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CYTOTOXIC EFFECTS OF METHANOL EXTRACT OF RAW, COOKED AND FERMENTED SPLIT BEANS OF *CANAVALIA* ON CANCER CELL LINES MCF-7 AND HT-29

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ABSTRACT

In vitro cytotoxicity evaluation of methanol extract of raw, cooked and solid-substrate fermented (Rhizopus oligosporus) split beans of wild legumes (Canavalia cathartica and C. maritima) of coastal sand dunes of the Southwest India was carried out. Cytotoxic activity (ED_{50} and cytotoxicity) of methanol extracts was tested by (3-(4, 5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Differential impacts on the cancer cell lines MCF–7 and HT–29 were seen even though both plant species grow in the same habitat. Methanol extract of cooked (C. maritima) and fermented (C. cathartica) split beans showed better in vitro anticancer activities compared to the raw beans. It is concluded that active principles of methanol extract of cooked and fermented Canavalia beans have potential to inhibit cancer cell lines MCF-7 and HT-29. Besides, it is possible to use extracts of cooked/fermented beans to control colon cancer by diet management.

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KEY WORDS

Cytotoxicity; Canavalia; Wild legumes; Solid-substrate fermentation; Rhizopus oligosporus; Cancer cell lines

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[I] INTRODUCTION

Out of 7.6 million deaths worldwide, cancer is one of the leading causes for human mortality in developed countries attained second place in developing countries after cardiovascular diseases [1,2]. Lung, stomach, liver, colon and breast cancers are the ailments for death every year. Most of the cancer deaths are caused largely because of aging and increasing adoption of cancer-causing behavioral and dietary risks including smoking, high body mass index (BMI), lack of physical activity and low fruit-vegetable intake [3]. The global burden of cancer is predicted to increase with an estimation of 13.1 million deaths during 2030 [1]. Present cancer treatments by radiation and chemotherapy pose serious side effects like fatigue, diarrhoea, nausea, hair loss, skin problems, malfunction of urinary bladder and decrease in RBCs due to cytotoxicity and genotoxicity of radiation and chemotherapeutic agents on the non-tumor cells [4].

Over the past few years, there has been growing interest in developing plant-based anticancer drugs due to their diverse pharmacological properties and benefits [5]. The use of natural products in anticancer therapy has a long history in folk medicine, which is part and parcel of traditional and allopathic medicines. Many drugs currently used in chemotherapy originated from different plant species or derivatives of a natural prototype. According to Cragg and Newman [6], more than 50% of drugs that undergo clinical trials for anticancer activity are derived from natural sources. Numerous epidemiological studies especially colorectal cancer have clearly showed an inverse relationship between the diet rich in vegetables/legumes and incidence of cancers [7]. The plant-derived anticancer drugs act via different pathways, which ultimately result in activation of apoptosis of cancer cells leading to cytotoxicity [8]. Legume grains play a major role in the fulfillment of diets of human beings throughout the world. Supplementation of legumes in the diets is reported to be one of the promising approaches (diet-management) to combat various free radical-mediated chronic diseases [9, 10].

The nutritional potency of underutilized legumes, *Canavalia cathartica* and *Canavalia maritima* of the coastal sand dunes (CSD) of Southwest coast of India has been reported by many researchers [11-14]. Xu et al. [15] have reported that pterocarpin derivative [(−)-mediacarpin] extracted from *C. maritima* inhibits the growth of HeLa cells *in vitro* by inducing apoptosis. Lectins derived from *Canavalia ensiformis* (ConA) and *Canavalia brasiliensis* (ConBr) showed anti-proliferative effects in human leukaemia cell lines (MOLT-4 and HL-60) [16]. Animal studies have suggested that L-canavanine present in *Canavalia* spp.

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possess potent antineoplastic activity, which can be used in treating pancreatic cancer [17, 18]. To consider *Canavalia* beans as potential nutraceutical agent, its effect on cancer lines are essential. Therefore, the current study reports differential cytotoxic effects of raw, cooked and solid-substrate fermented (SSF) (*Rhizopus oligosporus*) split beans of CSD *Canavalia* on cancer cell lines. As fermented beans showed high bioactive and antioxidant potential [19], it is hypothesised that the fermented beans also exhibit high cytotoxic activity on cancer cell lines.

