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ORIGINAL ARTICLE

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Rasagenthi lehyam (RL) a novel complementary and alternative medicine for prostate cancer

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Abstract *Purpose*: The use of complementary and alternative medicine (CAM) in cancer has been increasing. The therapeutic modalities which originated from India, viz., Ayurveda and Siddha, have phytotherapy as their fundamental basis and, therefore, produce few side effects. They are among the most ancient medicinal systems and are still being practiced in India and elsewhere, to cure cancer and other diseases. Many Siddha practitioners in the southern parts of India prescribe rasagenthi lehyam (RL) as a drug for cancer. RL contains 38 different botanicals, many of which have been shown to possess therapeutic efficacy, and 8 inorganic compounds, all prepared into a paste in a palm sugar and hen's egg base. The efficacy of RL in killing prostate cancer cells in vitro was investigated in this study to determine whether RL could be recommended as a CAM for prostate cancer. *Methods*: In order to scientifically validate the anticancer activity of RL on prostate cancer, a methanolic extract of RL was serially extracted with four organic solvents, and the extracts were tested for clonogenic inhibition and induction of apoptosis in PC-3 prostate cancer cells, with and without irradiation. *n*-Hexane, ethyl acetate and chloroform extracts of RL effectively killed PC-3 cells. *Results*: The IC_{50} values of *n*-hexane, ethyl acetate and chloroform extracts of RL were 3.84 μg/ml, 3.68 μg/ml and 75 ng/ml, respectively. All three extracts induced apoptosis in PC-3 cells. Further, all the three extracts when combined with radiation, caused enhanced effect on killing of PC-3 cells. Among the three extracts, the chloroform extract showed the most significant radiation-sensitizing effect. *Conclusion*: RL, either in its original formulation prepared under strict quality control or its chloroform extract, could potentially be an alternative medicine for prostate cancer, and also a sensitizing agent in the context of radiation therapy for prostate cancer, as a complementary medicine. A more directed study could lead to the identification of the active principle(s) in the chloroform extract of RL for use in prostate cancer therapy.

Keywords CAM for prostate cancer · Siddha medicine · Rasagenthi lehyam · Clonogenic inhibition · Apoptosis induction · Radiosensitization

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Introduction

Prostate cancer is among the cancers with the highest incidence among males in the US, and the incidence of prostate cancer has been increasing rapidly in recent years [16]. According to a WHO report, 36% of prostate cancer patients worldwide in the year 2000 belonged to the US population [55]. The precise causes of cancers in general and prostate cancer in particular are not yet known. Diagnosis of prostate cancer has been made easier in the recent years by the development of the prostate-specific antigen (PSA) test and awareness among the general population about the disease. Despite

an extensive effort, the underlying mechanisms involved in the onset of prostate cancer and its progression are not well established.

Current treatment options for early prostate cancer include radical prostatectomy, chemotherapy, radiotherapy, hormone therapy and watchful waiting. Potent curative procedures are normally offered to men with a life expectancy of at least 10 years [21]. In some patients, adjuvant therapy such as androgen deprivation has been shown to improve survival when given during, and for 3 years after, radiotherapy [3, 4], and there is increasing evidence that hormone therapy for prostate cancer patients with an intermediate or poor prognosis delays disease progression [11, 42]. Yet the fact remains that as of now there is no completely effective therapy for prostate cancer.

Alternative medicines have gained importance over the past decade, fueled in part by the public's desire to participate in their own health-care and the perception that the allopathic system of medicine has failed to find a reliable and definitive cure for cancer, despite almost three decades of virtual war against cancer. Thus, the patients, in their desire to keep themselves alive, go for ''unconventional medical therapies'' (UMTs). The use of unconventional/non-traditional therapies in the general population has increased dramatically in the past decade [22].

As medical care becomes more complex, technical and expensive, more cancer patients turn to complementary and alternative medicine (CAM). CAM is a group of medical and health systems, practices, and products that are not presently considered to be part of conventional medicine. While some scientific evidence exists regarding some CAM therapies, for most there are key questions that are yet to be answered through well-designed scientific studies—questions such as whether they are safe and whether they work for the disease or medical conditions for which they are used. Complementary medicine is used together with conventional medicine. Alternative medicine is used in place of conventional medicine. The two are together known as CAM. Interestingly, higher levels of education and income are associated with greater use of CAM. Among the patients who seek CAM treatment, 90% believe that it will help them live longer and improve their quality of life, 60% believe that it will relieve symptoms and 47% expect it to cure cancer [51]. About 80% of patients suffering from prostate cancer receive some form of CAM [47]. Some patients with advanced disease, after conventional treatment has failed, turn to CAM with the hope of keeping the disease under control, thereby extending their survival and improving their quality of life. Patients of high socioeconomic level and those who are clinically disease-free after radical treatment are most likely to turn to CAM, among which herbal medicine is the most commonly adopted by prostate cancer patients [23].

