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Priming of *Brassica napus* L. seeds with aqueous extract from mistletoe (*Viscum album* L.) boosts the content of photosynthetic pigments

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Abstract. The increasing occurrence and severity of drought periods due to climate change have a negative impact on crop yield worldwide. In the face of a rising human population, this poses a severe threat to food security. Natural products such as plant extracts have many uses, but the studies about their potential usage in seed pre-sowing treatments have been limited. In this study, we evaluated the potential of *Viscum album* L. extract as a *Brassica napus* L. seed coating and seed priming agent. Moreover, its potential to limit the growth of commonly occurring foodborne pathogens was verified. The results showed that seed treatments with mistletoe macerate did not significantly affect hypocotyl and root length and biomass. Chlorophyll and carotenoid content of the plants treated with mistletoe extracts increased – with the best results obtained for the seeds humidified with 20% extract and 50% for priming. *V. album* extract exhibits weak antibacterial properties towards *Escherichia coli* and *Salmonella typhimurium* and does not show antibacterial properties against *Staphylococcus aureus*. This study points to the new possible use of plant extracts as an ecological way for seed pre-sowing treatment.

Keywords: mistletoe, *Brassica napus*, plant growth, antibacterial properties, seed treatment, plant extracts, HPMO.

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1. Introduction

Brassica napus L. var. *napus* (canola) is one of the most important crops and the second, after soybean, most widely grown oilseed crop worldwide (Saeidnia & Gohari, 2012). In 2020/2021, global production of *B. napus* reached a substantial volume of 73.6 Mt (Borges et al., 2023). Canola is used in producing oil, fodder (Kowalska et al., 2020), and products classified as healthy food, rich in minerals, proteins, fiber, and compounds with anti-cancer properties (Ostrowska et al., 2018). Canola oil is characterized by high-quality protein content with a well-balanced amino acid composition and a favourable content of minerals (Na, Ca, Mg, Zn, Fe, Mn, Cu). These characteristics allow *B. napus* to be used as an alternative to legumes (e.g. soybean, pea, lupine) (Kowalska et al., 2020). Oilseed rape is also used in industry for the production of biofuels, paints, and polyurethanes (Lin et al., 2013), which contributes to a constant increase in the production of this crop (Iriarte et al., 2010).

Increasingly frequent drought events and climate change cause water shortages in crop plants with the consequences including disturbed metabolism, inhibited growth, reduced plant size, and lower yields (Anjum et al., 2011; Konieczna et al., 2023a; Chekol et al., 2023). These changes are caused primarily by a decrease in the efficiency of photosynthesis (Konieczna et al., 2023b) and changes in the expression of genes related to the functioning and protection of the photosynthesis apparatus (Stawski et al., 2008; Dąbrowska et al., 2020). Seed priming is one of the proposed techniques to improve plant health and increase their resistance to abiotic and biotic stress factors (Paparella et al., 2015). Improving seed quality and achieving rapid and uniform seedling emergence is crucial for crop cultivation as too slow germination exposes plants to adverse environmental conditions and infections (Osburn & Schroth, 1989). Seed conditioning can increase plant tolerance to stresses such as drought (Taherkhani et al., 2013), salinity (Elouaer & Hannachi, 2012), and temperature extremes (Chakraborty & Dwivedi, 2021). The effect of seed conditioning depends on plant species, physiology, and the method used (Parera & Cantliffe, 1994). Kaur et al. (2002) showed that *Cicer arietinum* L. seedlings obtained from seeds conditioned with 4% mannitol showed a fourfold increase in root and shoot length compared to non-conditioned seeds. Moreover, priming with hydrogen peroxide increased the germinability and biomass of *B. napus* seedlings. It was observed that 10.5% more seeds germinated when conditioned with H_2O_2 than the control seeds (Mierek-Adamska et al., 2019). Adinde et al. (2020) demonstrated a positive effect of water-based conditioning on the speed and uniformity of germination and germinability of basil (*Ocimum basilicum* L.). Another conditioning method is a technique that involves coating seeds with living microorganisms or their spores (Turkan et al., 2023). Inoculation of plants with plant growth-promoting fungi or bacteria can accelerate plant growth and increase their tolerance to biotic and/or abiotic stressors (Waller et al., 2005; Dąbrowska et al., 2021, 2021a; Garstecka et al., 2023). Beneficial effects of this method have been observed in tomato (*Lycopersicum esculentum* Mill.), cowpea (*Vigna unguiculata* L.) and bell pepper (*Capsicum annuum* L.) seeds using *Trichoderma harzianum*, *Trichoderma pseudoconingii*, *Bacillus* spp., *Gliocladium* spp., and *Pseudomonas fluorescens* (Ilyas, 2006). In *B. napus,* applying seed coating containing *Trichoderma viride* enhanced seedling growth and did not induce a stress response in plants (Turkan et al., 2023).