[II] MATERIALS AND METHODS

2.1. Seed samples and fermentation

Seed samples of *Canavalia cathartica* Thouars and *Canavalia maritima* Thouars were collected from three locations of the coastal sand dunes of Someshwara, Southwest India (12º47′N, 74º52′E) during summer (February–March, 2012). Undamaged seeds were separated from dry pods, sun-dried for two days and dehulled. First set of split beans (25 g) were transferred to conical flask (250 mL), soaked in distilled water (1:3 w/v) followed by pressure cooking (6.5 L, Deluxe stainless steel; *TTK* Prestige™, Prestige Ltd., India). The cooked split beans were spread on aluminium foil, dried in an incubator (45±2°C), milled (Wiley Mill, mesh # 30) and stored in air-tight glass containers. Another set of split beans were pressure-cooked, inoculated with two 5 mm plugs of 3-day-old cultures of *Rhizopus microsporus* var. *oligosporus* (Saito) Schipper and Stalpers (MTCC # 556; strain designation # 22959; Institute of Microbial Type Culture Collection, Chandigarh, India) and allowed for solid-substrate fermentation for 7 days at 37°C. Fermented split beans were spread on aluminium foil, oven dried at 45±2°C, powdered and preserved in air-tight glass containers for analysis.

2.2. Extraction

Samples were extracted in methanol using Soxhlet extractor [20]. Flours of raw, cooked and SSF beans were packed in thimbles, covered with glass wool and extracted with methanol (200 mL) (50-65ºC) in a Soxhlet extractor. The rate of condensation was fixed to 150 drops/min and the extraction was carried up to 7 hr. After recovering the solvent, the extract was concentrated by evaporating the solvent using flash evaporator and stored at –20ºC.

2.3. Cell lines

The cell lines, MCF–7 and HT–29 were procured from National Centre for Cell Sciences (NCCS), Pune, India. The MCF–7 is a human breast adenocarcinoma cell line [21]. It retains the characteristics of differentiated mammary epithelium including estradiol synthesis. This makes the MCF–7 cell line an estrogen receptor positive control cell line [22]. The HT–29 human colon carcinoma cells in culture show similar characteristics of enterocytes and these cells have been used to study intestinal drug, nutrient transport and metabolism [23,24].

2.4. Cytotoxicity assay

The *in vitro* cytotoxic activity of the extracts was tested by (3-(4, 5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay [25] with a slight modification. The MCF–7 and HT-29 (5×10^3 cells) in Dulbecco's Minimal Essential Medium (DMEM) (100 µL) with Foetal Bovine Serum (10%) were incubated overnight (37°C, 5% CO2) in a 96 well plate. Methanol extracts of test samples (*Rhizopus oligosporus*, raw, cooked and fermented flours of *C. cathartica* and *C. maritima*) at different concentrations (50, 100, 250 and 500 µg/mL; 1, 2.5, 5 and 10 mg/mL) were added to microtitre plate. Doxorubicin (5 µg/mL), an anticancer drug was used as internal positive control, DMEM served as negative control and the wells without any cells served as blank. It was incubated for 48 hr (37°C, 5% CO2, humidity 80-90%). After initial incubation, MTT (20 µL of 5 mg/mL) in phosphate buffered saline was added to each well and incubated further for 4 hr (37°C, 5% CO_2, humidity 80-90%). The medium together with MTT was aspirated and dimethylsulfoxide (DMSO) (200 µL) was added. The absorbance in each well was measured at 570 nm using micro-titre plate reader. Inhibition of cells (%) was calculated:

Inhibition (%) = 100 – [(Mean OD for test sample / mean OD for the control) × 100]

The concentration at which the sample exhibits 50% of its maximum activity (i.e., effective dosage: ED_{50}) was calculated using the ED_{50} plus v1.0 software.

2.5. Data analysis

The difference between the cytotoxic activity of methanol extracts of raw and processed (cooked and cooked + fermented) beans was assessed by One-way analysis of variance (ANOVA) (SigmaPlot 11; Systat Software Inc., USA).

