Acta Iguazu
ISSN: 2316-4093

Methods of preparing aqueous extract and bioactivity of *Mentha spicata* L. on *Raphanus sativus* L. seeds

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Artigo enviado em 25/08/2020, aceito em 13/12/2020

Abstract: The plant interaction knowledge can be applied in several sectors in agriculture such as crop rotation, weed management, green manure and the use of cover crops. Studies on the allelopathic potential of certain plants have been carried out with the application of vegetable extracts on the seeds of a sensitive plant, however, a consensus has not been reached regarding the best way to prepare such extracts. This study aimed to evaluate the efficacy of methods of extraction of allelopathic compounds and the bioactivity of different concentrations of the *Mentha spicata* L. aqueous extract on *Raphanus sativus* L. seeds. A completely randomized design was used in a 5x4 factorial scheme with four replications and four extraction methods (infusion, grinding in a blender, drying at 40 °C and 70 °C) and five concentrations (0; 25; 50; 75 and 100%). The variables investigated were: phenolic compounds quantification, germination, germination speed index (GSI), mean germination time (MGT), radish shoot and root length. The extract presenting the highest amount of phenolic compounds was prepared via infusion (724.36 μg/mL), followed by drying at 40 °C (605.07 μg/mL), blending (594.12 μg/mL) and drying at 70 °C (529.36 μg/mL). The *Mentha spicata* L. aqueous extracts interfered in the radish seed physiological quality, by inhibiting germination, GSI and MGT increase, mainly when in higher concentrations. There was a directly proportional relation between the quantity of phenolic compounds extracted through different extraction methods and the interference in the physiological quality of the radish seeds.

Key words: Allelopathy, phenolic compounds, spearmint.

Métodos de preparo de extrato aquoso e bioatividade de *Mentha spicata* L. em sementes de *Raphanus sativus* L.

Resumo: O conhecimento sobre as interações das plantas pode ser aplicado em muitos setores da agricultura, como na rotação de culturas, manejo de plantas daninhas, adubação verde e uso de cobertura vegetal. Os estudos sobre o potencial alelopático de plantas tem sido realizado com a aplicação de extratos vegetais sobre sementes de uma planta sensível, mas há divergências sobre a melhor forma de preparação de extratos. Objetivou-se avaliar a eficácia de métodos de extração de compostos alelopáticos e a bioatividade de diferentes concentrações do extrato aquoso de *Mentha spicata* L. em sementes de *Raphanus sativus* L.. Utilizou-se o delineamento inteiramente casualizado em esquema fatorial 5x4 com quatro repetições, sendo quatro métodos de extração (infusão, trituração em liquidificador, secagem a 40 °C e a 70 °C) e cinco concentrações (0; 25; 50; 75 e 100%). As variáveis analisadas foram: quantificação de compostos fenólicos, germinação, índice de velocidade de germinação (IVG), tempo médio de germinação (TMG), comprimento da parte aérea e raiz de plântulas de rabanete. O extrato que apresentou maior quantidade de compostos fenólicos foi o preparado por infusão (724,36 µg/mL), seguido da secagem a 40 °C (605,07 µg/mL), trituração em liquidificador (594,12 µg/mL) e secagem a 70 °C (529,36 µg/mL). Os extratos aquosos de *M. spicata* L. interferiram na qualidade fisiológica das sementes de rabanete, com inibição na germinação, IVG e aumento do TMG, sobretudo em maiores concentrações. Houve uma relação diretamente proporcional entre a quantidade de compostos fenólicos extraídos pelos métodos de extração e a interferência na qualidade fisiológica das sementes de rabanete.

Palavras chave: Alelopatia, compostos fenólicos, hortelã.

Introduction

Allelopathy is an ecological phenomenon of natural occurrence in which live organisms produce and release biochemical compounds (allelochemicals) in the environment, affecting growth, development, reproduction and survival of other live organisms in the neighboring environment (Nawaz et al., 2020).

