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Evaluation of Anti-cancer Potential of *Ragi* Bifunctional Inhibitor (RBI) from *Eleusine coracana* on Human Chronic Myeloid Leukemia Cells

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ABSTRACT

Finger millet (*ragi*), a staple food crop, is well known as a super cereal for its excellent nutritional value, long-term storage potential and ethno-medicinal characteristics. *Ragi* bifunctional inhibitor (RBI) from *Eleusine coracana* Gaertn. (*ragi*/finger millet) belonging to cereal alpha-amylase/protease inhibitor family, inhibits alpha-amylase and trypsin simultaneously. Several seed protease inhibitors are known for their protective as well as curative role against many types of human cancers. Recently, the anti-cancer activity of *ragi* seed extract on K562 chronic myeloid leukemia cells was explored. In the present study, RBI was purified from finger millet seeds by affinity chromatography followed by FPLC (Fast Protein Liquid Chromatography) size exclusion separation method. Purified RBI showed cytotoxicity against K562 chronic myeloid leukemia cells (IC_{50} = 20 µg/ml) but, not against normal human peripheral blood mononuclear cells. Reduction of cellular proliferation and induction of apoptosis of K562 cells by purified RBI was determined by MTT (3-4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium bromide) assay and flow cytometry analysis. This investigation being the first report on the anti-proliferative potential of RBI from edible *ragi* seeds, might provide a future preventive as well as curative natural solution for chronic myeloid leukemia.

Keywords: alpha-amylase-trypsin inhibitor; anti-proliferative activity; apoptosis; blood cancer, *ragi* or finger millet

INTRODUCTION

Leukemia is a malignant disease of blood forming tissues, bone marrow, lymph nodes and the spleen. The global incidence rate of leukemia vary from 0.6 to 2.0 cases per 100,000 people each year, that increase with age and are higher in men than in women (Rohrabacher and Hasford 2009). Chronic myeloid leukemia (CML) is a myeloproliferative disorder, characterized by its association with BCR-ABL oncogene, generated by Philadelphia chromosome. Philadelphia chromosome results from a (9;22) reciprocal translocation, that juxtaposes the Abelson murine leukemia viral oncogene homolog 1 (ABL) gene normally located on chromosome 9 with the Breakpoint cluster region (BCR) gene on chromosome 22 (Deininger *et al*. 2009). Formation of Philadelphia chromosome results in constitutive expression of BCR-ABL protein tyrosine kinase, which activates several proliferative pathways in CML cells (Deininger *et al*. 2000). Development of tyrosine kinase inhibitors (TKIs) like imatinib mesylate has revolutionized the treatment of CML (Alvarez *et al.* 2007). Resistance to such agents is now being reported in increasing numbers (Melo and Chua 2007). Second generation tyrosine kinase inhibitors like nilotinib and dasatinib have come up with much increased potency (Cortes *et al*. 2010a, 2010b). But, the CML stem cells could escape from imatinib therapy (Jiang *et al*. 2007), though, similar inhibitory potential of imatinib was reported against CML progenitor and CML stem cells. This observation indicated lack of dependence of CML stem cells on BCR-ABL for survival (Corbin *et al.* 2011; Perl and Carrol 2011). Other mechanisms causing imatinib resistance include mutations in the kinase domain of BCR-ABL and lack of adherence to medication, that cause emergence of resistant clones through suboptimal target inhibition (Marin *et al*. 2010; Ibrahim *et al*. 2011). Therefore, identification of new curative and preventive natural products against chronic myeloid leukemia is greatly needed.

Finger millet (*Eleusine coracana* Gaertn.), commonly known as *ragi* (India), is a staple food grain for many people of India, Sri Lanka and Africa for its excellent nutritional value (Barbeau and Hilu 1993). Finger millet is a wholesome food for diabetics and infants and is a well-known folk medicine for leprosy, liver disease, pleurisy, pneumonia and even small pox (Watt and Breyer-Brandwijk 1962). Recently, anti-proliferative activity of *ragi* seed extract on K562 chronic myeloid leukemia has been reported (Sen *et al.* 2011). *Ragi* seeds are known to contain a bifunctional alpha (α)-amylase-trypsin inhibitor (RBI), that simultaneously inhibits α-amylase (EC 3.2.1.1) and trypsin (EC 3.4.21.4) forming a ternary complex (Shivraj and Pattabiraman 1981; Maskos *et al.* 1996).

