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ANTI CANCER ACTIVITY OF HERBAL FORMULATION OF SIMAROUBA GLAUCA AND ANNONA MURICATA ON MCF- 7 CELL LINES

Microbiology

Josna George* Research Scholar, Department of Biochemistry, Dr. N.G.P. Arts and Science College, Coimbatore, India. *Corresponding Author

Dinesh. M.D Assistant Professor, Department of Microbiology, Pazhassiraja College, Pulpally, Wayanad, Kerala, India.

Dr. N. Kannikaparameswari Professor, Department of Biochemistry, Dr. N.G.P. Arts and Science College, Coimbatore, India.

Lijo Joy Assistant Professor, Department of Vocational studies, Pazhassiraja College, Pulpally, Wayanad, Kerala, India.

ABSTRACT

The lone and combinational effects of cytotoxicity of selected medicinal plants detected by using MTT assay, XTT assay, LDH assay and Tryphan Blue dye exclusion assay on MCF7 cell lines. Combination of two plant extracts shows more anti-cancer activity, because the combined extract may contains more phyto-consituents involved in anti-cancer activity of both plants. Thus our findings makes stronger that the extracts of *Simarouba glauca* and *Annona muricata* is more effective solution for the discovery of novel herbal formulations for cancer therapy.

KEYWORDS

Anti-cancer, In-vitro assay, *Simarouba glauca, Annona muricatais*.

Introduction

Various types of cancers are now identified and each of them named from where they started initially. Among all other types of cancers, Breast cancer is very common in females, basically due to lack of knowledge and negligence to preliminary symptoms. It has also been described as an orphan disease, in the sense that the very detailed knowledge about tumour characteristics and the necessary host biology capable of providing basic care is absent.[1] There are various treatment plans for cancer patient depending on the type and the stage at diagnosis. Surgical treatment for breast cancer has to do with breast-conserving surgery (BCS) or mastectomy and when this is done properly for localized or regional cancers, long-term survival is the same as with mastectomy [2].

Traditional drug practitioners use herbal medicines for prevention and treatment of cancer like diseases. *Simarouba glauca* and *Annona muricata* are traditional herbs used for formulation of traditional medicines for cancer treatment by traditional herbal practitioners specifically in Kerala. *Simarouba glauca* belongs under the family of Simaroubaceae and *Annona muricata* belongs under the family of Annonaciae. These plants carry mutli-medicinal properties. Many Indian herbal traditional practitioners especially in South India use combination of *Simarouba glauca* and *Annona muricata* against tumor and cancer treatment without any scientific clarification. World Health Organization suggested that the detailed scientific examinations for the traditional herbal drugs should be performed to understand the effects. In our present study we focused on the anti- cancer activity of *Simarouba glauca, Annona muricata's* lone and combined effect of both extracts against breast cancer cell lines (MCF 7).

2. Materials and methods

2.1. Collection of Medicinal Plants and parts used

Medicinal plants *Simarouba glauca* and *Annona muricata* were collected from in and around Wayanad District and authentically identified and certified by the senior scientist of M.S Swaminathan Foundation Research Centre, Wayanad, Kerala. Fresh leaves of *Simarouba glauca* and fruit of *Annona Muricata* were used for our study. The soxhlet extraction processes using distilled water as solvent were carried out for 6 hours. The obtained both liquid extract was evaporated to dryness using rotary flash evaporator. The dried extracts were scrapped and powdered to fineness using pestle and mortar. The fine powdered sample extracts were stored in a sterile air tight amber bottle at 4°C. Solvent extracts were separately analyzed to investigate the presence of phytochemical compounds.

2.2. *In-vitro* anti-cancer activity determination by different cytotoxicity assays

The anticancer activity of *Simarouba glauca* and *Annona muricata* were carried out by different cytototoxicity assay by diverse methods using MCF -7 cell lines. In our study we investigate the lone and combinational anticancer activity of *Simarouba glauca* and *Annona muricata* in order to prove the traditional knowledge widely used herbal practitioners in the Wayanad District. The cell line (MCF -7) was obtained from National Centre for Cell Science (NCCS), Pune, India and grown in Dulbeccos Modified Eagles Medium (DMEM) containing 10% foetal bovine serum (FBS).

