Antioxidant and antileukemic properties of selected fenugreek (*Trigonella foenum-graecum* L.) genotypes grown in western Canada

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Acharya, S. N., Acharya, K., Paul, S. and Basu S. K. 2011. **Antioxidant and antileukemic properties of selected fenugreek (*Trigonella foenum-graecum* L.) genotypes grown in western Canada**. Can. J. Plant Sci. **91**: 99–105. Fenugreek (*Trigonella foenum-graecum* L.) is an annual forage legume known to have a number of important medicinal properties such as being anti-diabetic and hyperchloesterolaemic among others. In this study we have investigated the anti-oxidant and antileukemic properties of five fenugreek genotypes (L3068, L3375, Tristar, PI143504 and Amber) grown in western Canada for their potential use as nutraceuticals. Our preliminary experiments conducted in two different laboratories showed that the seeds grown in western Canada have anti-oxidant and antileukemic properties and the genotypes differ in the two traits studied. All the genotypes were found to be good scavengers for hydroxyl and free radicals. Among all the varieties, L3068 showed a higher EC_{50} value i.e., lower inhibitory activity for lipid peroxidation than the standard catechin. Although all five extracts showed significant antioxidant activity, the crude extract of Tristar was the most effective. Out of the five cultivars of fenugreek, Amber and L3375 showed a robust antileukemic activity followed by Tristar. Hence we conclude that Tristar has the best potential among all the genotypes tested to be used as a future nutraceutical crop when grown in western Canada.

Key words: Fenugreek, nutraceutical, *Trigonella foenum-graecum* L., Tristar, antioxidant, antileukemic

Acharya, S. N., Acharya, K., Paul, S. et Basu S. K. 2011. **Propriétés anti-oxydantes et anti-leucémiques de certains génotypes de fénugrec (*Trigonella foenum-graecum* L.) cultivés dans l'Ouest canadien**. Can. J. Plant Sci. **91**: 99–105. Le fénugrec (*Trigonella foenum-graecum* L.) est une légumineuse fourragère annuelle qu'on sait posséder d'importantes vertus médicinales, notamment dans la lutte contre le diabète et l'hypercholestérolémie. Les auteurs ont examiné les propriétés anti-oxydantes et anti-leucémiques de cinq génotypes de fénugrec (3068, L3375, Tristar, PI143504 et Amber) cultivés dans l'Ouest canadien en vue d'en établir le potentiel pour la production de nutraceutiques. Les résultats des expériences préliminaires effectuées dans deux laboratoires indiquent que les graines recueillies dans l'Ouest canadien ont des propriétés anti-oxydantes et anti-leucémiques, et que les génotypes diffèrent pour chacun des caractères. Les génotypes combattent tous bien les radicaux hydroxyles et les radicaux libres. Parmi les variétés étudiées, L3068 présente la plus haute CE_{50}, c.-à-d. une plus faible inhibition de la peroxydation des lipides que la catéchine, qui sert d'étalon. Bien que les cinq extraits démontrent une activité sensible comme antioxydant, l'extrait brut de Tristar s'avère le plus efficace. Sur les cinq cultivars de fénugrec, Amber et L3375, puis Tristar, se démarquent par une robuste activité anti-leucémique. Les auteurs en concluent que, des génotypes testés, Tristar est celui qui présente les meilleures possibilités en tant qu'éventuelle culture nutraceutique dans l'ouest du Canada.

Mots clés: Fénugrec, nutraceutique, *Trigonella foenum-graecum* L., Tristar, antioxydant, anti-leucémique

Fenugreek (*Trigonella foenum-graecum* L.), an annual forage legume belonging to the family Fabaceae, is well known as a herb and spice crop (Petropoulos 2002). This crop is grown as a spice crop in parts of North Africa, Mediterranean Europe, West and South Asia and parts of Australia. India is the largest producer of fenugreek in the world. In North American fenugreek is grown in Canada and the Unites States, and in South America it has been grown in Argentina. Tristar, developed at Agriculture and Agri-Food Canada, Lethbridge Research Centre, is the first forage cultivar of fenugreek in North America. Tristar fenugreek is well adapted to the dry land growing conditions and the Dark Brown soil zone of western Canada (Petropoulos 2002; Acharya et al. 2007a, b, 2008).

