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Bioactivity-Guided Fractionation for Anti-Cancer Property of Leaves *of Justicia beddomei*

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Abstract
Aim: *The primary objective of the study was to evaluate the possible effects of ethanolic, chloroform extracts and isolated constituents of Justicia beddomei leaves (JB) on against Ehrlich's ascites carcinoma (EAC) induced cancer on male Swiss albino mice. Tumors were induced by Ehrlich's Aascites Carcinoma (EAC) at dose of 200 mg/kg (p.o).*
Materials and Methods: *The EAC was maintained in the ascetic form by the sequential passages in Swiss mice, by means of every 10 days i.p transplantation of $2x10^6$ tumour cells. All the groups except the control were inoculated with $2x10^6$ tumor cells. For tumour inoculation on day 0, the 0.5ml of a 1:10 Ehrlich Ascites Tumour crushed in Hank's balanced salt solution (HBBS) was inoculated by the i.p. route in albino mice. Test compounds were first dissolved in DMSO (10 mM) and then diluted in 0.9% NaCl. Treatment efficiency was assessed in terms of different extracts, and isolated fractions of effects on the tumor volumes of the tumor beard mice relative to the control group.*
Results: *The two parameters of Specific tumor growth delay (SGD) and percentage of life span (ILS %) was calculated. SGD of ethanolic extract, chloroform extract and isolated fractions (JBF2) of Justicia beddomei leaves was -0.83, -0.64 and -0.80 respectively. Similarly, the percentage of life span (ILS %) was 33.33%, 85.71% and 71.42% for ethanolic, chloroform and JBF2 fractions.*
Conclusion: *These finding indicates that the extracts and isolated fractions has significant anti proliferative action against Ehrlich Ascites Tumour- induced carcinoma in animal models. Thus, Justicia beddomei leaves and related products may provide a novel approach to the chemoprevention and treatment of cancer.*
Keywords: *Justicia beddomei, EAC, SGD, ILS,Bioactivity-guided*

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I. Introduction

Cancer is one of the leading causes of human death which is estimated at 8.2 million and will likely rise to 13 million worldwide per year till 2030[1].Cancer is a major public health burden in both developed and developing countries[2]. After cardiovascular disease, cancer is the second leading cause of death[3-4].Oncology has become the largest therapeutic area in the pharmaceutical industry in terms of the number of project, clinical trials and research and development spending[5]. As declining of expenditure on research and development by pharmaceutical companies while rising of the cost of bringing a new molecular entity to market, the so-called "Valley of Death" in anticancer drug development becomes a highly complex problem[5].By keeping this in the point of view of cancer protection, it has been estimated that diets rich in phytochemicals can reduce cancer. The old saying "Prevention is always better than cure" is particularly true in the case of cancer where a cure, if possible, is associated with high cytotoxic loads and/or invasive procedures. With our growing understanding of the molecular etiology of cancer, it has become apparent that strategies which limit DNA damage and/or increase the probability of DNA repair by inhibiting aberrant proliferation will decrease cancer incidence[6].

India so called 'Botanical garden of the World' for its rich natural resources has 15000-18000 species of flowering plants, 2500 Algae, 23000 Fungi, 1600 Types of Linches, 1800 Various types of Bryophytes and 30 Million of microbes of these about 15000-20000 plants have good medicinal value used traditional, folklore and herbal medicines. TheSiddha system of medicines uses around 600, Ayurveda; Unani-700 and modern medicines about 300 plant species[7].Scientists all over the world are concentrating on the herbal medicines to boost immune cells of the body against cancer. By understanding the complex synergistic interaction of various constituents of anticancer herbs, the herbal formulations can be designed to attack the cancerous cells without harming normal cells of the body. The plant, *Justicia beddomei* belongs to family-Acanthaceae is a large glabrous shrub or small tree grows up to 3-4 meters in height. It is native of India, Pakistan and Africa. In India,