Plant protease inhibitors are multifunctional proteins. Proteases are involved in various biological processes, including inflammation, infection, extracellular matrix degradation, blood coagulation, programmed cell death, tumor invasion and cancer metastasis (Mosolov *et al.* 2001; Pandey *et al.* 2007; reviewed in Raj 2012). The European Cancer Proteases Consortium (EUCPC) has proposed multidisciplinary research considering extracellular proteases as an attractive target for intervention against cancer (Pandey *et al.* 2007). Therefore, plant protease inhibitors regulating proteases have a very significant role to play in human health and disease management. Extensive *in vitro* and *in vivo* studies and clinical trials have established the potentiality of plant protease inhibitors in inhibiting different stages of carcinogenesis including initiation, promotion and progression. A Bowman-Birk inhibitor (BBI) from soybean, inhibiting trypsin and chymotrypsin simultaneously, could inhibit M5067 ovarian sarcoma by increasing expression of

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tumor-suppressor molecule Cx43 (Suzuki *et al.* 2005). BBI also suppressed the proteasomal chymotrypsin like activity in MCF-7 breast cancer cells (Chen *et al.* 2005). BBI and BBI concentrate could induce apoptosis of LNCaP prostate cancer cells, colorectal carcinogenesis and HT-29 colon cancer cells (Kennedy *et al.* 2002; Tang *et al.* 2009; Caccialupi *et al.* 2010). Antiproliferative and chemopreventive activity of the protease inhibitor was correlated with its trypsin inhibitory potential (Kato *et al.* 1998; Kennedy *et al.* 2002). A trypsin-chymotrypsin inhibitor from *Vigna unguiculata* caused induction of cell cycle arrest and apoptosis of MCF-7 breast cancer cells (Joanitti *et al.* 2010). A Kunitz-type trypsin inhibitor (TI) from soybean could suppress the invasion and metastasis of HRA ovarian cancer cells by blocking urokinase upregulation (Kobayashi *et al.* 2004). A trypsin-chymotrypsin inhibitor (BWI-1) from buckwheat (*Fagopyrum esculentum*) showed anti-proliferative effect on T-acute lymphoblastic leukemia cells, Jurkat cells (Park and Obha 2004) and solid tumor cells like hepatoma (HepG2), cervical carcinoma (HeLa) and esophageal squamous cell carcinoma (EC9706) cells (Li *et al.* 2009). The mechanism involved in the cytotoxic activity was found to be upregulation of Caspase-3, Caspase-9, disruption of mitochondrial membrane potential and increased DNA fragmentation (Li *et al.* 2009). Some other TIs from *Peltophorum dubium* and *Ipomoea batatas* also showed their potential in cancer prevention and therapy on rat lymphoma cells (Troncoso *et al*. 2007) and NB4 promyelocytic leukemia cells (Huang *et al*. 2007) respectively. Concisely, plant protease inhibitors are considered as neutraceuticals, providing both nutrition and pharmaceutics, specifically in the prevention and/or treatment of cancer.

Ragi bifunctional inhibitor (RBI) belongs to the cereal α-amylase trypsin inhibitor family. Its primary structure revealed a monomeric protein of 122 amino acids containing five intra-molecular disulfide bonds (Campos and Richardson 1983). Novel inhibitory strategy of RBI in complex with yellow meal worm α-amylase was determined at 2.5Å (Strobl *et al*. 1998). The crystal structure of RBI by X-ray diffraction analysis was reported at 2.2Å (Gourinath *et al*. 2000). Recently, the *Ragi* bifunctionl inhibitor (RBI) gene from *ragi* seeds has been cloned and expressed functionally in *E. coli* (Sen and Dutta 2012). As a member of non-specific lipid transfer protein family, RBI is expected to show anti-fungal activity (Garcia-Olmedo *et al.* 1995). *Ragi* α-amylase inhibitor exhibited pronounced inhibition of *Callosobruchus chinensis* (pulse beetle) α-amylase (Shivakumar *et al.* 2006). However, the functionality of *ragi* α-amylase-trypsin inhibitor (RBI) protein, regarding its anti-proliferative activity against cancer cells has yet to be evaluated. As trypsin and many serine proteases were found to be involved in the invasion of tumor cells and since several plant TIs showed anti-cancer potential, the present study was performed to evaluate the anti-proliferative and apoptosis-inducing potential of purified RBI against K562, a chronic myeloid leukemia cell line.