Abbreviations: **AAFC**, Agriculture and Agri-Food Canada; **BHT**, butylated hydroxylated toluene; **CLL**, chronic lymphocytic leukemia; **DPPH**, 1, 1-diphenyl-2-picrylhydrazyl; **EC_{50}**, half maximal effective concentration; **MDA**, malondialdehyde; **OH^-**, hydroxyl radicals; **PI**, propidium iodide; **RBC**, red blood cell; **TBARS**, thiobarbituric acid reacting substances; **TCA**, trichloroacetic acid

It is important to note that in addition to its traditional use as a spice and forage crop, fenugreek has been reported to be an important medicinal plant with a large number of medicinal properties such as being anti-diabetic, hypercholesterolemic, antileukemic, anti-nociceptive, antipyretic and antimicrobial, etc. [as reviewed in Acharya et al. (2008)]. A number of researchers have also reported its antioxidant properties (Genet et al. 2002; Thirunavukkarasu et al. 2003; Kaviarasan et al. 2004) and anti-lithogenic properties (Reddy and Srinivasan 2009a, b). The medicinal properties attributed to fenugreek have been reported to be associated with its unique phytochemicals, such as complex carbohydrates (galactomannans), steroidal sapogenins (diosgenin, yamogenin, tigogenin, neotigogenin), alkaloids (trigonelline) and amino acids (4-hydroxyisoleucine) [as reviewed in Acharya et al. (2007a, 2008)]. Our research group, while working on agronomic and yield traits of fenugreek, observed that the crop is significantly impacted by a strong genotype × environment interaction effect (Acharya et al. 2006, 2007a, b). We have also previously reported consistent yield variation and strong genotype × environment effects on different fenugreek accessions from our germplasm collection on both diosgenin content (Taylor et al. 1997, 2002) and antimicrobial properties (Thomas et al. 2006). Based on these studies and the observation of a significant genotype × environment effect we suggest that locally adapted cultivars need to be developed for any agronomic or medicinally important traits for optimal performance and performance consistency. It is from this perspective that we wanted to investigate the variations in antioxidant properties as well as antileukemic potential in the seed of different fenugreek lines grown in western Canada. Fenugreek as a crop has a great future in the nutraceutical and functional food industry due to its immense medicinal applications in human and animal health (Acharya et al. 2007a, b; Basu et al. 2007), and detecting suitable locally adapted genotypes with enhanced antioxidant and anti-lukemic properties could add to its usefulness.

Reactive oxygen species, which are produced continuously in cells and as accidental by-products of metabolism, are cytotoxic, and are important factors in several pathological conditions, such as cardiovascular diseases, cancer, inflammation, diabetes, etc. (Josh and Janardhanan 2000; Gomes et al. 2001). Antioxidants act as a major defence against radical-mediated toxicity by protecting against the damage caused by free radicals, such as reactive oxygen species (Manzi et al. 1999). The objective of this research was to understand the basic antioxidant property of fenugreek by looking at the crude extract of different fenugreek cultivars on hydroxyl and free radical scavenging activities, lipid peroxidation and DNA-sugar damage.