World Health Organization estimates that 1 in 10 people contracts foodborne pathogens yearly due to contaminated food, with 420,000 deaths annually from foodborne diseases (Awad et al., 2024). One of the major culprits are human pathogenic microorganisms (HPMOs), which can colonize and/or attach to the surface of plants. HPMOs can come in contact with the crops via contaminated surface water, soil, wastewater, wildlife faeces, and improperly prepared manure. Contamination may also occur during crop transportation, storage, and processing (Luna-Guevara et al., 2019; Jacob & Melotto, 2020). Human pathogenic microogranisms can colonize plants through stomata, fistulas, hydathodes, and sites of chemical or physical damage (Chahar et al., 2021). Consumption of raw vegetables has been linked to a higher incidence of foodborne diseases (Mogren et al., 2018). The most common pathogenic microorganisms on plants are noroviruses, *Salmonella enterica,* and Shiga toxin-producing strains of *Escherichia coli* (Bennett et al., 2018). The highest risk of HPMO infection is associated with the consumption of leafy vegetables, e.g., lettuce (*Lactuca sativa* L.), of which consumption in the United States was 12 kg per person in the last 10 years (Jacob & Melotto, 2020), and spinach (*Spinacea oleracea* L.) (Macarsin et al., 2013).

Common mistletoe (*Viscum album* L.) can exibit antibacterial properties against HPMO bacteria (Ekhaise et al., 2010; Mapfumari et al., 2022). Hussain at al. (2011) have demonstrated that leaves extract of *V. album* shown antibacterial activities against *Staphylococcus aureus, E. coli, Pseudomonas aeruginosa* and *Salmonella typhi*. Moreover Shah et al. (2017) confirmed this results by conducting research on antimicrobial activities of leaves and fruits of *V. album* extracts. Ethyl acetate, butanol, and water mistletoe extracts showed growth inhibitory effects against both Gram-positive (*Bacillus subtilis, S. aureus*) and Gram-negative (*E. coli, S. typhi*) bacteria. Ekhaise et al. (2010) showed that the methanolic extract of mistletoe inhibited the growth of microorganisms such as *S. aureus, Proteus mirabilis, Klebsiella pneumoniae, E. coli,* and *P. aeruginosa*. Besides antibacterial mistletoe can exhibits antiviral (Agbo et al., 2017), anti-cancer (Oei et al., 2019; Wrotek et al., 2014), antioxidant (Mapfumari et al., 2022), and cytotoxic (Kelter et al., 2007) properties. Mistletoe extracts contain biologically active substances, including lectins, viscotoxins, amino acids, flavonoids, polysaccharides, and lipids (Horneber et al., 2008). Mistletoe's chemical composition and biological activity are unstable and may be affected by environmental conditions, such as temperature, carbon dioxide concentration, pollution, soil fertility, and the season of the year (Stypiński, 1997). Pietrzak and Nowak (2021) showed that the chemical profile and biological activity of *V. album* was closely related to the climatic conditions and location of the harvested plant. Furthermore, authors showed that higher levels of phenolic compounds and high antioxidant activity were found in extracts obtained from plant materal collected in cold weather with the presence of snow and limited sunlight exposure. Turker et al. (2012) investigated the properties of *V. album* extracts from plants grown on different host trees and showed significant differences in antitumor activity between them. Belgüzar et al. (2020) have investigated the antibacterial effects of *V. album* collected from different woody host extracts on *Clavibacter michiganensis* subsp. *Michiganensis*, *Xanthomonas axonopodis* pv. *vesicatoria* and *Pseudomonas syringae* pv. *tomato* and observed no statistical differences in the antibacterial activity based on the host plant. Properties of the mistletoe extracts can also differ due to plant organs. Hussain et al. (2011) showed differences in the antimicrobial activity of twigs and leaves *V. album* extracts; however, both revealed substantial antimicrobial potential.