it is widely distributed in the hill regions of Kerala, and in dried parts of Katapadi village of Karnataka. The leaves of *Justicia beddomei* are simple, opposite, ovate -lanceolate, acute or acuminate, shiny, short-petioled up to 15 cms long, 3.75 cms broad, about 8 pairs of main nerves and are bitter in taste. A flower arises from axillary condensed spikes, flower heads short and white, and fruits capsules with a long solid base. Many Ayurvedic and Unani practioners have been using these medicinal plants for the treatment of various ailments. Leaves as an astringent, refrigerant, expectorant, diuretic, antispasmodic, febrifuge, styptic and tonic. Traditionally, the leaves are used for irritable cough and different bleeding conditions, flowers for ophthalmic, the roots along with leaves for phthisis, cough, haemoptysis and asthma[8-12].The objective of the present study is to investigate the anticancer property of the *Justicia beddomei* leave extracts. Here, both the ethanolic and chloroform extracts were used to evaluate *in vivo* anticancer activity.

II. Materials and Methods

1.*Collection and Identification*

Aerial part of *Justicia beddomei* was collected in the month of August 2009 from different parts Katapadi village, Udupi district, Karnataka. The plant material was authenticated by an eminent taxonomist Dr. P.M. Shivkumar, Department of Botany, DRM Science College, Kuvempu University, Karnataka, India. A voucher specimen (No: USJBL: 01/09/2011) was preserved in our research laboratory for future reference.

2.*Extraction of the plant material*

The powdered leaves of *Justicia beddomei* was taken separately into three 5000 ml round bottom flask, and extracted with solvents: petroleum ether (60-80^{o}C), chloroform and ethanol respectively in a ratio of 1:6 by successive solvent extraction process. Each time before extracting with next solvent the powdered material is dried, filtered, refluxed with all the organic solvents. The marc, finally obtained was macerated with chloroform water for seven days to obtain the aqueous extract. Each extract was evaporated to dryness in flash evaporator under reduced pressure and controlled temperature. The dried extracts were stored in airtight container in refrigerator below10^{o}C. The suspensions of petroleum ether, chloroform, ethanol (95%) and water extracts prepared using 1% Tween 80, which were used for the experiments[13-20].The percentage extractions of the extract are tabulated in the Table 1.

Table-1. Summary of yield, Colour and consistency of different extracts

S.No	Extract	Yield (in g) (for 800 g)	Colour	Consistency
1	Petroleum ether (60-80^{o}C)	50.00	Yellow	Oily
2	Chloroform	40.00	Dark Brown	Shining
3	Alcohol (95%)	35.00	Dark Green	Dry
4	Chloroform	25.00	Reddish brown	Dry

2.1*Phytochemical Studies*

All the extracts were then subjected to preliminary qualitative test by adapting standard methods to screen for presence of various phytoconstituents. The test and the report are summarized in the Table 2.Thevarious extracts of *Justicia beddomei* leaves show the presence of steroids, triterpenoids, alkaloids, carbohydrates, tannins and flavonoids.

Table-2. Preliminary qualitative investigation of extract

Phytoconstituents	Petroleum Extract	Chloroform Extract	Alcoholic Extract	Aqueous Extract
Alkaloids		+	+	+
Amino acids	-	-	-	-
Carbohydrates	-	-	+	+
Fats & Oils	-	-	-	-
Flavonoids	-	-	+	-
Glycosides	-	-	-	-
Saponins	-	-	-	-
Tannins	-	-	-	-
Triterpenoids	-	+	-	-
Coumarines	-	-	+	-

2.2 ***Isolation of alkaloids from column chromatography***

The ethanolic and chloroform extract was found to be biologically active, probably due to the presence of alkaloids, triterpenoids etc. This observation prompted us to take up the isolation of bioactive molecules by column chromatography methods. Hence, the powder from fresh leaves of this plant was prepared. The air dried coarse powdered material was subjected to Soxhlet extraction successively by using solvents of increasing polarity namely petroleum ether (60-80^{0}C), chloroform, ethanol and distilled water. All the extracts were evaporated to dryness by using rotary evaporator under reduced pressure and controlled temperature. The obtained chloroform extract material was subjected for further purification and isolation. The isolation of pure compounds from the plant extracts was done by column chromatographic separation using silica gel 100-200 mesh and 60-120 Mesh.