The role of natural products as a source for remedies has been recognized since ancient times (Farnsworth et al. 1985; Cragg et al. 1997). Despite major scientific and technological progress in combinatorial chemistry, natural products make an enormous contribution to the discovery of new drugs today. Nature is an attractive source of new candidate therapeutic compounds, as a tremendous chemical diversity is found in millions of species of plants, animals, marine organisms and microorganisms. An analysis of the number of chemotherapeutic agents and their sources indicates that more than 60% of approved drugs are derived from natural compounds (Cragg et al. 1997). To this day we do not have a cure for cancer, and the development of more effective antileukemic agents remains a viable goal. The most common forms of leukemia are resistant to the available antileukemic drugs (Yarbo et al. 1992; Sikora et al. 1999), and the majority of these agents have only limited anti-neoplastic activity. Furthermore, these agents have little impact on survival rates (Yarbo et al. 1992). This problem has recently been addressed in a contribution from medical oncologists from five continents, all arriving at the same conclusion on the inadequacy of current chemotherapeutic agents for the treatment of leukemia (Sikora et al. 1999). The other objective of this project was to unravel the antileukemic properties of fenugreek by looking at fenugreek extracts from different genotypes grown under uniform growing conditions so that good genotypes can be identified and included in the armament against chronic lymphocytic leukemia (CLL) in the future.

MATERIALS AND METHODS

Plant Material

Seeds of five different fenugreek lines with divergent origins grown in western Canada under dry land conditions were used for the study: L3068 (AAFC, Lethbridge collection CN# 19123, originated in Uttar Pradesh, India); L3375 (AAFC, Lethbridge collection, originated in China); Tristar (selected for forage production at AAFC, Lethbridge, Plant Gene Resource Canada, Saskatoon, SK, Canada, originated in the Shiraz area of Iran), PI143504 (Plant Gene Resource Canada, Saskatoon, SK, Canada, originated in the Hamadan area of Iran) and Amber (selected for seed production in AAFC, Morden, Manitoba, Canada, origin unknown) (Thomas et al. 2006).

Extraction

Crude extract was prepared from fenugreek seeds of the above lines by separately soaking 1 g of seed in 10 mL distilled water overnight. The next day, the water, along with the extracted materials, was filtered, lyophilized (Lyolab BII LSL Secfroid Lyophilizer; Aclens-Lausanne, Switzerland) and the lyophilized material was stored at –20°C for further use. The residue was resuspended in water or ethanol in concentrations as required.

Assay of Hydroxyl Radical

Hydroxyl radicals (OH^-) are generated from the Fe^{2+}-ascorbate-EDTA-H_2O_2 system (Fenton's reaction), which attack the deoxyribose and set off a series of reactions that eventually result in the formation of malondialdehyde (MDA), measured as a MDA- thiobarbituric acid (TBA) chromogen at 535 nm (Acharya et al. 2004; Halliwell et al. 1987). The reaction mixture (1 mL) containing deoxyribose (2.8 mM), KH_2PO_4-KOH (20 mM; pH 7.4), $FeCl_3$ (100 μM), EDTA (104 μM), H_2O_2 (1 mM) and ascorbate (100 μM) was incubated at 37°C for 1 h and colour developed as described above. The EC_{50} value of deoxyribose degradation by the crude extracts of different strains of fenugreek over the control was measured. Catechin was used as a positive control. All chemicals used for this and other assays were from Sigma.

Assay of Lipid Peroxidation

Lipid peroxidation was induced by the Fe^{2+} ascorbate system in human red blood cells (RBC) and estimated as thiobarbituric acid reacting substances (TBARS) following previously published protocols (Buege and Aust 1978; Acharya et al. 2004). The reaction mixture contained RBC-packed cell (10^8 cells mL^{-1}) in Tris-HCl buffer (20 mM; pH 7.0) with $CuCl_2$ (2 mM), ascorbic acid (10 mM) and the crude extracts of fenugreek in a final volume of 1 mL. The reaction mixture was incubated at 37°C for 1 h. Lipid peroxidation was measured as MDA equivalent using trichloroacetic acid (TCA), TBA and HCl (TBA-TCA reagent: 0.375% wt/vol TBA; 15% wt/vol TCA and 0.25 N HCl) (Acharya et al. 2002, 2004). The incubated reaction mixture was mixed with 2 mL of TBA-TCA reagent and heated in a boiling water bath for 15 min. After cooling, the flocculent precipitate was removed by centrifugation at $10\,000 \times g$ for 5 min. Finally, malondialdehyde concentration in the supernatant fraction was determined spectrophotometrically at 535 nm. The concentrations at which the reaction would be inhibited by 50%, the production of thiobarbituric acid reactive substances that is EC_{50} values, were calculated. Catechin, a synthetic antioxidant was used as a control.