Due to the urgent need to reduce the use of chemical pesticides, substances of natural origin are being sought as alternatives to promote plant growth. The aim of the research was to verify the potential of mistletoe extract for use in agriculture, namely for priming and coating *B. napus* seeds, and also to protect the plant at early stages of development from colonization by human pathogenic microorganisms.

2. Materials and methods

2.1. Plant material and seed treatment

Seeds of spring canola (cultivar Lumen) were used for this study. Based on the method described in Garstecka et al. (2023) primed or humidificated seeds were sown in the sterile Petri dishes (12 seeds per one Petri dish) on blotting paper moistened with 5 ml of sterile dH_2O and incubated in the dark at 24°C for 7 days. Germinability, hypocotyl length, root length, and fresh and dry biomass of seedlings were measured after 7 days of incubation. For measurement of the photysynthetic pigments content the plants after 2 days of germination were placed in a growth room at 8 h light and 16 h dark cycles, 22°C, and the light intensity of 250 μmol photons m^{-2} s^{-1} to evaluate photosynthetic pigments.

Seed priming: *B. napus* seeds were placed in flasks and primed with diluted mistletoe macerates (50% or 20%, v/v), or water (control). Seeds were stirred (160 rpm) for 3 hours at room temperature. Seeds were put on the blotting paper to drain off the solution and then dried in an incubator for 1 day at 37°C and 4 days at 24°C.

Seed humidification: canola seeds were placed in Petri dishes on the tissue paper and incubated for 15 minutes with diluted mistletoe macerates (50% or 20%, v/v) or water (control) at room temperature. Seeds were put on the blotting paper to drain off the solution and then dried in an incubator for one day at 37°C and 4 days at 24°C.

2.2. Mistletoe extract

The mistletoe was collected in early February 2023. Female and male specimens growing on several common maple (*Acer platanoides* L.) specimens were obtained. The leafy shoots without fruit were dried at room temperature, and thin fragments of twigs and leaves were ground using mechanical grounder. Mistletoe macerate was prepared according to the method presented by Najem et al. (2022) i.e. 50 g of ground dried mistletoe was soaked in 150 ml of dH_2O and shaken (120 rpm) for 72 h at room temperature. The extract was then filtered through blotting paper, sterilized via a syringe filter (⌀ 0.22; Genoplast, Poland), and stored at 4°C.

2.3. Bacterial strains

To analyse the antimicrobial properties of the mistletoe macerate, human pathogenic bacteria from the collection of the Department of Genetics at Nicolaus Copernicus University in Toruń – *Escherichia coli* DH5α (Gram-negative) and *Salmonella typhimurium* T98 (Gram-negative), as well as *Staphylococcus aureus* (ATCC 6538P; Gram-positive) purchased from the American Type Culture Collection (ATCC) were used. The strains were stored at -80°C in a solution of 80% glycerol (v/v).

2.4. Photosynthetic pigment content

The content of the photosynthetic pigments was determined using the modified method presented in Konieczna et al. (2023b). 1 ml of 80% ethanol was added to 100 mg of 7-day old cotyledons grounded in liquid nitrogen. Samples were then thoroughly vortexed and centrifuged at 4°C for 5 min (13.000 rpm). Absorbance was measured at $\lambda = 470$ nm, $\lambda = 648$ nm, and $\lambda = 664$ nm (Epoch Take 3 microplate reader, Agilent BioTek, Santa Clara, CA, USA). The concentration of photosynthetic pigments was calculated according to the formulas presented in Lichtenthaler and Wellburn (1983).

