IN VITRO CYTOTOXIC ACTIVITY OF ETHANOLIC EXTRACT OF FLOWER BUDS OF *Syzygium aromaticum* AGAINST HEPATOCARCINOMA CELL LINE (Hep G2)

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ABSTRACT
In vitro cytotoxic (liver cancer) activity of the Ethanolic extracts of the flower buds of *Syzygium aromaticum* was evaluated against liver HepG2 cell line. The percentage viability of the cell line and the cytotoxicity activity of *S.aromaticum* against HepG2 cell line was carried out by using MTT assay method. The ethanolic extract of *S.aromaticum* showed significant cytotoxicity effect on HepG2 cell line in the concentration range at 1000µg/mL produce 37.8 ± 3.5 of cell viability. The results indicate that the extract of the plant could be used against liver cancer. High IC_{50} values indicate the low toxicity of the extracts. Thus this plant could potentially be used as anti cancer drug. However it needs further investigation.

Key Words: *Syzygium aromaticum*,Phytochemicalanalysis,Hep G2,MTTassay

INTRODUCTION

Nature has provided a complete store house of remedies to cure all ailments of mankind. The human being appears to be afflicted with more diseases than any other animal species. About 80% of worlds inhabitants health problem' could be showed by using medicinal herbal drug for their primary health care [1,2] **(**Ekin, 1981; Sanjay Patel *et al*., 2009**)**. During the last decade, the use of plants in the treatment of cancer has been gaining moments in some of the plants used for the treatment of advanced stages of various malignancies were *Catharanthus roseus, Angelica gigas, Podophyllum pellatum, Taxus brevifolia, Campotheca acuminate* [3] **(**Zhong, 2006**).** Uncontrolled multiplication and spread within the body of abnormal form of body's own cells is called as "cancer". Hepatocellular carcinoma (malignant hepatoma; HCC) is an end-stage liver disease and is accompanied by yellowing of the skin, fluids build up in the abdomen, abnormalities in blood clotting, severe abdominal pain, vomiting/nausea and restlessness. While the majority of the cases of HCC occurs within injured or virally infected hepatocyte, other types of cells in the liver can route to the development of cancer, but they are less common. Cancers that arise in any other part of the body, such as lung, colon or breast and spread to the liver is called as metastatic cancer rather than liver cancer [4] (Mathew *et al*.,2014). People acquire liver cancer in the context of chronic liver disease (cirrhosis), which increases the risk of liver cancer with scars.

Medicinal plants have been the mainstay of traditional herbal medicine among rural dwellers worldwide since antiquity to date. India has rich medicinal plant flora of some 25,000 species, out of which 150 species are commercially used for extracting medicines or drug formulation. Over the last few years, researchers have aimed at identifying and validating plants derived substances for the treatment of various diseases. Interestingly, it is estimated that more than 25% of modern medicines are directly or indirectly derived from plants. In this context, it is worth mentioning that Indian plants are considered as vast source of several pharmacologically active principles and compounds, which are commonly used in home remedies against multiple ailments.

It is an unopened flower bud growing on a tree belonged to the family *Myrtaceae*. The plant have exhibited antibacterial activity [5,6] (Gupta *et al*., 2014; fu*et al*., 2007), anti -viral activity [7] (Chaieb*et al*., 2007), insecticidal activity [8] (Yang *et al*., 2003), hepato-protective activity [9] (Sallie *et al*., 1991), anti-diabetic

activity [10] (Prasad *et al*., 2005), anti-stress activity [11] (Singh *et al*., 2009) and aphrodisiac activity [12] (Tajuddin*et al*., 2004).

The present article deals with the determination of the cytotoxic activity of the methanolic extract of the flower buds of *S.aromaticum*

MATERIALS AND METHODS

Collection, Identification and Authentication of plant materials

The flower buds of *S.aromaticum* L. was collected in and around Koothanallur, Thiruvarur District, Tamil Nadu, India. The plant was identified with the help of the Flora of Presidency of Madras and authenticated by Dr. S. John Britto, RAPINAT Herbarium and Centre for Molecular Systematics, St. Joseph's college, Tiruchirappalli (Voucher number of the specimen, GTP 001) [13] (Gamble, 1997).

