Original Article

Phenolics, their antioxidant and antimicrobial activity in dark germinated fenugreek sprouts in response to peptide and phytochemical elicitors

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The phenylpropanoid pathway (PPP) was stimulated in fenugreek sprouts through the pentose phosphate and shikimate pathway, by natural elicitors such as Fish Protein Hydrolysates (FPH), Lactoferrin (LF) and Oregano Extract (OE). Among treatments 0.5 ml /L FPH elicited fenugreek sprouts had the highest phenolic content of 0.75 mg/g FW on day 3 of germination which was approximately 25 % higher than control on the same day. The antioxidant activity estimated by β-carotene assay was highest for LF and OE elicited sprouts on day 2 and 4, respectively with an antioxidant protection factor (APF) of 1.47 for both. In all treatments and control, higher antioxidant activity was observed during early germination, which correlates to higher phenolic content, suggesting that initially phenolics are antioxidant in nature. This increased activity also correlates with high guaiacol peroxidase (GPX) activity indicating that the polymerized phenolics required for lignification with growth have antioxidant function. The antioxidant activity as estimated by β-carotene and 1,1,-diphenyl-2-picryl hydrazyl (DPPH) assays indicate that fenugreek sprout extract can quench the superoxide free radical and also possibly scavenge the hydrogen peroxide generated in the reaction mix. OE elicited the highest levo dihydroxy phenylalanine (L-DOPA) synthesis of 1.59 mg/g FW, followed by FPH with 1.56 mg/g FW and LF 1.5 mg/g FW all on day 2 which was 24.5 %, 23 % and 20 % higher than control, respectively. Higher L-DOPA content was observed in the elicited fenugreek sprouts during early germination, correlating to high phenolics and antioxidant activity, suggesting that L-DOPA also contributes to the high antioxidant activity. The glucose-6-phosphate dehydrogenase (G6PDH) activity was higher during early germination (day 1-4) and gradually decreased during later stages (day 5-8) for all treatments and control. The early increase is possibly due to the carbohydrate mobilization from the cotyledons directed towards the high nutrient requirements of the growing sprout. As mobilization occurred, an allosteric feedback inhibition by sugar-phosphates is suggested, as lower G6PDH activity was observed on days 6-8. The elevated levels of GPX during early germination coincide with the higher phenolic synthesis; SOD activity and antioxidant activity suggests the elevated production and quenching of reactive oxygen species by elicitation. High antimicrobial activity against peptic ulcer-linked *Helicobacter pylori* was observed in the fenugreek sprout extract from control and LF treatments only. We hypothesized that in fenugreek sprouts, simple free phenolics that are less polymerized have more antimicrobial function.

Keywords: Fenugreek (*Trigonella foenum-graecum*), PPP (Pentose Phosphate Pathway), elicitors, FPH (Fish Protein Hydrosylates), lactoferrin, oregano extract, G6PDH (glucose-6-phosphate dehydrogenase), GPX (guaiacol peroxidase), L-DOPA (levo dihydroxy phenylalanine), SOD (superoxide dismutase), sprouts, phytochemicals, peptides, phenolics, antioxidants, antimicrobials

Introduction

Fenugreek (*Trigonella foenum-graecum*) being rich in phytochemicals has traditionally been used as a food, forage and medicinal plant.[1] Fenugreek seeds contain lysine and L-tryptophan rich proteins, mucilaginous fibre and other rare chemical constituents such as saponins, coumarin, fenugreekine, nicotinic acid, sapogenins, phytic acid, scopoletin and trigonelline, which are thought to account for many of its presumed therapeutic effects.[2] Various components of the seeds have varying activities. For example, the component called fenugreekine, a steroidal sapogenin peptide ester has hypoglycemic properties.[3] It is shown to delay gastric emptying, slow carbohydrate absorption, and inhibit glucose transport in humans.[4] It can increase the erythrocyte insulin receptors and peripheral glucose utilization, thus showing improved pancreatic function.[5] Trigonelline, another component is suggested to exert hypoglycemic effects in healthy patients without diabetes.[3] Thus the best-documented medical use of fenugreek is to control blood sugar in both insulin-dependent (type 1) and noninsulin - dependent (type 2) diabetics.[6-8] Treatment with fenugreek

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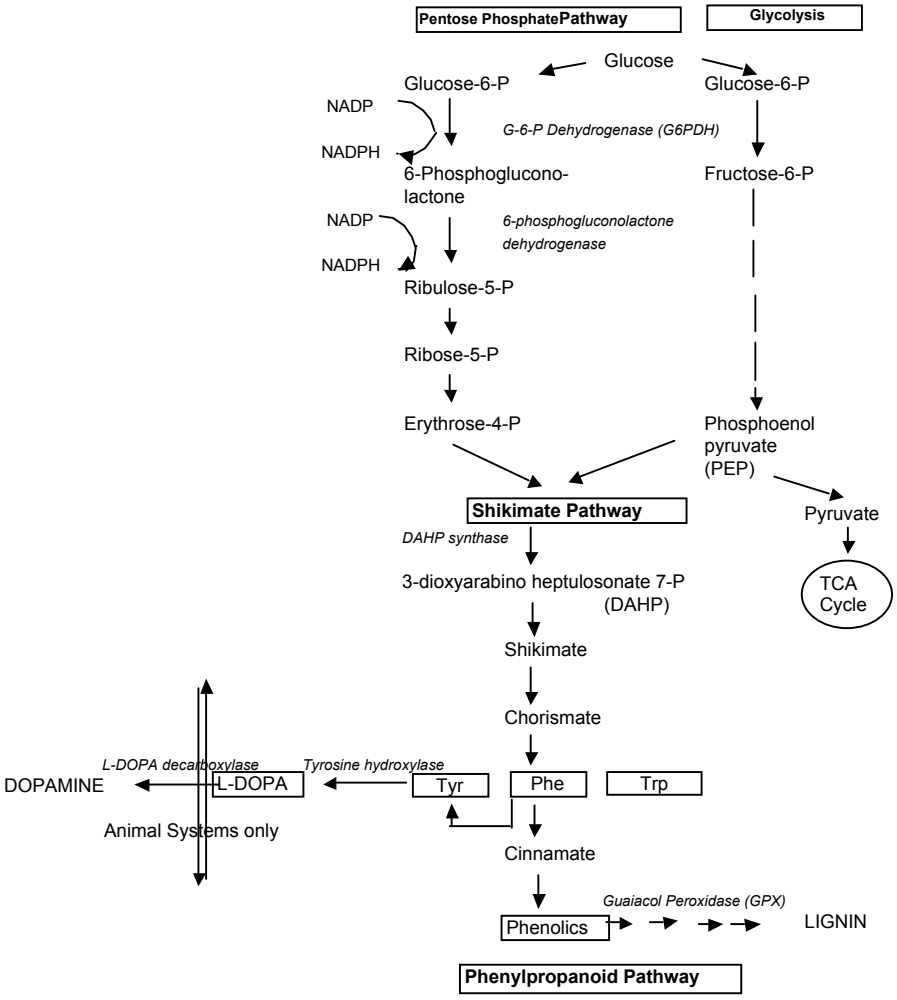