2.3 ***Chromatographic separation using silica gel (100-200 mesh)***

The chloroform extract (20g) was chromatograph over silica gel (100-200 mesh) on column 55 cm length and 6 cm diameter. Elution was carried out with solvent mixtures of increasing polarities. Fractions were collected in 100ml portions, and monitored by TLC [silicagel Gas adsorbent, solvent system dioxone: ammonia (9:1)] and the fractions showing similar spots are pooled together. The chromatography details are given in Table 3.Elution with ethyl acetate: chloroform (50:50) gave fluorescent crystalline solid (100 mg) and named as *Justicia beddomei*(JBI). Similarly, elution with ethyl acetate: water (80:11:11:47) yielded fluorescent colored crystalline solid (160 mg) and, was named as *Justicia beddomei* (JBII).

2.4 ***Isolation of pure components***

Since chloroform fraction of *Justicia beddomei* leaves showed better activity than the other fractions, we checked for the type of chemical constituents present therein from column chromatography. In the chloroform fraction, it was found that vasicine and vasicinone were the major compounds found in the chloroform: methanolic extract of *Justicia beddomei* leaves. After repeated re-crystallization of alkaloid fraction with ethanol, 150mg (0.26 % w/w) white solid needles (vasicine) were obtained. Remaining fraction was subjected to column chromatography using silicagel, and eluted with chloroform with increasing proportion of methanol. A white solid (vasicinone) was eluted from the column with chloroform: methanol (49:1) extract and was crystallized from chloroform: ethyl acetate (3:1)[21-22].

3. Chemicals & Reagents used in animal experiment

Hank's Balanced Salt Solution (HBBS), Dimethyl Sulphoxide (DMSO), 0.9% Sodium Chloride were from Hi-Media Laboratories Pvt. Ltd., Mumbai, India. Methanol, Chloroform, Ethyl acetate, Petroleum Ether, Dioxane and Ammonia, All other chemicals was of analytical grade.

4.Animal studies

Male Swiss albino mice (20-25g) were grouped and housed in polypropylene shoebox type cages with stainless steel grill top, bedded with rice husk under standard laboratory condition (temperature 22-25°C, humidity 50 ± 5% and dark/light cycle of 12 hours). The mice were allowed to acclimatize to laboratory condition for 7 days before commencement of the experiment as per the CPCSEA guidelines {Vide Reg. No. (0367/01/C/) India}.

4.1Acute toxicity test for animals

Acute toxicity study was performed using albino mice and doses were fixed as per OECD guideline No.423 and CPCSEA protocol. The albino mice male and female weighing 25-30 g were used for the experiment. The extract was administered orally to the different groups (n= 6) of overnight fasted mice at the dose of 50, 100, 250, 500, 1000, and 2000mg/kg body weight. After administration of drug extract, all the animals were observed continuously for signs of toxicity and mortality during 24 h, 48 h, and 72 h. Minimum lethal dose was observed as 1000mg[23]. Mortality was also noted every day, during 30 days of experiment and the MTD (Maximum tolerance dose) was determined.

4.2 Anticancer activity

Development and Treatment protocol in animals

The EAC (Ehrlich Ascites cell line) was maintained in the ascetic form by the sequential passages in Swiss mice, by means of every 10 days i.p transplantation of $2x10^6$ tumour cells. The animals were divided into ten groups and labeled accordingly. All the groups except the group-I (normal saline control) were inoculated with $2x10^6$ tumor cells. For tumour inoculation on day 0 (DO), the 0.5 ml of a 1:10 Ehrlich Ascites Tumour crushed in Hank's balanced salt solution (HBBS) was inoculated by the i.p. route in albino mice. Test compounds (JB extract) were first dissolved in DMSO (10 mM) and then diluted in 0.9% NaCl. Dosage given to animals in different groups has been shown in **Table 3**.

