Phytochemical Analysis & In Vitro Anticancer Activity of Methanol Extract of *Catharanthus Roseus* Leaves Using HEPG2 Cell Line

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Abstract:

The present study was formulated to understand the phytochemical analysis and *in vitro* anticancer properties *Catharanthus roseus* leaves. The preliminary phytochemical screening of the leaves of *Catharanthus roseus indicates* the presence of secondary metabolites, like alkaloids, tannins, saponins, Phytosterols and carbohydrates, having an essential role in medicine. Methanolic extract of *Catharanthus roseus* leaves was tested for its inhibitory effect on HepG2 cell line. The cytotoxicity of *Catharanthus roseus* leaves on HepG2 cell line in concentration range between 62.5to1000 µg/ml by using MTT assays.from this assay, methanolic extract of *Catharanthus roseus* leaves shows moderate therapeutic values on HepG$_2$ cell line with CTC$_{50}$ values 153.00±0.6respectively and that mean *Catharanthus roseus* leaves can be used in anticancer activity .

KEY WORDS: *cathalanthus roseus, HEPG2 Cell line, cytotoxicity, MTT Assay,* phytochemical

I. Introduction

Nature has been a source of medicinal agents for thousands of years and an impressive number of modern drugs have been isolated from natural sources. Many of these isolations were based on the uses of the agents in traditional medicine. The plant-based, traditional medicine systems continues to play an essential role in health care, with about 80% of the world's inhabitants relying mainly on traditional medicines for their primary health care [1].

According to the World Health Organization (WHO) "a medicinal plant" is any plant, which in one or more of its organ contains substances that can be used for the therapeutic purposes or which, are precursors for the synthesis of useful drugs [2].

Recently, the world health organization estimated 80% of people worldwide rely on herbal medicines partially for their primary health care. In Germany about 600-700 plant based medicines are available and are prescribed by some 70% of german physicians. In the last 20 years in the United States, public dissatisfaction with the cost of prescription medications, combined with an interest in returning to natural or organic remedies, has led to an increase in herbal medicine use. During the past three decades, the demand and utilization of medicinal plants has increased globally. There is now a condense regarding the importance of medicinal plants and traditional health systems in solving the health care problems, efficacy and safety of medicinal plants in curing various diseases. Because

of this growing awareness, the international trade in plants of medicinal importance is growing phenomenally, often to the detriment of natural habitats and mother populations in the countries of origin. There are less than 1% of some 250,000 higher plants that have been screened in depth for their Phytochemistry or pharmacology [3].

However, herbs are staging a comeback and 'herbal renaissance' is happening all over the globe. Herbal medicines are still being used in many countries, because of their compatibility with the human body, cheapness, and minimum side effects. During the last decade there has been a major increase in the use of medicinal plants all over the world particularly in U.S.A. and European countries [4] .

The herbal products today symbolise safety in contrast to the synthetics that are regarded as unsafe to human and environment. Although herbs have been priced for their medicinal, flavouring and aromatic qualities for centuries, the synthetic products of the modern age surpasses their importance, for a while. However, the blind dependence on synthetics is over and people are returning to the naturals with hope of safety and security. Over three-quarters of the world population nowadays relies mainly on plants and plant extracts for health care [5].

Herbal remedies and alternative medicines are used throughout the world and herb-dervied medications are still used extensively for the treatment of high blood pressure, diabetes and other illness. The use of herbal medicine is now wide spread for the treatment of various diseases and disorders, it is redundant [6].

The use of pharmaceuticals has led to unforeseen side effects such as genetic alterations, bio magnifications and even death. Unforeseen side effects often appear after a drug has been on the market for years and is taken by many. Drug testing does not find these effects, as the number of patients in trials is not generally high enough. Also, trials are controlled by the company that wants the medicine approved, they are slanted to find efficacy and safety [7]. On the other hand, the use of herbal medicines has several advantages. One advantage is its wide availability and simple in preparation. Plants can contain sugars, minerals, proteins and other chemicals that interact with the active chemical in a variety of ways viz. They may concentrate or intensify its effect, they may make it easier to digest or absorb, or they may lessen its harsh or toxic side effects [8]. Most herbs can be used as medicine by making decoctions. Traditional prescriptions generally include extracts and concentrated single active compound from plants [9]. Supporters of traditional herbal medicine feel that medicine is most effective in its natural state which contains all the active ingredients rather than the processed synthetic drug [10].

