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THE STUDY OF SOME FENUGREEK EXTRACTS BY GERMINATION BIOASSAY

Alina Perisoara[1.2*], Bianca-Maria Tihauan[5,6], Lucia Pirvu[3], Diana Manuela Ene[1], Marian Pavel[3], Stelica Cristea [2]

[1]Biotehnos S.A., Gorunului str., no. 3-5, 075100 Otopeni, Ilfov County, Romania

[2]Plant Pathology Departament, University of Agronomic Science and Veterinary Medicine Bucharest, District 1, Bucharest, Romania

[3]National Institute for Chemical-Pharmaceutical Research and Development, District 3, Bucharest, Romania

[4]Apa Nova Bucharest Waste Water Analysis Laboratory, Drumeţului str., no. 19, District 3, Bucharest, Romania

[5]National Institute of Research & Development for Food Bioresources-IBA Bucharest, Dinu Vintilă str., no. 6, District 2, Bucharest, Romania

[6]Research Institute of the University of Bucharest-ICUB, Splaiul Independenţei, no. 95, District 5, Bucharest, Romania

[*]Corresponding author. E-mail: pavel.alinaa@gmail.com

ABSTRACT

Since the constant use of traditional pesticides can have long-term adverse effects on human health, the environment and the fact that pests can develop resistance to them, it is of interest to develop natural alternatives with a role to protect plants and crops. Such an alternative would be the use of phytochemical compounds, with an antioxidant role, found in fenugreek seeds. This research paper aimed to evaluate the effect of three types of extracts obtained from fenugreek seeds (*Trigonella foenum-graecum*) on radish seeds through the germination bioassay. The extracts were obtained in three different organic solvents (ethanol 70%, propylene glycol - PG-50% and ethanol 40%), the phenolic profile was analyzed using the Folin Ciocalteu method and the antioxidant activity by the DPPH bioassay. The results showed that the variant in 40% ethanol was superior regarding the content of total polyphenols (TPC) and flavonoids (TFC) (2.667 CAE mg/ml and 3.199 RE mg/ml) compared to the extract variants obtained in 70% ethanol (1.635 CAE mg/ml and 1.863 RE mg/ml) and PG 50% (2.072 CAE mg/ml and 2.147 RE mg/ml), what particularly influenced the antioxidant activity. The Fenugreek extract in ethanol 40% has a higher redox potential compared to the other extract variants tested in our study. The phytotoxic profile of the extracts was achieved by applying the radish (*Raphanus sativus*) seed germination bioassay. According to the results obtained, the extracts in 40% ethanol showed moderately phytotoxic activity at the concentrations of 0.50% and 1.0% and strongly phytotoxic at the concentration of 1.5% extract, while the extract variant in 70% ethanol, showed moderately phytotoxic activity at the first three concentrations tested and a strong phytotoxic effect at the concentration of 1.5%; in the case of the extract obtained in PG, no phytotoxic activity was recorded on the tested seeds, the Gi being >80%.

Keywords: *Trigonella foenum-graecum*, phytotoxicity, germination bioassay, plant protection.

INTRODUCTION

Testing a possible toxic effect of extracts with phytosanitary potential through the biological testing method is extremely important for verifying their use in the field of agricultural applications (Mitelut and Popa, 2011), more precisely, the protection of plants from pests and the phytostimulation of germination. More precisely, phytotoxicity itself can be described as the poisoning of plants by natural chemicals present in the treatment environment, when they accumulate in the plant tissue (Chang et al., 1992; Araujo and Monteiro, 2005). The application of the seed germination bioassay is one of the most used methods for evaluating the phytotoxicity of different substances/extracts, to obtain products with phytosanitary properties (Kapanen and Itavaara, 2001). According to some data from the literature, the seed germination bioassay can be considered a less sensitive method, compared to the root length determination method, when it is used to evaluate phytotoxicity (Fuentes et al., 2004). However, there is a lot of scientific evidence

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that the application of the seed germination bioassay confers a relatively low potential for sensitization to a fairly large range of toxicants substance, because most chemicals substances cannot be taken up/extracted, seeds extract their nutritional substances from the substances stored in the seed and thus can be protected from the external environment (Kapustka, 1997).