Figure 1. Pentose Phosphate Pathway for synthesis of phenolic compounds

seed powder normalized the enhanced lipid peroxidation and increased susceptibility to oxidative stress associated with depletion of antioxidants in diabetic rats. In normal rats supple-mentation resulted in increased antioxidant status with reduction in peroxidation.[9]

The steroidal saponins (diosgenin, yamogenin, tigogenin and neotigogenin) are thought to inhibit cholesterol absorption and synthesis and hence its potential role in arteriosclerosis. Clinical studies demonstrated a statistically significant decline in human serum total cholesterol, triglycerides and LDL cholesterol by fenugreek consumption.[10-11] It is also used topically to treat inflammation, and to promote postpartum lactation in animals.[12] The beneficial gastroprotective effect of fenugreek seeds has been researched in gastric ulcers of rats.[13] There is considerable commercial interest in growing fenugreek for its high sapogenin content. At present diosgenin, a steroid sapogenin used in the manufacture of birth control pills is isolated from *Dioscorea* species. This is the starting compound for over 60% of the total steroids, hormones and cortisone production by the pharmaceutical industry. Fenugreek being an annual and easy to cultivate might one day replace the present commercial sources.[14]

Plant phenolics have potential health benefits mainly due to their antioxidant properties such as reactive oxygen species (ROS) scavenging and inhibition, electrophile scavenging and metal chelation.[15] Epidemiological studies support a relationship between the consumption of phenolic rich food products and a low incidence of coronary heart disease,[16] atherosclerosis,[17] certain forms of cancer[18] and stroke.[19] They have also been reported to exhibit pharmacological properties such as antitumor, antiviral, antimicrobial, anti-inflammatory, hypotensive and antioxidant activity.[20-21] They are plant secondary metabolites, primarily synthesized through the pentose phosphate pathway (PPP), shikimate and phenyl-propanoid pathways (Fig. 1). The oxidative PPP provides precursor erythrose-4-phosphate for the shikimate pathway. The shikimate pathway converts these sugar phosphates to aromatic amino acids like phenylalanine, which becomes the precursor for the phenylpropanoid pathway.

Considering the diverse uses of fenugreek we were interested in studying the stimulation of phenolics by natural elicitors. Previous research has demonstrated that the phenylpropanoid pathway can be stimulated through the pentose phosphate and shikimate pathway by natural elicitors such as Fish Protein Hydrolysates (FPH), Lacto-

ferrin (LF) and Oregano Extract (OE). FPH stimulated the total phenolics synthesis in fava bean and peas.[22-23] FPH are small soluble and partially hydrophobic peptides rich in proline and glutamic acid obtained from seafood waste processed with protein hydrolysates.[24] They have a wide spectrum of applications from high value peptones, food ingredients and fertilizer production. LF is an iron-binding glycoprotein found naturally in milk, saliva, mucosal surfaces and within white blood cells. Research has shown LF to be a natural antibiotic, antioxidant, anti-fungal, antiviral, antitumor and immune modulator. Other unique functions attributed to LF include protection from iron-induced lipid peroxidation, immunomodulation, cell growth regulation, DNA and RNA binding, RNAse activity and as a transcriptional factor.[25] Research shows that LF enters the cell and is transported to the nucleus where it binds to specific DNA sequences and induces transcription of the reporter gene.[26] Oregano Extract (OE) is high in phenolic compounds such as protocatechuic acid and its phenyl glucoside, caffeic acid, gallic acid, tocopherol and rosmarinic acid.[27] It has significant anti-bacterial, antiviral, antimutagenic and antioxidant activity. Elite clones of oregano that produce higher amounts of phenolics and rosmarinic acid in response to *Pseudomonas* inoculation have been developed.[28]

Preliminary experiments showed that dry fenugreek seeds were low in phenolics with poor antioxidant activity. The prime objective of this research was to improve the phenolic, antioxidant and antimicrobial properties of fenugreek through elicited sprouting. Experiments were designed to give an understanding of the metabolic interrelationships between total phenolics and their corresponding antioxidant/antimicrobial activity. Seed sprouting is gaining importance commercially because it improves the nutritional value of the seed. A multitude of chemical changes occur to mobilize the stored carbohydrate and protein reserve into the growing sprout. Sprouting also removes some anti-nutrients such as enzyme inhibitors in the seed that make sprouts safe for the diet. Sprouting in fenugreek is known to improve its soluble protein and fibre content and reduce the phytic, tannic acid and trypsin inhibitors.[29] Since previous research in our laboratory showed the stimulation of total phenolic content of germinating fava and mung bean by the above-mentioned elicitors, we hypothesized the same in germinating fenugreek sprouts with corresponding stimulation of antioxidant and antimicrobial activity against *Helicobacter pylori*. The parameters measured to characterize the effect of these elicitors were total phenolics, antioxidant activity, levo dihydroxy phenyl-alanine (L-DOPA), glucose-6-phosphate dehydrogenase (G6PDH), guaiacol peroxidase (GPX), superoxide dismutase (SOD), and antimicrobial activity against *Helicobacter pylori*.

Materials and methods

Elicitors and treatments

The three elicitors used in this study were Fish Protein Hydrolysates (FPH), Lactoferrin (LF) and Oregano Extract (OE). In order to determine the ideal concentration for maximum elicitor response various dilutions were tested. FPH emulsion, a byproduct of mackerel processing was obtained from Conolly Seafood, Gloucester, MA. The FPH dilution tested were 0.5ml, 1ml, 5ml and 10 ml/L. Lactoferrin was obtained from Sigma Chemical Co., St. Louis, MO. The LF dilutions tested were 50, 100, 250 and 500 ppm. The Oregano extract was prepared by soaking 1gram of the crushed dried oregano leaves in 50ml of 95% ethanol for three days. The mixture was pulverized in a blender and centrifuged at 13000 rpm for 10 minutes. The supernatant was transferred to a beaker and the ethanol was allowed to evaporate. The residue was then dissolved in 50ml of distilled water. The OE dilutions tested were 1ml, 2ml, 5ml and 10ml/L. The optimal concentration to elicit maximum phenolic levels was further used to study the antioxidant activity, L-DOPA, G6PDH, GPX, SOD and antimicrobial activity.

Seed treatment and dark germination

Dry seeds of fenugreek (*Trigonella foenum-graecum*) were purchased from Asian American Groceries, Hadley MA. The seeds were soaked in distilled water for the control and in distilled water plus FPH, LF or OE for the treatments. Ten grams of seeds were placed in 500ml of the soak solution in 1000ml conical flasks. The flasks were then placed in a rotary shaker at 120 rpm for 24 hours. The pre-soaked seeds were washed in distilled water and germinated in flats lined with moist paper towels. The flats were covered with aluminum foil and the seeds were germinated in the dark. The germinating seeds were kept moist with distilled water and the assays were performed daily for the next 8 days. Each experiment had three replications and each experiment was repeated three times. In all assays only the sprouts of fenugreek seeds were used because they contained higher concentrations of functional nutrients. [29]

Total phenolics assay

Total phenolics were measured following the protocol developed by Chandler and Dodds[30] and modified by Shetty.[31] Phenolics were measured as gallic acid equivalents. Approximately 50mg of the fenugreek sprouts was immersed in 2.5ml of 95% ethanol and kept in the freezer at –20^{o}C for 48-72 hours. The sample was homogenized using a tissue homogeniser (Biospec Products, Bartleville, OK) and centrifuged at 13000 rpm for 10 minutes. One ml of the supernatant was transferred to a test tube and 1ml of 95% ethanol, 5ml of distilled water and 0.5ml of Folin-Ciocalteu phenol reagent (Sigma Chemical Co, St. Louis, MO) were added. After an incubation period of 5 minutes 1ml of 5% Na_2CO_3 was added, mixed well and the solution was kept in the dark for 1 hour. Then the samples were vortexed and the absorbance measured at 725 nm using a UV spectrophotometer (Spectonic Genesys 5; Milton Roy Company, Rochester, NY).