Approximately 10,000 secondary compounds with allelopathic action are known, and the allelochemical action tends to occur more through the synergic interaction of different substances than the activity of an isolated product (Pires and Oliveira, 2011). The main compounds with allelopathic actions are those of phenolic nature, which are released in the environment in many different ways such as leaching, volatilization, plant residue decomposition and root exudation (Favaretto et al., 2018; Souza et al., 2019).

The knowledge of the allelopathic nature of certain plants and their actions on other organisms can be applied in many agricultural sectors such as weed control, crop rotation, green manure, use of mulch and integrated management of plagues and diseases. In addition, allelochemical compounds have the potential to be used to regulate growth, substitute herbicides and pesticides and to protect the crops against pathogens (Cheng and Cheng, 2015; Bachheti, 2020).

Spearmint (*Mentha spicata* L.) is a medicinal plant that has been studied for its allelopathic effect on other plants. Mairesse et al. (2007) and Parreiras et al. (2011) reported the spearmint extract negative effect on the germination of lettuce seeds (*Lactuca sativa* L.). Karkanis et al. (2019) identified that the cultivation of two spearmint cycles reduced chlorophyll content, photosynthetic rate, growth, dry biomass and yield of sweetcorn grains (*Zea mays L.*) planted subsequently.

Most of the studies involving allelopathy have used the method of applying extracts of one species on seeds of another species. However, the methods to obtain allelopathic extracts from the plants is varied, and can involve grinding the vegetable in organic extractor or water (Pires and Oliveira, 2011), grinding it in a blender (Periotto et al., 2004; Mairesse et al., 2007; Muniz et al., 2007; Corsato et al., 2010; Parreiras et al., 2011), using infusion (Souza et al., 2005; Souza et al., 2005b; Iganci et al., 2006), drying it at different temperatures ranging from 40 to 70 °C followed by grinding and dilution in water (Filho et al., 1996; Correa et al., 2005; Felix et al., 2007; Brass, 2009), among others. Such diversity of methods to prepare the vegetable extracts has generated divergences between research results and, with that, resulting in researchers' interest in seeking more efficient methods to extract bioactive compounds from vegetable sources.

Taking that into consideration, the objective of this study was to evaluate the efficacy of different allelopathic compound extraction methods and the bioactivity of different concentrations of spearmint (*Mentha spicata* L.) aqueous extract on radish (*Raphanus sativus* L.) seeds.

Material and methods

The study was developed between April and June 2018, at the Physiology Laboratory and Plant Germination and Growth Laboratory at the Federal University of Fronteira Sul (UFFS), in Laranjeiras do Sul-PR.

The experimental design used was completely randomized design (CRD) in a 5 x 4 factorial scheme, with five spearmint extract concentrations (0, 25, 50, 75 and 100%) and four extraction methods: drying at 40 °C, drying at 70 °C, grinding in blender and infusion, with four replications.

Spearmint (*Mentha spicata* L.) dried leaves were collected in the morning, on a family farm located at the *8 de Junho* Settlement in the municipality of Laranjeiras do Sul-PR, coordinates 25°26'28.86"S and 52°26'47.88"O. Next, the vegetable material was taken to the laboratory, washed in running water and fractioned in approximately 5 cm cuts. The aerial part of the plant (leaves and branches) was used, while the roots were disposed of.

To prepare the spearmint extract through drying at 40 °C, 210 g spearmint fresh material was dehydrated in oven at 40 °C up to the production of the desired weight. Next, the vegetable material was ground in a Willey cutting mill, with a 2 mm sieve. After obtaining the vegetable powder, it was diluted in distilled water at 20% (mass/volume). The sample was kept under agitation in a Shaker for 2 h, at 40 °C and 230 rpm rotation. Finally, the extract was filtered in a paper filter and funnel. For the drying at 70 °C extraction method, the same procedure was followed as the one at 40 °C, differing only the temperature.

