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Development of a Highly Potent Therapeutic Regimen for Chronic Myeloid Leukemia using the Extract of *Eleusine coracana* Seeds

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ABSTRACT

Leukemia, a virulent blood disease, is known to spread throughout the body asymptomatically. An increasing number of chronic myeloid leukemia patients showing resistance towards available treatments has created a substantial need for developing new natural therapeutic options. *Eleusine coracana* (commonly called *ragi* or finger millet) is a highly nutritive cereal and has long been used as a remedy for many infections. Since *ragi* is a prospective therapeutic cereal, this study was designed to elucidate the anti-proliferative and apoptosis-inducing activity of the protein extracted from seeds on chronic myeloid leukemia cell line K562. Peripheral blood mononuclear cells were obtained from healthy young volunteers. Cell growth inhibitory and anti-proliferative activity of the seed extract of *ragi* was studied by trypan blue dye exclusion method and MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay, using both K562 and peripheral blood mononuclear cells. Apoptosis inducing activity of the extract was determined by flow cytometric analysis. The protein extract of *ragi* seeds showed a dose-dependent reduction of proliferation and induction of apoptosis in the K562 cells. In contrast, normal human peripheral blood mononuclear cells were resistant to the seed extract mediated anti-proliferative activity. The IC_{50} value of the extract was found to be 2 mg/ml. Thus this study suggests the seed extract of *ragi* can target and inhibit K562 cells selectively, which may be claimed as its anticancer activity. As *ragi* is safe and relatively inexpensive and can be administered orally, there is a definite possibility that it will have a therapeutic role on patients with chronic myeloid leukemia.

Keywords: apoptosis, bioactive agents, chronic myeloid leukemia, finger millet, *ragi*
Abbreviations: CML, chronic myeloid leukemia; **TKI**, tyrosine kinase inhibitor

INTRODUCTION

Chronic myeloid leukemia (CML) is a form of leukemia characterized by increased and unregulated growth of myeloid cells in the bone marrow leading to their accumulation in the blood (Holyoake *et al*. 2002). This is a disorder involving proliferation of mature granulocytes and is associated with Philadelphia chromosome formed from a reciprocal translocation (Rowley 1973). The fusion gene encodes a constitutively active Bcr-Abl protein tyrosine kinase and drives several proliferative and antiapoptotic pathways (Deininger *et al*. 2000). Although the development of tyrosine kinase inhibitors (TKIs), like imatinib, has revolutionized the treatment of CML (Alvarez *et al*. 2007), resistance to such agents are now being reported in increasing number of patients (Melo and Chuah 2007). More potent second generation tyrosine kinase inhibitors, like nilotinib and dasatinib, have increased potency against Bcr-Abl (Cortes *et al*. 2010a, 2010b). Therapy with tyrosine kinase inhibitors has transformed CML from a life-threatening disease into a chronic condition. In most patients, after TKI therapy, residual leukemia cells persist; specifically, the CML stem cells are not eliminated and the patients remain Bcr-Abl+ (Konig *et al.* 2008). CML stem cells escape from imatinib and other TKIs either by altered Bcr-Abl expression level or due to the lack of CML stem cell dependence on Bcr-Abl expression (Jiang *et al.* 2007). Several groups have identified the Hedgehog signaling (Zhao *et al*. 2009), arachidonate 5-lipoxygenase (Alox5) (Chen *et al*. 2009), autophagy (Bellodi *et al.* 2009) or TGF beta/FOXO pathway combination (Naka *et al*. 2010) as novel regulators of CML stem cell survival. As imatinib shows similar inhibitory potential against CML progenitor and CML stem cells, it has been concluded that CML stem cells are not Bcr-Abl addicted and Bcr-Abl inhibition will not eliminate CML stem cells (Corbin *et al.* 2011; Perl and Carrol 2011). These observations thereby implicate the necessity for developing new curative therapeutic approaches to CML. Thus, desperate attempts are now being made to detect and recognize new preventive and curative natural products. Various plant extracts possessing anticancer activity have been reported from *Clausena lansium* seeds (Ng *et al.* 2003), *Plantago major* plant extract (Kazi *et al*. 2003), *Salix safsaf* leaf extract (Zahran *et al*. 2005), *Scutellaria baicalensis* roots (Scheck *et al*. 2006), *Trailliaedoxa gracilis* extract (Svejda *et al.* 2010), *Glochidion zeylanicum* roots (Shrama *et al.* 2011) and *Debregeasia salicifolia* extract (Nisa *et al.* 2011).