In other words, the roots of the seeds represent the component responsible for the absorption and accumulation of metals, so this can be evident in the way they develop (Mitelut and Popa, 2011). The alarming increase in the number of xenobiotic compounds that negatively influence the environment, many of the already existing chemical substances, represent toxicological threats to the biosphere and even more, a fact that shows the need for examinations of products and substances with phytosanitary potential through appropriate toxicological methods (Ciobanu, 2019). The application of germination bioassays is widely used to test for excess salinity or even the presence of potentially toxic substances, such as phenolic compounds in plant extracts (Zucconi et al., 1985). Taking into account the growing importance of using biologically active alternatives regarding plant protection, mainly in the production of vegetables and fruits, as well as other biological activities attributed to these types of products, this study evaluated the phytotoxic activity of seed extracts of Fenugreek. In addition, the total phenols and flavonoids in the extracts were quantified, the dry substance and the antioxidant activity were determined.

MATERIAL AND METHODS

Plant material and preparation of extract variants

The dried seeds of Fenugreek (*Trigonella foenum-graecum*) were purchased commercially, from AdNatura, country of origin, Egypt. To prepare the extracts, Fenugreek seeds were washed and dried in the oven at 40ºC, then grinded. Fenugreek seed extract in 40% ethanol was obtained by maceration in ethanol:water (70:30) solution at room temperature, with occasional stirring, for 10 days, extraction ratio 1:20 (w/v); after filtering, the sample was subjected to thermal treatment (35-40ºC in an ultrasonic water bath), to concentrate the extract. A homogeneous extract of fluid consistency was obtained, coloured reddish-brown, with specific smell, and a concentration in ethanol of 40%. Fenugreek (*Trigonella foenum-graecum*) seed extracts in propylene glycol: water (50:50) and hydroalcoholic ethanol: water (70:30), ratio 1:20, were obtained by maceration at room temperature for 10 days and occasional agitation; after filtering the extract was stored at 4ºC until the analyses were performed.

Quantitative phytochemical screening and evaluation of antiradicalic capacity

Polyphenol content **(TPC)** was assessed by applying the spectrophotometric method developed by Sidhu and Saxena (2017) which underwent minor modifications in house. This method is based on the property of polyphenols to reduce in alkaline medium the Folin-Ciocalteu reagent. The intensity of the colour resulting from the reductive process is determined at a wavelength of 765 nm (absorbance was read using a PerkinElmer Lambda 25 UV-VIS spectrophotometer). The concentration of polyphenols is extrapolated from the calibration curve recorded with caffeic acid over the concentration range 1-11 ug/ml. All reagents used (caffeic acid; Folin-Ciocalteu reagent diluted prior to use with distilled water in ratio 1:10 v/v; sodium carbonate; methanol) were purchased from Sigma-Aldrich Chemie GmbH, Germany.

Determination of flavones (**TFC)** from unknown samples is based on the property of flavonozides to form yellow chelated compounds with aluminium chloride, whose absorbance can be read at 415 nm wavelength. In this case, the concentration of flavones in the extracts analysed was determined by extrapolation from the calibration curve recorded with rutin (dose range 5-50 ug/ml). The applied analytical method followed the guidelines set by Chia-Chi et al. (2002). All reagents used

(rutin; aluminium chloride - working solution 10%; potassium acetate - working solution 1M) were purchased from Sigma-Aldrich Chemie GmbH, Germany.

The evaluation of the ***antiradical capacity*** of the studied plant extracts was carried out by applying the indirect spectrophotometric method using as DPPH generation system (stable free radical due to a delocalization of the electronic charge extended on the whole molecule, which generates a violet colour in solution). Basically, this method involves recording the decrease in absorbance at wavelength λ = 520 nm (DPPH absorption maximum) which is proportional to the concentration of reduced free radicals in solution. The ability of the compounds to trap DPPH* radical is determined by their electron or hydrogen yielding properties, i.e. the magnitude of the oxidation-reduction potential of the antioxidants studied. The antiradical activity (AAR) was defined as the amount of antioxidant required to decrease the initial DPPH* concentration by 50% and represents the effective concentration, EC_{50}. This method, often used in the analysis of both solid and liquid samples, due to its simplicity and rapidity, has recently been extended to studies of the antiradical activity of substances isolated and purified from sources other than plants, as it is not specific to a particular antioxidant component. Absorbance recording was carried out using a PerkinElmer Lambda 25 UV-Vis spectrophotometer equipped with a sample thermostatting system.

Determination of dry matter

The method for determining the dry substance used is modified and adapted according to the method - gravimetric method SR 7487 - Determination of extractable substances with solvents.

