

Comparison of two fenugreek seed genotypes: bitterness value, secondary metabolites contents and biological activities

H. ABDOULI^{1*}
H. MISSAOUI¹
S. JELLALI¹
G. TIBAOUI¹
L. TAYACHI¹

¹Laboratory of Improvement & Integrated Development of Animal Productivity & Food Resources, Higher School of Agriculture of Mateur, University of Carthage, Tunisia

*Corresponding authors: abdoulihedi@gmail.com

Abstract - In order to make fenugreek seeds more compatible with consumer acceptance, a claimed sweet genotype has been recently developed and registered in Tunisian seeds catalogue. Therefore, the objective of this study was to compare its bitterness value, secondary metabolites contents and biological activities with those of the commonly known bitter genotype. The sweet genotype had lower ($P<0.05$) bitterness value than bitter one (702 vs 5028 units/g). There were no differences ($P>0.05$) in total phenols (6.16 vs 5.92 mg/g), total tannins (2.59 vs 2.03 mg/g), condensed tannins (0.027 vs 0.023mg/g), total flavonoids (0.318 vs 0.313 mg/g), haemolytic and total antioxidant activities. Haemolytic and total antioxidant activities showed concentration dependent manner. At 2.5 mg fenugreek seed/ml, haemolytic activity was 80.2 vs 88.7 % of that of distilled water. Antioxidant activities varied ($P<0.05$) with the solvents, the highest was shown by aqueous followed by methanol and then ethanol extracts. Antioxidant activities of the aqueous extracts were 14 and 12.5% of that of vit.C for the bitter and the sweet genotypes, respectively. It was concluded that the findings confirm some key similarities between the bitter and the sweet genotypes.

Key words: Fenugreek seeds / bitterness value / haemolytic activity / antioxidant activity



1. Introduction

Fenugreek, *Trigonella foenum graecum*, has been receiving more and more attention as a multipurpose plant. It has been used since ancient times as a spice, forage and medicinal crop. More recently, fenugreek has been used as a functional food for human, and for diosgenin extraction to serve in the synthesis of steroidal sapogenins such as cortisone and sex hormones. These numerous uses are due to the high proteins, soluble fiber and phytochemicals contents of the seed. Fenugreek seed biologically active phytochemicals include tannins, flavonoids, terpenoids, carotenoids, saponin, sterols, alkaloids, fixed and volatile oils, minerals, vitamins etc. Some of these bioactive constituents convey a characteristic smell, a pungent aroma and a bitter taste to the seed. Generally, the bitter taste is mainly due to the oil, saponins, tannins and alkaloids while the odor is due to many compounds. In this regard, Shah and Mir (2004) and Al-Shaikh (2007) reported that the aroma and the taste of FS are not transmitted to milk and meat. Likewise, broiler chicks' carcass characteristics and sensory attributes have been found to be insensitive to fenugreek seed feeding (Yatoo et al. 2012). Thus, such components may be reduced by fenugreek seeds crushing and their exposure to air before ingestion or destroyed in the gastrointestinal tracts. Furthermore, the process of de-fatting, grinding, roasting and pre-germination can reduce not only raw fenugreek seeds flavor and taste but also its trypsin and α -chymotrypsin inhibitors activities (Al-Maiman 2004). Despite these alternatives, providing sweet or less bitter genotypes that preserve their biological activities would be more convenient for both consumer taste and health. In view of this, the objective of this study was to compare a claimed sweet genotype that has been recently developed and registered in Tunisian seeds catalogue with the commonly known bitter cultivated genotype in the country. The comparison was limited to the bitterness value and some secondary metabolites e.g. total phenol, tannins, flavonoids and biological activities e.g. total antioxidant and haemolytic activities.

2. Materials and methods

2.1. Material

Two fenugreek seed genotypes were used: a claimed sweet genotype registered in 2009 in Tunisian seeds catalogue as 'RIHANNA' and a bitter non identified genotype yet commonly cultivated and used for human and animal feeding. The later was kindly offered by a farmer from Mateur (60 km north Tunis, Tunisia). The seeds used in this study were harvested in July 2012.