MATERIALS AND METHODS

Plant material

Seeds of *Eleusine coracana* Gaertn. cv 'GPU-28' were procured from Gandhi Krishi Vignana Kendra, Bangalore, India. 'GPU-28' is one of the most popular and widely cultivated varieties of *ragi* growing all-year round and resistant to fungal infection by *Pyricularia grisea*. Materials used for the experiment are described in **Table 1**.

Purification of RBI protein from *ragi* seeds

100 g of powdered *ragi* seeds were homogenized with 1000 ml of 0.15 M NaCl (1:10 w/v) containing PMSF (Phynylmethylsulfonyl Fluoride) (0.5 mM) and protease inhibitor cocktail at 4°C, using an omni-mix homogenizer (Dupont) and filtered through cheese cloth. The filtrate was then centrifuged at 12,000 × *g* for 30 min (Sorval RC-5B). To precipitate the protein, chilled acetone (80%) was added slowly to the clear soup and kept at -20°C for 1 h and centrifuged at 10,000 × *g* for 20 min. The pellet was dissolved with 50 mM Tris-HCl (pH 8.0) containing 100 mM NaCl and centrifuged at 12,000 × *g* for 30 min at 4°C. To prepare a trypsin-sepharose column, trypsin was coupled with CNBr-activated Sepharose 4B (Amersham Biosciences, Piscataway, NJ, USA) as per manufacturers protocol. The activated sepharose 4B could bind 9.5 mg/ml of trypsin. The clean supernatant was applied to trypsin-Sepharose column (1 cm x 15 cm), pre-equilibrated with 50 mM Tris-HCl (pH 8.0) containing 100 mM NaCl. The column was washed extensively with the same buffer to remove unbound molecules and eluted with 0.1 M HCl at a flow rate of 1 ml/min (isocratic mode). Collected fractions (1 ml/fraction) were neutralized with 1 M Tris (pH 8.0). Active fractions were pooled and concentrated (10-fold) with Centriprep of 3 kDa cutoff (Amicon, Beverly, MA, USA) and loaded on to Superose 12 HR 10/30 (Pharmacia, NJ, USA) column for FPLC size exclusion chromatography. Peaks were separated with a buffer of 50 mM Tris-HCl (pH 8.0), 100 mM NaCl at a flow rate of 0.5 ml/min and chart speed of 0.2 cm/min. The homogeneity of the active peak fraction was determined by 15% SDS-PAGE. The peak fraction showing both α-amylase and trypsin inhibitory activity was concentrated and desalted with Centriprep 3 and the protein was dissolved in 50 mM NaCl and kept for further use.

Table 1 Materials used for the experiment and their manufacturers.

Manufacturing company	Product/products used for the experiment
Sigma-Aldrich, Hamburg, Germany	Trypsin, Alpha-amylase, Tris, TAME, PIPES, soluble starch, $CaCl_2$, SDS, β-ME, MTT, Histopaque, Antifoam3, BSA, and all solvents
Pharmacia, New Jersey, USA	Superose 12 HR 10/30 column
Millipore, MA, USA	Centriprep 3
Amersham Biosciences, PA, USA	CNBr activated sepharose 4B
SRL, Mumbai, India	Trypan Blue
Invitrogen, Paisley, UK	RPMI-1640 and Penstrep
Gibco BRL, Karlsruhe, Germany	Fetal bovine serum and phosphate buffered saline
Roche, IN, USA	Complete mini protease inhibitor cocktail tablets
Clontech, CA, USA	ApoAlert Annexin V-FITC Apoptosis kit
Fermentas, MD, USA	Bradford reagent
Piramal Healthcare, Hyderabad, India	Gentamicin sulphate (Genticyn)

Quantification of protein

Protein contents were estimated with Quick start Bradford reagent (Bio-Rad, CA, USA) with bovine serum albumin as the standard (1 mg/ml) (Bradford 1972).