For the grinding with blender extraction method, 210 g fresh spearmint was ground in blender with distilled water for 30 seconds. After that, two filtering procedures were carried out: the first, in a plastic sieve to remove the vegetable material larger particles; and the second in cotton cloth, to separate smaller particles. The 20% (mass/volume) aqueous extract was obtained.

In the infusion method, the water was heated up to the boiling point and 210 g of fresh spearmint was added. Next, the container was hermetically closed until the water reached room temperature. After this procedure, the material was filtered in a cotton cloth, obtaining the 20% (mass/volume) spearmint aqueous extract.

The 20% (mass/volume) spearmint aqueous extract was used as stock solution, from which dilutions were prepared to obtain the different concentrations, 25, 50, 75 and 100%, in which 100% was considered the spearmint aqueous extract at 20% without dilution. The witness contained only distilled water. To evaluate the biological activity of the spearmint aqueous extracts on the radish seeds, the following parameters were analyzed: germination percentage, germination speed index (GSI), mean germination time (MGT) shoot length and root length.

The germination, germination speed index and mean germination time tests were carried out jointly, with four replications of 50 seeds each, following the recommendations of the seed analysis rules (Brasil, 2009). The spearmint aqueous extract was used as the blotting paper humifying medium that covered the gerbox, and that paper was humified with the spearmint extract 2.5 times its dry mass. In the control, the blotting paper was humidified with distilled water.

The treatments were kept for 10 days in a germination chamber (B.O.D.) with a 12-hour photoperiod at 25 °C (Brasil, 2009). The radish seeds were evaluated daily and at the end of the tenth day, the count was carried out, considering normal, abnormal, dead and dormant seedlings.

The germination speed index (GSI) was obtained using the equation proposed by Maguire (1962):

$$IVG= G_1/N_1+G_2/N_2+...+G_n/N_n$$

Where: GSI= germination speed index; G= number of normal seedlings recorded in the first count, second count and the last count; N= number of days from the sowing to the first, second and last count; n= number of days.

The mean germination time (MGT) was calculated using the equation proposed by Edmond and Drapala (1958):

$$AGT = \Sigma(niti)/\Sigma ni$$

Where: MGT= mean germination time (days); ni = number of normal seedlings identified in each count; ti = incubation time.

The tests to evaluate the radish shoot and root lengths were carried out in the same conditions as those of the germination test, however, with 4 replications of 20 seeds each. The measurements were carried out using a digital pachymeter.

Total phenol determination was based on the methodology presented by Bittencourt (2017), in which, 50 mL of each aqueous extract was mixed to 150 mL acetone PA and agitated at room temperature (~22 °C) for 12 hours at ~15 rpm. With that, the supernatant protein and lipid content was precipitated and vacuum filtered in two layers of 25 µm porosity filter paper. The precipitate was discarded and the acetone removed from the extract by using the rotating evaporator at 40 °C. The resulting liquid was washed with three 100 mL hexane portions and again three times with 100 mL ether. The phase that contained hexane was discarded. The ether phase was dried with a spatula tip of anhydrous sodium sulfate (Na_2SO_4). The ether was removed with a rotating evaporator at 40 °C and the residue diluted in 20 mL distilled water.

The samples were read in a spectrophotometer and the cuvette containing 100 µL extract, 600 µL $NaCO_3$ (7.5% m/v), 700 µL distilled water and 200 µL Folin-Ciocalteau. The mixture was heated at 50 °C for 10 minutes and diluted with 1.0 mL distilled water before the spectrophotometer reading. The analytical curve was built as a function of the concentrations 0, 10, 20, 30 and 40 µg/mL for the gallic acid at 760 nm. The phenolic substances present in the sample under evaluation were reported in their equivalent of gallic acid micrograms per spearmint aqueous extract milliliter.

The quantitative data, whenever possible, was evaluated using regression analysis and the qualitative data was evaluated by employing the Tukey test at 5% probability. The data analysis was carried out using the Balanced Data Variance Analysis System, SISVAR, for microcomputers, developed by Ferreira (2014).

