Total phenolic content of the extracts were estimated using Folin-Ciocalteu method (Singelton *et al.,* 1999).

Chemicals used

Folin-Ciocalteu (FC) reagent, Sodium carbonate (Merck), Gallic acid (SRL).

Stock solutions: Gallic acid stock 1mg/ml in

methanol, FC reagent 1:2 dilution and Sodium carbonate solution 1.5% in water.

1ml of the extract (1mg/ml) in methanol was taken in a clean dry test tube. 0.0ml, 0.1ml, 0.2ml, 0.4ml, 0.6ml, 0.8ml and 1.0ml of the gallic acid (standered 1mg/ml) were taken in separate test tubes and made up to 1ml with distilled water. 5 ml of FC reagent (1:2 diluted with distilled water) and 4 ml of Sodium carbonate solution (1.5% in distilled water) were added to each test tube including the extract in methanol. Test tubes were then kept for incubation for **30'** at room temperature. Optical density (OD) was measured at 760nm using a UV-VIS spectrophotometer (UV-VIS 1604 Shimadzu).

A graph was plotted with concentration of gallic acid on X-axis and OD on Y-axis. The amount of total phenolic compound present in the extract was determined from the graph and expressed as gallic acid equivalents.

C= c x V/m where
C= Total phenolic (mg/ml)
c = concentration of gallic acid (mg/ml)
V = volume of the extract in ml
m = weight of the extract

This estimation was done in triplicate for each extract using gallic acid as standard. (Singelton *et al.,* 1992, Glucin *et al.,* 2002, Milisukas *et al.,* 2004, Irfan *et al.,* 2008 and Zexai *et al.,* 2008). Average ± SD value was calculated.

Estimation of total flavanoids

The total flavanoid content was estimated by the method described by Singelton *et al.,* 1992, and Kessler, 2003,

Stock solutions required: Quercetin standerd-0.1mg/ml in methanol, l.5% NaOH in water, 0.15% of $NaNO_2$

0.0ml, 0.2ml, 0.4ml, 0.6ml, 0.8ml, 1.0 ml of the quercetin standard and 0.5 ml of the extract were taken in 7 separate clean dry test tubes. Total volume in each test tube was made up to 2.0 ml with distilled water and 2.0 ml of 0.15% $NaNO_2$ was added in each test tube. Test tubes were kept in room temperature for 5min. Then 2ml of 5% NaOH was added to each test tube, mixed well and kept for 15' at room temperature. OD of each sample was taken at 510 nm using distilled water as blank. The experiment was carried out in triplicate. Mean ± SD value was calculated. This procedure was repeated for each and every extract.

A graph was plotted with concentration of the quercetin standard on X-axis and OD on Y-axis. The concentration of the total flavanoids present in each extract was thus determined using the graph and expressed as quercetin equivalents.

Reverse Phase High pressure liquid chromatography: RP-HPLC (Thara *et al.,* 2012)

Plant extracts which showed significant antimicrobial, antioxidant activities were analysed using HPLC. RP-HPLC-profiling was done using general method, changing the solvent system according to the plant material

HPLC system

HPLC unit with dual pump, rheo dyne injector, SPD photodiode array detector in combination with 6.12 SP5 integration software was used. The following chromatographic conditions were given for the present experiment.

Column: Lichrosper RP 18 e 5µm (Merck).

Detector: SPD PDA, Flow rate: 1ml/min, Injection volume: 20µl.

The mobile system and wavelength were selected according to the sample. The mobile phase used for different samples were as follows:

1. Strychnos potatorum:- Acetonitrile: methanol (50:50)

The finger prints were recorded in different wavelength and shown as overlaid chromatogram. A PDA detector was used for recording. The number of peaks and area of the peaks were noted.

Determination of cytotoxicity (Moseman *et al.*, 1983)

Cytotoxicity test using Mice Spleen cells was done by Tryphan blue exclusion method.