Trypsin inhibition assay

Trypsin inhibitory activity on bovine pancreatic trypsin was determined by measuring the hydrolytic activity towards the substrate *p*-toluene-sulfonyl-L-arginine methyl ester (TAME). Different concentrations of the inhibitor were incubated with 1 µg trypsin at 25°C for 10 min in 50 mM Tris-HCl, pH 8.0, 10 mM $CaCl_2$ in a final volume of 1 ml. After incubation of 10 min, 0.1 ml of TAME (0.01 M) was added (in 1 ml assay volume) and change in absorbance at 247 nm was measured, against a blank solution containing the buffer and substrate, with Varian Cary 50 Bio spectrophotometer. One trypsin unit (TU) is defined as 1 µmol of TAME hydrolyzed per minute at 25°C, pH 8.2. One inhibition unit is defined as a unit of enzyme inhibited. Trypsin inhibition assay was carried out to determine the specific activity of the inhibitor. Specific activity is defined as trypsin inhibition units (TIU)/mg of protein (Walker-Simmons and Ryan 1977).

α-Amylase inhibition assay

The α-Amylase inhibition assay was performed using soluble starch as substrate in 50 mM Pipes, pH 7.0, 100 mM NaCl, 1 mM $CaCl_2$ buffer, with a final concentration of 1.8 nM of porcine pancreatic α-amylase. Different concentrations of the inhibitor were pre-incubated with the buffer for 30 min at 37°C in 0.75 ml. Soluble starch (1%) (substrate) of 0.25 ml was added to start the enzymatic reaction and incubated at 37°C. Samples of 0.5 ml were removed after different incubation times (5-20 min), added with 0.5 ml 1% (w/v) dinitrosalicylic acid in 0.4 M NaOH, heated at 100°C for 5 min, cooled on ice and diluted to a final volume of 5 ml. Product formation was detected by the increase in the absorbance at 546 nm. Wheat α-amylase inhibitor (Sigma) was used as control. One unit of amylase inhibitor is defined as 1U of porcine pancreatic amylase inhibited under the assay conditions (Alam *et al.* 2001).

Human chronic myeloid leukemia cell line and its cultivation

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

Assay for reduction of cell viability

To determine the effect of RBI on K562 cells, 100 µl 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 RBI (5-40 µg/ml) (n = 5). The number of viable cells was counted after 24 h by the Trypan blue (0.4%) dye exclusion method under microscope (Ernst Leitz Wetzlar, Germany). The IC_{50} of RBI, defined as the concentration required for causing 50% reduction of viable cell count in 24 h, was measured (Reed and Muench 1938).

Determination of cell proliferation by MTT assay

Cellular proliferation was determined by MTT [3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetra-zolium bromide] assay, which measures the cleavage of the dye by mitochondrial dehydrogenase enzymes of viable cells forming purple formazan. Therefore, the concentration of formazan is proportional to the number of viable cells. K562 cells (100 µl from 10^6 cells/ml) were cultured in the absence and presence of varying amounts of purified RBI (5-40 µg/ml) (n = 4) in sterile 96-well plate. MTT reagent (20 µl from 5 mg/ml solution) were added to the wells, incubated for 4 h at 37°C and precipitated by centrifugation to discard the media. DMSO of 100 µl was added to dissolve the formazan crystal formed and to stop the reaction. The absorbance was recorded using a test wavelength of 570 nm and a reference wavelength of 630 nm, with automated microtiter plate reader (Bio-Rad model 550). Percent growth inhibition was calculated as [1- (A_{570} - A_{630} of treated / A_{570} - A_{630} of control)] × 100 and plotted onto the graph. Results represent the mean values of four independent experiments (P < 0.05) compared with the control (Yamaue *et al.* 1992).