1, 1-Diphenyl-2-Picryl Hydrazyl Assay

The hydrogen atom or electron donation abilities of the corresponding extracts and a pure compound were measured from the bleaching of the purple-colour methanol solution of 1, 1-diphenyl-2-picryl hydrazyl (DPPH). This spectrophotometric assay uses the stable radical DPPH as a reagent (Cuendet et al. 1997; Burits and Bukar 2000; Turkoglu et al. 2006). One millilitre of various concentrations of the crude, boiled and ehanolic extracts was added to 4 mL of 0.004% methanol solution of DPPH. After 30 min of incubation at room temperature the optical density was read against a blank at 517 nm. The scavenging ability was calculated as follows:

$$\text{Scavenging ability (\%)} = (A_{control} - A_{sample}/A_{control}) \times 100$$

Where $A_{control}$ is the absorbance of the control reaction (containing all reagents except the test compound), and A_{sample} is the absorbance of the test compound. EC_{50} value is the effective concentration at which DPPH radicals were scavenged by 50% and were obtained by interpolation from linear regression analyses. Bbutylated hydroxylated toluene (BHT; 2, 6- ditertiary-butyl-4-methyl phenol; Merck) was used as the control.

DNA Sugar Damage Assay

The iron-II dependent DNA sugar damage inhibition values were reported as the TBA reacting species in the presence of crude extracts of different strains of fenugreek in comparison with their absence (control). The reaction mixture contained 0.5 mL calf thymus DNA (1 mg mL^{-1} of 0.15 M NaCl), 0.5 mL sodium phosphate buffer (0.1 M; pH 7.4), 0.2 mL ferrous ammonium sulphate (4.8 mM), 1 mL of different extracts (100 μg mL^{-1} of ethanol) of fenugreek in a final volume of 2.2 mL. The reaction mixture was incubated for 1 h at 37°C in a water bath shaker. Then 1 mL 1% (wt/vol) TBA+1 mL 2.8% (wt/vol) TCA were added to the reaction mixture and kept in the boiling water bath for 15 min. The TBA reacting species (generated) were extracted into 2 mL n-butanol. The butanol extract was then centrifuged and fluorescence of the butanol layer was measured at 515 nm (excitation) and 555 nm (emission) (Halliwell and Gutteridge 1981; Imlay and Linn 1988).

Cell Culture

Freshly isolated CLL B-cells were resuspended at a density of $0.8 - 1.2 \times 107$ cells mL^{-1} in RPMI-1640 cell culture medium supplemented with 10% heat-inactivated foetal bovine serum, 100 U mL^{-1} penicillin, 0.1 mg mL^{-1} streptomycin, 2 mM L-glutamine and 1 mM sodium pyruvate (Invitrogen, Carlsbad, CA) and cultured at 37°C in a humified atmosphere containing 5% CO_2 (Pal et al. 2000).

Annexin V Staining

Freshly isolated CLL B cells were treated with different concentrations of fenugreek extracts. Cells were harvested and the percentage apoptosis was measured by flow cytometry after staining with flouresceinconjugated annexin-V and propidium iodide (PI) (Pal et al. 2004). Five concentrations (5, 10, 25, 50 and 100 μg mL^{-1}) of crude extract were used on five different cultivars of fenugreek seed. Chronic lymphocytic leukemia ($n = 6$) B cells were incubated with these crude extracts and then observed for the induction of

apoptosis. Viable cells were scored as those that were negative for annexin V and PI. The percentage of apoptosis was calculated as the reduction in the number of viable cells between the treated and untreated samples.

