Anti-Cancer potentials of the plant *Aquilaria malaccensis* leaves

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Abstract
In the present study the ethanolic extract of *Aquilaria malaccensis* (Agar wood) leaves before inoculation (BF) and after inoculation (AF)) was evaluated for its anticancer potentials. The leaf extracts (BF and AF) was subjected to in-vitro cytotoxicity studies on EAC cells, normal spleen cells and DLA cells. The leaf extracts showed dose dependent cytotoxicity with increase in dose. The IC50 value for BF and the AF leaf extract was 79.09µg/ml and 79.20µg/ml respectively. The effect on primary spleen cells was observed to be more than 100µg/ml. The study concluded that the extract of the said plant leaves has significant potential anti-cancer activity. All these results clearly indicate that the crude extract has a remarkable capacity to inhibit the growth of Ascites tumor induced by EAC cell line in animals.

Keywords: Anti-cancer, *Aquilaria malaccensis*, EAC cell line.

Introduction
Cancer is a group of disease characterized by uncontrolled growth and spread of atypical cells causing aggregation and producing tumors. Presently, cancer remains a major health problem, in spite of all advances in medical science. Recent reports from WHO indicates that approximately 15 million new cases and around 9 million cancer related deaths every year.[1] The number of new cases is expected to rise by about 70% over the next 2 decades. By 2030, it is estimated that there will be 26 million new cases diagnosed annually. This increase in the global cancer burden will be mainly due to a disproportionate rise of newly diagnosed cancer cases in the developing countries such as India. India, China and Russia are predicted to account for more than half (53%) of the cancer cases and 60% of the cancer related deaths.[2]

The development of new therapeutic approach remains as a challenge in cancer research. Therefore, identifying new anti-cancer drug with fewer side effects on the immune system has become a trend in most of the cancer research. Many synthetic chemotherapeutic agents have been developed; hitherto they exhibited various side effects. Hence, to overcome these untoward effects and to treat patient more conveniently and efficiently, herbal drugs have been developed. Many plants and natural products are used by the ancient healers for the cancer ailment. *Aquilaria malaccensis* (Agar wood) belonging to the family Thymelaeaceae, locally known as Eagle wood is distributed in south Asian countries. The fragrant oleoresin that permeates the heartwood of some trees is produced as a response to wounding and/or fungal infection.[3] The leaves are known to possess antidiabetic, antioxidant, antibacterial, antiviral and antidepressant activities.[4-6] Further the stem bark of agar wood reported to possess anticancer activity.[7] Moreover, the saponins, anthraquinones, steroidal glycosides and flavonoids can suppress cancer growth and spread.[8]

Hence, the ethanolic extract of the leaves of *Aquilaria malaccensis* was studied for its anti-cancer activities.

Materials and Methods
Plant Material and Extraction
The authenticated fresh leaves were collected and shade dried. The dried plant materials were coarsely powdered, weighed and stored in an air tight container till use. The fungal treated and non-treated leaf powder was exhaustively extracted in ethanol solvent for 2 days at room temperature (28±2ºC). The extraction of grounded leaves was further repeated with ethanol (twice). The filtrate was subjected to evaporate under reduced pressure to give concentrated crude extracts. The dried extract was kept in a glass container until further use.

Reagents and Chemicals
DPPH and NBT (Nitro blue tetrazolium) were purchased from SIGMA chemical company Inc., st. Louis, MO, USA. Ethylene di-amino tetra acetic acid (EDTA) was purchased from Sisco Research Laboratories Pvt. Ltd., Mumbai, India. Ascorbic acid and Vitamin E from MERCK Chemical Company Enzyme kits from Agappe Laboratories Ltd All other chemicals and reagents used in this study were of analytical grade and procured from reputed Indian manufacturers.

Experimental Animals
Wistar rats and mice of either sex maintained under standard room conditions with free access to food and water were used. The protocol was approved by the IAEC with reference number SCP/IAEC/F150/P20/ 2015.

Preliminary Phytochemical Analysis
Extract was subjected to phytochemical screening for the presence of active principles.