2.5. Antibacterial activity

The antibacterial activity of mistletoe macerate was analysed using modified agar diffusion techniques by Irobi (1992). Bacterial suspensions (100 μl) in LB broth (5 ml) was incubated overnight at 37°C with constant shaking at 180 rpm. Bacterial cultures were then diluted to an optical density OD_{600} of 0.2, which was determined using a DiluPhotometer spectrophotometer (Implen) at a wavelength of 600 nm. 100 μL of diluted cultures were applied onto agar LB medium and spreaded with cell spreader. Sterile paper disks soaked with 5 l of sterile dH_2O (control) or mistletoe macerate were placed at the centre of the plate. Plates were incubated for 16 h at 37°C and the zone of inhibition around the paper discs was measured. The experiment was performed in four replicates for each bacterial strain.

2.6. Statistical analysis

The results were analysed using PAST 4.12b software (Hammer et al., 2001) with a one-way analysis of variance ANOVA followed by Tuckey's *post-hoc* test ($p < 0.05$).

Arithmetic mean and standard deviation (SD) were determined using Microsoft Excel. In germination and evaluation of seedling growth parameters 30 plants were examined, in assessment of the photosynthetic pigments content 3 repetitions were performed.

3. Results and discussion

3.1. Seedlings growth parameters

There were no statistically significant differences in all tested growth parameters between treated plants and control plants (Fig. 1). However, there was an over 12% decrease in hypocotyl length in plants treated with 20% mistletoe macerate and an 8% decrease in seedlings treated with 50% mistletoe macerate compared to the control. The average root length of control seedlings was 9.46 cm, while that of plants treated with 20% mistletoe macerate was 8.20 cm. In primed seedlings, the root length was comparable in all tested variants. The fresh biomass of plants from the coated seeds was comparable, while the primed plants showed a slight decrease in fresh biomass compared to the control. Dry biomass values were similar, with the lowest value noted in plants primed with 50% mistletoe macerate. The germinability of seeds treated with 20% mistletoe extract decreased by 2% compared to the control, while seeds humidited with 50% mistletoe macerate decreased by more than 4% although the differences were not statistically significant. A similar trend was observed in primed seeds (Fig. 1).

Priming seeds with plant extracts remains poorly explored area, although in recent years there has been an increase of studies on that topic (Saini et al., 2023; Khan et al., 2022; Findura et al., 2020). Results obtained by Roy et al. (2012) have showed that treating *Ipomoea aquatica* Forssk and *Hibiscus esculentus* L. seeds with aqueous extract of *Terminalia bellirica* (Gaertn.) Roxb. leaves increased seed germination and enhanced the growth of shoot length and root length. Saini et al. (2023) showed that priming onion (*Allium cepa* L.) seeds with neem leaf (*Antelaea azadirachta* (L.) Adelb.) extract had positive effects on germination, seedling length, seedling dry weight and seedling vigour. It was also demonstrated that priming rice (*Oryza sativa* L.) seeds with moringa (*Moringa oleifera* Lam.) leaf extract enhanced seedling growth, photosynthetic pigments, gas exchange parameters and antioxidant activity, particulary under the water deficit regime (Khan et al., 2022). Findura et al. (2020) investigated allelopathic aqueous extracts from 20 plants for cauliflower (*Brassica oleracea convarietas* L. *botrytis var. botrytis*) seed dressing. Results showed that corn (*Zea mays* L.) extract was beneficial on seed germination and reduction of infestation by microbial pathogens.