Cytotoxicity assay on normal human peripheral blood mononuclear cells

After informed consent from a healthy human young volunteer (female, 27 years, Indian), 5 ml blood was drawn from cephalic vein, aseptically transferred to 10 U/ml heparin containing tube and diluted with equal volume of normal saline. Diluted blood of 10 ml was layered onto 5 ml of histopaque and centrifuged at 1500 rpm for 30 min. Peripheral blood mononuclear cells were collected from the histopaque-plasma interface, washed twice with normal saline, re-suspended in RPMI medium to a density of 10^6 cells/ml. Cells were cultured in CO_2 incubator for 24 h in the absence and presence of varying concentrations of RBI (5-40 µg/ml). The cytotoxic effect of purified RBI on peripheral blood mononuclear cells was analyzed by MTT assay and percent growth inhibition was measured in comparison to untreated control cells (n = 4) (Gomes *et al.* 2007).

Annexin V FITC/PI binding study by flow cytometry

To determine the effect of RBI protein on K562 cells, flow cytometry was carried out using ApoAlert Annexin V-FITC Apoptosis kit (Clontech). K562 cells (10^6 cells) in complete RPMI medium were added to a 24-well sterile tissue culture plate, and cultured in the presence and absence of purified RBI in a CO_2 incubator for 24 h. The cells were washed with 50 mM cold PBS (phosphate buffered saline) at pH 7.4, collected by centrifugation at 1000 rpm for 10 min at 4°C, rinsed and re-suspended in binding buffer. K562 cells were stained with Annexin V-FITC (0.1 mg) and propidium iodide (0.5 mg) in 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 (Becton Dickinson FACS Calibur single laser flow cytometer, NJ, USA) and analyzed with Cell Quest software program (n = 4). Flow cytometric reading of 10^4 cells was taken using 488 nm excitation and band pass filters of 530/30 nm (FITC detection) and 585/42 nm (for PI detection) at a coefficient of variation of < 3% (Vermes *et al.* 1995).

Statistical analysis

Data were expressed as the mean ± SD (standard deviation) of three independent experiments for Flow cytometry, and four independent experiments for Trypan blue dye exclusion assay and MTT assay. Statistical analyses were performed using Graph Pad Prism software (San Diego, USA). Student's *t*-test was performed. A P value of 0.05 was considered statistically significant.

Ethics declaration

This research was carried out in compliance with the Helsinki Declaration and was carried out under ethical approval granted by Indian Institute of Chemical Biology, India.

RESULTS

Purification of RBI from *ragi* seeds

Ragi seed extract was passed through a trypsin-sepharose column. The column eluent was concentrated and loaded onto a Superose 12 HR 10/30 FPLC column. The chromatogram obtained (**Fig. 1A**) showed three peaks. The horizontal bars represented the collection profile of the individual peaks. The peaks were collected separately and checked for α-amylase and trypsin inhibitory assays. The third peak showed both α-amylase and trypsin inhibitory activities and therefore considered as RBI. The third peak was pooled, concentrated and loaded onto FPLC column to check its purity. The column was calibrated with molecular mass standards. The retention time of the standards, as marked by vertical lines in the chromatogram, were 24.1 min for BSA (67 kDa) (**Fig. 1B**), 25.5 min for ovalbumin (45 kDa) (**Fig. 1C**), 28.9 min for chymotrypsinogen (25 kDa) (**Fig. 1D**) and 32.4 min for cytochrome C (12.4 kDa) (**Fig. 1E**). The retention time of the third peak was 32 min (**Fig. 1F**) in individual run, which corresponded to its retention time in earlier run (**Fig. 1A**). The homogeneity of the purified protein has been checked by SDS-PAGE (**Fig. 1G**).

Inhibition of cell viability by treatment with RBI

Mean inhibition (mean ± standard deviation, n = 5) percent of K562 cells after 24 h treatment with varying amounts of RBI (5, 10, 20, 30, 40 µg/ml) were 21 ± 2.2, 31 ± 4.1, 54 ± 3.6, 72 ± 4.8 and 84 ± 5.3%, respectively (**Table 2**). The