Results and discussion

The quantification of total phenolic compounds showed that when starting with the same fresh mass of spearmint leaves and branches, the extract prepared through infusion was the one presenting the highest amount of these compounds (724.36 μg/mL), followed by the drying at 40 °C (605.07 μg/mL), grinding in blender (594.12 μg/mL) and drying at 70 °C (529.36 μg/mL) methods (Figure 1).

The concentration of phenolic compounds in the aqueous extract prepared using the infusion method was 36.84%, 21.92% and 19.29% higher than the concentration found in the extract prepared via drying at 70 °C, grinding in blender and drying at 40 °C, respectively. These results emphasize the importance of the infusion extraction method, which in addition to demanding less work, is more efficient to extract phenolic compounds.

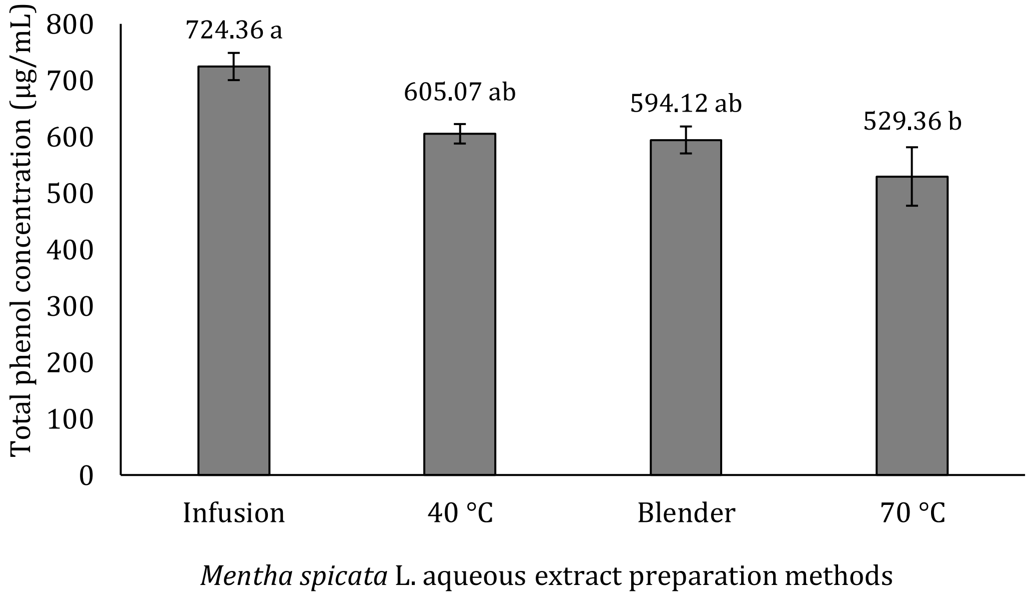

Figure 1. Amount of total phenolic compounds (μg/mL) in *Mentha spicata* L. aqueous extracts obtained through the methods: infusion (724.36 μg/mL, CV= 5.77%), drying in oven at 40 °C (605.07 μg/mL, CV= 4.95%) and 70 °C (529.36 μg/mL, CV= 16.97%); and grinding in blender (594.12 μg/mL, CV= 6.97%).

The low content of phenolic compounds in the extract prepared at 70 °C might be related to the degradation of these compounds at high temperatures. Salgaço and Sacramento (2019) verified a 16% reduction in phenolic compounds of *Capsicum baccatum* L., known as 'pimenta dedo-de-moça' in Brazil, submitted to pasteurization at 65 °C for 30 minutes and 11% reduction in *Capsicum chinense* Jacq., known as 'pimenta biquinho' in Brazil, cooked at 100 °C for 5 minutes.