The seeds of *Eleusine coracana* Gaertn. (commonly called *ragi* or finger millet) are a rich natural source of iron, calcium, carbohydrate and the valuable amino acid methionine, and are thereby considered as wholesome food for infants and diabetics. *Ragi* is an important cereal of Karnataka, Tamilnadu and Andhra Pradesh of India and some parts of Africa. It is less costly than wheat, rice or dairy milk (Lost Crops of Africa, 1996). In earlier days, *ragi* was used as a folk remedy for leprosy, liver disease, measles, pleurisy, pneumonia and even small pox (Watt and Breyer-Brandwijk 1962). The protein extract of *ragi* seed was known to contain *ragi* bifunctional inhibitor (RBI), capable of inhibiting alpha-amylase and trypsin simultaneously (Shivraj and Pattabiraman 1981; Campos and Richardson 1983).

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Table 1 Materials used for the experiments and their manufacturers.

Manufacturing company	Product/products used for the experiment
Sigma-Aldrich, St. Louis, MI, USA	MTT, Histopaque, Antifoam3, BSA and all solvents
Super Religare Laboratories (SRL), Mumbai, India	Trypan Blue
Invitrogen, Paisley, UK	RPMI-1640 and Penstrep
Gibco BRL, Karlruhe, Germany	Fetal bovine serum and Phosphate buffered saline
Roche, Mannheim, Germany USA	Complete mini protease inhibitor cocktail tablets
Clontech Laboratories, Mountain View, CA, USA	ApoAlert Annexin V-FITC Apoptosis kit
Bio-Rad, Hercules, CA, USA	Bradford reagent
Nicholas Piramal India Ltd., Ahmedabad, India	Gentamicin sulphate (Genticyn)

Even though *ragi* is cheap, it is a super cereal for its nutritional versatility and ethnomedicinal potential. However, its very high calcium content must be of importance because calcium has been identified as the messenger coordinating mitochondria and endoplasmic reticulum interactions to promote cellular apoptosis (Rizzuto *et al*. 2003). Additionally, the chemopreventive role of dietary calcium was reported against cancer cells (Lamprecht and Lipkin 2003). Therefore, exploration of the possible anticancer activity of *ragi* seed extract is reasonable. The seed protein extract was expected to contain protease inhibitors like *ragi* bifunctional trypsin-alpha amylase inhibitor (RBI). Buckwheat trypsin inhibitor showed antiproliferative activity against chronic myeloid leukemia cell line K562 (Wang *et al*. 2007). Consequently, the present study aimed to evaluate the possible effects of *ragi* seed extract on chronic myeloid leukemia cell line K562 in comparison with normal peripheral blood mononuclear cells.

MATERIALS AND METHODS

Plant material

Seeds of *E. coracana* variety 'GPU-28' were obtained from Gandhi Krishi Vignana Kendra, Bangalore, India. GPU-28 is one of the most popular and widely cultivated varieties of *ragi* that can be grown all-year round and is resistant to 'Finger blast' by *Pyricularia grisea*. Materials for the experiment are described in **Table 1**.