Statistical Analysis

Results were subjected to statistical analysis using Student's *t* test. Data are presented, as mean $\pm$ SD. Comparisons between groups were made with the paired Student's *t*-test. Values of $P < 0.05$ were considered statistically significant.

RESULTS AND DISCUSSION

Effect of Crude Extract on Hydroxyl Radical Scavenging Activity

When Ferric-EDTA was incubated with H_2O_2 and ascorbic acid at pH 7.4, hydroxyl radicals were formed in free solution and were detected by their ability to degrade 2-deoxy-2-ribose into fragments that formed a pink chromogen up to heating with TBA at very low pH ($\sim$0.2) (Aruoma et al. 1989). When the test extracts were added to the reaction mixture, they removed hydroxyl radicals from the sugar and prevented their degradation. All the crude extract of fenugreek lines showed potent hydroxyl radical scavenging activity (Table 1). The various extracts were effective in their ability to scavenge hydroxyl radicals in the following order of their EC_{50} value: Tristar > Amber $\geq$ L3375 > PI143504 > L3068. All the values were significantly lower ($P < 0.05$) than catechin (840 $\mu g\ mL^{-1}$), a synthetic antioxidant. These results indicate that all the varieties are good scavengers for hydroxyl radicals. Shi et al. (1991) reported scavenging activity of hydroxyl radicals of caffeine and attributed the alleged anticarcinogenic properties of caffeine to this activity. Accordingly, it is anticipated that the moderate to high scavenging effect of fenugreek may have an anticarcinogenic effect and can thus be expected to show some antimutagenic properties.

Effect of Crude Extract on Lipid Peroxidation

Lipid peroxidation, a process induced by free radicals, leads to oxidative deterioration of polyunsaturated lipids. A free radical prefers to steal electrons from the lipid membrane of the cell, initiating a free radical attack on the cell induced lipid peroxidation in polyunsaturated lipid rich areas like brain and liver (Coyle and Puttfarcken 1993). In this study the inhibitory effect of the crude extract of different fenugreek lines on lipid peroxidation as measured by the EC_{50} values of crude extract of various lines of fenugreek were in the following order: Tristar > L3375 > PI143504 > Amber > L3068 ($P < 0.01$) (Table 1). Among all the lines, only L3068 showed a higher EC_{50} value, i.e., a lower inhibitory activity for lipid peroxidation than the standard catechin. In general, polyphenols show much higher antioxidative activity (EC_{50}s in the range of $2.5 - 10\ \mu g\ mL^{-1}$). Considering the nature of the active ingredients in fenugreek (carbohydrates and steroidal components), this is not surprising. Also, the nature of the materials may affect their bioavailability, we are not sure of the plasma levels, and whether these components may show any significant antioxidative activity in vivo. Lipid peroxidation inactivates cellular components and thereby plays a key role in oxidative stress in biological systems. Several toxic by-products of lipid peroxidation can damage other biomolecules, including DNA, although these biomolecules are distant from the site of their generation (Box and Maccubbin 1997).

Free Radical Scavenging Activities of Crude Extract by DPPH Assay Technique

DPPH is a stable free radical possessing a characteristic absorbance at 517 nm, which decreases significantly on exposure to radical scavengers by providing a hydrogen atom or electron donation to become a stable diamagnetic molecule (Dash et al. 2005). The use of a stable DPPH radical has the advantage that it is unaffected by side reactions, such as enzyme inhibition and metal chelation (Wettasinghe and Shahidi 1999) and thus is a reliable measure of radical scavenging activity of the fenugreek extracts.