[III] RESULT AND DISCUSSION

Cytotoxic effects on human cancer cell lines was performed using MTT assay, which was based on the principle of conversion of purple tetrazolium salt into formazan by the methanol extracts of the test samples such as raw, cooked and solid-substrate fermented (*Rhizopus oligosporus*) beans of *C. cathartica* and *C. maritima* [25]. The results demonstrated that methanol extracts of *Canavalia* bean exhibited selective *in vitro* cytotoxic activity towards MCF–7 and HT–29 cell lines. Among the two cancer cell lines tested, viability of HT–29 cells was most affected especially by methanol extracts of cooked *C. maritima* beans followed by fermented *C. cathartica* beans **[Table- 1]**. Similarly, in bean extracts the ED_{50} value was lowest in cooked beans of *C. maritima* and fermented beans of *C. cathartica*. However, the ED_{50} values were the lowest and the cytotoxicity was highest in *R. oligosporus*. The quantity of bioactive compounds (total phenolics, tannin and vitamin C) and antioxidant potential of *C. maritima* fermented with *R. oligosporus* differed from that of *C. cathartica* [19]. Likewise, the *in vitro* cytotoxicity potential was also differed and thus did not correspond to the *in vitro* anti-cancer activity of two *Canavalia* spp. in the present study. On the contrary, the black soybean fermented with *R. oligosporus* exhibited higher phenolics, flavonoids and antioxidant activity, which corresponds to the effective cytotoxic activity against HeLa-S3 and Raji cell lines [26]. Similarly, fermented *R. oligosporus* soymilk showed selective cytotoxic effect on Hep 3B with ED_{50} value of 150.2 µg/mL [27]. It is interesting to note that certain strains of *Rhizopus microsporus* are known to produce rhizoxins, which shows anti-tumor activity (Jennessen et al. [28]. The ED_{50} of methanol extracts of cooked *C. maritima* was lower compared to methanol extracts of raw and fermented beans as well as *C. cathartica* (850-893 vs.1357-4063 µg/mL)

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on both cell lines **[Table- 1]**. Gazzani et al. [29] predicted that the environmental factors (climatic, growth conditions, ripening stage, temperature and duration of storage) and thermal treatment influence the antioxidant activity. Similarly, the cytotoxic potential might also varied exist in the same coastal sand dunes.

The initial hypothesis proposed (fermented beans show higher cytotoxic activity than the raw and cooked beans) was true only for *C. cathartica*. Future studies should focus on purification of anti-cancer compounds from *Canavalia* beans and their *in vivo* evaluation for therapeutic applications.

Table: 1. Effective dosage (ED_{50}) and cytotoxicity (% inhibition) of methanol extracts of raw, cooked, fermented beans of *C. cathartica* and *C. maritima* on cancer cell lines MCF–7 and HT–29 in culture in comparison with *Rhizopus oligosporus* (n=3; mean±SD) (low ED_{50} and high cytotoxicity are in bold-face)

	ED_{50} value (µg/mL)		Cytotoxicity at 1 mg/mL (% inhibition)	
	MCF–7	HT–29	MCF–7	HT–29
Doxorubicin	–	–	68.59	48.42
Rhizopus oligosporus	390.05±0.97	461.19±1.01	94.21±0.21	89.85±0.61
Canavalia cathartica				
Raw beans	3350.67±0.49^{a}	2844.41±0.03^{a}	NI	25.14±0.06^{a}
Cooked beans	3572.65±0.20^{ac}	4063.85±0.07^{ac}	NI	21.57±0.45^{ac}
Fermented beans	**2049.20±0.24^{ad}***	**2127.80±0.29^{ad}***	**14.04±0.16**	**34.85±0.13^{ad}***
Canavalia maritima				
Raw beans	1408.61±0.74^{a}	1747.71±0.27^{a}	31.90±0.92^{a}	27.14±0.04
Cooked beans	**892.89±0.06^{ac}**	**849.83±0.87^{b}*^{c}**	**63.63±0.69^{ac}**	**60.42±0.43**
Fermented beans	1505.35±0.86^{ad}*	1357.00±2.00^{bc}	26.11±0.03^{ad}*	NI

Different letters across the rows are significantly different (*, P < 0.05); –, Not determined; NI, No inhibition.

[IV] CONCLUSION

The active principles of methanol extract of cooked and fermented *Canavalia cathartica* and *C. maritima* beans of the coastal sand dunes of Southwest coast of India have potential to inhibit cancer cell lines MCF-7 and HT-29. Besides, it is possible to use extracts of cooked and fermented beans to control colon cancer by diet management. Further studies are necessary to assess purified bioactive compounds of *Canavalia* beans to improve the efficacy.

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CONFLICT OF INTERESTS

Authors declare that there are no conflicts of interest

FINANCIAL DISCLOSURE

Nil

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