Rasagenthi lehyam (RL), a Siddha medicine, is a formulation containing 38 different botanicals and 8 inorganic compounds. Siddha practitioners prescribe RL as a therapeutic modality for cancer. RL is claimed to cure prostate cancer, and many patients have complete remission after treatment. We interviewed 96 patients taking this therapy and were told that RL controlled the disease and comforted the patients without any side effects. In the context of the search for CAM modalities for cancers in general, and prostate cancer in particular, we carried out a few preliminary in vitro experiments using RL and found significant inhibition of growth of cells of the prostate cancer cell line, PC-3. This led us to hypothesize that one or more of the phytochemicals present in the herbal ingredients of RL may target prostate cancer cells and kill them through induction of the apoptotic pathway. The antitumor effect is most likely caused by a synergistic effect among some of the phytochemicals present in RL. Therefore, through a process of scientific validation either the entire lehyam or the cancer therapeutic compound(s) present in it could be brought into the mainstream of cancer therapy.

Material and methods

Preparation of RL

RL was prepared by one of the authors (S.T.) as prescribed (no. 168; IMPCOPS, 1988; Chennai, India). The composition is given in Table 1, and RL is the subject of an IPR from India.

Extraction of RL

RL was quantitatively extracted first with methanol. The methanol phase was evaporated under reduced pressure to obtain a dark brown residue (336 g of lehyam yielded 99.13 g of residue). This residue was suspended in water and extracted with four organic solvents of increasing polarity, viz., *n*-hexane, chloroform, ethyl acetate and *n*-butanol. In the next extraction step, a more polar solvent was always used on the aqueous water phase remaining from the previous step (Fig. 1). Each extract was concentrated in vacuo to dryness to yield 16.7 g of powder from the *n*-hexane extract, 0.34 g of powder from the chloroform extract, 1.15 g of powder from the ethyl acetate extract, 7.17 g of powder from the *n*-butanol extract and 73.77 g of powder from the aqueous extract remaining. All these extracts were tested for anticancer activity. Only the more lipophilic extracts (*n*-hexane, chloroform and ethyl acetate) were active. The HPLC chromatograms (RP-18, acetonitrile-water gradient, $UV_{254\ nm}$) of these active extracts (Fig. 2) showed that the ingredients of the paste could indeed be separated by such a series of extractions, although the most lipophilic extract (*n*-hexane) contained the bulk of the lipophilic ingredients of RL. While the *n*-hexane and chloroform extracts contained a plethora of diverse compounds, the ethyl acetate extract contained either only a single major compound or a mixture of compounds with similar characteristics.

HPLC analysis

All the fractions were subjected to HPLC on RP-18 silica gel at a flow rate 1 ml/min using acetonitrile and water as solvents. The following gradients were used: water/acetonitrile 7.3:3.7 over 30 min, then to 100% acetonitrile for 10 min for the *n*-hexane and chloroform fractions, and 100% water to 100% acetonitrile over 40 min for the ethyl acetate fraction. The HPLC chromatograms of the *n*-hexane and chloroform extracts showed at least 35–40