The radical scavenging activities of crude extracts from five different fenugreek lines are presented in Fig. 1. Results are expressed as the ratio of percentage of sample absorbance decrease and the absorbance of DPPH solution in the absence of extract at 517 nm. The DPPH radical scavenging activity of crude extracts of five different fenugreek lines were expressed as EC_{50} value. The radical scavenging activities (EC_{50} values) of

Table 1. In vitro hydroxyl radical scavenging and inhibition of lipid peroxidation assays as measured by EC_{50} ($\mu g\ mL^{-1}$) on the crude extracts of fenugreek genotypes

	Fenugreek genotypes					
	L3068	L3375	Tristar	PI143504	Amber	Standard (catechin)
Scavenging of OH radical	648.1 $\pm$ 85.1[z]	528.5 $\pm$ 49.4	502.4 $\pm$ 76.1	549.8 $\pm$ 73.0	526.5 $\pm$ 56.9	840.0 $\pm$ 45.0
Inhibition of lipid peroxidation	598.9 $\pm$ 30.0	280.7 $\pm$ 36.8	267.6 $\pm$ 12.5	297.8 $\pm$ 55.0	312.9 $\pm$ 40.5	450.0 $\pm$ 25.0

[z]Values are the means $\pm$ SD from three independent observations, each in triplicate.

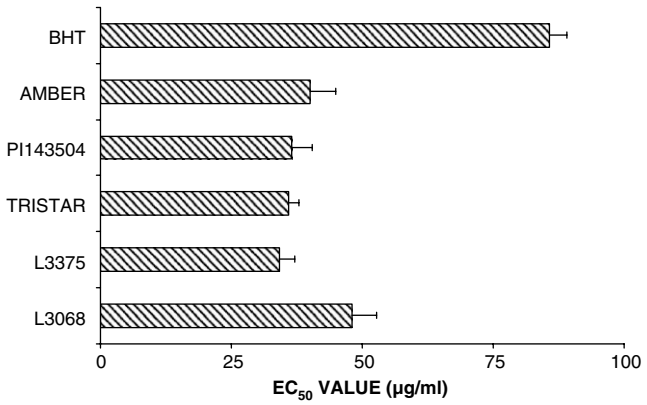

Fig. 1. The 1, 1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging capacity of crude extracts of five fenugreek genotypes. Results are mean ± SD of three separate experiments, each in triplicate.

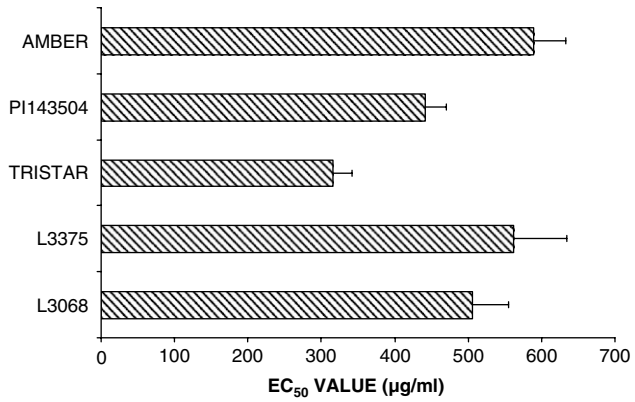

Fig. 2. The inhibition of DNA-sugar damage by crude extract of five fenugreek genotypes. Results are mean ± SD of three separate experiments, each in triplicate.

extracts were observed to be in the following order: L3375 (34.10 ± 3.21 µg mL^{-1}) > Tristar (35.90 ± 2.05 µg mL^{-1}) > PI143504 (36.51 ± 4.01 µg mL^{-1}) > Amber (39.99 ± 5.00 µg mL^{-1}) > L3068 (48.05 ± 4.63 µg mL^{-1}). The EC_{50} values for all the fenugreek lines grown in western Canada were significantly lower ($P < 0.01$) than that of standard synthetic antioxidant BHT ($EC_{50} = 85.70 \pm 3.40$ µg mL^{-1}) indicating that fenugreek extracts have a better ability to protect from impact of free radicals than BHT.