2.2. Analyses

Three separate samples from each genotype were milled to pass through a 0.5 mm sieve and then crashed in a glass mortar to a fine powder. They were stored during the study in tight containers at room temperature

2.2.1. Bitterness value.

The bitterness value was evaluated by comparing the threshold bitter concentration of aqueous seed extract (minimum concentration that still has a bitter taste) with that of a dilute solution of quinine hydrochloride as outlined in WHO (1998). Thus, the bitterness value of the solution containing 1 g of quinine hydrochloride in 2000 ml of drinking water is set at 2×10^5 units. Extraction was carried out by stirring 100 mg fine powder in 45 ml safe drinking-water for 1hr, filtering, diluting the filtrate to 50 ml and again diluting 1ml of the mixture to 100ml with safe drinking-water. A taste panel comprising 6 students was used. The students previously appreciated the bitter sensation of a solution containing 0.058 mg quinine hydrochloride in 10ml of water. The Bitterness value for each panel member was calculated from the expression:

$$\text{Bitterness Value (unit/g)} = (2000 \times C)/(A \times B)$$

Where:

A= mg FS/ml extract before dilution

B= ml of extract in the dilution of the lowest concentration that was judged to be bitter

C = mg quinine-HCl in the dilution of the lowest concentration that was judged to be bitter

2.2.2. Total phenolics and tannins contents.

Seed extract was prepared by twice stirring 100 mg powder in 5 ml diethyl ether containing 1% acetic acid for 15 min, centrifugation for 20 min at 2000rpm,



discarding the supernatant and then twice extracting the residue in 5 ml acetone (70%, v/v) for 1 hr. Total phenolic content was determined in acetone extract according to the Folin-Ciocalteu method reported by Makkar et al. (1993) and was expressed as gallic acid equivalent in mg/g dry matter. Total tannin content was determined as the difference between total phenolic contents before and after mixing 2ml of the diluted acetone extract with 100 mg polyethylene glycol (PEG4000) for 15 min at 4°C. Thus, the PEG was used to precipitate the tannins leaving other phenols in the supernatant. Condensed tannins content was estimated according to the method of Porter et al. (1986).

2.2.3. Total flavonoids content.

Total flavonoids content was determined using aluminum chloride method as reported by Patel et al. (2010). Fenugreek seed extract was prepared by twice stirring 100 mg of de-fatted powder in 5 ml of 80% methanol for 5 hr, and centrifugation for 20 min at 2000rpm. The supernatants were combined and their volume adjusted to 50 ml with distilled water and filtered on Wathmann 54. A standard calibration plot was generated using known concentrations of catechin (+catechin, cat No:88191-48-4, Sigma). Total flavonoids content of the extracts was expressed as mg catechin equivalent /100 g dry matter.

2.2.4. Total antioxidant activity.

Aqueous, methanolic and ethanolic seed extracts were prepared by separately stirring 1g powder in 40 ml of each solvent for 24 hr and centrifuging for 30 min at 3000 rpm. The supernatants were decanted, dried at 40 °C, weighed and dissolved in distilled water before determination. The phosphomolybdenum assay method (Prieto et al. 1999) was used for measuring the antioxidant activity of different volumes each seed extract and of different volumes of a solution of Ascorbic acid (vit.C, 100ug/ml). The method involves the reduction of Mo (VI) to Mo (V) with seed extracts or ascorbic acid and the subsequent formation of a green phosphate-Mo (V) complex in acidic condition. Different volumes of the seed extracts (0.1, 0.2 and 0.3 ml) and of the vit.C solution (0 to 1ml) were adjusted to 2 ml with distilled water and combined with 2 ml of reagent solution (0.6 M sulfuric acid, 28 mM sodium phosphate and 4 mM ammonium

molybdate). The reaction mixture was incubated at 95°C for 90 min and cooled to room temperature. Then the absorbance of the solution was measured at 695nm (A_{695nm}) using an UV-visible spectrophotometer against a blank. A straight line relating ascorbic acid concentrations (mg Vit. C /ml in Y-axis) to their corresponding absorbance (A_{695nm} in X-axis) was established. The slope of the line (A_{695nm} /mg vit.C/ ml) was used to express the antioxidant activity of seed extracts as the number of mg equivalent of ascorbic acid (mg vit.C Eq) as follows:

mg vit.C Eq. = slope x A_{695nm} of seed extract
Total antioxidant activity was then expressed as A_{695nm} /mg seed /ml and mg vit.C Eq./mg seed/ml using regression of A_{695nm} and mg vit. C Eq on mg seed in 1 ml of the reaction mixture used for absorbance measurement.