In-vitro Antioxidant Activities DPPH (2, 2-diphenyl-1-picrylhydrazyl) Radical Scavenging Assay

Freshly prepared DPPH (187µl) was taken in different test tubes protected from sunlight. To this solution different concentrations (0, 0.25, 0.5, 1, 2, 4, 6, 8, 10µg/ml) of crude leaf extract was added. The reaction mixture absorbance was measured at 515nm. The concentration of test materials to scavenge 50% DPPH radical (IC_{50} value) was calculated from the graph plotted with % inhibition against concentration.[9]

Hydroxyl Radical Scavenging Assay

About 100µg each of deoxyribose, Ferric chloride, EDTA, Ascorbic acid and Hydrogen peroxide were added to test tubes. Then the different concentrations (0, 0.25, 0.5, 1, 2, 4, 6, 8, 10µg/ml) of crude leaf extract was added. The OD of the reaction mixture was read at 532nm. The IC_{50} value of hydroxyl radical was calculated.[9]

Superoxide Radical Scavenging Assay

The topical density of before and after illuminated reaction mixture of EDTA (0.1M), 0.3mM NaCN, Riboflavin (0.12mM), NBT (1.5 n moles), phosphate buffer (67Mm, pH 7.8) and 0, 0.25, 0.5, 1, 2, 4, 6, 8, 10 µg/ml of the leaf extract was measured at 560nm. The inhibition of SOD radical generation was determined.[10]

In-vitro Anticancer Activity

Cytotoxicity Analysis of Leaf Extract Trypan Blue Dye Exclusion Method

Cytotoxicity of the *A. malaccensis* leaves extract towards cancer cells were assessed using Trypan blue exclusion method.[11] Approximately 100 µl cell suspension from a stock of $1x10^7$ EAC cells, normal spleen cells and DLA cells were diluted to 0.9 ml (by adding 0.8ml PBS, 0.2M, pH 7.4) containing various concentrations of extracts were incubated at 37ºC for 3 hours. The trypan blue treated cell suspension (10µl) were applied on to a haemocytometer and observed under microscope. Live cells (non-stained cells) and dead cells (blue stained cells) were separately counted and percentage cell death was determined. The percent cytotoxicity was calculated after comparing with the untreated control. The percentage values were plotted on a graph against concentrations and concentration needed to induce 50% cell death (IC_{50}) was determined using following formula,

$$\% \text{ Cell death} = \frac{\text{Number of cells}}{\text{Total no of cells}} \times 100$$

Acute Oral Toxicity Study

The extract was subjected to toxicity studies using Wistar rats as per OECD guidelines No. 425.[12] Animals were observed for first 3h and monitored for 14 days for any toxicity related manifestations.

In vivo anticancer activity Ehrlich Ascites Carcinoma (EAC) Induced Tumor Model

The EAC cells were implanted into the peritoneal cavity of each recipient mouse.[13] The ascitic fluid with viable cells was injected to each mouse by i.p. route to obtain ascitic tumor. The drug was administered 24 h after the tumor inoculation for 7 days daily and Cisplatin (1.25-15 µg/ml, i.p.) on only 1st day. The mean survival time (MST) and % increase in life span (% ILS) was assessed.

Statistical Analysis

Values are mean ± SEM of at least three consecutive experiments. The significance of data was analyzed by the one-way ANOVA.

Results

Preliminary Phytochemical Analysis

Phytochemical evaluation was performed with *A. malaccensis* leaves extracts. The extracts showed positive result for saponins, alkaloids, flavonoids, terpenoids, tannins, carbohydrate, glycosides, coumarin, emodins, anthraquinones, resins, phenols.

In-vitro Antioxidant Activities

The leaf extract showed concentration dependent free radical scavenging against DPPH, hydroxyl and superoxide radicals are depicted in (Table .1), (Fig 1-3).

