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ANTI-OXIDANT ANDANTI-CANCER POTENTIAL OF S*YMPLOCOS RACEMOSA* BARK AGAINST HEP3B CELL LINE

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ABSTRACT: Objective: The aim of this study is to evaluate anti-oxidant and anticancer activity of methanol extract and ethyl acetate soluble fraction of methanol extract of *Symplocos racemosa* bark against the hepatocellular carcinoma. **Materials and methods:** DPPH and H_2O_2 free radical scavenging assay were tested for determining the anti-oxidant activity. Rat normal liver cells (BRL-3A) and human hepatocellular carcinoma (Hep3B) cells were tested in-vitro for cytotoxicity using (3-4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium (MTT) assay. **Result:** Ethyl acetate soluble fraction of methanol extract of *Symplocos racemosa* bark exhibited significant DPPH and H2O2free radical scavenging activity withIC50 value (µg/ml) of 10.58 and 11.89 as compared to standard drug ascorbic acid. Ethyl acetate soluble fraction of methanol extract of *Symplocos racemosa* bark exhibited significant cytotoxicity against human hepatocellular carcinoma (Hep3B) cells in-vitrowithIC50 value (µg/ml) of 32.55 as compared to standard doxorubicin (55.43µg/ml), and not affected the normal liver (BRL-3A) cells. **Conclusion:** *Symplocos racemosa* bark showed the potent anti-oxidant and anticancer activity. It may be due to presence of phytochemicals which are responsible for the anticancer activity.

INTRODUCTION: Hepatocellluar carcinoma (HCC) is the one of the commonest cancer, ranking third with very high morbidity and mortality rates and poor prognosis. Therefore, there is an urgent quest for improvement of therapeutic activity and selectivity of anti-cancer agents or drug combinations from natural source with no or limited toxicity for developing cancer therapeutics.

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Considering the continuing need for effective anti-cancer agents, medicinal plants play inexhaustible source of anticancer drugs in term of both variety and mechanism of action [1]. Over 50% of anticancer drugs approved by United states Food and Drug Administration, originated from natural resources, especially from terrestrial plants. The major type of constituents such as phenolics, flavonoids, triterpenoids, steroids, tannins, lignans, coumarins may be responsible for the anticancer activity in the plant extract [2, 3].

Symplocos racemosa Roxb. belongs to a unigeneric family Symplocaceae; is a small evergreen tree reaching a height of 6-8.5 m and diameter of 15 cm. It is found commonly in the plains and hills of northern India and other Asian countries, up to a

height of 1400 m. The common names of *Symplocos racemosa* are astringent bark, lodha (means glorious, use in opthalmia),lodhra (means use in opthalmia), rodhra, lodhraka [4]. Ethnobotanical literature indicates that the bark of *Symplocos racemosa* has been widely used in liver complaints, bowel complaints such as diarrhoea, dysentery and dropsy, skin diseases, ear diseases, eye disease, uterine complaints, vaginal and menstrual disorders, tumors, fever, ulcers and scorpion-string [5, 6]. In Ayurveda pittaja arbuda and medoja arbuda tumors are treated withbark of *Symplocos racemosa* in combination with other various drugs. The powder of *Symplocos racemosa* in combination with *Curcuma domestica*, *Soymida febrifuga*, is mixed with honey which has been used as an external remedy for tumors. The bark of *Symplocos racemosa* has many phytochemicals with various bioactivities and also indicated in traditional system of medicines like ayurveda and unani. [7, 8, 9]. Phytochemical reports on this bark have revealed the many kinds of chemicals, such as phenolic glycosides, triterpenoids, steroids, alkaloids, tanninsand red coloring matter [10, 11, 12]. *S. racemosa* having rich source of various glycosides such as phenolic glycosides: benzoyl salireposide and salireposide which having phosphodiestrase inhibitory activity, ethyl substituted glycoside: 1-ethyl brachiose-3'-acetate, ketochaulmoogric acid, nonaeicosanol, triacontyl palmitate and methyl triacontanoate having lipoxygenase and urease inhibitory activity, phenolic glycosides: symplocomoside and symponoside having thymidine phosphorylase inhibitory activity [13, 14].