2.2.5. Haemolytic activity.

Haemolytic activity was evaluated by the method of Monkiedje et al. (1990). Erythrocyte suspensions were freshly prepared as follows: blood was collected from two ewes by jugular venupuncture into heparinized Vacutainer™ tubes which were placed in ice and transported to the laboratory and centrifuged within 15 min. Then erythrocytes pelleted by centrifugation (2000rpm, 15 min) were washed three times with isotonic pH 7.4 phosphate buffer (PB). Washed erythrocytes (2 ml) were suspended in 98 ml of this buffer. Fenugreek seed extract was prepared by twice stirring 100 mg of powder in 5 ml methanol for 1 hr on a hot plate (60°C) and centrifuging for 20 min at 3000rpm. The supernatants were combined and filtered on Wathmann 54. Methanol was evaporated in an oven at 62 °C and the remaining extract was dissolved in the PB. Different volumes (0.1, 0.3 and 0.5 ml) of seed extracts and of a solution of Quillaja saponin (15 mg/50 ml PB) adjusted to 4 ml with the PB, 4 ml of PB and 4ml of distilled water were mixed with 1 ml of 2% erythrocyte suspension and incubated for 1 h at 37°C. Following incubation, tubes were kept at room temperature for 5 min, then centrifuged (3000 rpm, 5 min) and the absorbance at 540 nm was measured in the supernatants. Absorbance values were corrected for blank (PB). Distilled water caused complete erythrocytes lysis and, therefore, haemolytic activity of fenugreek seed and of saponin was expressed as



absorbance (A_{540nm}) and as % of that of distilled water.

2.3. Statistical analysis

Data of secondary metabolites were subjected to statistical analysis using Student's t-test and those related to bitterness value and biological activities were subjected to a two-way ANOVA (fenugreek seed genotypes and either taste panel or solvents used for extraction). Comparisons between solvents were made with the DUNCAN test. Values of $P < 0.05$ were considered statistically significant.

3. Results and discussion

3.1. Bitterness value

Results from the determination of bitterness value are shown in Table 1. While the panel affected ($P > 0.05$) neither the volume of seed extract in the lowest dilution that was judged

to be bitter (B in Table 1) nor the bitterness value, these were both significantly different between the two seed genotypes. The mean bitterness value of the sweet genotype was more than 7 times lower than that of the bitter one. The panel members appeared to be of the same sensitivity to the bitterness of quinine hydrochloride (C in Table 1). Their appreciation of fenugreek seed bitterness was less homogenous as indicated by the wide bitterness value ranges in Table 1. However, although the determination of bitterness by taste might have been subjective, the difference between the mean values was large enough to qualify the tested seed genotype as sweet. Despite the many published studies on fenugreek seed, there was no reported its bitterness value.

Table1: Bitterness value of the two fenugreek seed genotypes.

Fenugreek seedS genoty]	*C (mg)	**B (ml)	***BV (Units $\times 10^{-3}/g$)			
			mean	minimum	maximum	SD
Bitter	0.039 \pm 0.003	5.11 \pm 2.32	5.028 ^a	2.25	9.50	2.865
Sweet	0.039 \pm 0.003	3.50 \pm 1.93	0.702 ^b	0.271	1.110	0.301

*C = mg quinine-HCl in the dilution of the lowest concentration that was judged to be bitter; **B= ml of extract in the dilution of the lowest concentration that was judged to be bitter; ***BV=bitterness value; ^{a,b} means of the same column are different ($P=0.0001$)

3.2. Secondary metabolites.

Fenugreek seeds are a rich source of bioactive phytochemicals including polyhenols and flavonoids. Table 2 shows such compounds in the two fenugreek seed genotypes. There was no difference ($P > 0.05$) between the two genotypes in total phenolics, total and condensed tannins and total flavonoids contents. The phenols levels were higher than those reported by Kochhar et al. (2006), Hooda and Jood (2002 and Amany et al. (2012) which were 1.05; 1.48 and 0.447 mg/g, respectively. They were lower than that (10 mg/g DM) found by Naseri et al. (2013) and in agreement with those of Bukhari et al. (2008) who showed total phenolics contents in the range of 1.35-6.85 mg/g of the fenugreek extract in different solvents. However, Fenugreek seeds also had been found to contain total phenols at

much higher levels varying throughout their maturity from 9.42 to 16.22 mg/g (singh et al.1994). In the current study, tannins in both fenugreek seed genotypes were near 99% in hydrolysable form. Their levels were close to that (3.8mg/g DM) reported in Iranian fenugreek seed genotype (Naseri et al. (2013) and lower than levels (20mg/g) reported in Yemen genotype (Yaser et al. 2013). Measured levels in the two seed genotypes were far below the threshold level (50 mg/g DM) in tropical tannin rich plants considered to have antinutritional effects for ruminants (Muller-Harvey, 2006). Consequently, tannins in fenugreek seed may have no adverse effect or even be beneficial. Total flavonoids contents in both genotypes were within the range (208-653 μ g/g) reported by Bukhari et al. (2008) and lower than those (1.36 to 2.74 mg/g) found by



Singh et al. (1994) in seeds of different maturity stages and the levels (49.9mg/g)

reported by Yaser et al. (2013).