Preparation of plant powder

The flower buds were air dried under shade for 10-15 days. Then the dried material was grinded to fine powder using an electric grinder and stored in air tight bottles. The powder matter was used for further analysis.

Preparation of Ethanol extract

Ethanolic extracts was prepared according to the methodology of Indian pharmacopoeia. The coarse powder material was subjected to Soxhlet extraction separately and successively with 210 ml ethanol and 90 ml distilled water. These extracts were concentrated to dryness in flash evaporator under reduced pressure controlled at a temperature (40ºC – 50ºC). The paste form of the extracts was put in an air tight container stored in refrigerator.

Preliminary phytochemical analysis

The preliminary phytochemical investigation of the flower bud of the *S.aromaticum* was carried out with the standard protocol. The extracts were subjected to preliminary phytochemical analysis

Cytotoxicity activity

Extract preparation

200 g of the ground powder was added with 500 ml of ethanol and kept at room temperature for 12 hours with 6-8 shakes. Compound extraction was carried out using Soxhlet apparatus at 77°C for 4 hours. The solvent was collected and evaporated using a Rotary Evaporator. Colloidal stage of the extract with ethanol was collected from Soxhlet and dried in vaccum chamber, and the dried extract was kept at 4°C until further use.

Cell lines

Cell lines, Hepatic carcinoma (HepG2 cell line) were used in this study, which were procured from National Centre for Cell Sciences (NCCS), Pune, India.

Culture medium

The liver cancer cell line HepG2 preserved in DMEM medium with 10% FBS, 100 units/mLpenicillin, and 100 mg/mL streptomycin tissue culture flasks at 37 °C under a humidified 5 % CO_2 and 95 % air.

Preparation of test solutions

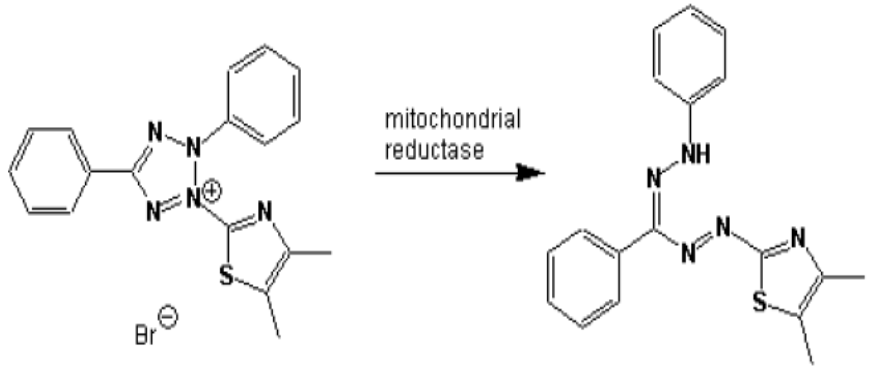
The ethanolic extract was separately dissolved in distilled DMSO and the volume was made up with medium supplemented with 2% inactivated FBS to obtain a stock solution of 1mg/ml concentration and sterilized by filtration. From this stock solution, six different lower dilutions (100, 200, 400,600,800, 1000 µg/ml) were prepared.

Microculture tetrazolium (MTT) assay

The evaluation of the cytotoxic activity against hepatocarcinoma cell lines was done through MTT assay by the method of Masters, (2000).