Antioxidant assay

The antioxidant activity of the fenugreek sprouts was determined by two methods: namely β-carotene oxidation and 1,1-diphenyl-2-picrylhydrazyl (DPPH) inhibition. The β-carotene oxidation model system as described by Miller was followed with some modifications.[32] The β-carotene solution was prepared by dissolving 10 mg of

β-carotene in 50 ml of chloroform in an amber coloured flask to prevent light oxidation. One ml of this solution was pipetted to a flask covered with aluminium foil. Chloroform was then evaporated under vacuum at 40°C for 5 min. The β-carotene was then dissolved in 20µl of linolenic acid and 184µl of Tween 40 emulsifier. Then 50 ml of H_2O_2 solution (176 µl H_2O_2 in 100 ml distilled water) was added and mixed thoroughly till the β-carotene was completely dissolved. Five ml of this prepared β-carotene solution was added to 100µl of the phenolic extracts. Control tubes contained 100 µl of 95% ethanol. As soon as the emulsion was added the zero time absorbance was read at 470 nm. Subsequent absorbance readings were recorded after a 30 min incubation period in a 50°C water bath. The antioxidant protection factor (APF) was used to express antioxidant activity as a ratio of sample absorbance at 30 min to that of the control.

The antioxidant activity of fenugreek sprouts was also determined by the 1,1- diphenyl-2-picrylhydrazyl (DPPH) assay as described by Cervato.[33] To 3ml of 60 µM DPPH, 100 µl of fenugreek sprout extract was added, mixed well and incubated at room temperature for 15 minutes. The absorbance was monitored at 517nm. The fenugreek sprout extract radical scavenging activity was compared with the activity of equivalent concentration of quercetin, a strong antioxidant standard.

Total protein assay

A cold pestle and mortar was used to thoroughly grind 100mg of the fenugreek sprouts in cold enzyme extraction buffer (0.5% polyvinylpyrrolidone (PVP), 3mM EDTA. 0.1 M potassium phosphate buffer of pH 7.5). The sample was centrifuged at 13000 rpm for 15 min at 2-5º C and stored on ice. The supernatant was used in the estimation of total protein, G6PDH and GPX enzyme assays.

The protein content was measured by the Bradford method.[34] Bradford dye reagent was prepared by diluting the commercial dye concentrate in a 1:4 ratio with distilled water. Five ml of the dye was added to 100 ml of the sample and blank (extraction buffer only) in test tubes and incubated at room temperature for 5 min. The samples were mixed and the absorbance was read at 595 nm using a UV spectrophotometer.

Glucose-6-phosphate dehydrogenase (G6PDH) assay

A modified version of the assay described by Deutsch[35] was followed. The enzyme reaction mixture containing 5.88µmol B-NADP, 88.5µmol MgCl2, 53.7µmol glucose-6-phosphate and 0.77mmol malemide was prepared. This mixture was used to obtain a basal blank reading at 339 nm. Then 1ml of this mixture was placed in 1.5 ml plastic cuvettes and 50µl of the enzyme extract was added. The change in absorbance was monitored over a period of 5 minutes. The rate of change in absorbance per minute was used to quantify the enzyme in the mixture using the extinction co-efficient of $NADPH_2$ (6.22 $mM^{-1}cm^{-1}$). The enzyme was quantified in nanomoles per minute per milligram of protein.

Guaiacol peroxidase (GPX) assay

The assay followed was a modified version developed by Laloue.[36] The enzyme reaction mixture containing 0.1M potassium phosphate buffer (pH 6.8), 50mM guaiacol and 0.2mM hydrogen peroxidase was prepared. This mixture was used as a blank reading at 470 nm. Then 1ml of this reaction mixture was placed in 1.5 ml plastic cuvettes and 50µl of the diluted enzyme extract (1:10) was added. The change in absorbance was monitored for a period of 5 minutes. The rate of change in absorbance per minute was used to quantify the enzyme in the mixture using the extinction co-efficient of the oxidized product tetra-guaiacol ($26.6mM^{-1}cm^{-1}$). The GPX enzyme was quantified in nanomoles per minute per milligram of protein.

Superoxide dismutase (SOD) assay

The SOD activity was determined as described by Spychalla and Desborough.[37] The enzyme reaction mixture containing 50 mM $Na_2CO_3/NaHCO_3$ buffer (pH 10.2), 0.1 mM EDTA, 0.015 mM ferricytochrome *c* and 0.05 mM xanthine was prepared. This mixture was used as a blank reading at 550 nm. The assay was initiated with the addition of sufficient xanthine oxidase to produce a basal rate of ferricytochrome *c* reduction. One unit of SOD was defined as the amount of enzyme that inhibited the rate of ferricytochrome *c* reduction by 50%.

HPLC analysis of L-DOPA

Fenugreek sprouts (200 mg) were soaked in 2.5ml 95% ethanol and kept in the freezer at –20°C for 48-72 hours. It was then homogenized in a macerator (Biospec Products, Bartleville, OK) and centrifuged at 13000 rpm for 10 min. The supernatant was transferred to a 25ml beaker and evaporated to dryness at room temperature. The residue was dissolved in 10ml of buffer solution (32 mM citric acid, 54.3 mM sodium acetate, 0.074 mM Na_2EDTA, 0.215 mM octyl sulphate pH 4). This solution was filtered through 0.45µm disposable syringe filters (Schleicher & Schuell, Keene, NH).

High Performance Liquid Chromatography (HPLC) was performed using an Agilent 1100 liquid chromatograph equipped with a variable wavelength detector. The analytical column was a reverse phase Supleco Discovery C18, 250 mm x 4.6 mm with a packing material of 5µm particle size. The total composition of the mobile phase was 18% methanol and 82% buffer (0.01 M ammonium acetate at pH 5.4) at a flow rate of 1ml/min. Tyrosine and catecholamine standards (L-DOPA, dopamine, norepinephrine and epinephrine) (Sigma chemicals, St. Louis, MO] were chromatographed separately and in a mixture. Retention time and spectra were compared with that of the standard L-DOPA. The amount of L-DOPA in fenugreek sprouts was measured from the peak height obtained at 280 nm, computed automatically using Agilent Chemstation 4.0 and was expressed in terms of milligrams per gram fresh weight.