Table 2: Total phenols, tannins and flavonoids (mg/g DM) (means \pm SD)

Fenugreek seed genotypes	Total phenols	Total Tannins	Condensed tannins	Total flavonoids
Bitter	5.92 \pm 0.45	2.03 \pm 0.57	0.023 \pm 0.0005	0.313 \pm 0.0167
Sweet	6.16 \pm 0.59	2.59 \pm 0.55	0.027 \pm 0.001	0.318 \pm 0.0321

The similarity in total phenols, tannins and flavonoids contents of the two fenugreek seed genotypes despite the large difference in their bitterness values was not expected as these compounds are known to be responsible for the bitter taste (Delcour et al. 1984; Chung 1998; Bravo 1998). Differences might have existed in detailed composition of each of these groups of phytochemicals or in other non measured bitter compounds like saponins and alkaloids (Rao et al. 1996). It is reported that while lower-molecular-weight phenolic compounds tend to be bitter, higher-molecular-weight polymers are more likely to be astringent, and while some flavonoids are very bitter others are not (Drewnowski and Gomez-Careros 2000). Amounts of phenolic compounds in plant foods and the level of bitterness are also reported to be influenced by genetic factors and by environmental conditions (Chung et al. 1998).

3.3. Biological activities.

Secondary metabolites are known to exert beneficial as well as adverse effects, depending on their chemical nature and concentrations in feeds and on the animal species. In the current study, measured fenugreek seed biological properties were limited to the total antioxidant activity and haemolytic activity as these properties may reflect differences in the nature and levels of the secondary metabolites of the two fenugreek seed genotypes.

3.3.1. Haemolytic activity.

Haemolytic activity of methanolic fenugreek seed extracts at three concentrations, of distilled water taken as reference (100%) and of Quillaja saponin at two concentrations are shown in table 3. Haemolytic activity of the bitter genotype was slightly higher than that of the sweet one. Unlike haemolytic activity of Quillaja saponins, those of the two fenugreek

seed genotypes showed concentration dependent manner. Haemolytic activity of both fenugreek seed extracts corresponding to 2.5 mg seed powder /ml of the final reaction mixture were close to those of Quillaja saponins at concentrations of 0.018 and 0.03 mg /ml of the final reaction mixture. Although, not all saponins exhibit haemolytic activity (Jenkins & Atwal 1994; Gestetner et al.1968), haemolysis due to fenugreek seed extracts measured in the present study might have been a reflection of saponins levels in the seed. Consequently, in concordance with the commonly accepted use of the haemolytic assay as an index of the biological activity of saponins (Price et al. 1987), the herein data suggested that both fenugreek seed genotypes had similar biological properties ascribable to saponins. To the authors knowledge there is no published study on haemolysis due to purified fenugreek seed saponins. In contrast, fenugreek seed extracts has been found to protect erythrocytes from oxidative damage induced by hydrogen peroxide (Kaviarasan et al. 2004). In vivo, fenugreek seed used as dietary supplement in clinical trial had been found to be safe and had good beneficial effects to raise blood hemoglobin (Doshi et al. 2012). To the authors knowledge, only one haemolysis case was reported in a patient with glucose -6- phosphate dehydrogenase (G6PD) deficiency and taking a teaspoonful of fenugreek seed once a day (Sadler et al. 2009).



Table 3: Haemolytic activities of fenugreek seeds methanolic extracts compared to those of distilled water and Quillaja Saponin (means \pm SD)

	Concentration, mg/ml	Haemolytic activity	
		A _{540nm}	%
Distilled water		0.123 \pm 0.01	100
Quillaja Saponin	0.018	0.100 \pm 0.014	82.00 \pm 11.53
	0.03	0.112 \pm 0.001	91.51 \pm 0.94
Bitter fenugreek seed	0.5	0.020 \pm 0.002	16.84 \pm 2.24
	1.5	0.066 \pm 0.007	54.06 \pm 5.96
	2.5	0.108 \pm 0.004	88.69 \pm 3.86
Sweet fenugreek seed	0.5	0.018 \pm 0.001	14.94 \pm 0.94
	1.5	0.052 \pm 0.007	42.38 \pm 6.15
	2.5	0.098 \pm 0.010	80.27 \pm 8.93