In recent years, there has been renewed interest in the treatment against different diseases using herbal drugs, as they are generally non-toxic and World Health Organization has recommended its effectiveness rather than the precarious modern drugs. Plant derivatives with hypoglycemic properties have been used in folk medicine and traditional healing systems around the world from ancient time [11]. Despite, the introduction of hypoglycemic agents from natural and synthetic sources, diabetes and its secondary complications continue to be a major medical problem to people [12].

Medicinal plants used to treat hypoglycemic and hyperglycemic conditions are of considerable interest to ethnobotanical community as the plants contain valuable medicinal properties in its different parts. In traditional medicine, physical exercise and medicinal plants. Even though, more than 1200 plants were used in the

control of diabetes mellitus, approximately 30% of the antidiabetic plants were pharmacologically and chemically investigated [13].

Medicinal plants in cancer prevention:

Cancer (malignant tumor) is an abnormal growth and proliferation of cells. It is a frightful disease because the patient suffers pain, disfigurement and loss of many physiological processes. Cancer may be uncontrollable and incurable, and may occur at any time at any age in any part of the body. It is caused by a complex, poorly understood interplay of genetic and environmental factors [14]. It continues to represent the largest cause of mortality in the world and claims over 6 million. Cancer kills annually about 3500 per million populations around the world. A large number of chemo preventive agents are used to cure various cancers, but they produce side effects that prevent their extensive usage. Although more than 1500 anticancer drugs are in active development with over 500 of the drugs under clinical trials, there is an urgent need to develop much effective and less toxic drugs [15].

The plant *Catharanthus roseus* has been used in diverse traditional medication for the treatment of diseases and illness of human beings. The crude latex extract obtained from the leaves *Catharanthus roseus* was evaluated for cytotoxic, antimicrobial and wound healing properties. Cell viability and cytotoxicity assays such as Colony Formation method and Enzyme based methods that determined cell viability with a colorimetric method were performed to evaluate the medicinal properties of *Catharanthus roseus*. Results showed that the latex crude extract of *Catharanthus roseus* showed potent Antioxidant and Anticancer properties, but the viability of the cells were unaffected [16].

The Present Study Phytochemicals analysis, anti-oxidant and anti-cancer activity of the Leaf extract of **"***Catharanthus roseus***"** was undertaken with the main objectives of the phytochemical analysis of methanol extract *Catharanthus roseus* and studying of the *in vitro* anticancer activity of methanol extract of *Catharanthus roseus* leaves using hepatocellular carcinoma cell line (HepG2).

II. Materials and Methods

A. Plant Material:

Catharanthus roseus plants were collected in and around Rasipuram, Namakkal district Tamilnadu, India.

***Fig. 1 Catharanthus roseus* plants**

B. Preparation of Plant Extracts:

Fresh leaves were collected from the plants, washed and shade dried and powdered. The powder was extracted using methanol solvent by soxhlet apparatus. The residue was filtered and the solvent were evaporated under reduced pressure and stored for further studies. The extract was used for the determination of phytochemical constituents and for *in vitro.*

C. Preliminary Phytochemical Screening:

The preliminary phytochemical screening were carried out to identify the useful constituents present in the methanol extract by standard method [14].

1) Tests for Carbohydrates (Benedict's Test): Equal volume of Benedict's reagent and test solution in test tube were mixed.Heated in boiling water bath for 5 min. Solution may be appeared green, yellow or red depending on amount of reducing sugar present in

test solution.

2) ***Tests for Carbohydrates (Fehling's Test):*** 1 ml of Fehling's A and 1 ml of Fehling's B solutions were mixed and boiled for one minute. Equal volume of test solution was added. The mixture was heated in boiling water bath for 5-10 min and tubes were observed for a yellow, then brick red precipitate.