The determination of dry matter content consists in determining the amount of product remaining after the mass of volatile compounds has been removed at a temperature of 105ºC. Before starting the process of determining the dry substance from each extract sample taken in the study, the heat-resistant weighing capsules were subjected to a drying-cooling process, in an oven and desiccator (105ºC-25ºC), and weighed. All these processes were carried out in triplicate to obtain a constant mass of the capsule. The final mass (m_1) of the capsule is expressed as the average of the three weighings. The method itself consists in weighing 20 ml of each variant of fenugreek seed extract (40% ethanol, 50% PG and 70% ethanol), in thermo-resistant glass weighing capsules. The extract samples are placed in the thermostatic water bath, at a temperature of 95ºC, to evaporate the solvent. After evaporation, the samples are subjected to a drying process at a temperature of 105ºC, for 60 minutes. After the drying process, the extract samples are cooled and weighed (m_2) to determine the dry substance and calculated according to the formula below:

(1) $[(m_2 - m_1)] / V \times 1000 \times 1000$ mg/l

where:

m_1 = mass of the empty capsule, in grams,

m_2 = mass of capsule with residue, in grams,

V = the volume of the sample used in the work in ml.

Seed Germination Bioassay

The method was modified and adapted following the procedure reported by Miteluț and Popa (2011) and Ghayal et al. (2018). The aim of the study was to evaluate the phytotoxic potential of extracts obtained from fenugreek seeds in ethanol 40%, 70% and propylene glycol 50%. Thus, four dilutions of the extracts were prepared using distilled water to obtain the following concentrations, 0.1%, 0.5%, 1.0% and 1.5%, the same dilutions were made for all solvents used in extraction, 40% ethanol, 50% propylene glycol and 70% ethanol. Results were reported as a negative control containing distilled water. Before starting the germination bioassay, Petri dishes were disinfected with 70% isopropyl alcohol and left to dry, and filter paper were placed in, and sterilized in a hood with a UV lamp, for 30 min and the radish seeds were passed through purified water and dried in the oven

for a few minutes at 40ºC. After the sterilization process of the filter papers was completed, they were placed in Petri dishes, on top of which were placed 10 seeds of close-sized Long White Icicle (*Raphanus sativus*) radishes and 5 ml of the mixture obtained after the dilutions were pipetted into each plate. The Petri plates were left to incubate, in the dark, for 5 days, at a temperature of 25ºC±1. The whole experiment was done in duplicate (P1, P1.1, ...P5, P5.1). After the end of the incubation period, germinated seeds (G) were counted and root length (L) was measured. The results were analyzed by determining the following indicators: germination percentage (G%), relative seed germination index (RSG), relative root growth index (RRG) and germination index (Gi), according to the formulas below:

$$GP = \frac{Number\ of\ germinated\ seeds}{Total\ number\ of\ seeds} \times 100\%; \quad (2)$$

$$RSG = \frac{Number\ of\ germinated\ seeds\ (sample)}{Total\ number\ of\ seeds\ (control)} \times 100\%; \quad (3)$$

$$RRE = \frac{Root\ length\ of\ germinated\ seeds\ (sample)}{Root\ length\ of\ germinated\ seeds\ (control)} \times 100\%; \quad (4)$$

$$Gi = \frac{G}{G0} \times \frac{L}{L0} / \times 100 \quad (5)$$

where:

- G is the number of normal germinated seeds on the sample substrate,
- G_0 is the number of normal germinated seeds in the control,
- L is the average of plant roots per substrate sample,
- L_0 is the average length of the plant roots on the control substrate.

The germination index (GI) indicates the effect of the substances/extracts on the seeds under study. Quantification of GI values is done by processing the results obtained RRE and RSG. A GI level lower than 50% indicates a strong phytotoxic activity, a level between 50-80% indicates moderate phytotoxic activity, a level between 80-100% indicates a non-phytotoxic activity, and over 100% indicates a phyto-stimulating activity (Ravindran et al., 2017). The test time will be ended when more than 65% of the seeds from the control sample have germinated or developed roots of at least 20 mm in length (EPA, 1996).

Statistical analysis

Data for TPC and TFC were performed in duplicate, and antioxidant activity in triplicate. Results were reported as mean and standard deviation of the mean (SD). The statistical analysis was performed using Microsoft Excel 2019. The data were analyzed using a regular t-test: Paired Two Sample for Means to compare the results of extracts and solvents obtained following the germination bioassay. A *p*-value of less than 0.05 was interpreted as statistically significant.

RESULTS AND DISCUSSION

Quantitative phytochemical screening and evaluation of antiradicalic capacity

The content of total polyphenols determined from all three varieties of Fenugreek seed extract was expressed in caffeic acid equivalents (CAE) and was quantified by the Folin-Ciocalteu method. As can be seen from Table 1, the highest concentration of polyphenols was found in the case of the Fenugreek extract variant in 40% ethanol, with an amount of 2,667 ± 0,397 CAE mg/ml, followed by the extract variant obtained by cold maceration in propylene glycol 50% (2.072 ± 0,445 CAE mg/ml), and in the case of the variant of the extract obtained in ethanol 70%, recorded the lowest amount of polyphenols (1.635 ± 0,259 CAE mg/ml).