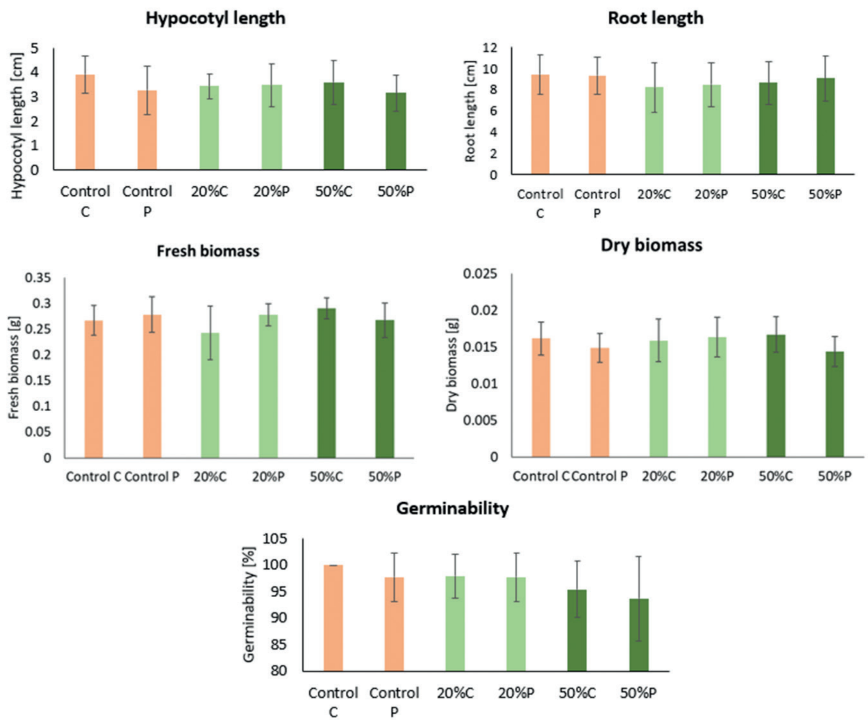

Figure 1. Seed germination and growth parameters of primed (P) and humidified (C) *B. napus* seedlings with 50% and 20% mistletoe macerate (green bars) or water (control, orange bars). Bars represent mean values (n = 30) ± SD. One-way analysis of variance ANOVA ($p < 0.05$)

3.2. Photosynthetic pigment content

The content of photosynthetic pigments was measured to further assess the growth of *B. napus* seedlings after the treatment of seeds with mistletoe extract (Fig. 2). The content of chlorophyll a was 11% higher in plants primed with 50% mistletoe macerate than in control plants. Plants coated with 20% mistletoe macerate showed a 34% increase in chlorophyll a content compared to the control. The lowest content of chlorophyll b was recorded in plants primed with 20% mistletoe macerate. In primed seeds, there were significant differences in the total chlorophyll content of the tested variants, while in coated seeds, the highest value was obtained for plants treated with 20% mistletoe macerate. The content of carotenoids in plants primed with 20% mistletoe macerate was significantly lower than in seeds coated with the same concentration of mistletoe macerate (Fig. 2).

Among the metabolic processes occurring in the plant, photosynthesis is the most sensitive to stress conditions (Szymańska et al., 2019). Chlorophyll fluorescence measurements enable a precise assessment of plant responses to disruption of the photosynthesis process by stress factors. At the same time, it allows to determine the ability of plants to coordinate and regulate life processes during stress (Staniak & Baca, 2018). Seed coating with mistletoe macerate increased chlorophyll a and b content in *B. napus* seedlings while priming plants with mistletoe macerate did not show statistically significant differences in photosynthetic pigment content. Amutenya et al. (2023) examined the chlorophyll content of *Boscia albitrunca* (Burch.) Gilg & Ben. host trees for *Viscum rotundifolium* L.f. *B. albitrunca* which showed lower chlorophyll content than uninfected trees, suggesting that mistletoe may induce chlorophyll synthesis in infected trees. A similar result was obtained by Johnson and Choinski (1993), showing lower chlorophyll content in parasitized *Agelanthus zizyphifolius* (Engl.) Polhill & Wiens than in its host *Diplorhynchus condylocarpon* (Müll. Arg) Pichon. Mistletoe leaves tend to have lower photosynthetic rates than their hosts. *Viscum* ssp. obtain large amounts of heterotrophic carbohydrates from the phloem of the tree to allow expansion of the mistletoe leaf surface (Matsubara et al., 2002; Mathiasen et al., 2008) leading to reduced carbon content in the host, reduced growth rate and reduced host leaf biomass (Meinzer et al., 2004; Rigling et al., 2010).