2.2.1. *In vitro* anti-cancer activity determination by MTT assay

15mg of MTT (Sigma, M-5655) was reconstituted in 3 ml of PBS until completely dissolved and sterilized by filter sterilization. After 24 hours of incubation period, the sample content in wells were removed and 30µl of reconstituted MTT solution was added to all test and cell control wells, the plate was gently shaken well, then incubated at 37ºC in a humidified 5% CO_2 incubator for 4 hours. After the incubation period, the supernatant was removed and 100µl of MTT Solubilization Solution (DMSO) was added and the wells were mixed gently by pipetting up and down in order to solubilze the formazan crystals. The absorbance values were measured by using micro-plate reader at a wavelength of 570 nm [4]. The percentage of growth inhibition was calculated using the formula:

% Cell viability = Abs (sample)/Abs (control) x100.

2.2.2. *In vitro* anti-cancer activity determination by Lactic Dehydrogenase Assay (LDH assay)

Transfer 200 µl cell suspension in a 96-well plate at required cell density (20,000 cells per well), without the test agent. Allow the cells to grow for about 12 hours. Add appropriate concentrations of the test agents which were dissolved initially with DMSO and further dilutions with culture medium. (The higher concentration of test agent must contain less than 5% of DMSO in its dilution). Incubate the plate for 24 hrs at 37°C in a 5% CO_2 atmosphere. After the incubation period, takeout the plates from incubator, and remove spent media and add 50uL of LDH assay reagent and wrap the plate with aluminum foil to avoid exposure to light. Kept the plates to the incubator and incubate for 30 min. (Incubation time varies for different cell lines. Within one experiment, incubation time should be kept constant while making comparisons). Read the absorbance on a spectrophotometer or an ELISA reader at 490 nm and 630nm used as reference wavelength. The IC_{50} value was determined by using linear regression equation i.e. Y =Mx+C.

Here, Y = 50, M and C values were derived from the viability graph.

2.2.3. *In vitro* anti-cancer activity determination by lactic dehydrogenase activity (LDH) assay method:

Lactic dehydrogenase activity is spectro-photometrically measured in the culture medium and in the cellular lysates at 340 nm by analyzing

NADH reduction during the pyruvate to lactate transformation. Cells are lyzed with 50mM Tris-HCl buffer, pH 7.4 + 20 mM EDTA + 0.5 % Sodium Dodecyl Sulfate (SDS), further disrupted by sonication and centrifuged at 13,000 X g for 15 min. The assay mixture (1ml final volume) for the enzymatic analysis consists of 33 µl of sample in 48 mM PBS, pH 7.5 + 1 mM pyruvate and 0.2 mM NADH. The percentage of LDH released is calculated as percentage of the total amount, considered as the sum of the enzymatic activity present in the cellular lyzate and that in the culture medium.

2.2.4.- 2,3Bis- (2 methoxy-4-nitro-5-sulfophenyl)- 2 H-tetrazolium- 5- carboxanilide assay (XTT assay)

Transfer 200 µl cell suspension in a 96-well plate at required cell density (20,000 cells per well), without the test agent. Allow the cells to grow for about 12 hours. Add appropriate concentrations of the test agents which were dissolved initially with DMSO and further dilutions with culture medium (The higher concentration of test agent must contain less than 5% of DMSO in its dilution). Incubate the plate for 24 hrs at 37°C in a 5% CO2 atmosphere. An XTT assay is performed at the end of incubation. Briefly, 50 ml of XTT labeling mixture solution is add to each well, and the cells are incubated at 37 °C for 4 h. The formazan dye formed is soluble in aqueous solutions and the optical density at 450 nm is compared with that of control wells with a screening multiwall spectro-photometer enzyme linked immune sorbent assay (ELISA) reader at 500nm and 630nm used as reference wavelength. The Ic_{50} value was determined by using linear regression equation i.e. Y =Mx+C.

Here, Y = 50, M and C values were derived from the viability graph.

2.2.5. Tryphan blue dye exclusion assay.

Transfer 200 µl cell suspension in a 96- well plate at required cell density (20,000 cells per well), without the test agent. Allow the cells to grow for about 12 hours. Add appropriate concentrations of the test agent and incubate the plate for 24 hrs at 37°C in a 5% CO_2 atmosphere. After the incubation period, takeout the plates from incubator remove spent media and add 20ul of 0.4% trypan blue and take the images. The viable cells and dead cells were counted in each image and percentage of viability was calculate
/ Total cells counted) X 100

3. Results and Discussion

3.1. Collection of Medicinal plants and extracts preparation

Medicinal plants *Simarouba glauca* and *Annona muricata* collected from in and around Wayanad District and authentically identified from M.S Swaminathan Foundation Research Centre, Puthurvayal, Kalpetta,Wayand district, Kerala (Figure- 1 and 2). After soxhlet extraction 8% of *Simarouba glauca* and 10 % of dried powder of *Annona muricata* was collected and stored in airtight container for further analysis. Within the present study we investigated the components present in *Simarouba glauca* are Tannin, Flavanoids, Phenol, Steroids and Reducing sugars. The *Annona muricata* contains Tannin, Flavanoids, Steroid, Sugars and Reducing sugars.