Table 1 Composition of RL

No.	Chemical/botanical name	Common name	Family	Part	Quantity (g)
1	Pure mercury				10
2	Pure sulfur				10
3	Pure mercuric chloride				10
4	Pure arsenic triphosphate				10
5	Pure iron ore (magnesite)				10
6	Pure copper sulfate				10
7	Pure zinc sulfate				10
8	Pure lead oxide				10
9	*Zingiber officinale*	Ginger	Zingiberaceae	Dry rhizome	10
10	*Trachyspermum roxburghianum* and *T. ammi*	Indian celery tree	Apiaceae	Rhizome	10
11	*Curcuma longa*	Turmeric	Zingiberaceae	Rhizome	10
12	*Embelia ribes*	False pepper	Myrsinaceae	Fruit berry	10
13	*Acorus calamus*	Sweet flag	Acoraceae	Rhizome	10
14	*Cinnamomum zeylanicum*	Cinnamon	Lauraceae	Bark	10
15	*Smilax chinensis*/*S. china*	Chopchini	Liliaceae	Rhizome/bark	10
16	*Semecarpus anacardium*	Marany nut	Anacardiaceae	Nut	10
17	*Terminalia chebula*	Hirola	Combretaceae	Fruit	10
18	*Nigella sativa*	Black cumin	Ranunculaceae	Seed	10
19	*Vernonia anthelmintica*	Ironweed	Asteraceae	Seed	10
20	*Clerodendrum serratam*	Barangi	Verbenaceae	Root and leaf	10
21	*Abies spectabilis*	Himalayan fir	Pinacea	Cone	10
22	*Vitis vinifera*	Grape	Vitaceae	Fruit	10
23	*Piper longum*	Long pepper	Piperaceae	Seed	10
24	*Alpina galanga*	Greater galangale	Zingiberaceae	Rhizome	10
25	*Saussurea lappa*	Costus	Asteraceae	Root	10
26	*Celastrus paniculatus*	Staff tree	Celastraceae	Seed	10
27	*Foeniculum vulgare*	Fennel	Apiaceae	Seed	10
28	*Elelttaria cardamomum*	Cardamom	Zingiberaceae	Seed	10
29	*Myristica fragrans*	Nutmeg	Myrsinaceae	Aryls of fruit	10
30	*Piper nigrum*	Pepper	Piperaceae	Seed	10
31	*Cuminum cyminum*	Cumin	Apiaceae	Seed	10
32	*Psoralea corylifolia*	Babchi seed	Fabaceae	Seed	10
33	*Quercus infectoria*	Oak galls	Fabaceae	Galls	10
34	*Piper longum*	Long pepper	Piperaceae	Bark	10
35	*Calamus verus*		Arecaceae	Tuber	10
36	*Strychnos nux-vomica*	Poison nut	Loganiaceae	Seed	10
37	*Strychnos potatorium*	Indian gum nut	Loganiaceae	Seed	10
38	*Hygrophila auriculata*	Long-leaved baruria	Acanthaceae	Seed	10
39	*Sesamum indicum*	Sesame seed	Pedaliaceae	Seed	10
40	*Dolichos biflorus*	Horse gram	Fabaceae	Seed	10
41	*Cocos nucifera*	Coconut	Palmae	Kernel	10
42	*Acalypha betulina*	Indian nettle	Euphorbiaceae	Root	10
43	*Azima tetracantha*	Azima	Salvadoraceae	Root	10
44	*Withania somnifera*	Winter cherry	Solanaceae	Tuber	10
45	*Bryonia epigoea*	Bryoms	Cucurbitaceae	Tuber	10
46	*Plumbago indica*	Fire plant	Plumbaginaceae	Root bark	10
47	Hen's egg				400
48	Palm sugar				400

compounds, while the HPLC chromatogram of the ethyl acetate extract showed only one large elution mixture as well as five or six other compounds.

Cell culture

PC-3 cells were obtained from the American Type Culture Collection, and maintained as adherent monolayer cultures in RPMI-1640 medium supplemented with 10% fetal bovine serum.

Clonogenic inhibition assay

All the RL extracts were dried and dissolved in dimethyl sulphoxide (DMSO; Sigma Aldrich, St. Louis, Mo.). In our experience, as also reported in the published literature, DMSO does not alter the growth of prostate cancer cells and, therefore, data on cells treated with DMSO alone are not included. Survival curves of PC-3 cells in the presence of the various RL extracts dissolved in DMSO were obtained from a colony-forming assay and analyzed by the single-hit multitarget (SHMT) method. The colony-forming assay was performed as described previously [6]. In brief, two different cell concentrations in quadruplicate sets were used for each concentration of RL extract. Cell lines were left untreated or exposed to different extracts of RL. For clonogenic cell survival studies, two different cell concentrations in quadruplicate sets were used for each concentration of RL extract. PC-3 cells were left untreated or treated with various RL extracts. After incubation for ten or more days, each flask was stained with crystal violet and colonies containing more than 50 cells were counted. The surviving fraction (SF) was calculated as the ratio of the number of colonies