Phenolic compounds form a chemically heterogeneous group of molecules with varied size and complexity (Taiz et al., 2017) and they are the main substances responsible for the allelopathic inhibiting action on plants (Pires and Oliveira, 2011), although this action can also be obtained from substances in the terpenoids group (Maia et al., 2011; Farooq et al., 2020).

The composition of phenolic compounds in a plant is influenced by several factors such as its genetic structure, stage of development, organ under analysis (root, leave, stem, flower), environmental conditions and farming practices (Telci et al, 2010; Farooq et al., 2020). Cirlini et al. (2016) reported that the main phenolic compounds found in the *Mentha spicata* L. extract were: rosmarinic acids (88%), salvianolic acids (5.6%), caffeic acids (1.2%), hydroxycinnamic acids (1.1%), in addition to other groups such as flavonols, flavonones, flavones, hydroxybenzoic acids and hydroxyphenylpropanoic acids (1%).

The Table 1 shows the mean squares and the significance for the different parameters evaluated: germination, germination speed index (GSI), mean germination time (MGT), root and shoot length.

Table 1. Mean square and significance for germination, germination speed index (GSI), mean germination time (MGT), shoot and root length for 4 different methods of preparing aqueous extract in 5 different concentrations.

VS	DF	Mean square				
		Germination	GSI	MGT	Shoot length	Root length
Methods (M)	3	449.6500*	68.9904*	4.0776*	229.4597*	376.664*
Concentration (C)	4	3368.5500*	113.4235*	5.3814*	150.3455^{ns}	232.049^{ns}
M x C	12	94.4833^{ns}	10.5190*	0.9908*	51.0175^{ns}	170.191^{ns}
Error	60	61.6500	1.5880	0.2918	60.3463	113.2392
CV (%)		11.38	18.87	11.39	17.48	20.78

*Significant at the 5% probability level; ns = Not significant

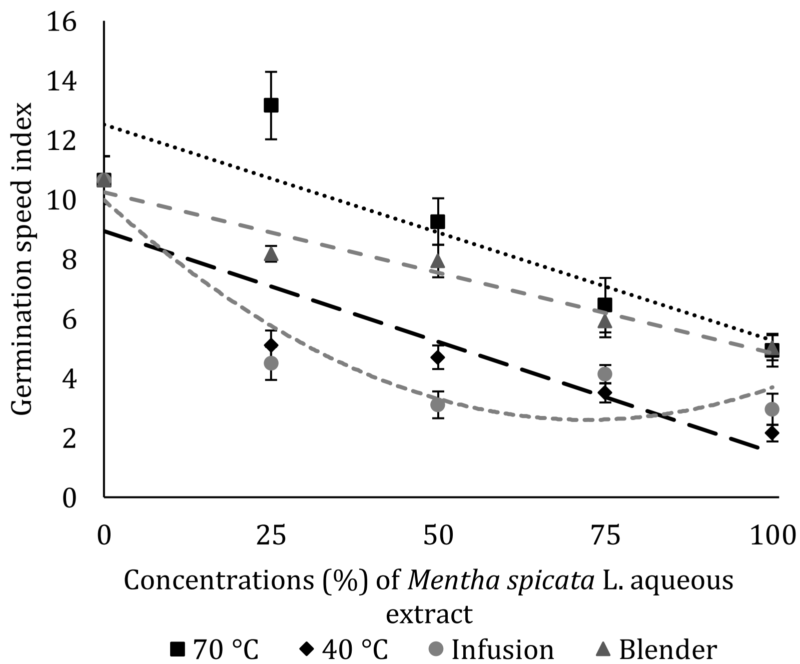

Figure 2. Radish (*Raphanus sativus* L.) seed germination speed index (GSI) as a function of different spearmint (*Mentha spicata* L.) aqueous extract preparation methods and different concentrations. Regression analysis: (70 °C) y = -0.0725x+12.515 *0.0000, R^2 = 0.7645; (grinding in blender) y = -0.054x + 10.242 *0.0000, R^2 = 0.9512; (40 °C) y = -0.0744x + 8.9389 *0.0000, R^2 = 0.8205; (infusion) y = 0.0014x^2– 0.2044x + 9.985 *0.0000, R^2 = 0.8786.