Antimicrobial assay

The anti-microbial efficacy of fenugreek sprout extracts to inhibit the growth of the bacteria *Helicobacter pylori*

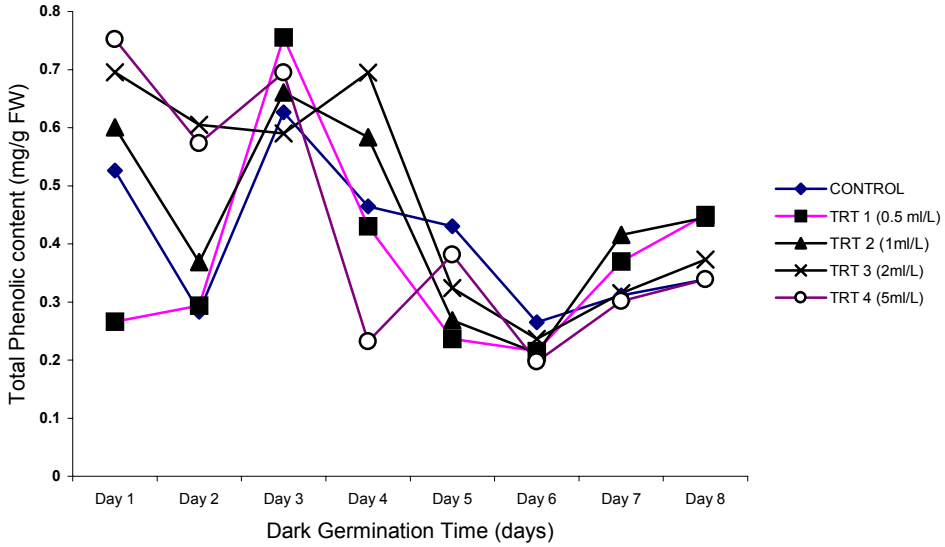

Figure 2. Effect of FPH on the total phenolic content of dark germinating fenugreek sprouts; all treatments were significant at $P = 0.05$; ANOVA P value = 0.27; least significant difference at $P<0.05 = 2413521$

was studied. *H. pylori* is one of the most common bacterial infections in human beings causing gastro-duodenal disease. *H. pylori* was cultured on peptone agar plates containing 10g peptone, 15g granulated agar, 5g sodium chloride, 5g yeast extract, 5g beef extract (Becton Dickinson and Co., Cockeysville, MD.) and 0.5g of pyruvic acid in 1L of water. *H. pylori* were maintained in a broth medium (same media mentioned above without agar) at 4°C. Stock cultures were grown at 37°C for 48 hours prior to use. The plates were inoculated with 100µl of overnight active culture and smeared for even bacterial growth.

Agar-diffusion test was done aseptically using sterile 12.7mm diameter paper (susceptibility) disks purchased from Schleicher & Schuell, Inc., (Keene, NH 03431, USA). The fenugreek phenolic extracts produced in response to optimal elicitor concentrations of FPH (0.5 ml/L), LF (500 ppm) and OE (1ml/L) were used in this study. Fenugreek sprout extract was prepared by grinding 1g sprouts in 10 ml of distilled water. The extract was then centrifuged and the supernatant was filter sterilized. The extracts were diluted such that the total phenolic content was approximately 500 µg/ml. Round paper disks were sterilized and loaded with 50µl, 100µl, 150µl and 200µl of the extract. The positive control in this study was oregano extract (1mg/ml ddH_2O), which has been previously determined to have high antimicrobial activity against *H. pylori*. Saturated disks were then placed on top of the bacterial growth. Three disks per concentration tested were plated. Treated plates were inverted and immediately placed in an anaerobe jar using a *Campylo-bacter* microaerophilic gas generator with the catalyst in place (Microaerophilic Systems Envelops, Becton Dickinson & Company, Sparks, MD). Samples were incubated at 37°C for 18 hours. The diameter of clear inhibition zone surrounding each disk was measured in centimeters.

Statistical analysis

Analysis of variance was conducted using ANOVA single factor test using Microsoft Excel 2000. Statistical *P* values were calculated to quantify levels of significance for each treatment type. A significant *P* value ($P = 0.05$) means that there exists significant difference between the two sets of data being tested. Measurement of Least Significant Difference (LSD) between any two groups was also determined using a multiple group analysis of variance using the Tukey's method.[38] This is the standard measurement of least significant difference between any two groups after a multiple group analysis of variance, when the variance within the group is known. The LSD test is used to test the null hypothesis that there existed no difference between treatment means at 5 percent level of significance. A rejection of the null hypothesis indicates significance in difference among treatment means.

Results

The total phenolic content of fenugreek sprouts was estimated for 8 days of dark germination. The highest stimulation was observed during different days for the different elicitors used in this study. Only those days where the maximal stimulation was observed are discussed. The different FPH concentrations tested were 0.5, 1, 2, and 5ml/L of the priming solution. For all treatments and control higher phenolic levels was observed during early germination (days 1-4) followed by declining levels on days 5-8. Among the different FPH elicitor concentrations tested 0.5ml/L gave the highest phenolic content of 0.75mg/g FW on day 3 of germination (Fig.2). This level was approximately 25% higher than control on the same day. For all treatments the phenolic content was higher compared to control on days 1-3. However, on days 5-6 after germination control was higher than all treatments. The different LF concentrations tested were 50, 100, 250 and 500 ppms. Maximum phenolic stimulation of 0.68 mg/g FW was observed for the 500 ppm treatment on day 2 of dark germination, which was 61 % higher than control the same day (Fig.3). Other treatments of 250 and 500 ppm showed higher phenolic content during early germination and decreased on later days (4-8). Interestingly, in the case of control

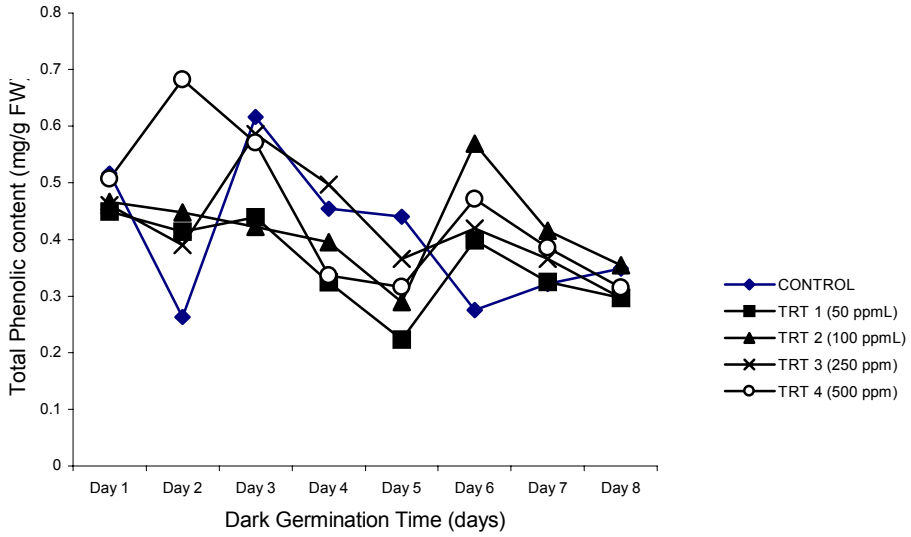

Figure 3. Effect of LF on the total phenolic content of dark germinating fenugreek sprouts; all treatments were significant at $P = 0.05$; ANOVA P value = 0.029; least significant difference at $P<0.05$ = 142.0439