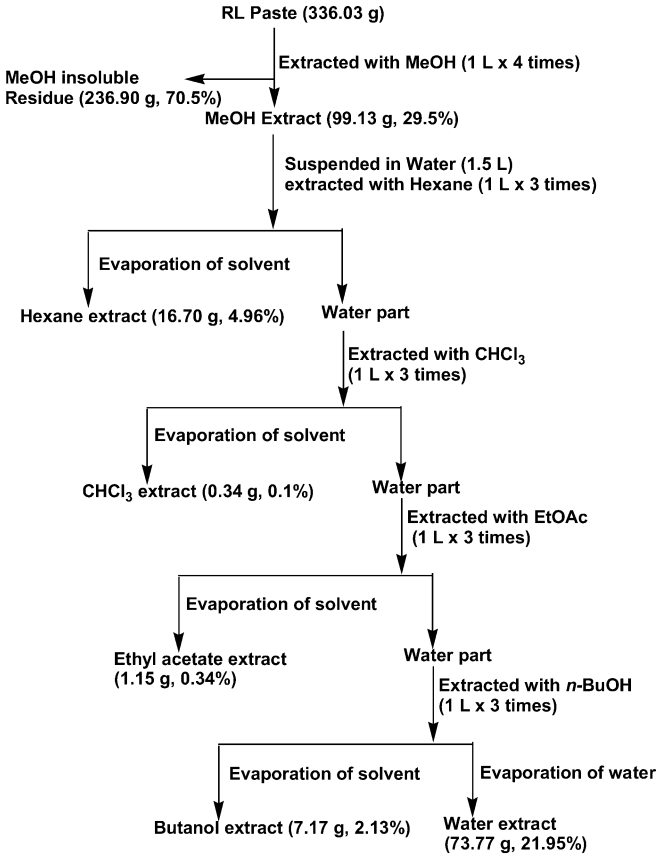

Fig. 1 Extraction and solvent partitioning of RL

formed to the product of the number of cells plated and the plating efficiency. The curves were plotted using *x–y*log scatter (Delta Graph 4.0) and D_0 (the dose required to reduce the fraction of cells to 37%, indicating single-event killing) was calculated adopting the formula of the single-hit multitarget (SHMT) model. Since the effects of a single treatment were being investigated, one log inhibition of cell survival was determined. Clinically, the drug is given to patients for 6 months or more, depending upon response.

RL extracts and radiation-induced clonogenic inhibition

To determine whether the various RL extracts would enhance the radiosensitivity of PC-3 cells, two different assays, each in quadruplicate, were carried out. In the first one, to determine whether the addition of radiation at the clinical dose of 2 Gy would enhance the killing by RL extract of prostate cancer cells, different concentrations of each extract of RL were combined with radiation at 2 Gy and the clonogenic inhibition was calculated. A 100 kV industrial X-ray machine (Philips, Eindhoven, Netherlands) was used to irradiate the cultures at room temperature. The dose rate with a 2-mm Al plus 1-mm Be filter was about 2.64 Gy/min at a focus–surface distance of 10 cm.

In the second assay, to determine whether RL extract would enhance radiation-induced clonogenic inhibition, each RL extract at its IC_{50} was combined with radiation at doses of 1–6 Gy and the clonogenic inhibition was calculated. SF_2 is the survival fraction of exponentially growing cells that were irradiated at the clinically relevant dose of 2 Gy.

Detection of apoptosis and its quantification

Apoptosis was detected by TUNEL staining. An ApopTag in situ apoptosis detection kit (Oncor, Gaithersburg, Md.), that detects DNA strand breaks by terminal transferase-mediated dUTP-digoxigenin nick end labeling (TUNEL), was used as described previously [7]. Briefly, the cells were seeded in chamber slides and the next day were left untreated or were treated with extract, radiation or extract and radiation in combination. After 24 and 48 h the DNA was tailed with digoxigenin-dUTP and conjugated with an anti-digoxigenin fluorescein. The specimens were counterstained with propidium iodide and then Antifade was applied. The stained cells were viewed under a Nikon-microphot epifluorescence microscope using a triple bandpass filter. To determine the percentage of cells showing apoptosis and for statistical analysis of the data, four experiments in total were performed and approximately 1000 cells were counted in each experiment.

Results

RL extract-induced clonogenic inhibition of PC-3 cells

Water and *n*-butanol extracts of RL (50 μg/ml) failed to inhibit the growth of PC-3 cells. However, the *n*-hexane extract (IC_{50} 3.84 μg/ml), ethyl acetate extract (IC_{50} 3.68 μg/ml) and chloroform extract (IC_{50} 75 ng/ml) significantly inhibited the growth of PC-3 cells (Table 2). Further, all three extracts (*n*-hexane, ethyl acetate and chloroform) produced significant clonogenic inhibition of PC-3 cells. The chloroform extract (D_0 105 ng/ml) produced a more significant inhibition than the *n*-hexane (D_0 5.76 μg/ml) and ethyl acetate (D_0 4.74 μg/ml) extracts (Fig. 3). Thus, the chloroform extract had a much more potent effect on PC-3 cells than the *n*-hexane and ethyl acetate extracts.