The germination speed index (GSI) showed an decreasing trend according to the extract concentration. When the extract prepared via infusion was investigated, a sharp initial reduction of 25% was observed and little variation between it and higher concentrations (50, 75 and 100%). The extracts prepared via infusion and drying at 40 °C promoted higher GSI reduction, followed by the extract prepared via grinding in blender and the one prepared via drying at 70 °C, which presented the lowest effect on GSI. This result evidences a directly proportional relation between the extracts with higher presence of phenolic compounds (Figure 1) and GSI reduction (Figure 2).

A tendency to increase the mean germination time (MGT) was observed with the increase in the extract concentrations. Increased MGT in seeds submitted to the extract prepared via infusion was higher up to the 25% concentration, following with smaller variation in the remaining concentrations (50, 75 and 100%). The extracts prepared via infusion and drying at 40 °C were observed to be the ones that most affected MGT, followed by the extract prepared in blender and that prepared via drying at 70 °C (Figure 3).

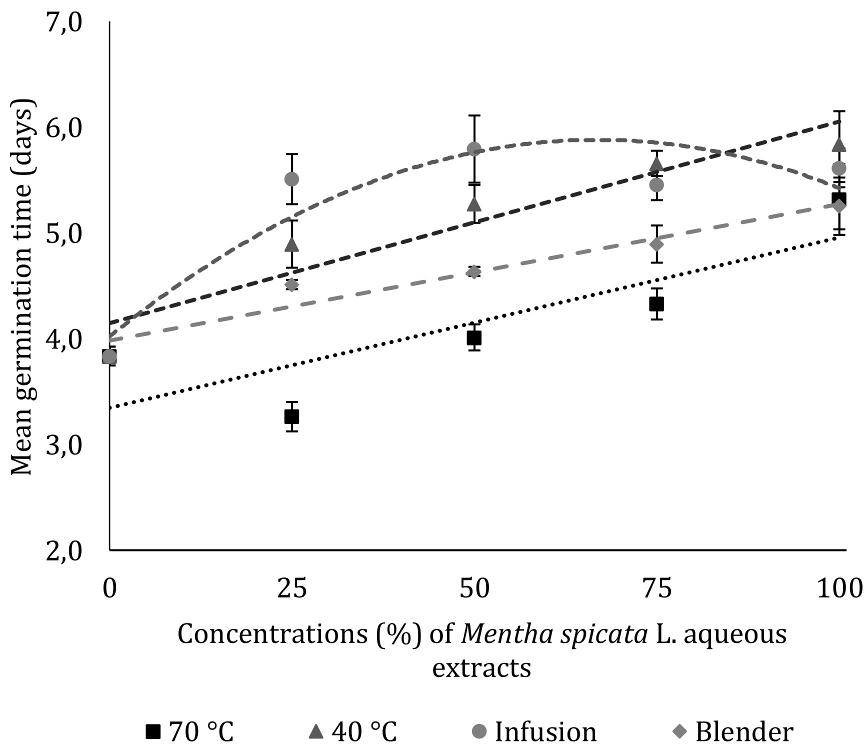

Figure 3. Variation of radish (*Raphanus sativus* L.) seed mean germination time (MGT) as a function of different spearmint (*Mentha spicata* L.) aqueous extract preparation methods and different concentrations. Regression analysis: (70 °C) y =0.0161x + 3.3456 *0.0062, R^2 =0.7058; (grinding in blender) y =0.0129x + 3.9815 *0.0415, R^2 =0.9391; (40 °C) y =0.0191x + 4.1462 *0.0000, R^2 =0.8996; (infusion) y =-0.0004x^2 + 0.0556x + 4.0195 *0.0251, R^2 =0.8585.