3.3.2. Total antioxidant activity.

The yields of fenugreek seed extractions and the antioxidant activity of the extracts and of vit. C used as standard reference are shown in Tables 4 and 5 and in fig.1. The reducing activity which is related to the antioxidant activity is one of the numerous fenugreek seed virtues. Such property is due to the presence of many active phytochemicals which act as reducing agents (hydrogen atom donors and thereby free radical terminators). Because the extraction of these compounds is sensitive to the solvent, three solvents of different polarities, namely, water, ethanol and methanol were used in the present study. Also among the many in vitro methods that can be used to measure the antioxidant activity, the phosphomolybdenum assay method involving the reduction of Mo (VI) to Mo (V) was used. The yields of the extracts (table 4) were different ($P < 0.05$) between the solvents but not

($P > 0.05$) between the fenugreek seed genotypes. Percentage yields in water were the highest and those in methanol were the lowest. Bukhari et al. (2008) found close yield in ethanol (25.32%) and higher yield in methanol (25.89%) than the current ones. Successive extraction of fenugreek seed in different solvents showed the presence of saponins in water and methanolic extracts, flavonoids in methanolic extract, and the absence of tannins and alkaloids in these extracts (Chauhan et al. 2011). Subhashini et al. (2011) found alkaloids, flavonoids, saponins, and tannins in fenugreek seed 70 % ethanolic extracts. Water should extract polar compounds like flavonoids, tannins, alkaloids and saponins (Snyder and Kirk 1979) and organic solvents extract less polar compounds (Ali-Emmanuel et al. 2002).

Table 4: Yield of fenugreek seed extraction (g DM/100 g DM; means \pm SD)

Fenugreek seed	Solvents		
	Ethanol	Water	Methanol
Bitter	27.02 \pm 0.54	43.04 \pm 9.02	17.09 \pm 1.43
Sweet	29.07 \pm 0.18	43.27 \pm 7.024	18.68 \pm 1.06

The plot of vit C concentrations vs its antioxidant activity expressed in terms of absorbance (A_{695nm}) resulted in a straight line with a slope (A_{695nm}/mg vit.C ml⁻¹) of 0.089. Such value was used to calculate the antioxidant capacity of fenugreek seed extracts

in terms of mg equivalent of vit.C. As illustrated in Fig1, shown as an example, the antioxidant activity of the two fenugreek seed genotypes measured in the three solvents increased with fenugreek seed concentrations corresponding to extracts volumes used for



absorbance reading. Individual data were not shown for all fenugreek seed concentrations. Instead, slopes of lines relating antioxidant

activity to fenugreek seed concentrations were presented in table 5.