MTT Assay is a colorimetric assay that measures the reduction of yellow 3-(4, 5dimethythiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT) by mitochondrial succinate dehydrogenase. Cancer HepG2 cells were seeded at the density of 2×105 cells/well were plated on into 6 well plates and treated with extract for 24 and 48 h. The cells were permitted to adhere for 24 hours, and the growth medium (MEM) was removed using micropipette, and the monolayer of cells were washed twice with MEM without FBS to remove dead cells and excess FBS. 1ml of the medium (without FBS) containing different dilution of drugs were added to respective wells, 200 µl of MTT (5 mg/ml in PBS) was added to each well, and the cells were incubated for 6-7 hrs in 5% CO_2 incubator. After removal of the medium, 1ml of DMSO was added to each well. The effect of extracts on cell growth inhibition was assessed as percent cell viability, where vehicle treated cells

were taken as 100% viable. The cells were then exposed to the medium alone (as positive control). Concentrations of the SAE in the range of 100-1000 µg/ml and doxorubicin 100 µg/ml were used in the study. The supernatant was removed and 50 µl of propanol was added and the plates were gently shaken to solubilize the formazan. The MTT enters the cells and passes into the mitochondria where it was reduced to an insoluble, coloured (dark purple) formazan product.



MTT FORMAZAN CRYSTALS

The cells were then solubilised with an organic solvent (eg. isopropanol) and the released, solubilised formazan reagent. Since reduction of MTT could only occur in metabolically active cells the level of activity is a measure of the viability of the cells. The cells were incubated with the extract for 24h and 48 h and the cell mortality was checked. The plates were placed on a shaker for 15 min and the absorbance was read on an enzyme-linked immunosorbent assay (ELISA) reader at 570 nm. Each experiment was carried out in triplicate, and the half maximal inhibitory concentration (IC_{50}) of each extract as the percentage survival of the cells was calculated according to the formula provided below:

Percentage of viable cell concentration was calculated thus:

Viability (%) = (Test Sample OD/Control OD) ×100

RESULTS AND DISCUSSION

Phytochemical analysis of ethanol extract of *Syzygiumaromaticum*

The phytochemical test of the ethanol extract of the flower buds of *S.aromaticum* revealed the presence of alkaloids, carbohydrates, proteins, amino acids, fixed oils, flavonoids and phenolic compounds (Table 1).

In vitro cytotoxic activity

The 70% ethanolic extract of the flower buds of *S.aromaticum*was studied for their *in vitro* effects on liver HepG2 cell lines MTT asaay. The selection of the crude plant extracts for screening has the potential of being more successful in initial steps than the screening of pure compounds which are isolated from natural products. In this present cytotoxic study the 70% ethanolic extract exhibited the most effective cytotoxicity at 1000µg/mL (98.83%) which is depicted in Table 2 and Figure 4**.**

The inhibitory concentration (IC_{50}) value was calculated using the regression analysis and was found to be 728.59µg/ml. The amount of formazan crystals produced by MTT is directly proportional to the number of viable cells.

A decrease in the cell count was observed with the increase in the concentration of the extract. There was a dose depended increase in the cytotoxic activity. The extract at low concentration (100µg/mL) showed 86.7 ± 7.4% cell viability and at high concentration (1000µg/mL) 37.8 ± 3.5% cell viability and the same is seen in Table 2 and Figure 1.

Table 1: Phytochemical analysis of ethanol extract of the flower buds of *S.aromaticum*

S.No.	Phytochemicals	Ethanolic extracts
1.	Carbohydrate	+
2.	Tannin	-
3.	Phlobatannin	-
4.	Flavonoids	+
5.	Alkaloids	+
6.	Quinones	-
7.	Anthroquinones	-
8.	Saponin	-
9.	Steroids	-
10.	Protein and Amino acids	+
11.	Phenolic compounds	+
12.	Fixed oil	+
13.	Terpenoids	-
14.	Triterpenoids	-
15.	Glycosides	-
16.	Cardiac glycosides	-
17.	Coumarin	-