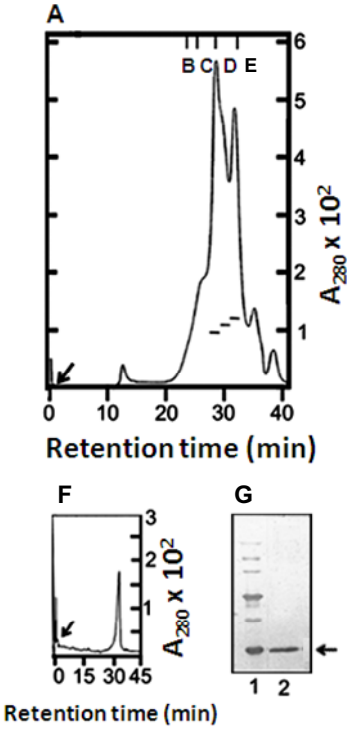

Fig. 1 Purification of RBI protein from ragi seeds. (**A**) Chromatogram showing peaks of protein from Superose 12 HR 10/30 column for FPLC size exclusion chromatography (**B, C, D, F**) represent molecular mass standards BSA (67 kDa), ovalbumin (45 kDa), chymotrypsinogen 25 kDa and cytochrome c (12.4 kDa), respectively; (**F**) Third peak obtained with trypsin and alpha-amylase inhibitory activity is concentrated and loaded onto the column separately showing single peak of purified protein; (**G**) SDS-PAGE showing protein molecular weight marker (lane 1) containing 92.2, 66.4, 44.3, 29.0, 20.1 and 14.3 KDa proteins and purified RBI protein (lane 2) of 14 kDa (as shown by the arrow).

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

RBI added (µg/ml)	% Growth inhibition at 24 h
5	21 ± 2.2
10	31 ± 4.1
20	54 ± 3.6
30	72 ± 2.6
40	80 ± 2.1

Values shown as percentage mean ± SEM, n = 5.

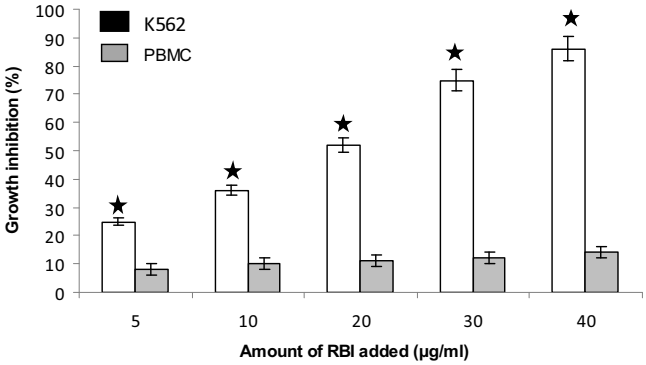

Fig. 2 Effect of K562 cells and peripheral blood mononuclear cells with varying concentrations of RBI by MTT assay. Values represent Mean ± standard deviation (n = 4) of three independent experiments. Student's *t*-test was performed. Asterisk indicates statistically significant difference. $P < 0.05$ was considered statistically significant in treated cells with respect to untreated cells.

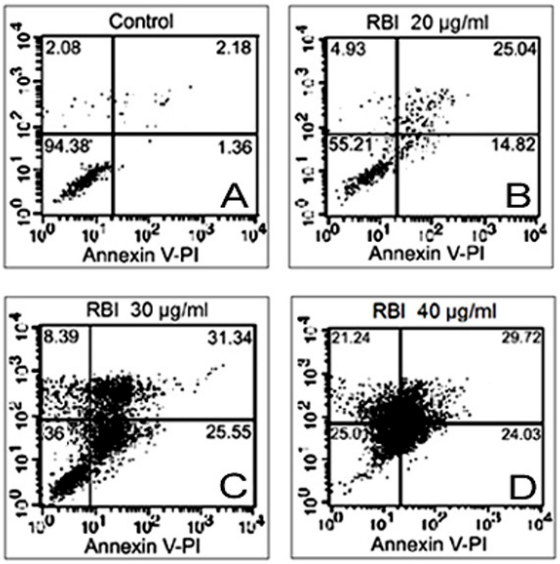

Fig. 3 Apoptosis inducing activity of RBI on Annexin-V/PI binding of K562 cells by flow cytometry. (**A**) K562 cells without RBI (control); (**B**) K562 cells treated with 20 µg/ml for 24 h; (**C**) K562 cells treated with 30 µg/ml for 24 h; (**D**) K562 cells treated with 40 µg/ml for 24 h. The relative percentage of intact/apoptotic/necrotic cells was analyzed by flow cytometry (n = 4).

IC_{50} value of RBI protein was 20 µg/ml, causing approx. 50% reduction in cell growth after 24 h.