Table 1: IC_{50} values for *A. malaccensis* leaf extract in various in vitro anti-oxidant assay

	IC_{50} values		
	DPPH radical scavenging assay	**Hydroxyl radical scavenging assay**	**Superoxide radical scavenging assay**
BF	4.21±1.16µg/ml	6.03±1.26 µg/ml	6.62±1.60 µg/ml
AF	4.33±0.89µg/ml	5.88±1.06 µg/ml	6.59±1.42 µg/ml
Standard	2.88±0.56µg/ml (Vit. C)	289.35±7.36ng/ml (Vit. E)	54.17±3.15µg/ml (Vit. C)

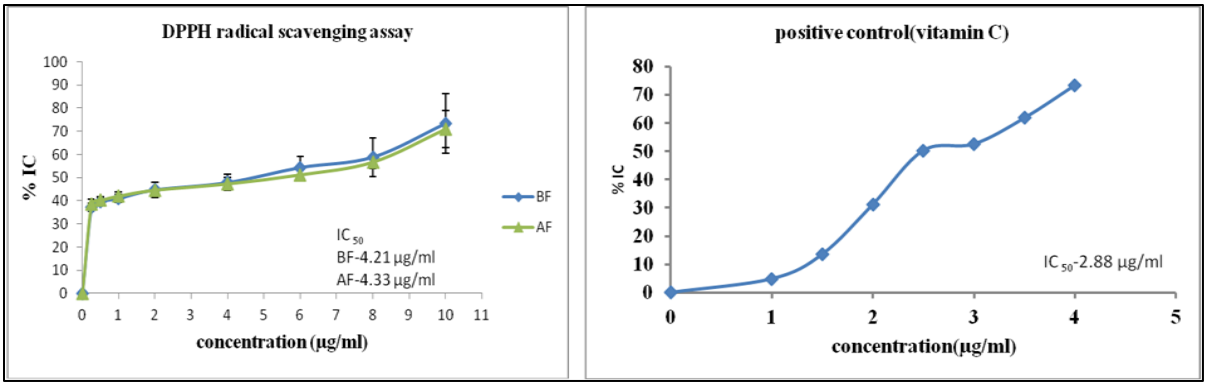

Fig. 1: Effect of leaf extracts on DPPH radical

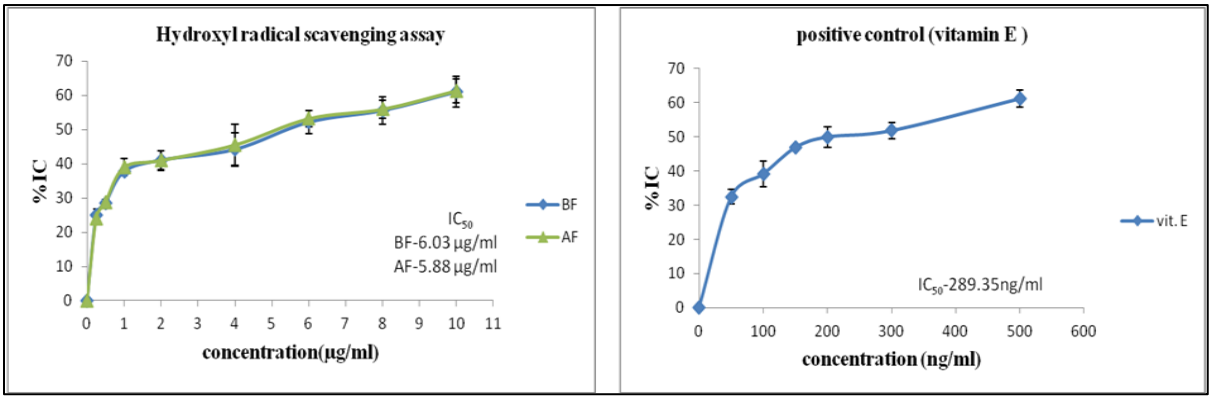

Fig. 2: Effect of leaf extracts on hydroxyl radical

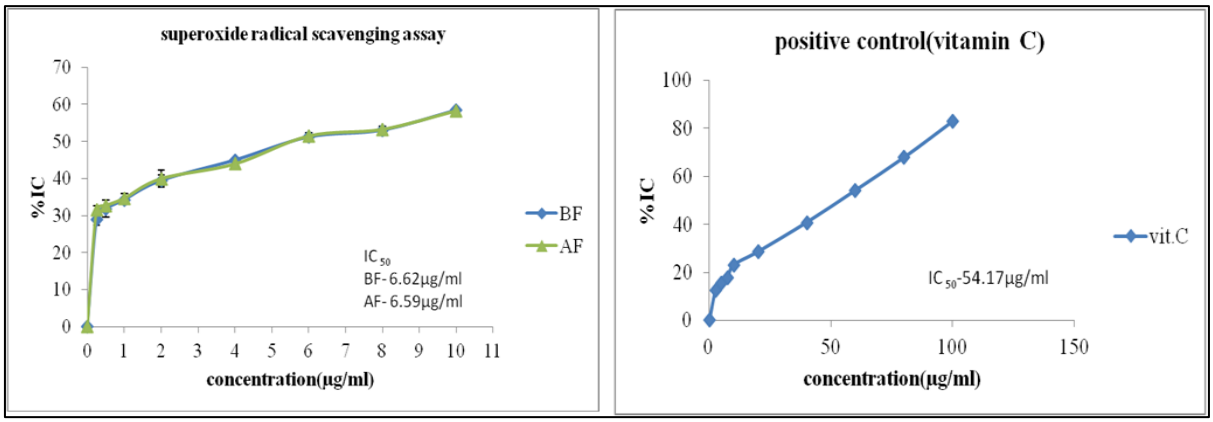

Fig. 3: Effect of leaf extracts on superoxide radical

In-vitro Cytotoxicity Analysis

Effect of Leaf Extracts on DLA cell Lines

The leaf extracts showed an increase in cytotoxicity with increase in dose. Maximum cytotoxicity wasobserved at 100 µg/ml. The BF and AF extract showed. 72.24 and 75.07 µg/ml respectively (Fig. 4, Table 2).

Effect of Leaf Extracts on EAC Cell Lines

In the EAC cell population, the leaf extracts showed significant cytotoxicity. The BF leaf extract had an IC50 value of 79.09 µg/ml. Similarly the IC50 value.for AF leaf extract were found to be 79.20 µg/ml (Fig. 5, Table 2).

Table 2: IC_{50} values of *A. Malaccensis* leaf extracts

S. No.	Test compound	IC_{50}values (µg/ml)		
		DLA	EAC	Spleen
1	BF	72.24±1.59	79.09±2.16	Above 100
2	AF	75.07±0.48	79.20±0.51	