Effect of Crude Extract on DNA Sugar Damage

DNA is also a major target of free-radical-induced damage. Under physiological conditions, the constant and endogenous rate of production of free radicals may lead to minimal DNA damage, which is needed to induce the defensive systems and DNA repair mechanisms. However, if the production of free radicals increases, they may attack DNA at either the sugar (deoxyribose) or the base level, giving rise to a large number of toxic products. Attack at the sugar level ultimately leads to a strand break with terminal fragmented sugar residues (Imlay and Linn 1988). Protection by crude extracts of fenugreek against DNA damage was determined in terms of the damage of its deoxyribose sugar moiety. Although all the five extracts showed significant antioxidant activity, the crude extract of Tristar was the most effective (Fig. 2). EC_{50} values in DNA sugar damage assay were 589.57, 562.32, 505.57, 441.89 and 316.00 µg mL^{-1} for Amber, L3375, L3068, PI143504 and Tristar, respectively.

From the above results it is clear that crude extract of Tristar fenugreek seed has good in vitro and ex vivo (lipid peroxidation test using RBC in test tubes) antioxidant activity, suggesting its therapeutic value for human health. Although the cultivar was developed for forage production its seeds have medicinal value for several killer diseases.

Apoptotic Effect of Fenugreek Seed Extract on CLL B Cells

To explore the antileukemic potential of crude extracts of fenugreek in chronic lymphocytic leukemia B cells, we conducted flow analysis. We examined the cytotoxic effects of fenugreek seed extracts varying in concentrations from 0 to 100 µg mL^{-1} (48-h treatment) on the CLL B cells. A dose-dependent induction of apoptosis was observed in CLL B cells treated with fenugreek seed extract (Fig. 3). Out of the five fenugreek lines, Amber and L3375 showed a robust antileukemic activity. Other lines, including Tristar, showed a modest effect as far as apoptosis of CLL B cells is concerned. The IC_{50} values of Amber and L3375 were 25.2 and 29.5 µg mL^{-1}, respectively. This preliminary study opens up the

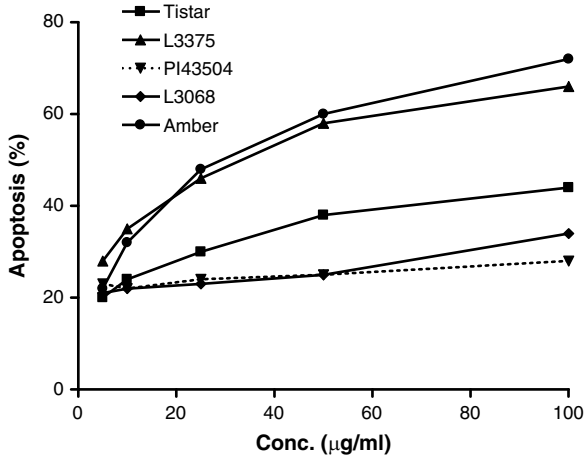

Fig. 3. Induction of apoptosis by five fenugreek seed extracts at 48 h of induction as evidenced by Annexin-PI staining. Results are mean of three separate experiments, each in triplicate.

possibility of some compounds present in western Canada grown Amber and L3375 seeds, which may be a potential drug for the treatment of CLL.

Finally we conclude that Tristar fenugreek shows great potential as a locally adapted cultivar suited to the dry land region of the prairies in western Canada for future nutraceutical use. Although our study is preliminary and needs to be validated by studies conducted over a few more years and locations, it is clear that the fenugreek seed has antioxidant and antileukemic (or anti-proliferative/cytotoxic) effects. More importantly the five fenugreek lines with diverse origin showed genotypic difference for both traits studied, indicating the possibility of genetic improvement of this crop through selection.

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