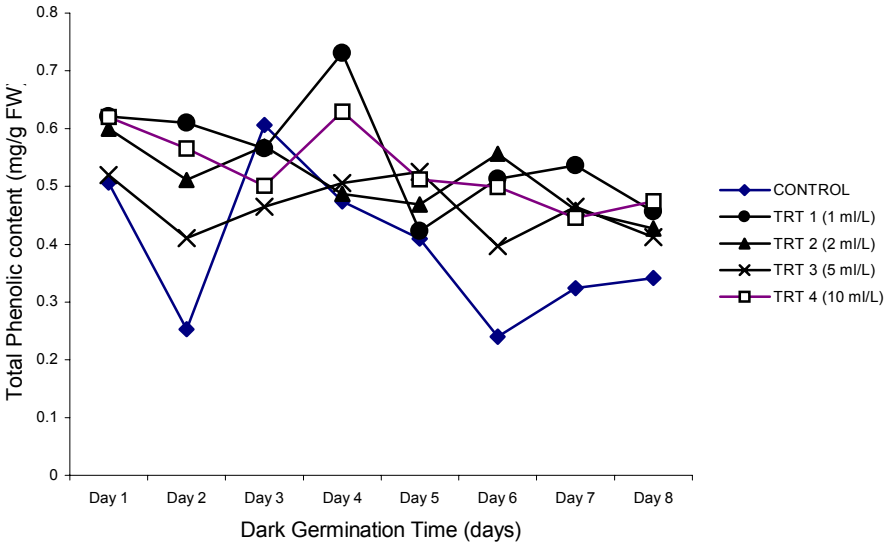

Figure 4. The effect of OE on the total phenolic content of dark germinating fenugreek sprouts; all treatments were significant at $P = 0.05$; ANOVA P = 0.031. Least significant difference at $P<0.05$ = 117.0318

that yielded lower phenolics than treatments in general, however it showed higher phenolics compared to treatments only on day 3 of germination. The different OE concentrations tested were 1, 2, 5 and 10 ml/L of soak solution. The phenolic content of all treatments and control fluctuated throughout all stages of germination. The elicitor treatment of 1 ml/L OE showed the highest phenolic content of 0.53 mg/g FW on day 4 of dark germination that was 25% higher than control on the same day (Fig.4).

The antioxidant activity in elicited fenugreek sprout extract was substantially improved over control (Fig. 5, 6). The activity of dry fenugreek seeds as measured by β-carotene assay (APF- Antioxidant Protection Factor) was less than one (0.91) and 27% DPPH inhibition. The antioxidant activity estimated by β-carotene assay was highest for LF and OE elicited sprouts on day 2 and 4, respectively with an APF of 1.47 for both (Fig.5). For all treatments and control higher activities were observed between days 1-4 of dark germination, slowly declining on days 5-7 and increasing again on day 8. The general trend of the antioxidant activity measured by DPPH inhibition was similar compared to β-carotene assay (Fig. 6). The antioxidant activity of control was maintained between 35 and 45% throughout the dark germination period. Oregano extract (OE) elicited fenugreek sprouts had the highest antioxidant activity of 61.3 % on day 4 of germination, which was 19.5 % higher than control the same day. The FPH elicited sprouts had increased activity of 58.5 % on day 3 and 58.6 % by LF on day 2.

The FPH, LF and OE treatments also elicited the L-DOPA content in the dark germinating sprouts (Fig. 7). The L-DOPA content of dry fenugreek seeds is only 0.47 mg/g FW after soaking. Elicited sprouting substantially improved the L-DOPA content in control and all treatments on day 2 of dark germination. OE elicited the

highest L-DOPA synthesis of 1.59 mg/g FW, followed by FPH with 1.56 mg/g FW and LF 1.5 mg/g FW, all on day 2 (Fig. 7) which is 24.5%, 23% and 20% higher than control, respectively. The L-DOPA content reduced as the germination progressed with time for all treatments and control. A similar response was also observed in the case of fava bean elicited with the same elicitors.[22]

In general, the G6PDH activity was higher during early germination (day 1-4) and gradually decreased during later stages (day 5-8) for all treatments and control. The activity for FPH and OE elicitor treatments and control peaked during day 3 of dark germination after lower levels on day 1-2, whereas LF showed stimulated G6PDH activity on day 2 (Fig. 8). The highest activity was observed in sprouts elicited by OE on day 3 that was 16 % higher than control the same day. The activity started to reduce from day 5 onwards for all treatments and control. The activity of control, however, was higher than LF/FPH treatments during certain days (days 3, 4 & 6). In the case of GPX, higher activity was observed in the elicited fenugreek sprouts than the control during early germination (days 1-3) (Fig. 9). Highest GPX activity was elicited by FPH on day 3, which was 20% higher than control on the same day. Interestingly, in the case of control a steady increase in GPX activity that was higher than all treatments from day 5 onwards was observed. High GPX activity coincides with high G6PDH activity during early germination (Figs. 8, 9). The general trend of SOD activity was similar to the GPX activity during early germination (Fig. 10) where the elicited sprouts showed a substantial increase in activity compared to control. In the case of control, slightly higher levels were observed during day 1- 2 and from then on the levels were maintained at almost steady levels. In the case of treated samples higher levels were observed during early germination days 1-3 with lower levels on day 4 and 5 and increasing again from day 6. Among treatments, OE elicited the highest SOD activity on day 2 of dark germination, which was 15% higher than control the same day. The antimicrobial activity of varied amounts of the

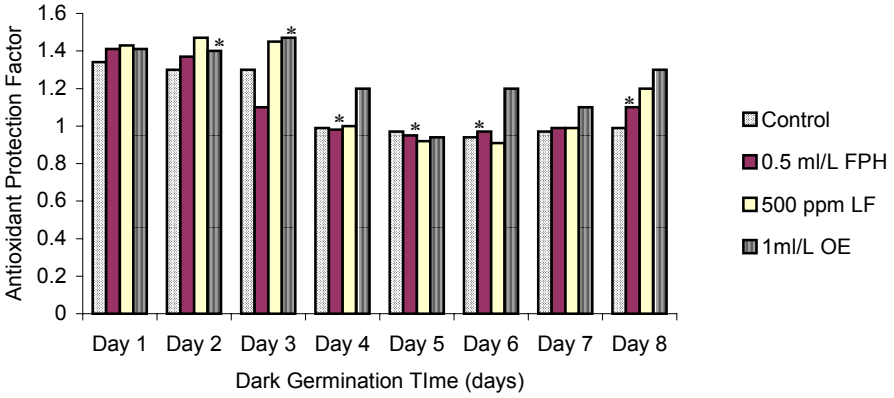

Figure 5. The effect of elicitor treatments on the average antioxidant activity of dark germinating fenugreek sprouts—β-carotene method. Marked data are significantly different than control at $P<0.01$(*) or $P<0.05$ (+) by ANOVA single factor F test. Least significant difference at $P<0.05 = 0.2516$