Human chronic myeloid leukemia cell line and the method for its cultivation

Human chronic myeloid leukemia cell line K562 was obtained from the Indian institute of Chemical Biology, Kolkata, India. The cells were cultured in RPMI 1640, supplemented with 10% heat-inactivated Fetal bovine serum, penicillin (100 units/ml), streptomycin (100 µg/ml) and gentamicin (100 µg/ml), and maintained in a humidified incubator containing 5% CO_2 and 95% humidity at 37°C.

Preparation of *ragi* seed protein extract

To prepare the total protein extract from 0.1 kg of ground *ragi* seeds, the seed powder was homogenized with 0.15 M NaCl (1:10 w/v) containing protease inhibitor cocktail tablets (1 tablet/10 ml, Roche, Mannheim, Germany) and antifoam 3 (1:0.005, v/v). After centrifugation at 10,000 rpm for 30 min at 4°C, clear supernatant was collected. Total protein content was precipitated with chilled acetone (75%), incubated at -20°C for 1 h and centrifuged at 10,000 rpm for 20 min at 4°C. The pellet was dissolved in 100 mM NaCl buffer (pH 8.0) ($A_{280} \approx 50$).

Method for protein quantification

Protein contents were estimated by the Coomassie blue dye-binding method (Bradford 1976) with bovine serum albumin (Bio-Rad, Hercules, CA, USA) as the standard (1 mg/ml).

Assay for the reduction of cell viability

To determine the effect of *ragi* seed extract on the K562 cell line, 100 µl of cell suspension from 10^6 cells/ml in RPMI 1640 medium was added to each well in a sterile 96-well tissue culture plate. Cells were cultured in a CO_2 incubator supplied with 5% CO_2, 95% humidity at 37°C for 24 h, in the presence and absence of varying concentrations of *ragi* crude extract (0.5-2.5 mg/ml; n=5). The number of viable cells was counted after 24 h by the trypan blue (0.4%) dye exclusion method under a microscope (Ernst Leitz Wetzlar, Germany, Model no. 324869). The IC_{50} of *ragi* seed extract, defined as the concentration required for causing 50% reduction in viable cell count in 24 h was measured (Reed and Muench 1938).

Assay for the detection of cellular proliferation with the help of MTT

For the MTT [3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium bromide] assay (Yamaue *et al*. 1992) the yellow tetrazolium dye that can be cleared by mitochondrial dehydrogenase enzymes of viable cells to form purple formazan, was used. The concentration of formazan formed is proportional to the number of viable cells in a tissue culture system. K562 cells (100 µ1 from 10^6 cells/ml) were cultured in the presence and absence of varying amounts of *ragi* seed extract (0.5-2.5 mg/ml; n = 4), in a sterile 96-well plate and incubated in a CO_2 incubator supplied with 5% CO_2 with 95% humidity at 37°C for 24 h. Cells were added to 10 µl of MTT reagent (5 mg/ml) and incubated for 4 h at 37°C and precipitated by centrifugation in order to discard the media. Then, 100 µl of dimethyl sulfoxide (DMSO) was added to dissolve the formazan crystals formed and also to terminate the reaction. For this assay, control wells contained no seed extract. The absorbance was recorded using a test wavelength of 570 nm and a reference wavelength of 630 nm, with an automated microtiter plate reader (model 550, Bio-Rad, Hercules, CA, USA). Percent growth inhibition was calculated as [1- (A_{570} - A_{630} of treated / A_{570} - A_{630} of control)] × 100 and plotted to produce a graph. All experiments were performed in triplicate. Statistical analyses in all studies followed standard methods given next.