3) ***Test for Proteins (Biuret Test)*****:** To 3 ml Test solution few drops 4% NaOH and 1% $CuSO_4$ solution were added and observed for violet or pink colour.

4) ***Test for Proteins (Millon's Test):*** To that 3 ml test solution 5 ml of Millon's reagent was added and observed for white precipitate.

5) ***Test for Proteins (Xanthoprotein Test):*** To 3 ml of test solution 1 ml of concentrated H_2SO_4 was added and observed for white precipitate.

6*) Test for amino acids (Ninhydrin Test, General test***):** 3ml test solution and 3 drops 5% Ninhydrin solution were heated in water bath for 10 min, observed for purple or bluish colour.

7) ***Test for Flavonoids (Lead Acetate Test):*** The extracts were treated with few drops of 10% lead acetate solution. The formation of yellow precipitate confirmed the presence of flavonoids.

8) ***Test for Flavonoids (Shinoda Test):*** To 0.5 ml of alcoholic extract added 5 - 10 drops of dilute hydrochloric acid and small piece of magnesium or zinc and content was boiled for some minutes. Dirty brown color or reddish pink showed the presence of flavonoids

9) ***Tests for Glycosides:*** Part A**:** Extract 200 mg of the drug by warming in a test tube with 5 ml of dilute (10%) H_2SO_4 (sulphuric acid) on awater bath at 100^o C for 2 min, centrifuge or filter, pipette off supernatant or filtrate. Neutralize the acid extract with 5% solution of sodium hydroxide (noting the volume of NaOH added). Add 0.1 ml of Fehling's solution A and then Fehling's solution B until alkaline (test with pH paper) and heat on a water bath for 2 min. Note the quantity of red precipitate formed and compare with that formed in part B.

9) Tests for Glycosides: **Part B:** Extract 200 mg of the drug using 5 ml of water instead of sulphuric acid. After boiling add volume of water equal to the volume of NaOH used in the above test. Add 0.1 ml of Fehling's solution A & B until alkaline (test with pH paper) and heat on a water bath for 2 min. Note the quantity of red precipitate formed. Compare the quantity of precipitate formed in Part B with that of formed in part A. If the precipitate in part-A is greater than in part B then glycoside may be present. Since part-B represents the amount of free reducing sugar already present in the crude drug, whereas part-A represents free reducing sugar plus those resulted on acid hydrolysis on any sides in the crude drug.

10*) Test for tannins (Ferric chloride test***):**
The extract was diluted to 5 ml with distilled water. To this a few drop of neutral 5% ferric chloride solution was added. A dark green colour indicates the presences of phenolic compounds.

11) Test for tannins (Acetic acid test): To 3 ml test solution, add 2 to 3 drops of acetic acid solution. Red solution is observed.

12) Test for Saponin (Foam Test): The drug extract or dry powder was shaking vigorously with water. Persistent foam was observed.

13) Test for Alkaloids (Dragendroff's Test): To 0.5 ml of alcoholic extract, added 2 ml of hydrochloric acid and 1 ml of Dragendroff's reagent. Orange red

precipitate or reddish brown color was obtained which showed the presence of alkaloids.

14) Test for Phenolic Compounds (Ferric Chloride Test): To 3 ml test solution, add 2 to 3 drops of ferric chloride solution. Deep blue black colour is observed.

15) ***Test for steroids***: Crude extract was mixed with 1ml of chloroform, few drops of acetic anhydride and two drops of concentrated H2SO4. The development of a greenish coloration indicated the presence of steroids

16) Test for Terpenoids (Hirshorn Test): To 1 ml of the extract added 2 ml of trichloroacetic acid and heated and gives yellow color which changes to red showed the presence of terpenoids.

***17) Test for Terpenoids (Lieberman Starch Morasky Test*)**: To 1 ml of the extract, 3 ml of warm acetic acid was added with few drops of concentrated sulfuric acid. Color changes from red to blue indicated the presence of terpenoids.