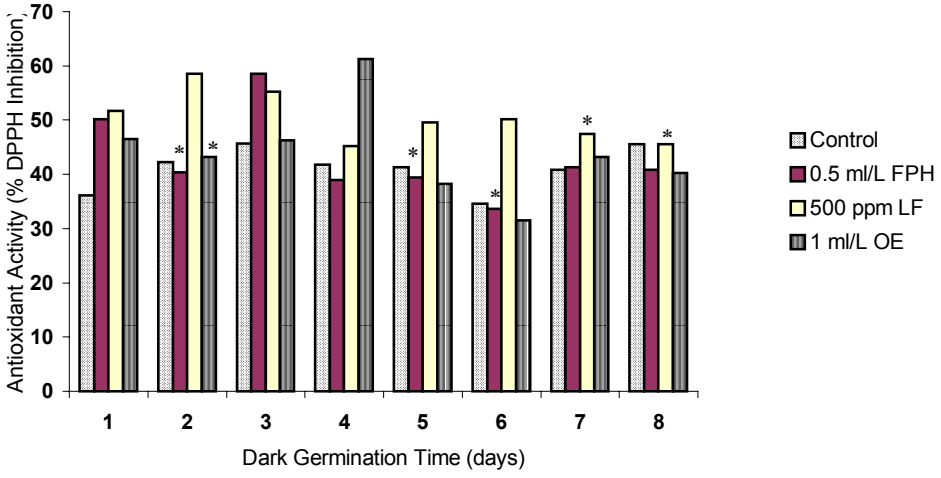

Figure 6. The effect of elicitor treatments on the average antioxidant activity of dark germinating fenugreek sprouts - DPPH inhibition method. Marked data are significantly different than control at $P<0.01$(*) by ANOVA single factor F test. Least significant difference at $P<0.05 = 8.2753$

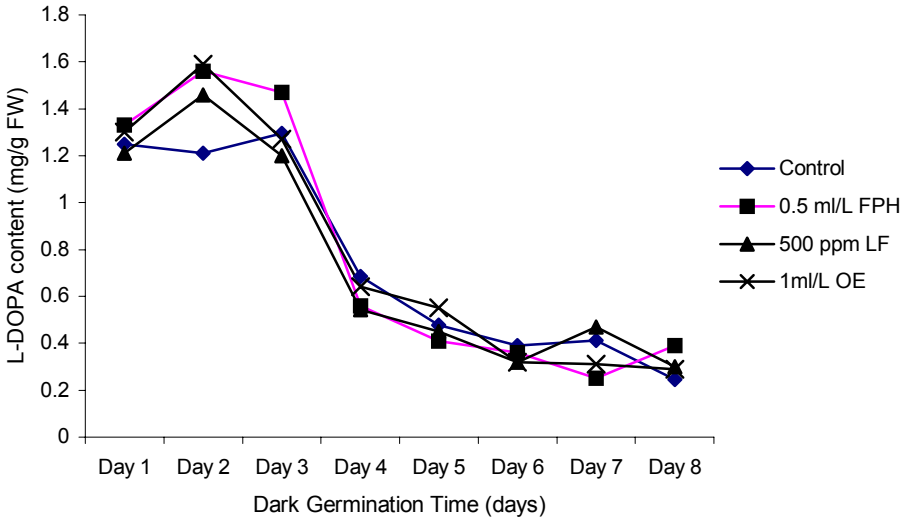

Figure 7. The effect of elicitor treatments on the average L-DOPA content of dark germinating fenugreek sprouts. All treatments are significant at $P = 0.05$; ANOVA $P = 0.013$. Least significant difference at $P<0.05$ =142.0439

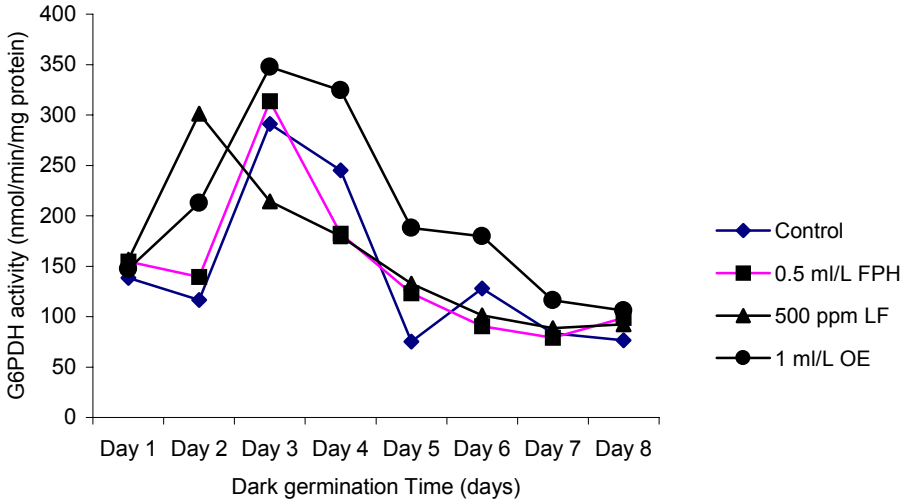

Figure 8. The effect of elicitor treatments on the average G6PDH activity of dark germinating fenugreek sprouts. All treatments were significant at $P = 0.05$; ANOVA $P = 0.0033$. Least significant difference at $P<0.05 = 101.3507$

fenugreek sprout extract (50, 100, 150, 200 µls) was tested against *Helicobacter pylori* (Fig. 11). Oregano leaf extract that was used as an internal positive control showed very high inhibition at all amounts tested. In all treatments, higher concentrations resulted in higher inhibition zones. Interestingly, the control sprout extract showed the highest inhibition, followed by the LF elicited sprouts. Very low activity was observed in the case of FPH and OE elicited sprouts with no activity at lower concentrations.

Discussion

The potential health benefits of plant phenolics are widely investigated. In humans they are known to be strong antioxidants, which might prevent oxidative damage to biomolecules like DNA, lipids and proteins that play a role in chronic diseases, such as cancer and cardiovascular diseases.[39] The present research aimed at improving the phenolic content and related antioxidant properties of fenugreek through elicited sprouting. The elicitors stimulated the PPP pathway towards increased phenolic production. Among all treatments, FPH elicited the highest total phenolic content that was 20% higher than control on day 3. We speculate that the glutamate and/or proline present in FPH accumulated in the seeds during the priming period and elicited the PPP, triggering the increased production of phenolics. Proline-induced PPP pathway has been reported earlier in plants.[40] In the case of LF, 500 ppm elicitation concentration stimulated a 61% higher phenolic content than control on day 2. In the case of OE, 1 ml/L elicitation concentration stimulated a 25% higher phenolic content than control on day 4. The high phenolic content observed during early germination for all treatments corresponds to high antioxidant activity (days 1- 4) during the same period. This suggests that the phenolics are antioxidant in nature during early germination when the oxidative stress in the germinating sprouts is naturally high due to the multitude of biological processes that are initiated with seed imbibition and growth. The increased oxidative stress in treated sprouts

is also evident by the elevated SOD activity during early germination. Previous research indicated that in fava beans subjected to abiotic stress (such as microwave heat) before sprouting an abrupt increase in antioxidant activity was observed.[41] Thus, in the present study it is possible that the biotic elicitors tested had a similar function of inducing a stress response in fenugreek sprouts.