The main aim of this study is to investigate anti-oxidant and anticancer potential of methanol extract and ethyl acetate soluble fraction of methanol extract of *Symplocos racemosa* bark against the(Hep3B) cells.

MATERIAL AND METHODS:

Reagents:

Dulbecco's Modified Eagle medium (DMEM), Fetal bovine serum (FBS), RNase A, ethidium bromide, penicillin and streptomycin solution were purchased from Himedia laboratories, Mumbai, India. Trypsin and 3-(4,5-dimethylthiazol-2-yl-)-2,5-diphenyltertazolium bromide (MTT), 1,1-diphenyl-2-picrylhydrazyl (DPPH) were obtained from Sigma aldhrich, Bangalore. Dimethyl sulfoxide, H_2O_2, other chemicals and reagents obtained from Merck, Mumbai.

Preparation of extract:

The dried bark material was purchased from local market of Ramnivas Darak & bros; Mansh; district Neemuch (M.P., India); authenticated by Pharmanza Herbal Pvt. Ltd. Dist. Anand, India (PHPL/HB/060). The dried bark material was powdered (500g) extracted with methanol using hot extraction method. Methanol extract fractionated with ethyl acetate (ESME). The extract and fraction were cooled at room temperature, filtered, evaporated to dryness under reduced pressure in a rotary evaporator and used for the study.

DPPH free radical scavenging assay:

0.1mM DPPH solution was prepared in ethanol.1 ml of this solution mixed with 3 ml of test solutions (5-25µg/ml). The mixture was vigorously shaken and allowed to stand for 30min at room temperature. Absorbance was measured at 516nm [15].

% Scavenging activity = $(A_0\text{-}A_t/A_0) \times 100$;

Where, A_0=Abs. of control; A_t=Abs. of test

H_2O_2 free radical scavenging assay:

20mM H_2O_2 solution was prepared in phosphate buffer saline (pH 7.4). 2 ml of this solution mixed with 1 ml of test solutions (5-25µg/ml). The mixture was vigorously shaken and allowed to keep for 10min at room temperature. Absorbance was measured at 230nm [16].

% Scavenging activity = $(A_0\text{-}A_t/A_0) \times 100$;

Where, A_0=Abs. of control; A_t=Abs. of test

Cell lines and Culture Medium:

BRL 3A (normal rat liver cell) and Hep3B (human hepatoma cell) cell line were used in the experiment, have been obtained from National Centre for Cell Science (NCCS), Pune. The cells were matained in DMEM, supplemented with 10% FBS, penicillin (100IU/ml), streptomycin (100µg/ml) and amphotericin-B (5µg/ml) in a humidified atmosphere of 5 % CO_2 at 37 °C.

In-vitro assay for cytotoxic activity (MTT assay):

Both normal and cancer cells were pre incubated at a concentration of 2×10^6 cells/ml in culture

medium for 3 hrs at 37°C and 6.5 % CO_2, 75 % Relative Humidity. Cells were seeded at a concentration of 5×10^4 cells/well in 100 µl culture medium and various concentration of compound (1000 µg/ml-0.05 µg/ml) were added into microplates (tissue culture grade, 96 wells, flat bottom). Cell cultures were incubated for 24 hrs at 37 °C and 6.5% CO_2. 10 µl of MTT labeling mixture was added and incubated for 4 hrs. 100 µl of DMSO was added to each well and incubate for overnight.

Absorbance of the samples was measured using a microplate (ELISA) reader at wavelength 570nm. Three independent experiments were performed. The effect of the ethyl acetate soluble fraction of methanol extract of *Symplocos racemosa* on the viability of normal liver and hepatoma cells were as the % of viability using following formula.

% Viability = (A_{570} of treated cells – A_{570} of blank cells) / (A_{570} of contolled cells – A_{570} of blank cells) × 100.

Percentage cell growth inhibition or percentage cytotoxicity was calculated by following formula % cytotoxicity = 100 – % cell Viability [17]. IC_{50} values have been determined from plot of Dose Response curve between log of compound concentration and percentage growth inhibition. IC_{50} value has been derived using curve fitting methods with Graph Pad Prism as statistical software (Ver. 5.02).