Enhancement of radiation-induced clonogenic inhibition of PC-3 cells by RL extracts

Combination of radiation at 2 Gy with all three RL extracts enhanced the killing of PC-3 cells (Table 3). Radiation at 2 Gy in combination with the chloroform extract enhanced the clonogenic inhibition of PC-3 cells (D_0 48 ng) more than in combination with ethyl acetate and *n*-hexane extracts (D_0 2.57 and 1.8 μg, respectively, Fig. 4). The D_0 enhancement ratio in PC-3 cells treated with the chloroform extract and radiation at 2 Gy was 2.18, and equivalent values for the *n*-hexane and ethyl acetate extracts plus 2 Gy radiation were 2.63 and 2.24, respectively. The three extracts showed similar radio-sensitization effects on PC-3 cells.

In the assay to determine whether RL extracts would enhance the radiation-induced clonogenic inhibition as compared to radiation alone (D_0 226 cGy, SF_2 0.63), the chloroform extract showed a greater radiosensitization effect (D_0 88 cGy, SF_2 0.026; Fig. 4) than ethyl acetate (D_0 139 cGy, SF_2 0.029) and *n*-hexane extracts (D_0 143 cGy, SF_2 0.030). The comparison of the SF_2 values showed that the chloroform extract of RL produced a significantly greater enhancement ratio (2.42) when compared to the *n*-hexane (2.1) and ethyl acetate (2.2) extracts (Table 3).

RL extract-induced apoptosis in PC-3 cells

After 24 h of treatment of PC-3 cells with the extracts of RL, the increase in apoptosis over the untreated population was 7.93% in *n*-hexane extract-treated cells, 11.47% in chloroform extract-treated cells and 13.18% in ethyl acetate extract-treated cells (Fig. 5). Thus, all three potent extracts of RL induced apoptosis in PC-3 cells.

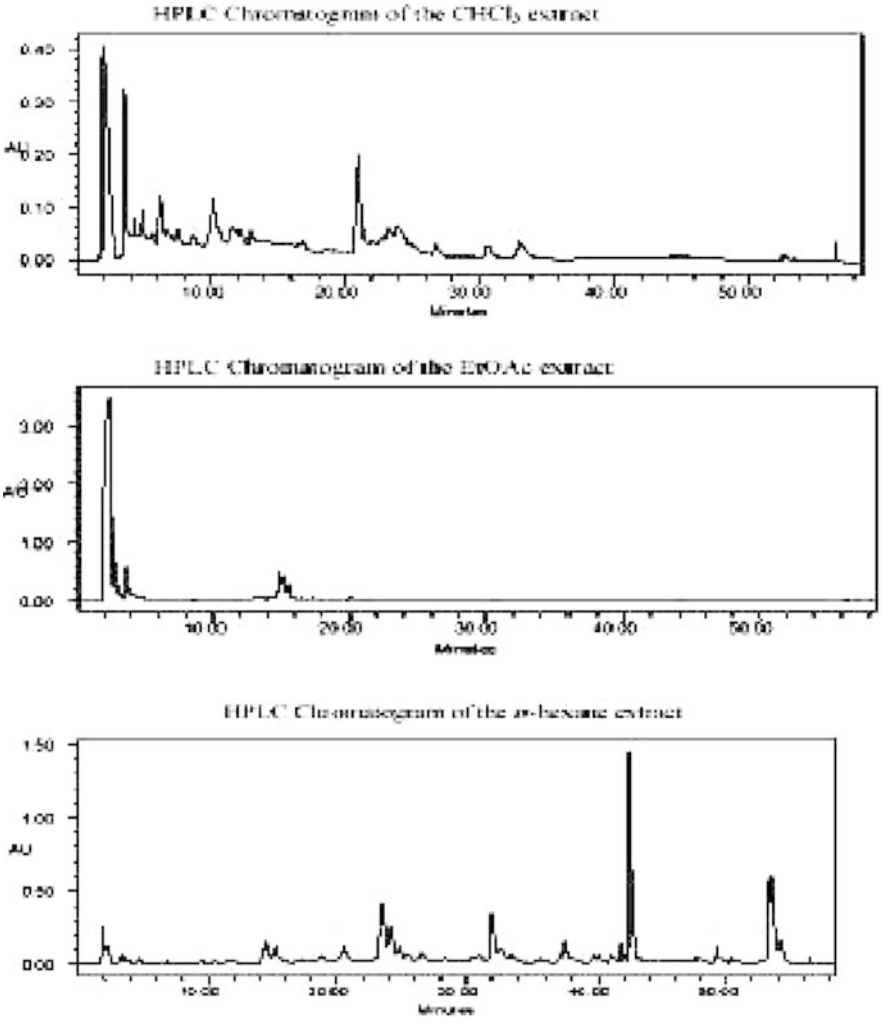

Fig. 2 HPLC separation of chloroform, ethyl acetate and *n*-hexane extracts of RL