Analysis of anti-proliferative activity of RBI by MTT Assay

K562 cells, after treatment with varying concentrations of RBI (5, 10, 20, 30 and 40 µg/ml for 24 h), showed distinct % growth reduction in absorbance, viz., 25 ± 1.8, 36 ± 2.7, 52 ± 4.3, 75 ± 3.8 and 86 ± 5.4, respectively in comparison to the untreated control cells. The mean values (mean ± standard deviation, n = 4) have been plotted in the graph. Human peripheral blood mononuclear cells, treated with varying concentrations of purified RBI for 24 h showed very negligible reduction in cellular proliferation. The IC_{50} value of RBI was determined as 20 µg/ml on K562 cells (**Fig. 2**).

Analysis of apoptotic activity by flow cytometry

The cellular toxicity of RBI towards K562 cells was analyzed by flow cytometry. The effect of varying concentrations of RBI on the induction of apoptosis of K562 cells was also examined (**Fig. 3**). After treating K562 cells with varying dose of RBI (20, 30, 40 µg/ml, 24 h) it was observed that 14.82, 25.55 and 24.03% of cells, respectively were in the lower right (LR) quadrant ($AnnexinV^+/PI^-$, early apoptotic cells) as compared to only 1.36% for the untreated control cells. Again, 25.04, 31.34 and 29.72% of cells, respectively were in the upper right (UR) quadrant ($AnnexinV^+/PI^+$, late apoptotic cells), in comparison to only 2.18% for the untreated control cells. This result clearly indicated the potentiality of RBI to induce apoptosis of K562 cells in a dose-dependent manner (**Fig. 3; Table 3**).

DISCUSSION

Ragi Bifunctional Inhibitor (RBI) from *ragi* (finger millet) seeds, inhibiting trypsin and α-amylase belongs to the cereal trypsin α-amylase inhibitor family. Several plant protease and α amylase inhibitors are known as biopesticides and plant defense molecules (Raj 2012). On the contrary, interestingly, several plant protease inhibitors have also been reported as anti-proliferative, apoptosis inducing and chemopreventive agents against human cancer cell lines. The present study has therefore been intended to explore the anti-proliferative activity *Ragi* bifunctional inhibitor (RBI) on K562 human chronic myeloid leukemia cells.

Table 3 Flow cytometric data showing the effect of treatment of K562 cells (10^6 cells/ml for 24 h) with RBI stained with AnnexinV and PI.

	LL quadrant	LR quadrant	UL quadrant	UR quadrant
Control K562	94.38 ± 1.12	1.36 ± 0.10	2.08 ± 0.04	2.18 ± 0.05
RBI (20 µg/ml)	55.21 ± 1.16*	14.82 ± 0.58*	4.93 ± 0.87	25.04 ± 0.07*
RBI (30 µg/ml)	36.7 ± 0.07*	25.55 ± 0.43*	8.39 ± 0.38*	31.34 ± 0.32*
RBI (40 µg/ml)	25.01 ± 0.75*	24.03 ± 0.73*	21.24 ± 0.56*	29.72 ± 0.27*

Values shown as percentage mean ± SEM; Student's *t*-test was performed. * $P < 0.05$ 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