Assay for detection of cytotoxicity on normal human peripheral blood mononuclear cells

Five ml blood was drawn from 5 healthy human young volunteers after obtaining their consent. Each of these was transferred aseptically to a tube that contained 10 U/ml of heparin and diluted with an equal volume of normal saline. This diluted blood (10 ml) was layered over 5 ml of histopaque and centrifuged at 1500 rpm for 30 min at room temperature. Peripheral blood mononuclear cells were collected from the histopaque-plasma interface, washed twice with normal saline and re-suspended in complete RPMI medium (containing 10% FBS) to a density of 10^6 cells/ml. Cells were cultured in CO_2 incubator supplied with 5% CO_2, 95% humidity at 37°C for 24 h in the presence and absence of the *ragi* seed extract (IC_{50}). Cell viability was determined using the trypan blue (0.4%) dye exclusion method using an Ernst Leitz Wetzlar microscope. The percentage of inhibition of growth was calculated compared to the untreated control (n = 5). The cytotoxic effect of the extract on peripheral blood mononuclear cells was analysed by the MTT assay and the % growth inhibition was measured as mentioned above (Gomes *et al.* 2007).

Determination of apoptosis-inducing potential by flow cytometry

To determine the effect of the protein extract of *ragi* seeds on K562 cells, flow cytometry was carried out using an ApoAlert Annexin V-FITC Apoptosis kit (Clontech). The complete RPMI medium containing K562 cells (1×10^6) was added to a 24-well sterile tissue culture plate, and allowed to grow in the presence and absence of *ragi* seed extract in an CO_2 incubator supplied with 5% CO_2, 95% humidity at 37°C for 24 h. The cells were washed with 50 mM cold phosphate buffered saline (PBS) at pH 7.4. The cells were collected by centrifugation at 1000 rpm for 10 min at 4°C, rinsed and re-suspended in binding buffer. K562 cells were stained with 0.1 µg Annexin V-FITC and 0.5 µg propidium iodide in the binding buffer. After an incubation period of 15 min in the dark at room temperature, the relative percentage of live/apoptotic/necrotic cells was determined by flow cytometry on a Becton Dickinson FACS Calibur single laser flow cytometer and analyzed with Cell Quest Pro software (n = 4). The flow cytometric reading was taken using 488 nm excitation and band pass filters of 530/30 nm (FITC detection) and 585/42 nm (for PI detection; Vermes *et al*. 1995).

Statistical analysis

Data are expressed as the mean ± standard deviation (SD) of three independent experiments. All statistical analyses were evaluated using Graph Pad Prism software (San Diego, USA). Statistical analysis was performed using the Student's *t*-test. A *P*-value of 0.05 or less was considered as statistically significant.

RESULTS

Inhibition of cell viability by treatment of the seed extract of *ragi*

Mean inhibition % of K562 cell growth at 24 and 48 h after treatment with the extract (0.5-2.5 mg/ml) was studied in comparison with untreated control cells (**Table 2**). The mean inhibition % of K562 cell growth (mean ± SD, n = 5) in the seed extract was 27.2 ± 2.3 to 53.8 ± 2.6 for 24 h and 29. 5 ± 1.7 to 55.6 ± 3.3 for 48 h. The IC_{50} value of the protein extract of the seed on K562 cells was 2 mg/ml, causing approx. 50% reduction of cell growth in 24 h.

Analysis of anti-proliferative activity of *ragi* seed extract by MTT assay

Distinct inhibition of cellular proliferation of K562 cells was observed after treatment with protein extract of seed compared with the untreated cells (control). Growth reduction % of K562 cells after 24 h treatment with varying amounts of the extract (0.5, 1, 1.5, 2, 2.5 mg/ml) was 27.6 ± 2.1, 37.1 ± 2.3, 41.6 ± 2.7, 50.3 ± 3.2 and 51.6 ± 2.5, respectively (n = 4; **Fig. 1**). No significant anti-proliferative effect of the seed extract could be observed in peripheral blood mononuclear cells.