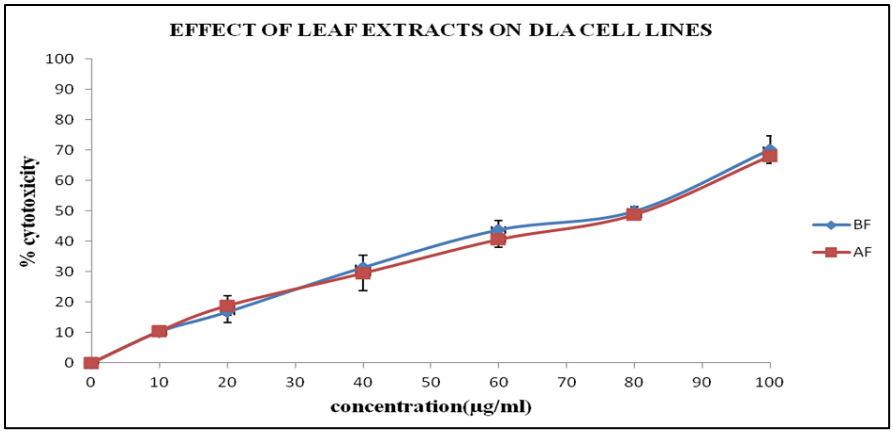

Fig. 4: Effect of extracts on DLA

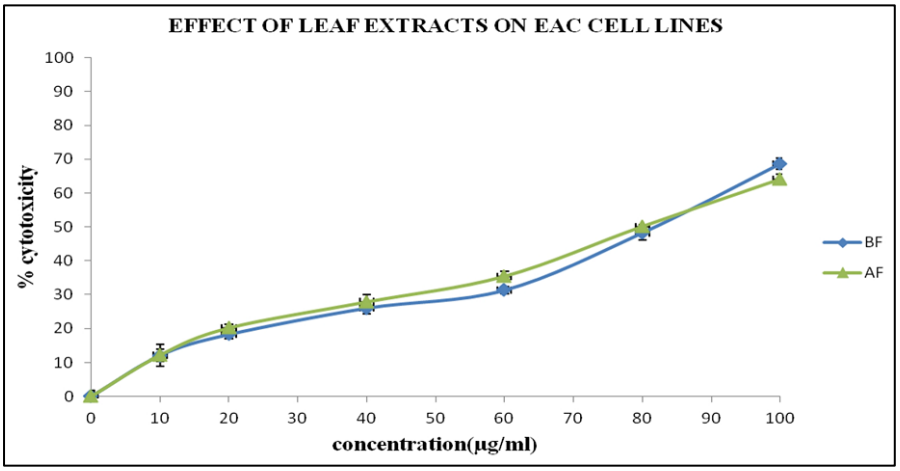

Fig. 5: Effect of extract on EAC

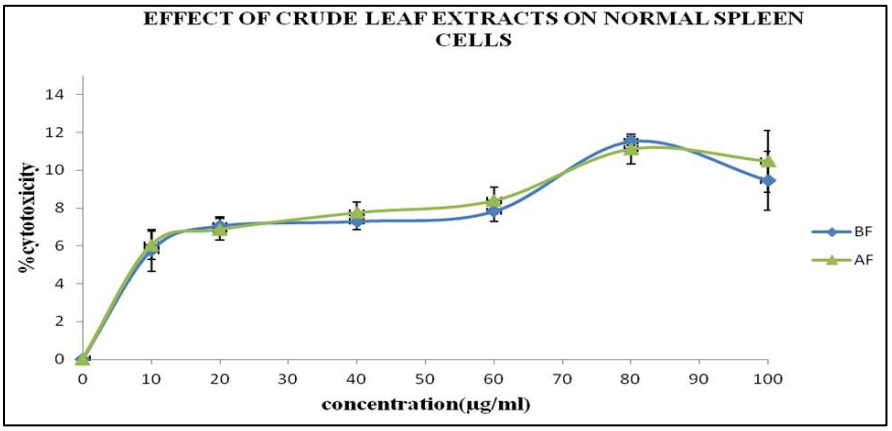

Fig. 6: Effect of extract on normal spleen cells

Acute Toxicity Study (LD_{50})

The oral acute toxicity was found to be safe up to 2000 mg/kg body wt. In vivo antitumor studies by EAC. The animals treated with 200 mg/kg, 400 mg/kg and Cisplatin, shown a significant increase in survival period.

The average life span of animals was 27.50 and 25.83 days respectively with BF and AF extracts. The % increase in life span of BF and AF treated animals was 30.33 % and 22.43 % respectively compared to untreated control (Fig .7, Table .3).

Table 3: Effect of leaf extracts on EAC mice

Treatment	MST (days)	%Increase in Life span
Tumor control (EAC cells)	21.10±1.26	-------
Standard (Cisplatin)	31.17±1.98	47.71±2.92
High dose (BF)	27.50±1.56	30.33±2.23
Lower dose (BF)	25.83±2.13	22.43±1.84
High dose (AF)	25.83±1.53	22.43±1.62
Lower dose (AF)	31.00±2.36	46.92±2.56