From *ragi* seeds, RBI protein was purified to homogeneity by a unique method using affinity chromatography followed by FPLC size exclusion chromatography and its α-amylase and trypsin inhibitory activities were assayed. As the effect of purified RBI has been tested on K562 cells, growth inhibition of K562 cells by RBI in a dose dependent manner has been found. The IC_{50} has been determined as 20 µg/ml. The cytotoxic activity of RBI on K562 cells was determined by the MTT assay. MTT is a water-soluble tetrazolium salt. Mitochondrial succinate dehydrogenase enzyme of viable cells cleaves the tetrazolium ring and converts this to insoluble purple formazan. The disruption of mitochondrial dehydrogenase system due to the mitochondrial dysfunction or apoptosis reduces the colour production by formazan in the MTT assay. Interestingly, RBI induced growth inhibition and cytotoxicity was specific for cancer cells, as peripheral blood mononuclear cells remained unaffected by the treatment of varying concentrations of RBI. It indicates that RBI protein, obtained from a cereal, is safe and nontoxic to normal human cells. A critical stage of apoptosis involves the acquisition of surface changes by dying cells that eventually results in the recognition and uptake of these cells by phagocytes. Among the plasma membrane phospholipids, phosphatidylserine pre-dominates the inner surface facing the cytosol. In apoptotic cells the membrane phospholipid phosphatidylserine (PS) is translocated from the inner to the outer leaflet of the plasma membrane, thereby exposing PS to the external cellular environment. AnnexinV, having high affinity for PS, binds to cells with exposed PS. On the other hand, propidium iodide (PI) penetrates the permeable cell membrane of the dead or damaged cells. Staining cells simultaneously with AnnexinV-FITC (green fluorescence) and propidium iodide (red fluorescence) allows the discrimination of intact cells (FITC-PI-), early apoptotic (FITC+PI-) and late apoptotic or necrotic cells (FITC+PI+). In comparison to the untreated K562 cells, the cells treated with increasing concentration of RBI, showed gradual shift from viable to early apoptotic and then to late apoptotic phase, establishing the apoptosis inducing activity of RBI. This observation may be explained by the ability of protease inhibitors to regulate proteases, involved in various biological processes like cancer metastasis, tumor invasion, programmed cell death, etc. This observation is supported by the fact that *ragi* is not only a staple food grain for many people of India, Africa and Sri Lanka, but, also a wholesome food for diabetics and infants and a folk remedy for leprosy, liver disease, pleurisy, pneumonia and even small pox (Watt and Breyer-Brandwijk 1962). This study may be further supported by anti-cancer activity of plant protease inhibitors, viz., Bowman-Birk TI from Hokkaido large soybean (*Glycine max*) against breast cancer (MCF-7) and Hepatoma (HepG2) cells (Ho and Ng 2008; Ye and Ng 2009), and TI from *Fagopyrum esculentum* (buckwheat) against human T-acute lymphoblastic leukemia and K562 chronic myeloid leukemia cells (Park and Obha 2004; Wang *et al.* 2007). Additionally, Bowman-Birk protease inhibitor from *Vigna unguiculata* seeds was found to induce apoptosis and membrane permeabilization of breast cancer cells (Joanitti *et al*. 2010), Kunitz type TI from *Glycine max* (soybean), inhibited growth of ovarian cancer cells (Kobayashi *et al*. 2004) and Bowman-Birk inhibitors from soybean exerted cytotoxicity to HT29 colorectal cancer cells (Clemente *et al*. 2010) and prostrate adenocarcinoma and prostrate carcinoma cells (Tang *et al*. 2009). Furthermore, anti-proliferative activity of Bowman-Birk inhibitor of *Lens culinaris* (lentil) on human colon cancer cells has been reported recently (Caccialupi *et al*. 2010). These evidences have made the plant protease inhibitors to be considered as neutraceutical proteins, owing to prevenion and treatment of angiogenesis and metastasis of cancer cells (de Mejia and Dia 2010).

Drug resistance is a serious problem in cancer therapy. Therefore, medical and pharmaceutical companies need new drug candidates. Though imatinib mesylate, inhibiting BCR-ABL, has emerged as the frontline therapy for chronic myeloid leukemia (Deninger *et al.* 2005), secondary imatinib resistance has led to the development of second generation inhibitors nilotinib and dasatinib, capable of targeting most of the imatinib-resistant BCR-ABL variants (Deininger *et al*. 2005; Quintas-Cardama *et al.* 2007). However, some problems still persist: i) long-term tolerance of BCR-ABL inhibitors, ii) inability of BCR-ABL inhibitors to act against leukemia stem cells and iii) primary and secondary resistance, especially in advanced disease stages (O'Hare *et al*. 2007; Quintas-Cardama *et al.* 2007; Schiffer 2007). Like many other protein drug targets, BCR-ABL tyrosine kinase acts as a part of a large multi-protein complex forming a molecular network. However, instead of targeting BCR-ABL only by a drug, pleiotropic effects on a number of proteins of the molecular network by the action of a drug or a drug combination is more preferred today (Brehme *et al*. 2009). So, establishing new natural products for single or combinational therapy against chronic myeloid leukemia must be of ample interest.

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