It is believed that the effectiveness of plant phenolics in protecting against oxidative stress depends on their reactivity towards reactive oxygen species (ROS). Reduction of phenoxyl radicals by intracellular reductants is known to recycle phenolic antioxidants, thus enhancing antioxidant protection.[42] Antioxidant activity was measured by the β-carotene bleaching method and DPPH free radical scavenging method. The β-carotene method reflects the ability of the fenugreek sprout extract to function at a lipid water interface through prevention of H_2O_2 catalyzed β-carotene oxidation. The DPPH method estimates the ability of the fenugreek extract to quench the DPPH free radical. The antioxidant activity of dry fenugreek seeds measured by β-carotene assay (APF-Antioxidant Protection Factor) was less than one (0.91) and 27% DPPH inhibition. Sprouting substantially improved the antioxidant activity for control and all treatments (Fig.5 and 6). Highest antioxidant activity was observed during early germination showing that initially phenolics are antioxidant in nature. Another reason could be that, during early stages of germination there is a higher demand for oxygen and therefore phenolics might be protecting the cells from potential oxidation-induced deterioration. This increased activity correlates with high GPX activity indicating that the polymerized phenolics

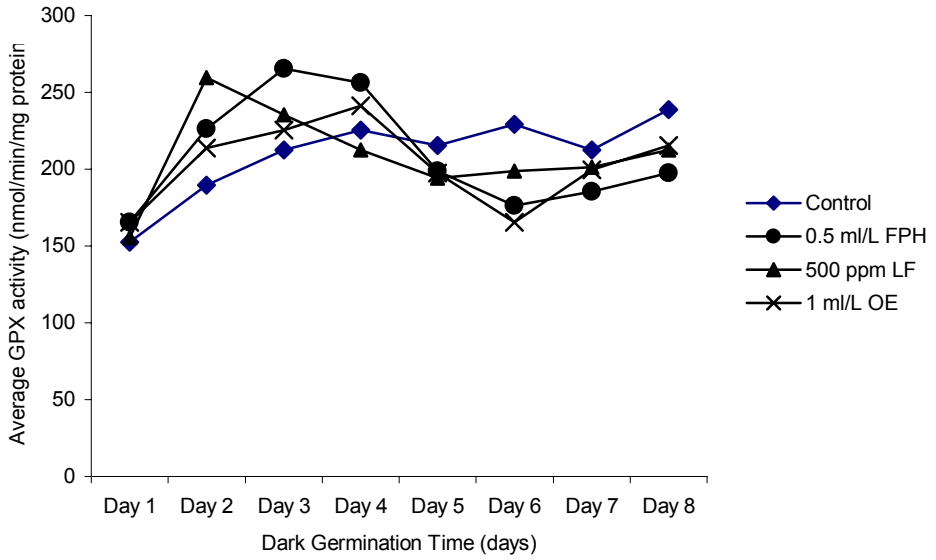

Figure 9. The effect of elicitor treatments on the average GPX activity of dark germinating fenugreek sprouts. All treatments were significant at $P = 0.05$; ANOVA $P = 0.0019$. Least significant difference at $P<0.05 = 38.8061$

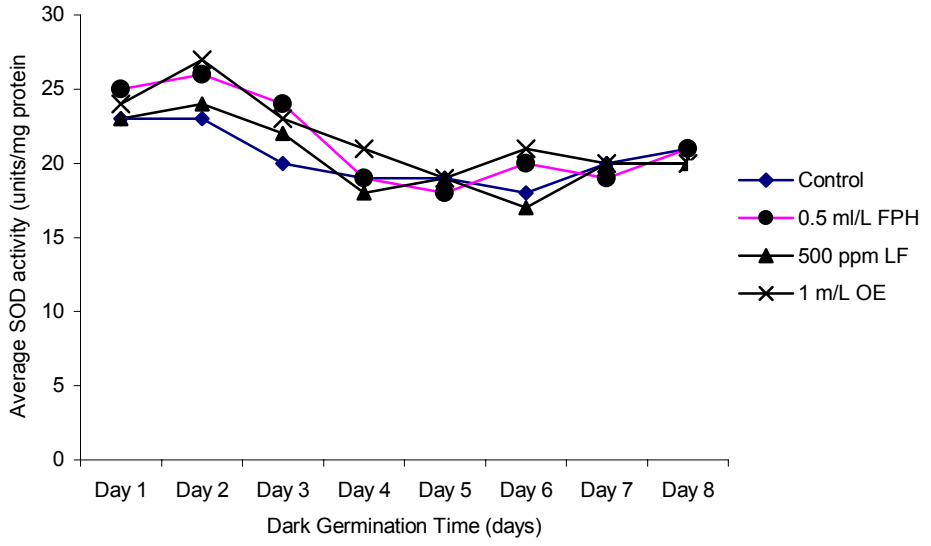

Figure 10. The effect of elicitor treatments on the average SOD activity of dark germinating fenugreek sprouts. All treatments were significant at $P = 0.05$; ANOVA $P = 0.017$. Least significant difference at $P<0.05 = 3.2148$

required for lignification with growth have antioxidant function. Higher GPX activity observed in control compared to treatments during late stages of germination indicates that most phenolics under natural conditions are diverted towards lignification with growth. Whereas in the case of treatments perhaps the stress imposed by the elicitors reduce the amount of phenolics being partitioned for lignification because some are utilized for antioxidant function against the ROS generated. The antioxidant activity as estimated by the above two methods, indicate that fenugreek sprout extract can quench the superoxide free radical and also likely scavenge the hydrogen peroxide generated in the reaction mix.

Oxidative damage to neuronal cells and DNA is implicated in the pathogenesis of neurodegenerative diseases such as Parkinsons. It is widely considered that one of the suggested treatments for early Parkinson's disease is L-DOPA which is converted to dopamine in the brain.[43] Treatment with synthetic L-DOPA is known to have side effects and so a search for a natural source is becoming more significant and necessary.[44] L-DOPA in the natural fava bean background was observed to show positive clinical benefits.[45] However, the use of fenugreek as a source of natural L-DOPA has not yet been harnessed to treat neurological deficiencies. In the present research higher L-DOPA content was observed in the fenugreek sprouts during early germination correlating to high phenolics and antioxidant activity suggesting that perhaps L-DOPA contributes to the high antioxidant activity. Since one of the current hypotheses is that oxidative stress in humans contributes to neurological diseases, the elevated L-DOPA content with accompanying high antioxidant function looks promising. This increased activity correlates with high SOD activity during day 2 of dark germination confirming the antioxidant role of L-DOPA in fenugreek sprouts.

The stimulatory effect of the elicitors on the total phenolic content and antioxidant activity lead to the investigation of the role of key regulatory enzymes in phenolic mobilization. Glucose-6-phosphate dehydrogenase (G6PDH), the rate-limiting enzyme of the pentose phosphate pathway (Fig. 1), determines the amount of $NADPH_2$ by controlling the metabolism of glucose via the PPP.[46] In plant tissues, two G6PDH isoforms exist in two different cell compartments, one in the cytosol and one in the chloroplast stroma.[47,48] In the present study higher G6PDH activity was observed during early germination (days 3-4) in general and slowly declined during late germination (days 6-) for control and all treatments. The early increase is possibly due to the carbohydrate mobilization from the cotyledons directed towards the high nutrient requirements of the growing sprout. We also hypothesize that the elicitor instantaneously stimulated G6PDH during the soaking period, which is reflected in high levels of phenolics and L-DOPA during days 1-4 of germination. As mobilization occurred an allosteric feedback inhibition by sugar-phosphates was possible thus G6PDH activity was low on days 6-8.