Table 1. The total polyphenol content (caffeic acid equivalents mg/ml), total flavonoids content (rutin mg/mL) and antioxidant activity (DPPH)

Extraction variant	Total polyphenols - caffeic acid (CAE) mg/mL	Total flavonoids rutin (RE) mg/mL	DPPH
Fenugreek Seed Extract in 50% PG	2,072± 0,445	2,147 ± 0,284	5,033 ± 0.156; *RSD = 3.09
Fenugreek seed extract in 70% ethanol.	1,635± 0,259	1,863 ± 0,109	6,557 ± 0.091; *RSD = 1.4
Fenugreek seed extract in 40% ethanol.	2,667± 0,397	3,199 ± 0,235	3.018 ± 0.109; *RSD = 3.6

*relative standard deviation

Obtained results indicate that the extract variant in 40% ethanol had the best extraction yield of phenolic compounds, followed by the extract in 50% PG and 70% ethanol. The yield regarding the amount of phytochemical compounds obtained depends on the concentration of water used in the extraction mixture. Contrary to the results obtained by Lohvina et al. (2021), where the concentration of phenolic compounds found in the variants of fenugreek extract in developed ethanol (30%, 50%, 70% and 96%) was higher in 70% ethanol, these decreasing as the concentration of water in the extractive mixture increases.

The content of flavonoids in the three types of extracts obtained from Fenugreek seeds was expressed in rutin equivalents (RE) mg/mL sample-extract test, using the spectrophotometric method. Thus, in the case of Fenugreek seed extract in 40% ethanol, a flavonoid concentration, expressed in rutin, of 3,199±0,235 mg/mL was obtained, a significantly higher concentration, compared to the variants of Fenugreek seed extracts in 70% ethanol and PG 50 %, as shown in Table 1.

It can be observed that the variant of extract from fenugreek seeds in 40% ethanol, has a net higher content in both flavonoids and total polyphenols, compared to the variants of extract in ethanol 70% and PG 50%, obtained by cold maceration without applying no heat treatment.

Norziah et al. (2015), obtained the highest amount of flavonoids and polyphenols (38.5 mg CE/g; 156.3 mg GAE/g) in the case of the aqueous extract from germinated fenugreek seeds, compared to the extracts obtained in 75% ethanol and 75% methanol. However, ethanol is considered to be more efficient in the extraction of polyphenols with small molecular sizes, while water is efficient in the extraction of phenolic compounds with large molecular sizes (Norziah et al., 2015). As it appears from our study, the concentration of flavonoids is higher compared to the concentration of polyphenols, this fact being applicable in all three tested extract variants. The results correlating with the concentration of water found in the extraction mixture.

The indirect spectrophotometric method that uses DPPH as a generation system (stable free radical due to a delocalization of the electronic charge extended over the entire molecule, which generates a purple colour in the solution) involves measuring the decrease in absorbance at the wavelength $\lambda = 520$ nm (absorption maximum of DPPH) which is proportional to the concentration of reduced free radicals in the solution. Antiradical activity (AAR) was defined as the number of antioxidants required to decrease the initial concentration of DPPH* by 50% and represents the effective concentration, EC_{50}. For all methods described, at least three determinations were performed for each sample and the results were reported as means. The results are presented in Table 1, as % inhibition depending on the *Log* of the applied dose, thus calculating the effective concentration, EC_{50}.

According to the results obtained and reproduced in Table 1, the most pronounced antiradical effect is noted in the version of fenugreek seed extract in 40% ethanol, demonstrating a significant redox potential. In the specialized literature, there are a series of studies on the determination of the content of polyphenols and flavonoids in extracts obtained from fenugreek seeds and their

correlation with antioxidant activity (Selma et al., 2014).

As it appears from our study, the extract obtained from Fenugreek in 40% ethanol, presented a higher antioxidant activity, precisely due to the presence of a higher content of polyphenols and flavonoids in the extract. A similar study was carried out by the researchers M. Rahmani and his collaborators (Rahmani et al., 2018), where the best results regarding the antioxidant activity (IC_{50}: 80.98) of fenugreek extract were given by the variant in which the highest concentration of polyphenols was found (TPC: 2.083±0.01mg GAE/gExt) and flavonoids (TFC: 3.778±0.13mg CE/gExt). Fenugreek extract in general presented a strong antioxidant activity, due to its composition rich in phenolic compounds, especially polyphenols (Ojha et al., 2018).