Analysis of apoptotic activity by flow cytometry

K562 cells treated and untreated with *ragi* seed extract were examined after 24 h for analysis by Flow cytometry (**Fig. 2, Table 3**). Untreated K562 cells showed the presence of 94.38 ± 1.12% intact viable cells (FITC-PI-). On the contrary, K562 cells treated with *ragi* seed extract (1 mg/ml and 2 mg/ml, IC_{50}= 2 mg/ml) showed 68.77 ± 1.14% and 55.41 ± 0.75% of intact viable cells respectively (n=4). Additionally, apoptotic cells (FITC+PI-) obtained (including early and late apoptotic cells) were 28.30 ± 0.58% and 33.46 ± 0.65% respectively due to treatment by the seed extract (1 mg/ml and 2 mg/ml); however, the value in the untreated cells (control) was 3.54 ± 0.15%.

Table 2 Effect of *ragi* seed extract on the inhibition of K562 cells (10^6 cells/ml) determined by Trypan blue dye exclusion method.

Ragi extract added to 1 ml of K562 cells (mg)	Percent inhibition of growth at 24 h of incubation	Percent inhibition of growth at 48 h of incubation
0.5	27.2 ± 2.3	29.5 ± 1.7
1	35.4 ± 3.2	37.2 ± 2.9
1.5	41.7 ± 1.9	43.3 ± 2.3
2	50.3 ± 2.7	53.1 ± 3.2
2.5	53.8 ± 2.6	55.6 ± 3.3

Values shown as percentage Mean ± SD, n= 5.

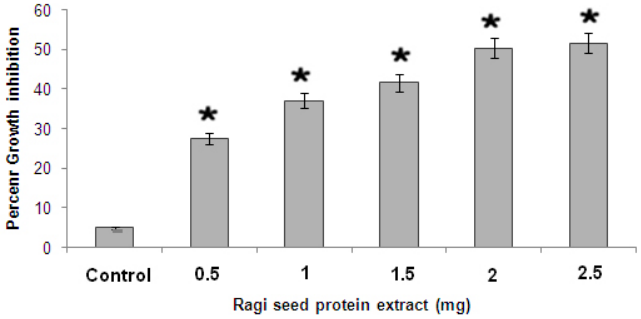

Fig. 1 Effects of K562 cells with various concentrations of *ragi* (*Eleusine coracana* Gaertn.) cv. 'GPU 28' seed protein extract after 24 h of treatment. Values represent mean ± standard deviation (n = 4) of three independent experiments. Asterisk indicates statistically significant difference. $P < 0.05$ in treated cells with respect to the untreated (control) cells.

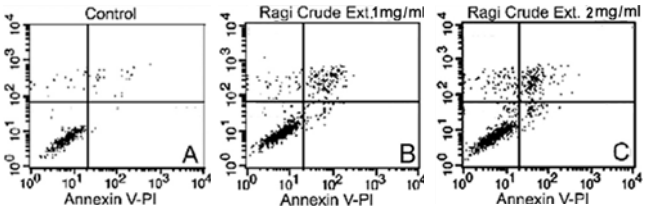

Fig. 2 Flow cytometric determination of effect of *ragi* seed extract on the Annexin-V/PI binding of K562 cells. (**A**) K562 cells without *ragi* seed extract (control); (**B**) K562 cells treated with *ragi* seed extract (1 mg/ml) for 24 h; (**C**) K562 cells treated with *ragi* seed extract (2 mg/ml) for 24 h. The relative percentage of intact/ apoptotic/ necrotic cells was analyzed using flow cytometry (n = 4). Refer to Materials and Methods for details of the AnnexinV/PI staining procedure.

DISCUSSION

Leukemia is a malignancy of blood-forming tissues, bone marrow, lymph nodes and the spleen, and according to the WHO, the global incidence of leukemia is about 8-9 per 10,000 persons each year (Wang *et al*. 2007). CML cells express the Philadelphia chromosome and the related fusion oncoprotein Bcr-Abl. Deregulated tyrosine kinase activity

Table 3 Flow cytometric data showing the effect of *ragi* crude extract on AnnexinV/PI binding of K562 cells.