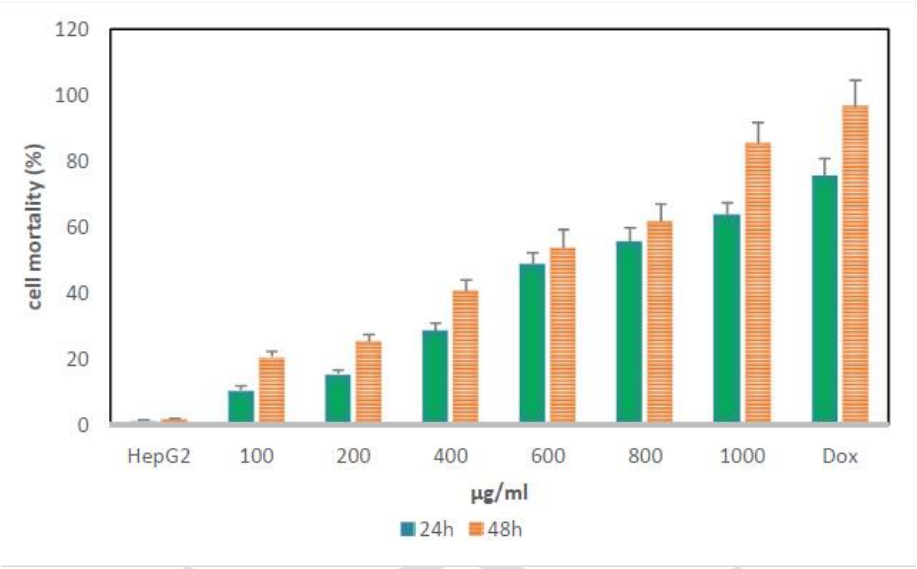
(+) Indicates presence
(-) Indicates absence

Table 2: Cytotoxic activity (% cell viability) against Liver HepG2 cell lines by MTT assay method

S.No.	Treatment	Conc (µg/ml)	Absorbance 570 nm	% of Cell viability
1.	HepG2untreated cells	-	0.317	100 ± 5.9*
2.	Ethanolic Extract of *Syzygiumaromaticum*Treated	100	0.275	86.7 ± 7.4*
3.		200	0.234	73.8 ± 5.6*
4.		400	0.215	67.8 ± 5.1 *
5.		600	0.174	54.9 ± 3.9*
6.		800	0.148	46.7 ± 4.1*
7.		1000	0.120	37.8 ± 3.5 *
8.	Doxorubicin	100	0.085	26.8 ± 1.9*

Values are mean ± SEM expressed as (n=3); *P<0.001, as compared with HepG2incubated cells

Figure 1: Cytotoxic activity (% cell mortality) against Liver HepG2 cell lines by MTT assay method



The crude extract showed pharmacological effect which could be due to the synergistic effects of the various components present in the crude extract. The active constituent namely flavonoids could be responsible for reducing the cancer risk factors. Thus the 70% ethanolic extract of *S.aromaticum* exhibited significant cytotoxic effect particularly for liver cancer. The *in vitro* cytotoxic effect particularly against liver cancer on HepG2cell lines showed that the plant possesses anti proliferative effect comparable to that of Doxorubicin

Phytochemical screening of ethanolic extracts of *S.aromaticum* showed the presence of biologically active phytochemicals such as alkaloids, carbohydrates, proteins, amino acids, fixed oils, flavonoids and phenolic compounds. *S.aromaticum* was subjected to *in vitro* study for the evaluation of cytotoxicity using MTT assay. The study was carried out in HepG2 hepatocellular carcinoma cell lines. *S.aromaticum* exerted antiproliferative effect on HepG2 cells. The IC_{50} values of *S.aromaticum* extract in HepG2 cell was 728.59µg/ml.*In vitro* assay on HepG2 proved that *S.aromaticum* possessed antiproliferative property. This study validates the traditional use of the plant in the management of cancer.

On the basis of the results obtained in the present study, it is concluded that the ethanolic extracts of *S.aromaticum* has potential anticancer activity. Further, investigation on the mechanism of action of *S.aromaticum* extracts at molecular level could pave the way for the development of better therapeutic regimen for hepatocellular carcinoma in future.

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