18) Test for Resin: To 2 ml of extract, added 5 - 10 ml acetic anhydride, gentle heating, cooled and then added 0.5 ml of concentrated sulfuric acid leads to the formation of bright purple color showed the presence of resins

D. In Vitro Cytotoxicity of Methanol Extract of Catharanthus Roseus on HEPG2 Cell Line

1) ***Chemicals:*** 3-(4,5–dimethyl thiazol–2–yl)–5–diphenyl tetrazolium bromide (MTT), Fetal Bovine serum (FBS), Phosphate Buffered Saline (PBS), Dulbecco's Modified Eagle's Medium (DMEM) and Trypsin were obtained from Sigma Aldrich Co, St Louis, USA. EDTA, Glucose and antibiotics from Hi-Media Laboratories Ltd., Mumbai. Dimethyl Sulfoxide (DMSO) and Propanol from E.Merck Ltd., Mumbai, India.

2) ***Cell lines and Culture Medium:*** HepG2 (Human, liver hepatocellular cells) cell line were procured from National Centre for Cell Sciences (NCCS), Pune, India. Stock cells were cultured in DMEM supplemented with 10% inactivated Fetal Bovine Serum (FBS), penicillin (100 IU/ml), streptomycin (100 µg/ml) and amphotericin B (5 µg/ml) in an humidified atmosphere of 5% CO2 at 37ºC until confluent. The cells were dissociated with TPVG solution (0.2% trypsin, 0.02% EDTA, 0.05% glucose in PBS). The stock cultures were grown in 25 cm2 culture flasks and all experiments were carried out in 96 microtitre plates (Tarsons India Pvt. Ltd., Kolkata, India).

3) ***Preparation of Test Solutions:*** For Cytotoxicity studies, each weighed test drugs were separately dissolved in distilled DMSO and volume was made up with DMEM supplemented with 2% inactivated FBS to obtain a stock solution of 1 mg/ml concentration and sterilized by filtration. Serial dilutions were prepared from this for carrying out cytotoxic studies.

E. Determination of Cell Viability by MTT Assay:

1) ***Principle:*** The ability of the cells to survive a toxic insult has been the basis of most Cytotoxicity assays. This assay is based on the assumption that dead cells or their products do not reduce tetrazolium. The assay depends both on the number of cells present and on the mitochondrial activity per cell. The principle involved is the cleavage of tetrazolium salt 3-(4, 5 dimethyl thiazole-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT) into a blue coloured product (formazan) by mitochondrial enzyme succinate dehydrogenase. The number of

cells was found to be proportional to the extent of formazan production by the cells used [15].

2) Procedure: The monolayer cell culture was trypsinized and the cell count was adjusted to 1.0 x 105 cells/ml using DMEM containing 10% FBS.

To each well of the 96 well microtitre plate, 0.1 ml of the diluted cell suspension (approximately 10,000 cells) was added. After 24 h, when a partial monolayer was formed, the supernatant was flicked off, washed the monolayer once with medium and 100 µl of different test concentrations of test drugs were added on to the partial monolayer in microtitre plates.

The plates were then incubated at 37º C for 3 days in 5% CO2 atmosphere, and microscopic examination was carried out and observations were noted every 24 h interval.

After 72 h, the drug solutions in the wells were discarded and 50 µl of MTT in PBS was added to each well.

The plates were gently shaken and incubated for 3 h at 37º C in 5% CO2 atmosphere.

The supernatant was removed and 100 µl of propanol was added and the plates were gently shaken to solubilize the formed formazan.

The absorbance was measured using a microplate reader at a wavelength of 540 nm.

The percentage growth inhibition was calculated using the following formula and concentration of test drug needed to inhibit cell growth by 50% (CTC50) values is generated from the dose-response curves for each cell line.

$$\% \text{ Growth Inhibition} = 100\left(\frac{Mean\ of\ OD\ of\ individual\ test\ group}{Mean\ OD\ of\ control\ group} X100\right)$$

III. Results

A. *Preliminary Phytochemical Screening*

The results of phytochemical screening of the leaves of *Catharanthus roseus* were presented in Table 1. Phytochemical analysis showed the presence of alkaloids,tannins, saponins, Phyto sterols and carbohydrates.