A study worth mentioning is that of the researcher Sytar (2015), who highlighted the antioxidant potential of the species buckwheat and tartary buckwheat, where were found high concentrations of some phenolic compounds (*p*-anisic acid, chlorogenic acid, vanillic acid…) in their component (Sytar, 2015).

Dry matter determination

The determination of dry matter from the three variants of fenugreek extract obtained in different solvents from the category of concentrated alcohols, revealed that the extract in 40% ethanol recorded the highest amount of residue (31465 ± 0,005 mg/L) compared to the other varieties of fenugreek extract studied (Table 2).

Table 2. Results regarding the final residue obtained for each variant of Fenugreek

Sample	Dray matter m_1 (g)	Dray matter m_2 (g)	Sample volume (ml)	Residue (mg)	Final residue (mg/l)
Fenugreek seed extract in et. 70 %	27.7467	28.0582	20	3115	15575± 0,001
Fenugreek seed extract in et. 40%	28.0270	28.6563	20	6293	31465± 0,005
Fenugreek Seed Extract in PG 50%	24.5941	25.0369	20	4428	22140± 0,001

It should be noted that these results correlate with the concentrations of polyphenols and flavonoids recorded in the composition of the extracts. The higher the concentrations of these compounds, the higher the residual mass was obtained.

Seed Germination Bioassay

Seeds can adsorb from the environment various substances that are found in their composition (e.g. allelopathic substances, nutrients) using them further in various metabolic processes, and here we can call cell division, elongation and differentiation (Ravindran et al., 2017). The effects of these substances can be particularly observed in the development and growth of the roots of the tested seeds.

In our study, the effect of the three variants of *Trigonella foenum-graecum* seed extract was compared with solvents used in the process of extraction in similar concentrations to highlight the germination capacity of the seeds under the treatment of the extracts taken in the study. The results regarding the relative germination index (RSG%) fell within the following ranges: 90-100% for the 40% ethanol extract variant and the solvent 84-105%; the extract in propylene glycol registered an RSG % of 100%, at all tested doses and for the solvent was 90-100%, and in the case of the extract variant obtained in 70% ethanol, the RSG % fell between 95-100% and for solvent 95-100% at the tested doses.

Regarding the results of the elongation index (Figure 1), the results are the following: in the case of the 40% ethanol extract variant, the RRE% fell between 89.84-39.09% and for solvent the RRE % value was: 96.17-81.29%, the lowest value is registered at the concentration of 1.0%; for the variant of extract in PG 50%, the RRE% values were between 98.95-83.69% and for solvent 74.13-33.74%, where the lowest concentration was registered at the concentration of 1%; and in the case of the

70% ethanol variant, the lowest RRE% values were recorded, these being between 55.83% and 41.62%, compared to the solvent where the RRE% values fell between 88.56-52.64%, where the development of the roots was statistically significantly improved in the case of the solvent at the concentration of 1.5% ($p<0.05$). In all three extract variants, it appears that the RRE percentage values decrease with the tested extract concentration (1.5%), the lowest value being recorded in the case of the extract in ethanol 40% (39.09%). In the case of the extracts in 40% and 70% ethanol, the chemical influences present in the compound extract can be observed, because the elongation index is lower compared to the elongation index obtained in the solvent treatment of the seeds. On the other hand, in the case of the extract in propylene glycol, it was observed how the treatment of the seeds with the extract as such did not affect the development of the roots, the percentage of elongation being higher compared to that recorded after the treatment of the seeds with solvent (PG 50%).