RESULTS:

Anti-oxidant activity:

The anti-oxidant activity of methanol extract and ethyl acetate soluble fraction of methanol extract of *Symplocos racemosa* bark have been determined using DPPH and H_2O_2free radical scavenging assay methodas compared to standard drug ascorbic acid. **Table 1** showed the IC_{50} value of methanol extract and ethyl acetate soluble fraction of methanol extract on DPPH and H_2O_2 free radical scavenging assay method (**Fig.1** and **2**).

TABLE 1: SHOWED THE IC_{50} VALUE OF METHANOL EXTRACT AND ESME ON DPPH AND H_2O_2 FREE RADICAL SCAVENGING ASSAY METHOD.

Name of extract	IC_{50} value (µg/ml)	
	DPPH	H_2O_2
Methanol extract	16.03	25.26
ESME	10.58	11.89
Ascorbic acid	7.65	10.59

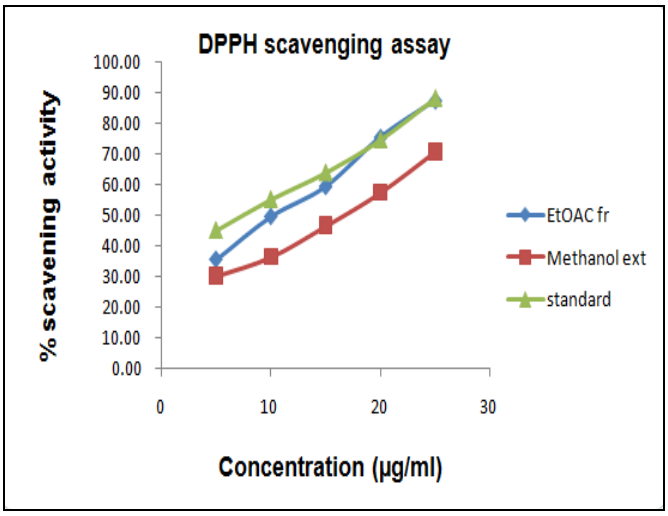

FIG.1: DPPH SCAVENGING ACTIVITY OF METHANOL EXTRACT AND ESME

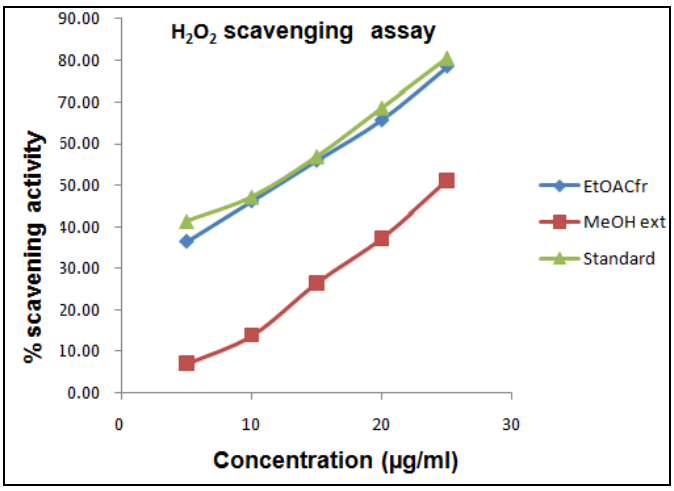

FIG.2: H_2O_2 SCAVENGING ACTIVITY OF METHANOL EXTRACT AND ESME

In-vitro assay for cytotoxic activity (MTT assay):

The cytotoxic effect of methanol extract and ethyl acetate soluble fraction of methanol extract of *Symplocos racemosa* bark against human hepatoma cell (Hep3B) and normal liver cell (BRL-3A) have been evaluated as compared to standard drug doxorubicin. The results revealed that methanol extract and ethyl acetate soluble fraction of methanol extract of *Symplocos racemosa* bark showed the effectiveness against Hep3B cell line withIC_{50} value (µg/ml) of 73.91 and 32.55 respectively, and not affected the normal cells BRL-3A (>1000 µg/ml). **Fig. 3, 4** showed the dose response curve of standard doxorubicin, methanol extract and ESME of *S. racemosa* on Hep3B cell line and BRL 3A using MTT assay. **Table 1** showed the IC_{50} value of doxorubicin, methanol extract and ESME of *S. racemosa* against Hep3B cell line.