Table 2 IC_{50} values of various extracts of RL in PC-3 cells

Extract	IC_{50}
n-Hexane	3.84 μg/ml
Ethyl acetate	3.68 μg/ml
Chloroform	75 ng/ml

Discussion

The use of CAMs in the management of cancers of different kinds is on the increase, and patients adopt CAM practices in the frantic attempt and hope to keep themselves alive. Even though the CAM medicines are claimed to cure the disease, and perhaps they do, many of them have not yet been subjected to scientific validation. Therefore, it is pertinent that a potential CAM formulation is subjected to scientific validation before it is recommended for therapy.

RL is a traditional Indian medicine comprising eight inorganic substances and 38 botanicals, all in a hen's egg and palm sugar base. There is evidence in the scientific literature to the effect that some of the herbs/herbals in RL possess anticancer properties and could be useful in cancer therapy. These include *Zingiber officinale*[14, 25, 33, 52], *Trachyspermum roxburghianum* and *T. ammi*[49], *Curcuma longa* [5, 13, 20, 48, 54], *Acorus calamus* [1, 45],*Smilax chinensis* or *S. china*[27],*Semecarpus anacardium* [38, 39, 50], *Terminalia chebula* [36, 40], *Nigella sativa* [41], *Vitis vinifera* [8, 20, 35, 54], *Saussurea lappa* [15, 32, 56], *Celastrus paniculatus* [26], *Myristica fragrans* [17, 34],*Cuminum cyminum* [2], *Psoralea corylifolia* [1], *Hygroglypha auriculata* [29] and *Withania somnifera* [12].

Since several of the herbals in RL have been shown to possess anticancer properties, in one way or another, the necessity to test the whole drug for its efficacy in CAM cancer therapy could be questioned. An appropriate extract of a polyherbal formulation may be more potent in therapy than an extract of a single plant or the active secondary chemical in it because the former would contain several compounds, each targeting a different molecular mechanism in the etiology and/or progression of the disease, which might be of benefit in cancer treatment. Further, the different compounds in an

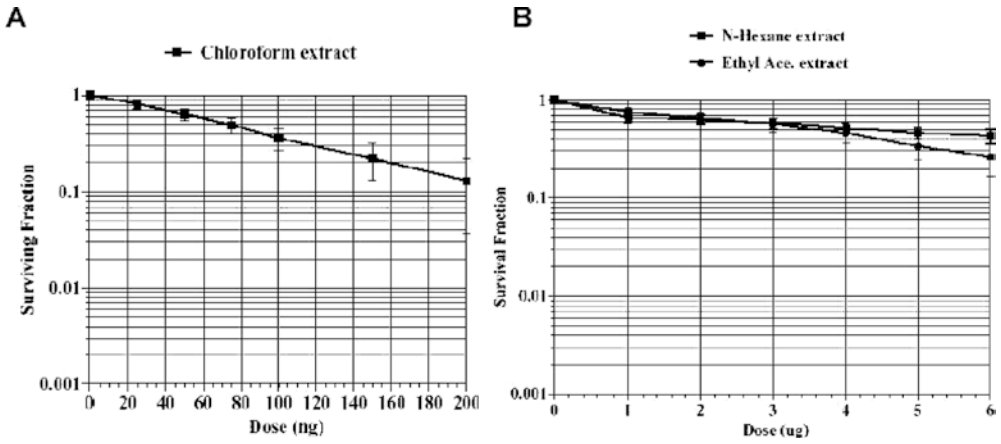

Fig. 3A, B RL-induced clonogenic inhibition in PC-3 cells. **A** Chloroform extract; doses ranged from 0 to 200 ng. **B***n*-Hexane and ethyl acetate extracts; doses ranged from1 to 6 μg. The data shown are means from two individual experiments

Table 3 SF_2 and D_0 values of RL extracts, radiation, the combination of RL extracts & radiation and enhancement ratios in PC-3 cells