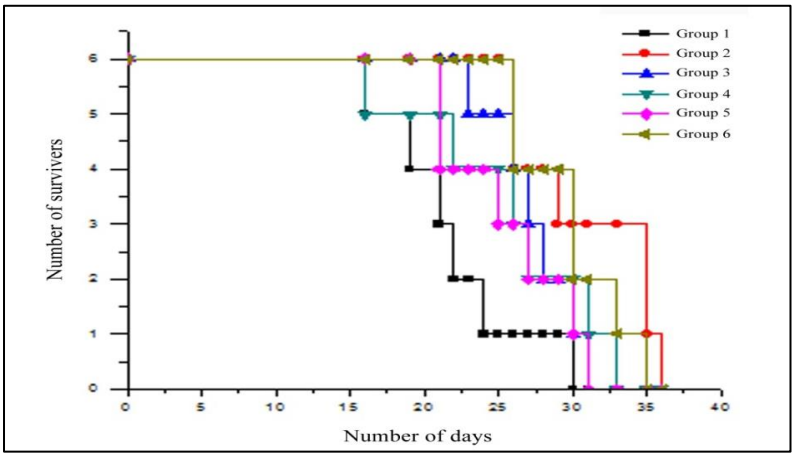

Fig. 7 Effect on MST and % increase in life span

Discussion

Cancer is one of the most life threatening disease and serious health problem in both developing and developed countries. Many synthetic and chemotherapeutic agents used in cancer therapy involve the risk of life threatening host toxicity. Plants are important source of biologically active secondary metabolites. For instance triterpenes found to have hypotensive and cardio depressant properties.[14] Anthraquinones possess astringent, purgative, anti-inflammatory, moderate antitumor and bactericidal effects,[15] whereas alkaloids having antimicrobial activity.[16] Tannins reduce the risk of coronary heart diseases and phenolic compounds are potential antioxidants and free radical scavengers.[17] Alkaloids and terpenoids are the class of compounds, predominant in the currently using plant derived chemotherapeutic compounds. In oral drug delivery system, the co-administration of therapeutic agents with natural compounds is reported to improve absorption activities.[18] Based on this, the present work was aimed to study the leaves of the plant *A. malaccensis* for its potential anticancer properties.

The effect of crude leaf extract on the mean survival period was monitored. The EAC cells induced and treated animals showed increased survival time compared to untreated animals. Tumor bearing animals treated with lower dose of 200 mg/kg and higher dose of 400 mg/kg of BF showed a significant increase in survival period of 25.83 and 27.50 days when compared to control animals without any treatment having a survival period of nearly 21.10 days. Animal treated with AF low dose and high dose have an increase in survival days as 31.00 and 25.83 days. Cisplatin single dose (1.25µg/ml) used as standard at its therapeutic dose and the survival dose observed in this group is 31.17 days. In animals treated with AF (lower dose), have more average life span than that in BF and also the % increase in life span also more for AF extract.

In the present study, the extracts of *A. malaccensis* leaves showed considerable cytotoxicity towards neoplastic cells (DLA and EAC). However towards the primary spleen cells, cytotoxicity was minimal. This suggests the specificity of the leaf extracts towards cancerous cells without affecting the normal cells. As it does not affect the normal spleen cells, there is strong indication that the leaves extract may not have immunosuppressive effect *in vivo.*

In addition the extracts revealed good radical scavenging efficacy. In the present study the crude extracts showed almost same radical scavenging effect which is more or less similar to vitamin C. In the hydroxyl radical generation assay crude leaf extracts and vitamin C have been found to possess similar efficacy which is highly significant. The highly reactive Hydroxyl radicals can cause membrane damage.[19] By inhibiting hydroxyl radical mediated damage, the *A. malaccensis* leaf extract find the promising role in various degenerative conditions where lipid peroxides and carbonyl are involved. A number of antioxidants have been isolated from the plant sources. However they require in higher amounts in many of the in vitro assays mentioned above. Exceptions are curcumin, ellagic acid,

ferulic acid, etc. The crude extracts in the present study is less moderate cytotoxic but possesses significant radical reducing and scavenging activities requiring less than 10 µg/ml in all the in vitro assays. Since it is a crude extract, further purification may yield better nontoxic antioxidants.

The result showed the presence of various bioactive secondary metabolites such as alkaloids, anthraquinones, terpenoids, tannins and phenolics. Terpenoids and alkaloid class of compounds are good anti-cancer agents.[20] It is likely that these compounds might be responsible for its cytotoxic property. However, the exact mechanism for the anticancer activity *Aquilaria malaccensis* leaves is still unclear, which needs further studies.

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