Figure: 1 - ***Simarouba glauca***



Figure: 2 – ***Annona muricata***

3.3. Lone and combinational Effect of *Simarouba glauca* and *Annona muricata* extract on MCF -7 cancer cell lines.

Four different cytotoxicity assays were performed on order to prove the anti-cancer activity of medicinal plants. Lone and combinational action of both plants analyzed using MTT assay, XTT assay, LDH assay and Tryphan Blue dye exclusion assay.

On MTT assay the IC_{50} value was calculated as 36.7955µl/ml for *Simarouba glauca,* 37.6159µl/ml for *Annona muricata* and 18.7665µl/ml for combinational extract of both *Simarouba glauca* and *Annona muricata.* 18.7665µl/ml of extracts needed for the 50% of cell inhibition. Results indicated that *Simarouba glauca* and *Annona muricata* hold anti-cancer activity in lone and combinational level, but in combinational level, the anticancer activity is more evident than in lone level. The findings of Sridevi *et al.,* 2017 [5] stated that *Simarouba glauca* leave extracts possess anticancer activity on T 24 bladder cell lines.

Cell lines study using LDH assay IC_{50} value of *Annona muricata* was 51.96µl/ml and 52.87µl/ml for *Simarouba glauca*. The combinational effect of both plant extracts showed 36.57µl/ml. These results suggested that the combinational extracts of selected medicinal plants can more effectively inhibit the cell viability. According to the report of [6] Asare *et al*., (2004) the extract of *Annona muricata* on the prostate cancer cell line to act as anti-proliferate agent. That can show anticancer activity.

In-vitro anticancer activity via XTT assay addition of increased concentration of plant extract showed decreased cell viability. The IC_{50} value was calculated as 49.60µl/ml for *Simarouba glauca,* 64.18µl/ml for *Annona muricata* and 44.02 µl/ml for combinational effect of both *Simarouba glauca* and *Annona muricata.* These results suggested that combinational doses can more effectively reduce the cell viability. Report of Rivero-Cruz in 2005 [7] confirmed the cytotoxic activity of 4 canthine 6- one derived alkaloids, isolated from *Simarouba glauca,* against human colon cancer, human oral epidermoid cancer.

In our study Tryphan blue dye exclusion assay for in-vitro screening of cytotoxicity the control or untreated cell showed 96.90% viability, whereas treated cells showed 90.38, 86.75, 75.65, 60.21 and 47.63 % for 6.25, 12.5, 25, 50 and 100µl/ml, respectively. Decreased concentration of extracts showed the number of dead cells decreased and higher concentration of extract showed the presence of increased dead cell. The viability of cells will decrease depending up on the concentration of extracts. *Annoana muricata* showed the presence of dead cell was 114 and the percentage of viability remains 47%. 145 dead cells was detected the cell treated with *Simaroba glauca* and the viability was 45%. The combinational effect of both extracts on MCF- 7 cell lines was significant increase in the dead cell was 146 and viability remains 34.49%. These results suggested that lone and combinational effect of both plant extracts possess good anticancer activity on MCF -7 cell lines. In 2005 Umesh [8] and his co-workers reported the free radical and cytotoxic activity of *Simaroba gluca* extract is high level,due to the presence of plant metabolites.

Conclusion

In our present study we identified some active photochemical compounds such tannin, flavanoids, phenol, steroid, protein, sugars and reducing sugar in *Simarouba glauca*, Tannin, flavanoids phenols, steroid, protein,amino acid, sugars , reducing sugar and alkaloid in *Annona muricata* by phytochemical screening. The lone and combinational effects of cytotoxicity of selected medicinal plants detected by using MTT assay, XTT assay, LDH assay and Tryphan Blue dye exclusion assay on MCF7 cell lines. These assays indicated that the inhibitory concentration (IC50) is less in combinational dosage of two plant extract when compared to lone dosage of individual plant extracts. According to the in-vitro studies *Simarouba glauca* and *Annona muricata* are natural anti-cancer agent. Further investigations are needed in order to screen the Phyto-compounds which involved the anti-cancer activity. Moreover we require the evidence of *In vivo* results in order to prove the scientific evidences. Further investigation (*in vivo*, pre-clinical studies) is needed to prove the action of *Simarouba glauca* and *Annona muricata* for formulating as anticancer drug.

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