3.3. Antibacterial activity of mistletoe extract

The antibacterial properties was tested to verify the potential of mistletoe extract as an antimicrobial factor protecting plants against colonization by pathogenic microorganisms (Fig. 3). The mistletoe extract showed low antibacterial properties against Gram-negative bacteria *E. coli* and *S. typhimurium*. *E. coli* was more susceptible to mistletoe extract compared to *S. typhimurium* by 33%. For Gram-positive bacteria *S. aureus*, no growth inhibition was observed in the presence of the mistletoe extract.

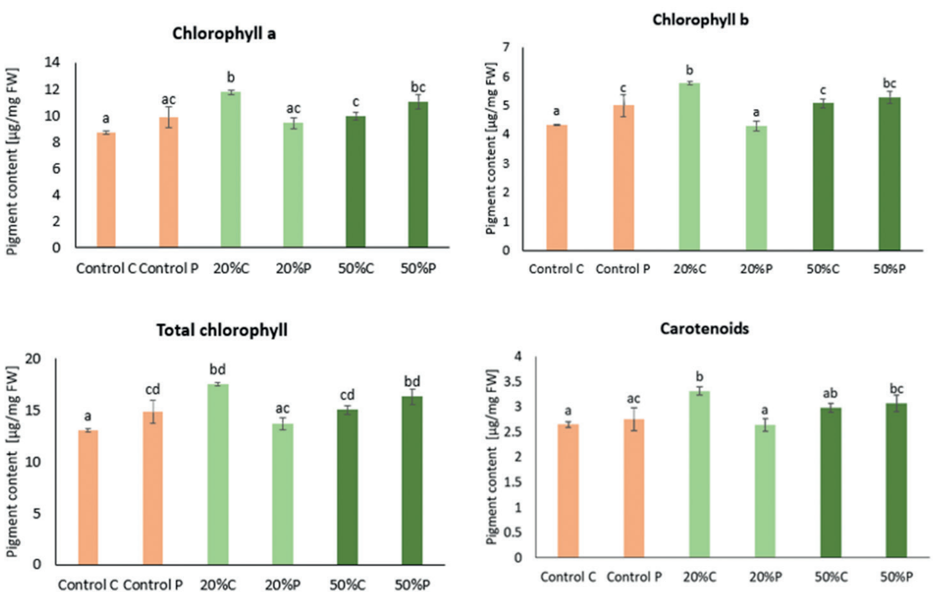

Figure 2. Content of photosynthetic pigments in primed (P) and humidified (C) canola seedlings with 50% and 20% mistletoe macerate (green bars) and control seedlings (orange bars). Bars represent mean values ($n = 3$) ± SD. Different letters indicate statistically significant differences (one-way analysis of variance ANOVA with Tuckey's *post-hoc* test, $p < 0.05$)