Treatments	SF_2 value	D_0 value	Enhancement ratio	
			SF_2	D_0
Chloroform extract	–	105 ng/ml	–	–
n-Hexane extract	–	4.74 μg/ml	–	–
Ethyl Acetate extract	–	5.76 μg/ml	–	–
Radiation alone	0.63	226 cGy	–	–
Chloroform extract +IR (2 Gy)	–	48 ng/ml	–	2.18
n-Hexane extract + IR (2Gy)	–	1.8 μg/ml	–	2.63
Ethyl Acetate extract + IR (2Gy)	–	2.57 μg/ml	–	2.24
Chloroform extract (IC_{50}) + IR	0.26	88 cGy	2.42	2.56
n-Hexane extract (IC_{50}) + IR	0.30	143 cGy	2.1	1.58
Ethyl Acetate extract (IC_{50}) + IR	0.29	139 cGy	2.2	1.62

extract of an entire formulation may have additive or synergistic effects against the disease. For example, the herbal medicine of sho-saiko-to contains several herbal plants, and it is more effective against cancer cells [30] when compared to the individual components which are moderately cytotoxic [57]. It has been speculated that synergy in a herbal medicine results from the existence of ''redundancy and back-up mechanisms found in the key regulatory and metabolic pathways of the cell'' [10]. A combination of a number of different compounds may have synergistic activity by targeting both primary and back-up mechanisms simultaneously. Also, practitioners of herbal medicines have long claimed that the use of an entire formulation reduces toxicity because of ''buffering'' between different constituents [53].

The RL preparation contains eight inorganic compounds. The question may be raised as to whether these inorganic compounds could be toxic and cause side effects. There is information that among the inorganic compounds in RL, sulfur [31], arsenic [28, 44], iron [24] and copper [9] could be of use in the management of cancer. Toxicities observed in recent clinical trials of herbal medicines in cancer populations are far less severe than those reported in the early phase trials of conventional cancer agents [37, 46]. Nevertheless, several studies have implicated heavy metals such as mercury and lead in toxic side effects [18, 43] and, therefore, in the design of the experiments in the present study one of the purposes of serial extraction was to eliminate the possibility of heavy metal toxicity, if any.

In the present study,*n*-hexane, ethyl acetate and chloroform extracts of RL significantly inhibited the growth of PC-3 cells, whereas *n*-butanol and water extracts did not. This indicates that the lipophilic compounds in RL are more potent in cancer therapy than the hydrophilic compounds. As seen from HPLC analysis of the extracts, *n*-butanol and water extracts of RL contained mainly constituents of palm sugar and little, if any, of the cancer therapeutic compounds. The results of the present study indicate that all the three lipophilic extracts may contain diverse compounds of which one or more may possess anticancer properties.

It is known that PC-3 cells are resistant to radiation [7, 19]. As seen in the present study, all three lipophilic extracts of RL enhanced the radiation sensitivity of PC-3 cells. Among the three, the chloroform extract showed the highest enhancement of radiation sensitivity of PC-3 cells (4.12-fold). Patients undergoing radiation therapy for prostate carcinoma frequently rely on complementary health practices [23]. We are currently exploring the molecular mechanisms of RL-mediated increases in radiation sensitivity of PC-3 cells, so as to recommend it as a complementary therapy for prostate cancer.