Principle

The dead and damaged cells stain blue inside on treating with trypan blue and can be distinguished from viable cells. This is due to the toxic effect of the drug which makes pores on the membrane and trypan blue can enter inside the cells (Mossman *et al.*, 1983).

Materials required

PBS (Phosphate buffered saline): NaCl-4.0 gm, KCl- 0.1gm, Na_2HPO_4- 0.72 gm, KH_2PO_4- 0.1 g dissolved in 500ml of double distilled water, gentamicin-50µg/ml, trypan blue-1.0% and sterile distilled water. Chemicals used were purchased from M/s. SRL, Mumbai.

Preparation of cell lines

Mice spleen cells were collected and cultured in RPM1 1640 (Roswell Park Memorial Institute) medium. Mice were dissected and spllen was collected and crushed in RPM1 medium. It was then centrifuged at 1000 rpm. Pellet was collected, washed and diluted with phosphate buffer to get concentration of 10^6cells/ml. It was then incubated in RPM1 medium at 36^0C for 18 hrs in CO_2 incubator. Cytotoxicity of plant extracts under study was tested using these mice spleen cells cultured in RPM1 1640 (Roswell Park Memorial Institute medium). The number of cells at 10^{-3} dilution was counted using a haemocytometer. Different dilutions (10^{-1}, 10^{-2}, 10^{-3}) of the extract were made in sterile water (10mg/ml). Incubated the different concentration of the extract like 0.0µg, 5.0, 10.0, 50.0, 100, 200, 500 and 1000 µg/ml with the cell lines at concentration of 10^6cells/ml per well. The final volume was made up to 1.0 ml with PBS and was incubated at 37^0c for 4hrs. 1 ml of 1% trypan blue (in distilled water) was added to each tube and mixed well. One drop was placed on each side of the haemocytometer and the number of dead cells were counted (10x) (Table.4.5).

% toxicity = (Number of dead cells/Total number of cells) x 100

The experiment was repeated for 12 extracts and % cytotoxicity was determined for each extract. Data obtained were plotted on a graph with concentration of extract on X-axis and % toxicity on Y-axis for calculating IC_{50} values of each extract (Fig 24).

Determination of antioxidant activity

Diphenyl di picryl phenyl hydrazyl (DPPH) method

This method was used to determine the antioxidant activity of different extracts (Kolleva, 2002).

Principle

Antioxidants can scavenge the DPPH and reduce to hydrazine. The colour or absorbance

of the DPPH will be reduced on reduction by an antioxidant compound. So a decrease in absorbance of the reaction mixture is the indication of the antioxidant activity of the compound added. The difference in absorbance can be calculated by measuring the OD using a spectrophotometer.

Materials required

DPPH (SRL) - 0.1mM in methanol, extract at concentrations of 10, 25, 50, 100, and 200 µg/ ml in methanol, ascorbic acid standard (100µg/ml) and distilled water. Chemicals used were of AR grade.

Procedure

500 µl of the sample was taken in a test tube. DPPH was prepared in methanol at a concentration of 0.1 Mm. 1 ml of the DPPH was then added to the extract and mixed well. It was kept at room temperature for 20 min., OD was measured at 517nm using a UV-VIS spectrophotometer.

Ascorbic acid was taken as control. Negative control was DPPH without the extract. Percentage activity was calculated using the formula given below,

% activity =1-([A_{sample}/$A_{control}$] x 100).

The experiment was done in triplicate for each extract. Mean value ± SD was calculated. IC_{50} (Inhibitory concentration for 50% activity) value for each extract was determined utilizing linear regression formula by plotting graph with concentration on X axis and percentage of inhibition on Y-axis.