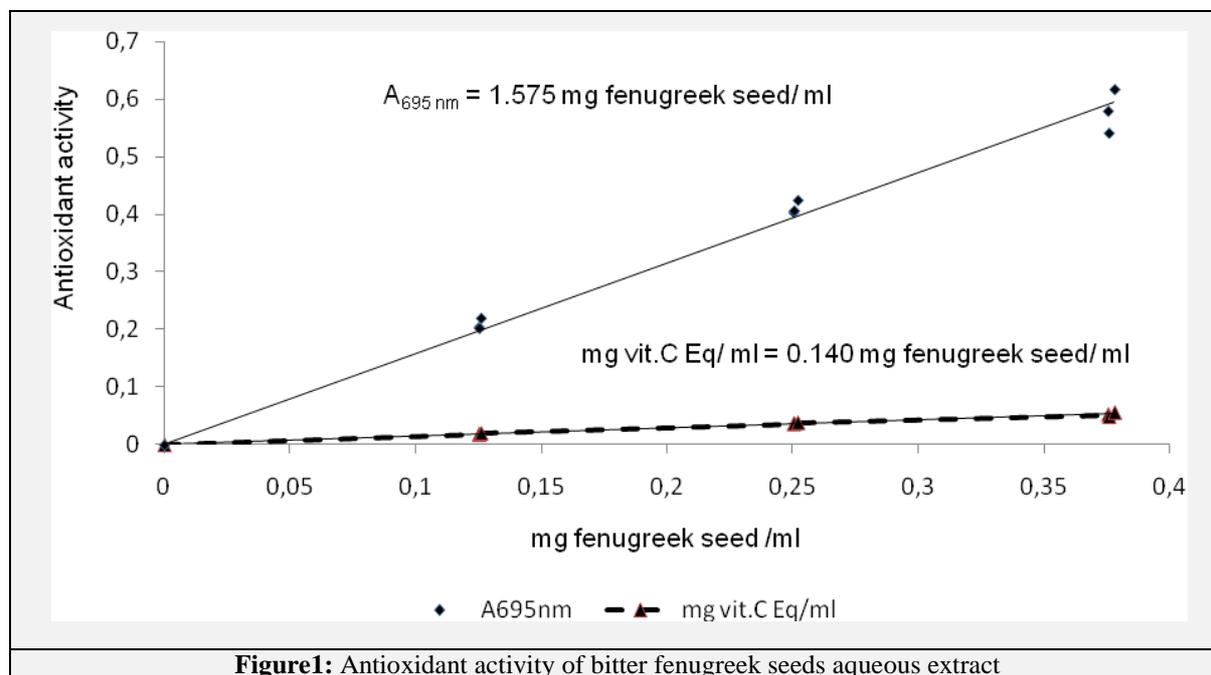


Figure1: Antioxidant activity of bitter fenugreek seeds aqueous extract

Antioxidant activity was not different ($P > 0.05$) between the two fenugreek seed genotypes and varied ($P < 0.05$) with the solvents. The highest antioxidant activity was shown by aqueous followed by methanol and then ethanol extracts. Thus, aqueous extract corresponding to 1 mg fenugreek seed per ml reaction mixture had an antioxidant activity measured by the absorbance of 1.557 and 1.405 for the bitter and the sweet genotypes, respectively. These values were translated to 0.140 and 0.125 mg vit.C equivalent, meaning that the antioxidant activity of fenugreek seed was 14 and 12.5% of that of vit.C for the bitter and the sweet genotypes, respectively. The antioxidant activity was around 6.6 and 1.9 % of that of vit.C for both seed genotypes in ethanol and methanol extracts, respectively. Differences in the antioxidant activity between the three solvents suggested the presence of differences in the nature and amounts of antioxidant compounds in the extracts. These results were in agreement with that reported by Meghwal and Goswami (2012) who cited: 'an aqueous fraction of fenugreek exhibits the highest antioxidant activity compared to other fractions.' Bukhari et al. (2008) used the ferric reducing antioxidant power method to compare the antioxidant activity of butylated

hydroxyanisole (BHA) to those of fenugreek seed extracts in ethanol, methanol, dichloromethane, acetone, hexane and ethyl acetate. They found that the antioxidant activity increased with an increase in the amount of the extracts, antioxidant activity of the extracts was markedly lower than that of BHA, and the ethanol extract showed the highest antioxidant activity. They also found the highest amount of phenolic compounds in ethanolic extract indicating that total phenolics play a major role in the antioxidant activity of fenugreek seed. In this regard, antioxidant activity of methanolic extract of fenugreek seed using various in vitro assay systems was found to be correlated with the polyphenolic components present in the extract (Kaviarasan et al. 2007). In other studies, flavonoids and polyphenols were found to be responsible for fenugreek antioxidant activity (Rababah et al. 2004; Dixit et al. 2005). Whether phenols or flavonoids were the main fenugreek seed constituents responsible for the antioxidant activity, these were in similar amounts in the two fenugreek seed genotypes (see above) as was the observed similarity of their total antioxidant activity. Hence, it was suggested that other related properties would have been similar in both fenugreek seed.

Solvents	Fenugreek seed genotypes	Total antioxidant activity	
		A _{695nm} / mg fenugreek seed/ ml	mg vit. C Eq./mg fenugreek seede/ ml
water	Bitter	1.557 \pm 0.076	0.1406 \pm 0.006
	Sweet	1.405 \pm 0.386	0.1255 \pm 0.0346
Ethanol	Bitter	0.2157 \pm 0.014	0.0192 \pm 0.0016
	Sweet	0.2145 \pm 0.012	0.0191 \pm 0.0010
Methanol	Bitter	0.7396 \pm 0.0097	0.066 \pm 0.0008
	Sweet	0.7686 \pm 0.020	0.0686 \pm 0.0019

4. Conclusion

Put together, the aforementioned results provided evidence to support the preservation of some of the well documented desirable properties of common bitter fenugreek seed through selection for a less bitter genotype. However, further studies shall be made to determine the chemical structure and in vivo biological activities of the compounds that have been selectively bred out from the sweet genotype.

5. References

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