Guaiacol peroxidase (GPX) is one of the diverse groups of plant peroxidases, which catalyze the oxidoreduction reactions between H_2O_2 and various cell reductants.[49] In the present research the GPX activity increased in the elicited treatments compared to control. In the case of control a steady increase of GPX activity with germination was observed reflecting the developmental demand for lignification with growth. In elicited treatments the activity increased during days 2-4, declined on days 5 and 6 followed by an increase on days 7-8. Among the different elicitors, LF evoked the highest GPX activity on day 2, followed by FPH on day 3 of germination. The elevated levels of GPX during early germination coincide with the higher phenolic synthesis, SOD

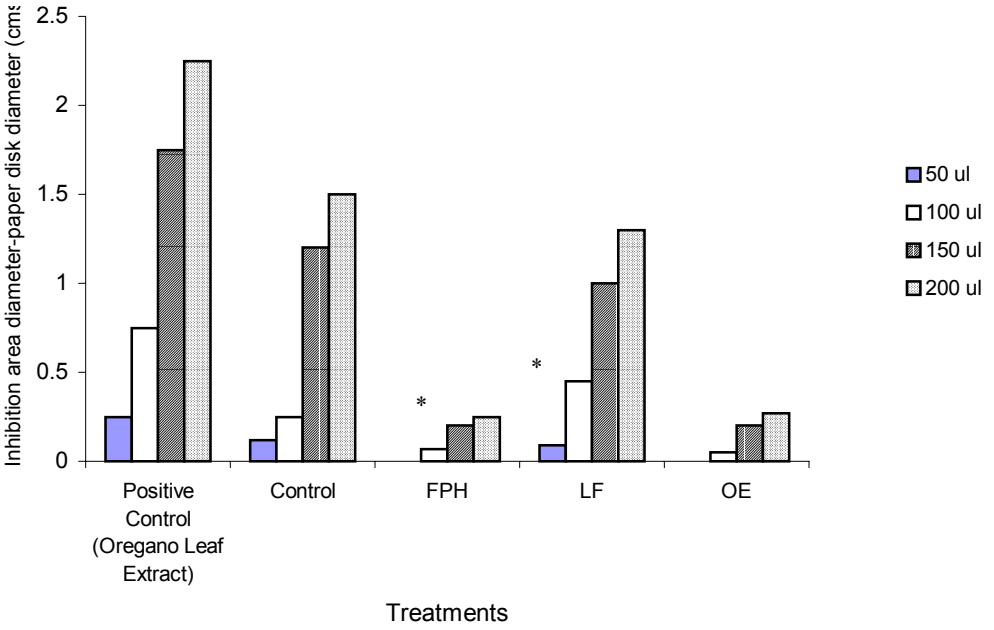

Figure 11. The effect of fenugreek sprout extracts on the growth of *Helicobacter pylori*. Marked data are significantly different than control at $P<0.01$(*) by ANOVA single factor F test. Least significant difference at $P<0.05 = 1.1724$

activity and antioxidant activity suggests the production of reactive oxygen species by elicitation. This response is very similar to a stress response where peroxidase activity is triggered accompanied by an accumulation of phenolics.[41] The late stage increase in activity on day 8 might be due to a developmental requirement for polymerization of simple phenolics to lignin with growth.

Superoxide dismutases (SOD) are a group of metalloenzymes that protect cells from superoxide radicals by catalyzing the dismutation of the superoxide radical to molecular O_2 and H_2O_2.[50] A number of environmental stresses can lead to enhanced production of O_2 within plant tissues, and plants are believed to rely on the enzyme SOD to detoxify this Reactive Oxygen Species (ROS). In the present research elevated SOD activity compared to control was observed in the elicited sprouts during early germination. We hypothesize that the elicitors actually impose a biotic stress on the germinating fenugreek seeds and evoke the ROS that in turn triggered the elevated SOD expression. In the case of control, the almost steady levels of SOD activity correlate to steady levels of antioxidant function throughout dark germination. This further suggests that the elicitors have a stress inducing action in the germinating sprouts.

Phenolic-type antimicrobial agents have long been used for their antiseptic, disinfectant, or preservative properties, depending on the compound.[51,52] Phenolics are known to have anti-bacterial properties by disrupting the membrane Proton Motive Force (PMF) thereby causing lysis and leakage of intracellular constituents,[53] perturbation of cell homeostasis,[54] inhibition of enzymes, electron transport, oxidative phosphorylation,[55] coagulation of cytoplasmic constituents[56] and effects on macromolecular biosynthetic processes.[57] In the present research a link between higher antioxidant activity during early germination and antimicrobial activity was suspected, therefore antimicrobial disk assays were performed. We targeted *Helicobacter pylori* for evaluation of antimicrobial activity of our phenolics rich fenugreek sprout extracts. The majority of peptic ulcers in humans are caused by *Helicobacter pylori.*[58] High antimicrobial activity against *Helicobacter pylori* was observed in the fenugreek sprout extract from control and LF treatments only. The FPH and OE elicited sprout extracts had minimal activity. Among treatments the control sprout extract of day 3, correlating with low GPX and SOD activity showed the maximal activity. This could be because those simple free phenolics, which are less polymerized, have more antimicrobial function in the case of fenugreek. This result is opposite to the antimicrobial activity observed in mung bean extracts, which probably have a different array of antimicrobial agents.[59] In the case of mung bean higher antimicrobial activity was linked to higher stimulation of G6PDH and GPX activity during early stages of germination. It was hypothesized that enhanced mobilization of carbohydrates (as indicated by elevated G6PDH activity) accompanied by enhanced polymerization of simple phenols which are known to destabilize the membrane proton ATPase (as indicated by GPX) were contributing to the high antioxidant activity producing intermediary phenolic metabolites that had antimicrobial function.

The antimicrobial activity observed in fenugreek could also be due to presence of scopoletin, a coumarin derivative of coumaric acid. Scopoletin is a lactonized phenolic, which has the potential to inhibit the electron transport chain in prokaryotes. Both control and the LF treatment showed reduced SOD activity suggesting that perhaps at these unstressed situations, the free phenolics such as scopoletin were synthesized and therefore contributed to higher antimicrobial activity. Lactoferrin which has antimicrobial activity on its own showed the second best inhibition, suggesting perhaps trace quantities of the elicitor was still present on the sprouts that may have contributed to the antimicrobial activity against *H. pylori*. It is interesting to observe that the FPH and OE stimulated fenugreek extract had minimal antimicrobial activity in spite of its comparable phenolic profile. This correlates to high GPX activity suggesting that polymerized phenolics in the case of fenugreek might not be effective as an antimicrobial. The high SOD activity seen in FPH and OE suggests that the sprouts are stressed and all biomolecules are directed towards neutralizing this stress with minimal antimicrobial function. Another reason could be that the elicitor inducible phenolics in fenugreek unlike mung bean sprouts are a different type than the control phenolics thus exhibiting a reduced antimicrobial function. However, the exact mechanisms of cellular damage by phenolics have not been elucidated and will be further investigated in future studies.

In conclusion, the present study has illustrated significant increase of phenolic antioxidants with accompanying L-DOPA content by elicited sprouting in fenugreek. The antimicrobial activity was high under non-elicited conditions and therefore reflecting the specific type of phenolics and its mode of mobilization. This knowledge can be used to design fenugreek sprouts for specific applications. If antioxidant activity with high L-DOPA were needed then elicitation would be preferred and if antimicrobial activity were needed then simple non-elicited sprouting would be useful.

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