Table.1 Percentage yield for each plant extract

Name of the plant	Plant part used for the extraction *	Solvent used	Dry mass (gm)	%yield **	Extraction method used ***	Designated as
Strychnos potatorum	**S**	**Methanol** **Ethanol** **Water** **Chloroform**	**0.12** **ND** **ND** **ND**	**0.5** **ND** **ND** **ND**	**C**	**CP1**

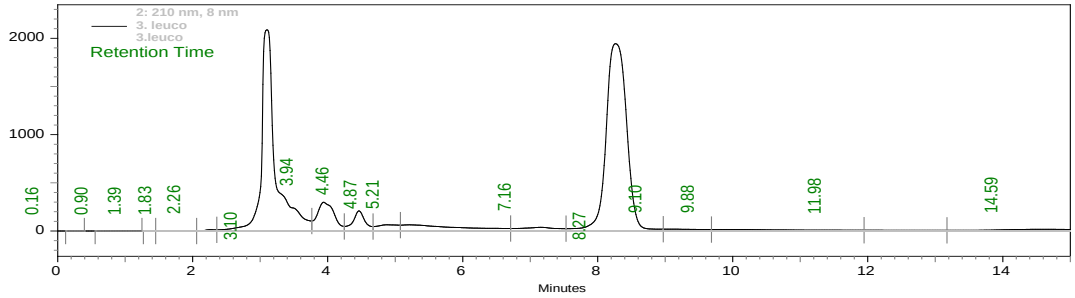
Various metabolites present in the extracts

Name of the plant	Extract tested	Phenolic acids	Flavanoids	Alkaloids	Saponi	Glycosides	Terpenoids	Protein
Strychnos potatorum	CP1 CP3	+-	++					

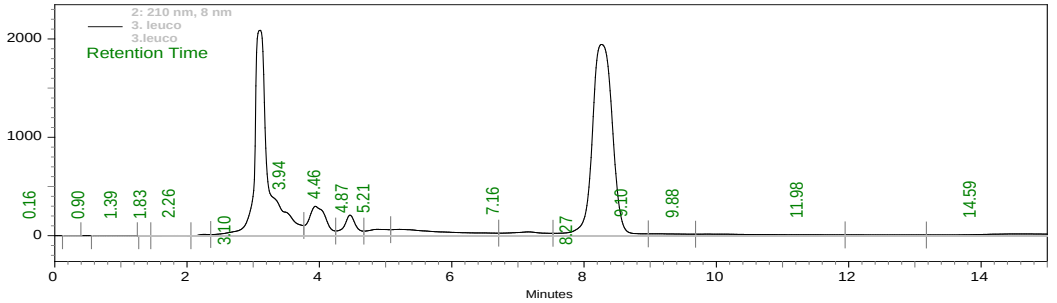
Table.3 Total phenolics and flavanoids in the extracts

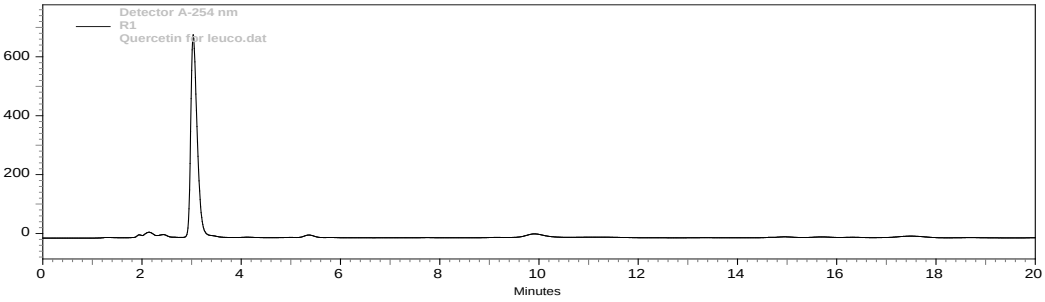
Name of the plant	**Extract used for the estimation**	**Total phenolics as Gallic acid equivalents**	**Total flavanoids as Quercetin equivalents**
Strychnos potatorum	**CP1**	**25.1 + 3.2**	**ND**