Table 3: Dosing of Different Groups

Groups	Dose	Extract	Route of Administration
Group I	Control	Solvent	Oral
Group II	Study group	EAC Tumor	Intraperitoneal
Group III	Low dose 50mg/kg	Ethanolic	Oral
Group IV	Moderate dose 100mg/kg	Ethanolic	Oral
Group V	Low dose 50mg/kg	Chloroform	Oral
Group VI	Moderate dose 100mg/kg	Chloroform	Oral
Group VII	Single dose 50mg/kg	Chloroform	Oral
Group VIII	Single dose 100mg/kg	Chloroform	Oral
Group IX	Low dose of 50mg/kg	Fraction-1	Oral
Group X	Low dose of 100mg/kg	Fraction-1	Oral

The treatment began the day after tumour inoculation, and test compounds along vehicle were administered i.p. using six mice per test group. Routinely, the test compound was administered six times every alternate day after tumor inoculation on day D3, D5, D7, D9 and D11, except on day one (D1) single dose was administered. In each chemotherapy trail, mice were checked with any adverse clinical reactions noted and deaths recorded. Mice are weighed 2-4 times weekly during treatment and once weekly thereafter. Tumours were measured by Vernier calipers twice weekly and tumour volume were estimated at $0.5(mm^3)$ (Length x $Width^2$).

4.3 Calculation and Analysis

Evaluation of antitumor activity: Median Life Span[24]

Mortality was noted every day and the median life span (MLS) was calculated as

$$MLS = \frac{Dm + (Mm\text{-number of mice dead before Dm})}{\text{Number of mice dead on (Dm)}}$$

The median mouse (Mm) separates into two identical groups (one group, including the MLS =Mice that died before Dm, the other group including those who died after the median day (Dm). The Median day (Dm) is the day Mm died. Mice surviving for at least 45 days were considered as cured and were included in the calculation of the median life span.

4.4 Drug efficiency

Drug efficiency was expressed by T/C as follows:

$$T/C\% = (MLS \text{ of treated animals}/ MLS \text{ of animals})$$

$$\text{Increase in life span } ILS\% = 100 \times (T\text{-}C) / C$$

The therapeutic index (defined as the ratio of the dose that kills 10% of tumor-free mice to the dose that gives a 50% increase in the life span in tumor-bearing mice) will be determined for each experiment.

4.5 Tumor growth

Treatment efficiency was assessed in terms of the compound effects on the tumor volumes of tumor bearing mice relative to the control vehicle-treated mice.

For evaluation criteria following SGD were used.

4.6 *Specific tumor growth delay (SGD)*,

Ehrlich Ascites Tumor model was calculated as follows:

$$SGD = Td \text{ (drug-treated group)}/ Td \text{ (vehicle-treated)}$$

Where Td, is the tumor doubling time of drug or placebo, defined as the time in days required for the tumors volume to double.

III. Results

Treatment efficacy was assessed with two parameters: SGD (Specific Tumor growth delay) and ILS% (Increase in Life Span). Drug was administered (p.o.) in different schedules and SGD and ILS was then determined. All the extracts and fractions were evaluated for anticancer activity at the dose of 50 mg/kg and 100 mg/kg of body weight. No toxic effects were observed in treated groups either by gross visual examinations or in the weight of the mice. After the discontinuation of the treatment, all the treated groups were observed for one month and measured the tumour volume in all the groups twice in a week. SGD of ethanolic extract, chloroform extract and isolated fractions (JBF2) of *Justicia beddomei* leaves was -0.83, - 0.64 and -0.80 respectively which is summarized in **Table 4a-4c.** Similarly, the percentage of life span (ILS %) was 33.33%, 85.71% and 71.42% of ethanolic, chloroform and JBF2 fractions.