The results shown in Figures 2 and 3 are better understood when related to the quantification of the phenolic compounds in the extracts (Figure 1), since the infusion and drying at 40 °C methods were the ones that resulted in the highest amount of total phenols, which are the main substances responsible for their allelopathic inhibiting action (Pires and Oliveira, 2011). Germination speed reduction in seeds of a certain species might limit considerably its ability to survive, since the seeds that germinate more slowly might result in seedlings of reduced size and, consequently, they might be more susceptible to stress and predators, presenting fewer chances to compete for resources such as water, light and mineral nutrients (Jefferson and Pennachio, 2005).

Regarding germination, no significant interaction was found between the extraction methods and the aqueous extract concentrations, appearing only as significant isolated factors.

The spearmint aqueous extracts affected negatively the radish germination, and this effect was directly proportional to the extract concentrations (Figure 4). The 100% concentration provoked 43.13% reduction in germination when compared to the control, indicating the spearmint aqueous extract allelopathic potential on radish.

Pereira et al. (2015) demonstrated that exudates from some aromatic plants, including species of the genus *Mentha*, presented antagonist substances that reduced in 84% the survival rate of lettuce seedlings, indicating allelopathic action. Karkanis et al. (2019) observed reduction in the photosynthetic rate, stomatal conductance, height and dry mass of sweet corn plants and yield, when sowed in succession to *Mentha spicata* L. and *Mentha x piperita* L..Those authors ascribed this effect to the allelopathic activity of the plants used in the crop rotation and called attention to the importance of knowing the interaction between plant species to obtain greater efficiency from the rotation system.

The extract prepared in blender was the one that showed the lowest inhibition of radish seed germination, while the other extracts (infusion and drying at 40 °C and 70 °C) did not differ one from another (Table 2). This effect might be related to the concentration of the phenolic compounds, since that was the second extract with the lowest concentration of such compounds (Figure 1).

When the variables shoot and root length were investigated, no significant difference was found between the concentrations of spearmint aqueous extracts. The seeds submitted to extraction via grinding in blender were the ones that resulted in plants with the longest root and shoot length, however, it did not differ from the infusion method for both variables and from the drying at 40 °C method for the root length variable (Table 2).

The variables related to seedlings vigor (germination speed index and mean germination time) obtained higher values in response to the extract preparation methods and the different concentrations when compared to the germination variable. This might have occurred because germination is less sensitive to the allelochemical effect when compared to the vigor parameters, since allelopathic substances might not affect the final germination percentage, but may influence the germination speed or other characteristics that precede the germination process (Ferreira and Aquila, 2000). According to Krzyzanowski et al. (1999), vigor loss precedes germination reduction, so that seed lots with similar germination might differ regarding the level of deterioration. Similar results were obtained in the study developed by Nariai et al. (2013), which demonstrated that the spearmint aqueous extract affected negatively the germination speed index in lettuce seedlings, delaying the germination process.