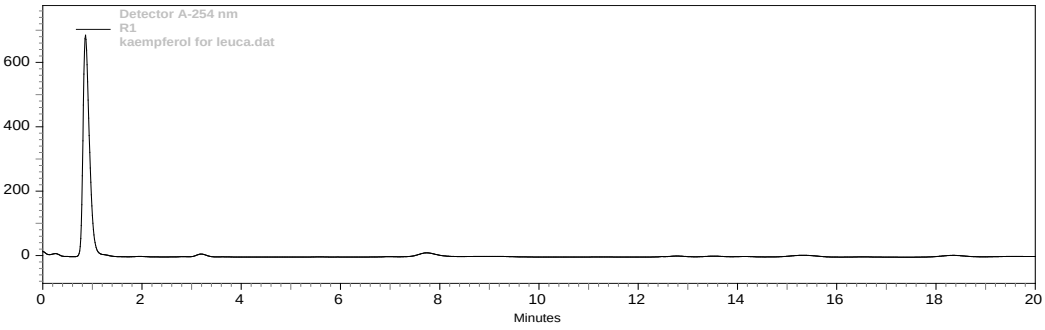
Figure.1 HPLC profiling of methanol extracts of seeds of *Strchnos potatorum*



Quantification of Quercetin and Kaempferol in *Strychnos potatorum*







Quantification of Quercetin and Kaempferol in *Strychnos potatorum*

Concentration of Sample: **20mg/ml** (wrt dry extract weight)
Concentration of Std Quercetin**: 100ug/ml (1 mg dissolved in 10ml)**
Concentration of Std Kaempferol: **100ug/ml (1 mg dissolved in 10ml)**
Retention time of Quercetin: **3.08**
Retention Time of Kaempferol: **0.78**
Percentage purity of injected Quercetin: 98%
Percentage purity of injected Kaempferol: 99%
Area given by Standard Quercetin in the standard profile: **68954758**
Area given by Standard Kaempferol in Standard profile: **568592634**
Area given by Quercetin in sample profile: **12242573**
Area given by Kaempferol in sample profile: **9093**

Result

The percentage of Quercetin in the sample: 0.85 %(w/w)

The percentage of Kaempferol in the sample: 0.0065 %(w/w)

0.85% of quercetin was present in the extract of *Strychnos potatorum*, while it contained kaempherol in comparatively very low level (0.0065%). The medicinal properties of the above three extracts can be due to the presence of these flavanoid compounds- quercetin, kaempherol or both.

Table.6 Antioxidant activity of extracts

Name of the plant	Extract used	IC_{50} VALUE DPPH ACTIVITY µg/ml
***Strychnos potatorum* seed**	**Water**	**70**
	Methanol	**50.5**
	Chloroform	**95.5**

Table.7 Cytotoxicity assay for various extracts

Name of the plant	Extract used for the estimation	Normal Mice spleen cells % inhibition	IC_{50} (concentration for 50% inhibition)
7) Strychnos potatorum	CP1	**10**	**1079.85**
	Methanol	**12**	**1260.5**
	Chloroform	**5**	**1300.0**

Strychnos potatorum: This has been traditionally used against diarrhea, eye diseases, diabetes and bronchitis (Shanmugapriya, 2008). But as per this study, this plant extract has not been a successful antimicrobial agent. The MIC value obtained was above 5000 µg/ml for all the pathogens under study. The aqueous extract of *S. potatorum* has shown the presence of alkaloids, glycosides and proteins, though no flavanoid was present. HPLC choromatogram at 210nm showed the presence of two major and two minor peaks. There was 90 % viability shown on EAC cell lines, in the MTT assay, indicating that the extract is less toxic or inhibitory on cancer cells, thus being a weak antiproliferating agent.

In conclusion, Strychnos potatorum is can be used in alternative medicine for relieve life style diseses,in functional food etc. It has good antioxidant effect with good flavaniod content.

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