Table-4a.Effect of ethanolic extract of JB on EAC

Treatment Schedule (day)	MTD[a] (mg/kg^{-1})	TI[b]	Dose (mg/kg^{-1})	SGD[c]	ILS (%)	Wt.(gm)changes in treatment Groups		Survival
						0-Day	After 21 Days	
D1	>2000	16.66	50	4.00	31.83	31.83	30.83	3/6
			100	0.16	31.50	31.50		6/6
D 1, 3, 5, 7, 9,11	>2000	16.66	50	1.0	30.33	30.33	31.00	5/6
			100	-0.50	31.33	31.33	32.16	6/6

Table-4b. Effect of chloroform extract of JB on EAC

Treatment Schedule	MTD[a] (mg/kg^{-1})	TI[b]	Dose (mg/kg^{-1})	SGD[c]	ILS (%)	Wt.(gm)changes in treatment Groups		Survival
						0-Day	After 21Days	
D1	>2000	10	50 100	0.66 0.08	14.281 09.04	32.00 30.83	29.16 28.33	3/6 4/6
D 1, 3, 5, 7, 9,11	>2000	10	50 100	-0.12 -0.58	47.61 57.14	30.33 31.16	31.00 31.66	5/6 6/6

Table-4c. Effect of isolated Fractions of JB on EAC

Treatment Schedule	MTD[a] (mg/kg^{-1})	TI[b]	Dose (mg/kg^{-1})	SGD[c]	ILS (%)	Wt.(gm)changes in treatment Groups		Survival
						0-Day	After 21Days	
D1	>2000	10	50 100	0.50 0.33	14.20 19.04	30.00 31.16	31.00 31.50	3/6 3/6
D 1, 3, 5, 7, 9,11	>2000	10	50 100	-0.20 -0.41	52.38 57.14	31.16 31.33	32.00 32.66	6/6 6/6

a) MTD: Maximum tolerance dose. b) TI: Therapeutic index, SGD: Specific tumor growth delay [SGD >1 corresponds to minimal level of activity.The tabulated results shown significant decrease in tumour volume and increase in ILS% at the dose of 100 mg/kg body weight of chloroform extract and isolated fraction.

IV. Discussion

Cancer prevention was generally associated with inhibition, reversion or retardation of cellular hyperproliferation. The presence of flavonoids/terpenoids of JB also exhibited inhibition of proliferation. The previous studies have shown that *Justicia beddomei* leaves contains the alkaloids like vasicine, which possess antifertility activity[14] andany bioactive compounds having antifertility property, can also possess good antiproliferation activity. In the present study, the ethanolic and chloroform extracts has also shown to contain quinozoline type of alkaloids. The new quinozoline alkaloid rutacecarpine (1-methoxy-7, 8-dehydrorutaecarpine) proved to reasonably cytotoxic towards murine leukemia p-388 cell line and HT-29 cells[15]. The Rf ~0.8 values of our isolated alkaloid from *Justicia beddomei* leaves was near to that of quinozoline alkaloid, hence based on the research reference we attempted to investigation of anticancer activity of *Justicia beddomei* leaves extracts and fractions against Ehrlich Ascites Tumour- induced mice. The secondary metabolites of leaves of *Justicia beddomei* among them a number of quinozoline type of alkaloids possessing diversified pharmacological properties like bronchodilatory, respiratory stimulant, uterine stimulant and moderate hypotensive activity[16-18].

V. Conclusion

The present study confirms that ethanolic extract of Justicia beddomei leaves shown dose dependent anticancer activity. The present study demonstrated the potent anticancer properties of Justicia beddomei leaves. Leaves of *Justicia beddomei* extracts and isolated fractions showed a significant clinical efficacy related to anti proliferative action against Ehrlich Ascites Tumour- induced carcinoma in animal models. Data reported here provide a rational basis for the use of Justicia beddomei leaves in the treatment of tumors in traditional medicine. The observed effect of this plant extracts may be due to the presence of alkaloids, tannins, triterpenoids and carbohydrates as evidenced by our in vivo model. Constituents of this plant may represent a new line of anti-tumor agents and can serve as a tool to fight against cancer. These findings may give an optimistic result and needs further investigation for the isolation of its active constituents to reveal the molecular level of mechanism of action of pure isolated compounds from leaves of *Justicia beddomei.*

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Conflict of interest

The author declares there is no conflict of interest.

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