Materials	LL quadrant	LR quadrant	UL quadrant	UR quadrant
Control K562 (24 h)	94.38 ± 1.12	1.36 ± 0.10	2.08 ± 0.04	2.18 ± 0.05
Ragi extract (1 mg/ml, 24 h)	68.77 ± 1.14*	11.19 ± 0.47*	5.93 ± 0.09*	17.11 ± 0.11*
Ragi extract (2 mg/ml, 24 h; IC_{50})	55.41 ± 0.75*	13.59 ± 0.58*	11.13 ± 0.87*	19.87 ± 0.07*

Values shown as percentage Mean ± SD

* $P < 0.05$ significant as compared to respective controls, n=4

LL quadrant = viable cells, LR quadrant = early apoptotic cells, UL quadrant = necrotic cells; UR quadrant = late apoptotic cells

of Bcr-Abl is the biochemical hallmark of CML and it drives several proliferative and antiapoptotic pathways. The central role of Bcr-Abl in the pathogenesis of CML has made it a suitable drug target (Alvarez *et al.* 2007). The tyrosine kinase inhibitor imatinib forms the first line of treatment for CML. Although most patients show excellent responses to imatinib treatment, resistance to imatinib has been developed by point mutations in Abl kinase and a proportion of patients remain unresponsive (Deininger and Holyoake 2005). Two potent second generation tyrosine kinase inhibitors, nilotinib and dasatinib, recently have been developed (Cortes *et al.* 2010a, 2010b). However, it has been found that Bcr-Abl cannot act as the regulator for CML stem cells (Corbin *et al.* 2011; Perl and Carrol 2011). Hence there is a dire need for the development of new therapeutic strategies. Since plant-derived compounds may have a great potential to be developed into anticancer drugs due to their multiple mechanisms and low side-effects (Kazi *et al*. 2003), anti-apoptotic effects of various natural products have been focused in recent years (Wang *et al*. 2007; Svejda *et al*. 2010; Nisa *et al*. 2011; Sharma *et al.* 2011).

The present study has clearly indicated the anti-proliferative effect of the extract of *ragi* seeds on CML cell line K562. Cellular growth of K562 cells was inhibited by the extract in a time- and dose-dependent manner, with an IC_{50} value of 2 mg/ml. It is known that in the MTT assay the reduction of OD value bears a direct correlation with the growth inhibitory rate and an inverse relation with the anti-proliferative rate (Joe *et al*. 2002). MTT is a water-soluble tetrazolium salt. Mitochondrial succinate dehydrogenase enzyme of viable cells can cleave the tetrazolium ring and can convert this to insoluble purple formazan. The disruption of the mitochondrial dehydrogenase system due to mitochondrial dysfunction or apoptosis reduces the colour production by formazan in the MTT assay. The extract-induced growth inhibition of K562 cells and cytotoxicity was found to be specific for cancer cells. Peripheral blood mononuclear cells remained unaffected by the application of this seed extract.

An altered phospholipid distribution over the plasma membrane was observed in the K562 cells treated with *ragi* seed extract. The phosphatidylserine expression of apoptotic cells could be detected using AnnexinV-FITC and the propidium iodide double staining method (Vermes *et al*. 1995). A dose-dependent increase in the number of early and late apoptotic cells of K562 cell line could confirm the growth inhibitory property of the extract, mediated by apoptosis.

Ragi seeds are known to contain α-amylase-trypsin bifunctional inhibitor (Shivraj and Pattabiraman 1981; Campos and Richardson 1983). There is accumulated evidence that consumption of seeds containing protease inhibitors can lower the incidence of breast, colon, prostrate, oral and pharyngeal cancers (Kennedy 1998; Meyskens and Szabo 2005). These reports suggest a possible positive contribution of such inhibitors to the bioactivity of this extract. As the seeds of *E. coracana* are now plentifully available in open market almost throughout the World, this may be considered for routine administration to patients suffering from CML in the form of a nutritive and potential supplement to conventional therapy. Further studies are in progress for the detection of the actual molecular action of this extract on K562 cells.

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