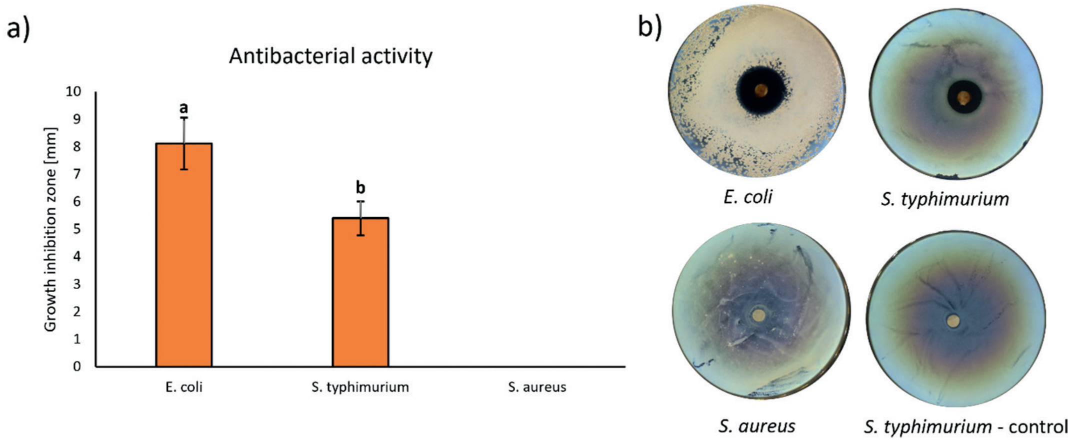

Figure 3. a): Growth inhibiton zone of bacteria treated with mistletoe extract. Bars represent mean values (n = 4) ± SD. Different letters indicate statistically significant differences (one-way analysis of variance ANOVA with Tuckey's *post-hoc* test, $p < 0.05$). b): Bacterial growth in the presence of paper disks soaked with mistletoe extract on LB agar medium

HPMO are the cause of many human infections linked to the consumption of raw leafy vegetables and other foods. The spread of HPMO infections is caused, among other things, by the lack of heat treatment in food preparation (Mogren et al., 2018). In the present study, we demonstrated that mistletoe extract exhibits weak antibacterial properties against *E. coli* and *S. typhimurium* bacteria, but does not show antibacterial properties against *S. aureus*. Interestingly, Najem et al. (2022) tested a water-based extract of *V. album* on Muller Hinton agar via well diffusion method with positive results of the tested extract on the growth inhibition of *Staphylococcus* spp., including *S. aureus*. Research conducted by Orhue et al. (2014) on extracts of *Tapinanthus dodoneifollus* (Dc) Dancer, a mistletoe annotated to the Loranthaceae family, native to Africa, showed antibacterial effect against *S. aureus, P. aeruginosa*, and *K. aerugenes*. Ekhaise et al. (2010) investigated the antibacterial properties of *Tapinanthus bangwensis* (Engl. & K.Krause) leaves methanolic extract on *S. aureus, E. coli, P. aeruginosa, P. mirabilis*, and *K. pneumoniae* and showed that growth of all of these microorganisms was inhibited, with minimum inhibitory concentration ranged from 10 to 50 mg/ml. Moreover, Tarfa et al. (2004) conducted research on aqueous, methanolic, hexane, and ethylacetate *Tapinanthus sessilifolius* (P. Beauv) van Tiegh extracts. They showed that water-based extract did not show any antimicrobial effect on *S. aureus, E. coli, B. subtilis, P. aeruginosa*, and *Candida albicans*. However, the other extracts showed antibacterial effects on tested microorganisms with different minimum inhibitory concentrations ranging from 62.5 to 500 μg/ml. These findings indicate that method of extract preparation, including chosen solvent, have a significant impact on the antibacterial activity of the obtained extracts.

Average monthly temperatures correlate with the rate of spread of *V. album*; consequently, it is likely that climate warming will result in a faster spread of these semi-parasitic plants (Varga et al., 2014). With the growing challenge to ensure food security and protect the environment, sustainable agriculture is becoming increasingly important. One of the critical elements of this approach is the search for new uses for plants. The expansion of mistletoe associated with a warming climate could negatively impact the environment by limiting growth and increasing tree mortality during periods of prolonged drought. Finding new uses for mistletoe could help reduce its spread and occurrence on trees by harvesting wild-growing mistletoe. Although seed priming and coating did not significantly affect the seedling growth parameters studied, the treatment raised chlorophyll levels. The potential of using mistletoe macerate was evident in the reduction of the growth of some microorganisms, which highlights the untapped potential of plant extracts to be used as elements of seed coatings and seed treatments, especially in leafy vegetables (e.g., *L. sativ*a L., *S. oleracea* L.) and other vegetables (e.g. *Raphanus sativus* L.) and herbs (e.g. *Petroselinum crispum* Mill.) that do not undergo the heat treatment before consumption.

4. Conclusion

The application of mistletoe aqueous extract to canola seeds did not significantly affect the growth of seedlings. However, notable increase in chlorophyll and carotenoid content was observed, suggesting a potential enhancement in the photosynthetic efficiency of the plants. Additionally, the extract demonstrated weak antibacterial activity, indicating a possible secondaray benefit for priming the seeds with mistletoe extract. Further research is recommended to explore the mechanisms behind these observations and the potential applications of mistletoe extract in agriculture.

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