Table 1 phytochemical screening of methanol extract of *Catharanthus roseus*

S.NO	Phytoconstituents	Solvents
		Methonal
1	Carbohydrates	Present
2	Proteins	Absent
3	Amino acids	Absent
4	Alkaloides	Present
5	Flavonoids	Absent
6	Glycosides	Absent
7	Tannins	present
8	Steroids	Absent
9	Phyto sterols	Present
10	Terpenoids	Absent
11	Phenols	Absent
12	Saponins	present
13	Resins	Absent

Table 2 Cytotoxic properties of test drugs against HepG2 cell line

SI. No	Name of test sample	Test concn. (µg/ml)	% Cytotoxicity	CTC_{50} (µg/ml)
1	Test drug	1000	78.86±0.4	153.00±0.6
		500	72.28±0.5	
		250	56.73±0.4	
		125	48.57±0.3	
		62.5	40.55±0.2	

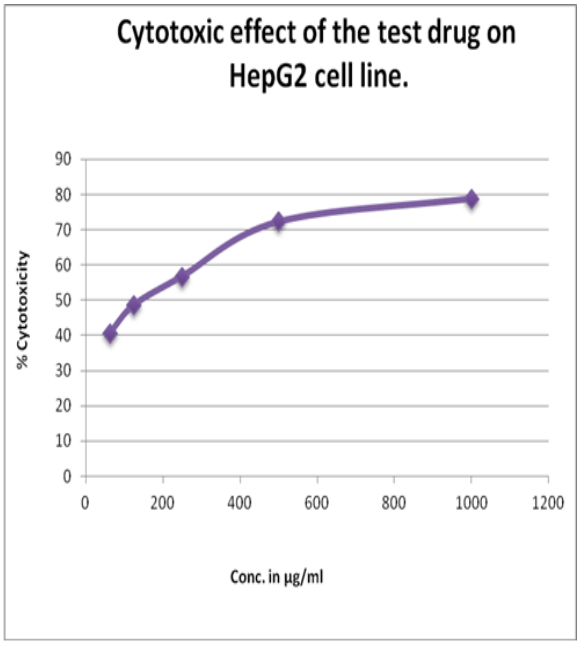

Fig. 2 Cytotoxic effect of the test drug on HepG2 cell line

IV. Discussion

The present work discusses the phytochemical investigation and the evaluation of *Catharanthus roseus* leaves of studies for anti-cancer activity in the light of recent research on medicinal properties.

In the present days of modernization, Ayurveda no longer can afford to remain confined to use of conventional conservative norms of medication. It has to accept the new challenges and be prepared to answer the queries of the modern man about the quality and efficacy of the herbal drugs administered to him and also how they are collected, processed, preserved and used. To meet this new thrust inquisitiveness, standardization of Ayurvedic drugs is mandatory which may help in understanding and solving some of the controversies with regard to their therapeutically active ingredients and action.

The biological selective activity of any compound might depend on the type of chemical composition and the concentration of active constituents as well as types and developmental stages of the cancer. The screening of plants of their anticancer properties use cell-based assays and established cell lines, in which the cytotoxic effects of plants extracts or isolated compounds could be measured. In the present study, we used MTT (3-(4,5- dimethyl-thiazol-2-yl)-2, 5-diphenyltetrazolium bromide) assay. The result of our study revealed that methanol extract of leaves of *Catharanthus roseus* has a cytotoxic effect on Hep G2 (Human liver hepatocellular cells) cell line in a concentration dependent manner. The extract showed moderate therapeutic values Hep G2 cell line with CTC_{50} values 153.00±0.6 respectively. Morphological studies also confirmed that the methanol extract of leaves of *catharanthus roseus* has got potential cytotoxic effect **.**

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