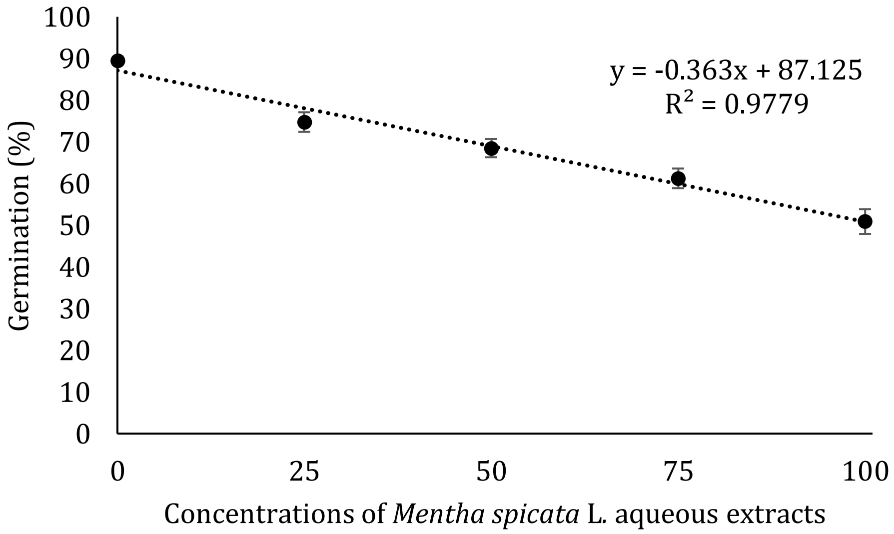

Figure 4. Percentage of *Raphanus sativus* L. seed germination as a function of the applications of different concentrations of *Mentha spicata* L. aqueous extract.

Table 2. Germination rate (%), shoot (mm) and root (mm) length of *Raphanus sativus* L. seedlings as a function of the *Mentha spicata* L. aqueous extract preparation.

Spearmint aqueous extract preparation method	Germination (%)	Shoot length (mm)	Root length (mm)
70° C	69.00 a	41.93 a	46.17 a
40° C	66.50 a	42.86 a	49.09 ab
Infusion	64.80 a	43.56 ab	54.05 ab
Grinding in blender	75.60 b	49.43 b	55.51 b
C.V. (%)	11.38	17.48	20.78

Means followed by the same small letter in the column did not differ one from another, considering the 5% level of probability in the Tukey test.

Mahdavikia and Saharkhiz (2015) developed an experiment using different concentrations (0, 2, 4, 6, 8 and 10%) (v/v) of the aromatic species peppermint (*Mentha x piperita*) aqueous extractin radish (*Raphanus sativus* L.) seed germination and seedlings development. They observed that increased concentrations of the aqueous extract impacted negatively the germination percentage as well as shoot and root length. In our study, when the variables root and shoot length were analyzed, no significant difference was found between the different concentrations of the spearmint aqueous extract.

Several factors might influence the allelopathic effect of extracts, such as the vegetable species used, preparation methods, formula (powder from leaves, aqueous extracts and essential oils), application methods, composition and the concentrations used (Cruz et al., 2000; Saharkhiz et al., 2010; Bichand Kato-Noguchi, 2012; Poonpaiboonpipat et al., 2013).

The preparation methods of spearmint (*Mentha spicata* L.) aqueous extracts that resulted in higher amounts of phenolic compounds and better effect on the parameters related to the radish (*Raphanus sativus* L.) seed vigor and germination were the infusion and drying at 40 °C methods. The results obtained in this work are in accordance with the study developed by Gião et al. (2007), which evaluated the effect of the method of preparation of different plant extracts regarding their phenolic compound content. Those authors demonstrated that the powder infusion extraction method is the most efficient to extract phenolic compounds.

The results of this study provided information to support the choice of a method of preparation of extracts in allelopathic potential research. After learning that the extract obtained via infusion resulted in the largest quantity of phenolic compounds, it seems reasonable to recommend its use in similar studies. The preparation of extracts via infusion also demands less work than the methods that require drying, crushing or grinding in blender.

Conclusions

The extract presenting the highest amount of phenolic compounds was the one prepared via infusion (724.36 µg/mL), followed by drying at 40 °C (605.07 µg/mL), grinding in blender (594.12 µg/mL) and drying at 70 °C (529.36µg/mL).

The *Mentha spicata* L. aqueous extracts impacted the physiological quality of radish seeds, by inhibiting germination, germination speed index and increasing mean germination time, mainly when in higher concentrations.

A directly proportional relation was observed between the amount of phenolic compounds extracted by the different extraction methods and the effect on the physiological quality of radish seeds.

Acknowledgments

The authors are thankful to the Universidade Federal da Fronteira Sul (Federal University of Fronteira Sul) for the approval of the research project PES 2019-0597 in the call 459/2019 – Financial Support to the Post-Graduation Program Research and the Coordination for Improvement of Higher Education Personnel (CAPES) for the scholarship provided for the first author.

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