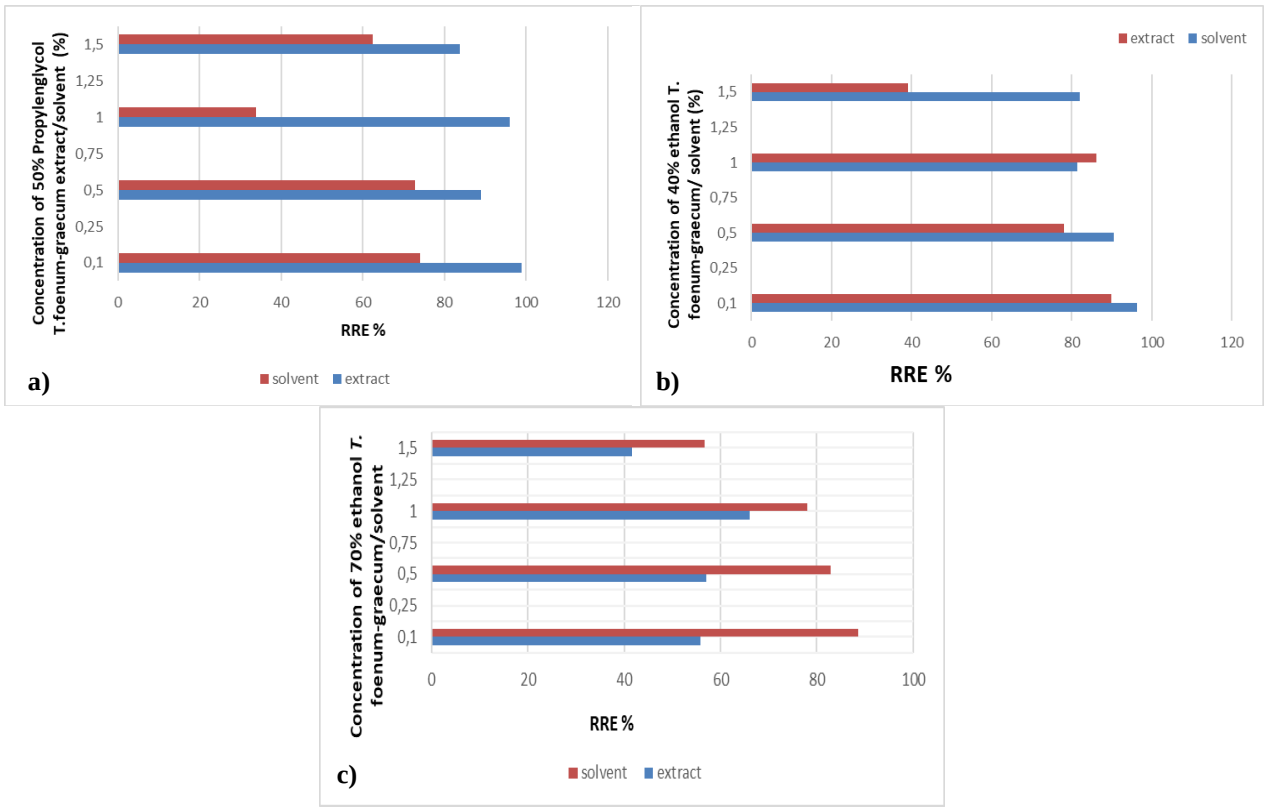

Figure 1. The effect of Fenugreek extracts on relative root elongation ofradish seeds;
a) Fenugreek seed extract in 50% PG; b) Fenugreek seed extract in 40% ethanol; c) fenugreek seed extract in 70% ethanol