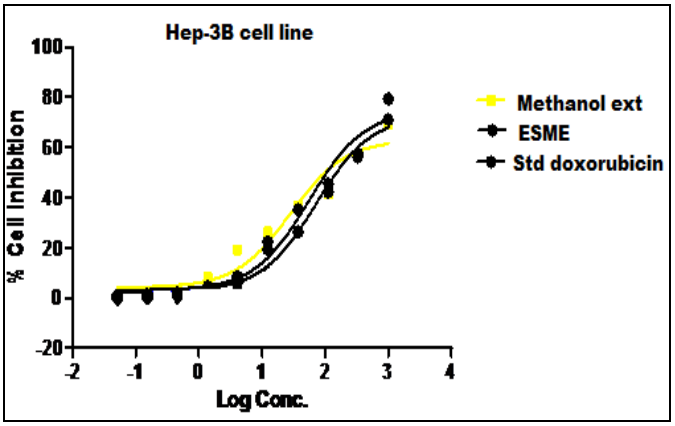

FIG.3: DOSE RESPONSE CURVE OF DOXORUBICIN, METHANOL EXTRACT AND ESME ON HEP3B CELL LINE USING MTT ASSAY

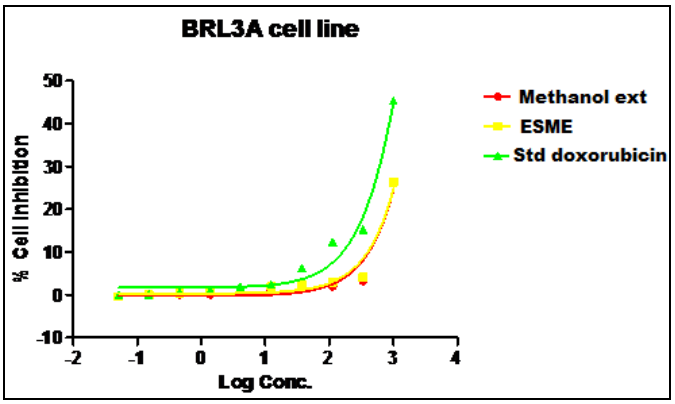

FIG. 4: DOSE RESPONSE CURVE OF DOXORUBICIN, METHANOL EXTRACT AND ESME ON BRL-3A CELL LINE USING MTT ASSAY

TABLE 1: IC_{50} VALUE OF STANDARD, METHANOL EXTRACT AND ESMEON HEP3B CELL LINE USING MTT ASSAY

Name of extract	IC_{50} value (µg/ml)
Methanol extract	73.91
ESME	32.55
Doxorubicin	55.43

Role of anti oxidant in chemoprevention:

Free radicals are highly reactive species produced in the body during normal metabolic function or introduced from the environment. These are the atoms or groups of atoms that have at least one unpaired electron, which makes them highly reactive. Reactive oxygen species (ROS) react with free radicals to become radical themselves. Anti oxidants counteract these cellular by products called as free radicals, and bind them before they cause the damage. Free radicals believed to play role in different health conditions such as cancer, ageing process and atherosclerosis [18]. Exogenous source of free radicals include smoking, ionizing radiation, certain pollutants, organic solvents and pesticides. As far as, ROS initiate the peroxidation of membrane lipids leading to accumulation of lipid peroxidation. Therefore, much attention has been focused on natural anti oxidants which inhibit the lipid peroxidation and protect the tissue from damage due to free radicals [19]. Ethyl acetate soluble fraction showed the potent anti oxidant activity by DPPH free radicals and hydrogen peroxide free radical scavenging assay method. Due to this evident, ethyl acetate soluble fraction showed the prominent *in vitro* anti cancer activity against hepatocellular carcinoma (Hep3B cell) indicating the chemoprevention.

CONCLUSION: Ethyl acetate soluble fraction of methanol extract showed the potent anti-oxidant activity and cytotoxic potential against the human hepatocellular carcinoma cells, whereas not affected the normal cells. It may be due to the presence of phytochemicals such as phenolic glycosides, steroids and triterpenoids. The further research work has been going on the phytoconstituents which have been responsible for the anti-cancer activity. The phytochemicals may serve as a novel therapeutic agent in the treatment or prevention of hepatocellular carcinoma, and deserve to be investigated further.

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