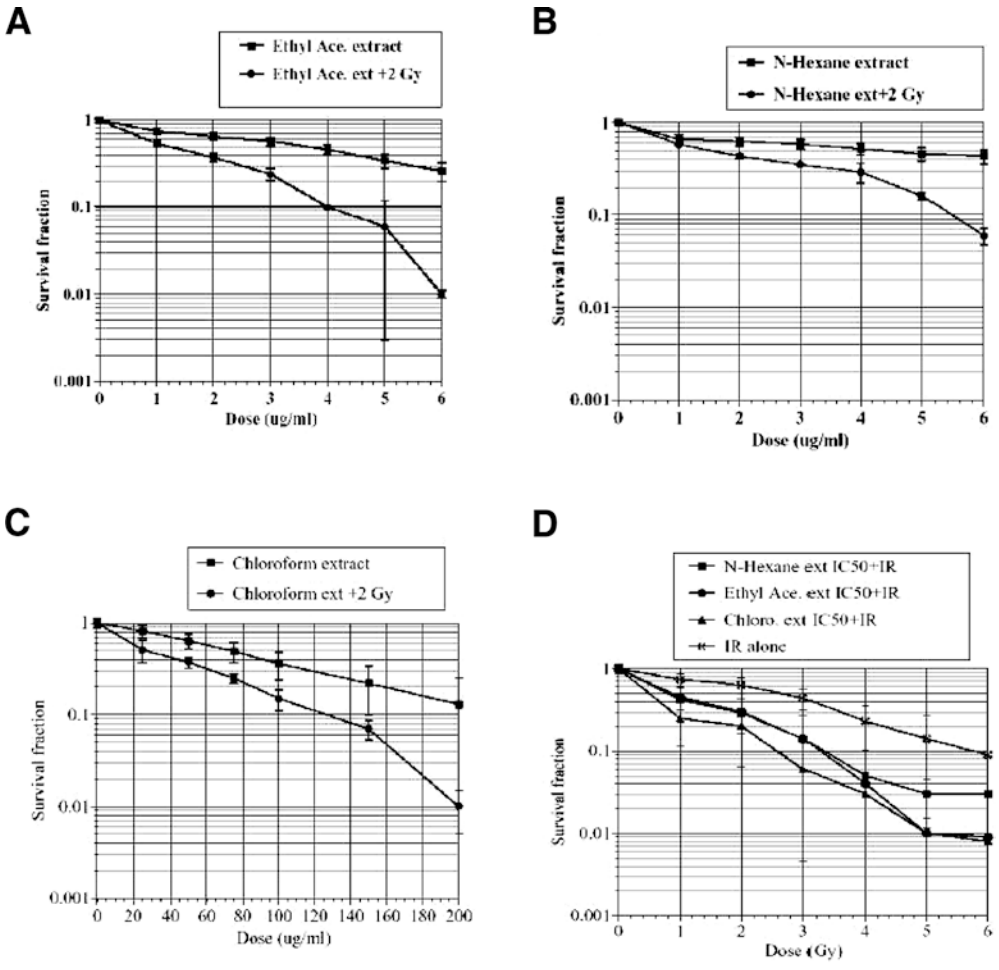

Fig. 4A–D Effect of RL extract on radiation-induced clonogenic inhibition in PC-3 cells. **A–C**Effects RL extracts (1–6 μg/ml) alone and RL extracts plus radiation at 2 Gy. **D** Effects of radiation at 1–6 Gy alone and radiation plus RL extracts. The data shown are the means from two individual experiments

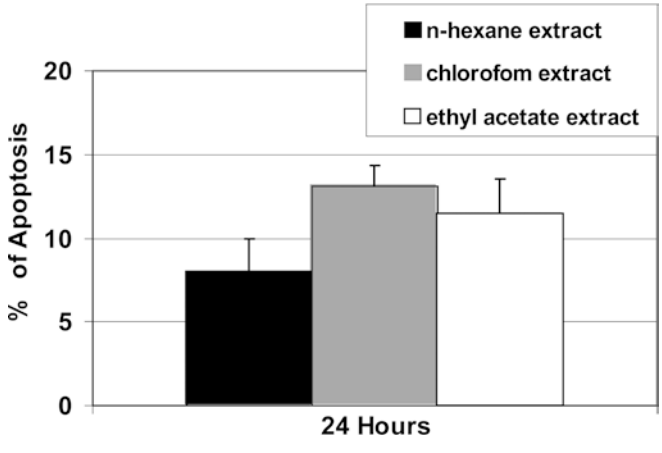

Fig. 5 RL extracts induced apoptosis in PC-3 cells. The bar graphs show the percentage of TUNEL-positive cells. Apoptosis in the treated cells was normalized to the baseline apoptosis in the untreated cells. The data shown are the means from two independent experiments. Vertical bars represent standard error

Thus, against the background of a lack of scientific information on many CAMs, the present study was a preliminary scientific validation of the anticancer properties of RL. We propose to conduct further studies to find the relevance of this Siddha therapeutic modality in the modern context, with special reference to prostate cancer therapy, and to establish RL or a variant of it (i.e. one containing all the ingredients other than the potentially toxic heavy metals) as an effective alternative medicine for prostate cancer and as a potential radiation sensitizer, as a complementary medicine, in the context of radiation therapy for prostate cancer. We further propose to (1) test the potent extracts on several other prostate cancer cell lines, (2) carry out biochemical studies using the extracts in animal models to establish their therapeutic efficacy as well as toxicity, and (3) subject the most potent extracts to HPLC and MS analyses to identify and characterize the efficacious phytotherapeutic and/or radiation-sensitizing compound(s) in RL.

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