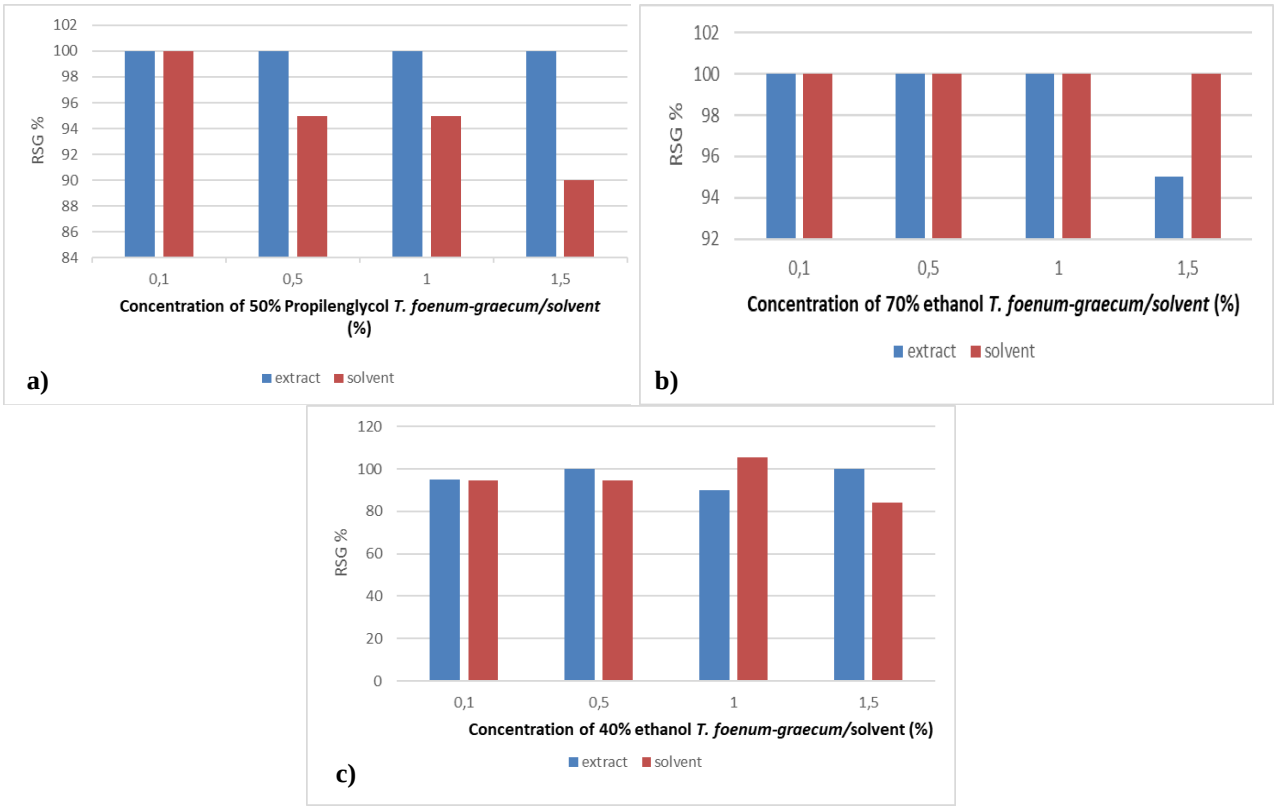

Figure 2. The effect of Fenugreek extracts on relative seed germination rate of radish seeds;
a) Fenugreek seed extract in 50% PG; b) Fenugreek seed extract in 70% ethanol; c) Fenugreek seed extract in 40% ethanol

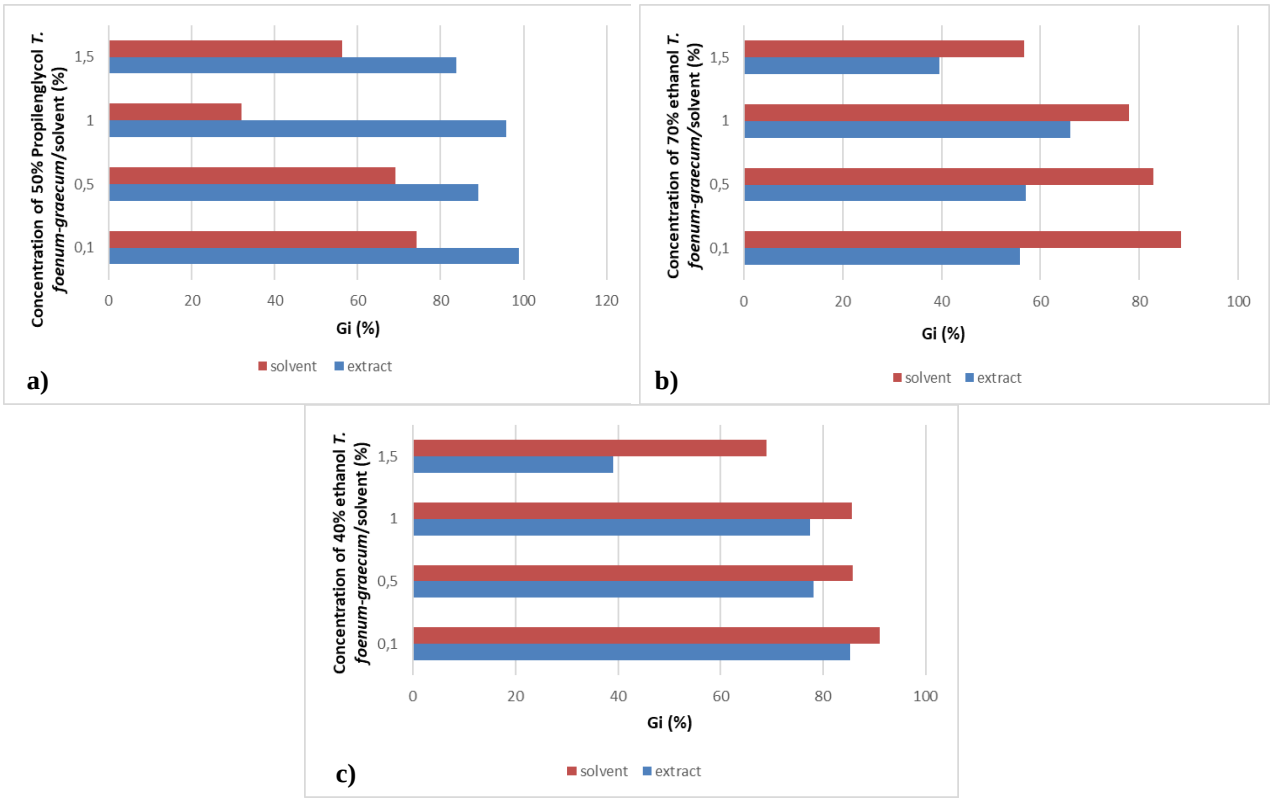

Figure 3. The effect of Fenugreek extract on the germination index (Gi)
a) Fenugreek seed extract in 50% PG; b) Fenugreek seed extract in 70% ethanol; c) Fenugreek seed extract in 40% ethanol

It can be seen that the RSG% (Figure 2) and RRE %, results are close to the concentration of antioxidants found in the composition of each variant of the extract. A good example is given by the results obtained in the case of fenugreek seed extract in 40% ethanol regarding the elongation index, at a concentration of 1.5%. This is lower than in the case of the extract in 70% ethanol (1.5%), where the concentration of phenolic compounds recorded was the lowest.

Following the analysis of the results obtained on the three varieties of fenugreek seed extract, a strong phytotoxic activity of the extracts in 70% and 40% ethanol was observed at the tested concentration of 1.5% (Figure 3), recording a germination index (Gi) under 50%, 39.54%, respectively, 39.09%. In the case of the treatment applied with 40% ethanol, Gi (%) fell between 91.11-69.02%. On the other hand, when treating the seeds with 70% ethanol (solvent), the Gi values were statistically significantly improved ($p<0.05$) at all tested concentrations, feeling between 88.56-56.63%. Results were influenced by the presence of the high concentration of polyphenols and flavonoids present in the variant of the extract obtained in 40% ethanol, which could explain the recorded Gi value (39.06%). This being the lowest Gi value of all the three extract variants tested. It is already known from the specialized literature that these phenolic compounds exhibit a strong allelopathic character. The phytotoxic responses of the extracts also appear to be dependent on the concentration tested, as can be seen from Figure 9. Regarding the fenugreek variant obtained in PG 50%, the percentage of Gi was over 80% (98.95-83.64%), which means that it does not show phytotoxic activity on the tested plant, and in the case of solvent treatment, it was observed. a strong phytotoxic effect at a concentration of 1% (Gi - 32.05%). Compared to the solvent, the Gi index was improved in the presence of the extract sample.

As we noticed in the case of the elongation index and the relative germination index, the recorded Gi values decrease with the concentration of the tested extract, the lowest values being recorded at a concentration of 1.5%. The results are dependent on the tested doses.

The strong allelopathic potential of the extract obtained in 40% ethanol may be related to a higher content of phenolic substances and, consequently, to a high antioxidant potential, since most of these compounds are reported as phytotoxic compounds (El-Gawad et al., 2015; Elshamy et al., 2019). A study conducted by Golsoomeh et al. (2011), regarding the allelopathic effect of the extracts obtained from different parts of the Fenugreek plant, on sesame, soybean, pigweed and velvetleaf seeds, showed that the extracts obtained from *Trigonella foenum-graecum* seeds and leaves presented the strongest allelopathic effect in all tested seeds, significantly reducing the germination yield and the elongation index. This effect was stronger at higher extract concentrations. The higher the extract concentration, the lower the germination rate and elongation index. Phytotoxic effects of phenolic compounds may include inhibition of respiration, nutrient substance absorption, photosynthesis process and receptor enzyme activities (Li et al., 2010; Anwar et al., 2021). According to the results obtained, we can say to some extent that there is a link between the allelopathic activity of plants with antioxidant potential and the content of phenolic compounds, but also the type of solvent used for extraction.

CONCLUSIONS

Three variants of extract from fenugreek seeds were obtained. In the extraction process, concentrated alcohols were used to preserve the main active compounds with a possible insecticide-pesticide-fungicide effect, but also because these compounds show high solubility in concentrated alcohols. According to obtained results, the most effective extraction method of phytochemical compounds from Fenugreek (*Trigonella foenum-graecum*) seeds was the thermal assisted one. This aspect was highlighted by the increased concentrations of polyphenols

and flavonoids obtained. In correlation with literature findings, these types of actives are associated with strong antimicrobial properties and present encouraging hallmarks for further use in development of biopesticides products. At the same time, the present work offers additional information in order to support the use of an economic, easy, safe and effective extraction process of bioactive compounds from plants.

Authors' contributions: Conceptualization, A.P; methodology, A.P., M.P., D.M.E. and L.P.; validation, A.P., M.P., D.M.E. and L.P.; investigation, A.P., M.P., E.M.D. and P.L.; writing - original draft preparation A.P.; writing - review and editing, A.P. and B.M.T.; visualization S.C. and L.P.; supervision, S.C.; project administration, A.P.; funding acquisition, A.P.

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