Original Research

Allelopathic effect of aqueous extract from selected invasive plants on germination and growth of Tartary buckwheat

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Abstract

Allelopathic compounds released by invasive plants can directly affect neighbouring plants by interfering with their germination and/or suppressing their growth. In this study, we investigated the allelopathic effect of aqueous extract from three invasive plants: Japanese knotweed (*Fallopia japonica*), Canadian goldenrod (*Solidago canadensis*) and stinkwort (*Dittrichia graveolens*) on the germination and early growth of Tartary buckwheat (*Fagopyrum tataricum*). All three aqueous extracts had almost no effect on grain germination but significantly reduced the growth of buckwheat seedlings. In addition, aqueous extracts obtained from a 2-fold serial dilution of a 10% extract of *D. graveolens* inhibited the growth of buckwheat seedlings in a dose-dependent manner. The results showed that root length was significantly more reduced than shoot length, while grain germination remained largely unaffected. The roots were more severely damaged than the shoots and were not only shorter but also thicker and darker in colour. The effect was dose-dependent.

Keywords

allelopathy, *Fagopyrum tataricum*, invasive plant extract, *Fallopia japonica*, *Solidago canadensis*, *Dittrichia graveolens*

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Alelopatski učinek nekaterih invazivnih rastlin na kalivost in rast tatarske ajde

Izvleček

Alelopatske spojine invazivnih rastlin lahko neposredno vplivajo na sosednje rastline tako, da motijo njihovo kalitev in/ali zavirajo njihovo rast. V tej študiji smo raziskovali alelopatsko delovanje vodnih izvlečkov treh invazivnih rastlin: japonskega dresnika (*Fallopia japonica*), kanadske zlate rozge (*Solidago canadensis*) in smrdljivke (*Dittrichia graveolens*) na kalitev in zgodnjo rast tatarske ajde (*Fagopyrum tataricum*). Vsi trije vodni izvlečki skoraj niso vplivali na kalitev semen, poleg tega pa so vodni izvlečki, pridobljeni iz 2-kratne serijske redčitve 10-odstotnega ekstrakta *D. graveolens*, zavirali kalitev ajde v odvisnosti od koncentracije. Pri tretiranih rastlinah je bila rast korenin močneje prizadeta kot rast poganjka, pri čemer smo opazili tudi zadebelitev in rjavenje korenin. Učinek je bil odvisen od koncentracije.

Ključne besede

alelopatija, Fagopyrum tataricum, izvlečki invazivnih rastlin, Fallopia japonica, Solidago canadensis, Dittrichia graveolens

Introduction

Invasive plants are an important environmental management problem exacerbated by climate change and socioeconomic globalization. They contribute to biodiversity loss and ecosystem degradation, and their occurrence is increasing (Pyšek and Richardson, 2010).

Allelopathy is defined as a phenomenon that encompasses both the positive and negative effects of plants on other organisms through chemical substances, described as allelochemicals (secondary metabolites), that plants produce to obtain a competitive advantage over other plants, animals and microbes (Schandry and Becker, 2020). Allelopathy is a common invasion mechanism across the plant phylogeny. Anti-plant allelopathic compounds can directly affect neighbouring plant tissues, disrupting germination and/or the growth of seedlings or mature plants (Kalisz et al., 2021). Since the invasive nature of plants is likely related to their ability to produce specialized allelopathic compounds that can inhibit the growth of neighbouring native plants (Kalisz et al. 2021), their large biomass could be used as a source of substances that inhibit or slow the growth of plants, so they could also be used to minimize the growth and spread of other invasive plants.

We tested three invasive plants: Japanese knotweed (*Fallopia japonica*), Canadian goldenrod (*Solidago canadensis*) and stinkwort (*Dittrichia graveolens*). These species are aggressive colonizers in new environments

and form dense monospecific stands. The allelopathic character of S. canadensis and F. japonica has been well documented (Kato-Noguchi, 2021; Kato-Noguchi and Kato, 2022). The root exudates, extracts, essential oil, and rhizosphere soil of S. canadensis suppressed the germination, growth and arbuscular mycorrhizal colonization of several plants (Kato-Noguchi and Kato, 2022). Allelochemicals such as flavanols, stilbenes and quinones were also detected in the knotweed methanol root extract. Some of these allelochemicals may be released into the rhizosphere soil through the decomposition process of their parts and the exudates of their rhizomes and roots (Kato-Noguchi, 2021). D. graveolens is a plant native to the Mediterranean region that is spreading northwards in Europe. Due to its chemical composition and biological activities, especially its terpene and phenolic content, it has attracted increasing research interest. Moreover, the invasive nature of D. graveolens seems to be related to its ability to produce specialized metabolites that can inhibit the growth of other species, leading to the elimination of competing vegetation and the spread of climate change. (Ponticelli et al., 2022)

The leaf extract of *S. canadensis*, the rhizome extract of *F. japonica* and the shoot extract of *D. graveolens* were used for the experiments. Sun et al. (2022) demonstrated that the aqueous extract (50 g/L) from the aerial parts of *S. canadensis* significantly inhibited the germination rate of *Zoysia* grass, while root and litter extracts showed no significant effect on germination. In *Fallopia*, most of the secondary metabolites are stored in the underground rhizomes (Chen et al., 2013; Frantík et al., 2013). The extract from the shoots of *D. graveolens* was selected because its allelopathic effect on the germination and growth of some weed species is already known (Almhened et al., 2021).

Tartary buckwheat is a very resistant plant that accumulates abundant bioactive compounds that help it survive in harsh environments, such as cooler climates and higher altitudes. In many mountainous regions where other crops cannot survive, Tartary buckwheat is grown as a staple crop because of its short growing season, high ecological adaptability, and tolerance to nutrient-poor conditions (Zou et al., 2023). Tartary buckwheat is more bitter and contains more rutin than common buckwheat (Fagopyrum esculentum). It also contains a wide range of bioactive compounds such as flavonoids, phenolic acids, triterpenoids, phenylpropanoid glycosides, bioactive polysaccharides, bioactive proteins and peptides, as well as D-chiro-inositol and its derivatives (Zou et al., 2023). In addition, Tartary buckwheat has been reported to have an allelopathic activity against weeds (Vieites-Álvarez et al., 2023). The results obtained by Vieites-Álvarez et al. (2024) indicate that Tartary buckwheat can sustainably control weeds through plant interference, such as competition or allelopathy and is effective against both monocot and dicot weeds. Therefore, Tartary buckwheat can be considered a very suitable candidate for testing the strength of the allelopathic effect of invasive plants.

Materials and Methods

Plant material

Shoots of *S. canadensis* were collected during the flowering period in Ljubljana, Slovenia (N 46° 4' 19.86", E 14° 25' 52.25"). Fresh leaves were separated from the shoot and air dried at room temperature in the dark and ground in the mill (IKA, IKA-Werke M20, IKA, Germany).

Fresh shoots of *D. graveolens* were collected during the flowering period in September (det. S. Strgulc Krajšek) in Ljubljana, Slovenia (N 46°6'17.17", E 14°28'54.36"). The shoots were air-dried at room temperature in the dark and ground in the mill.

Rhizomes of F. japonica were collected in November

in a dense *F. japonica* stands next to stream Mali Graben, Ljubljana, Slovenia (N 46°02'33.9"; E 14°27'00.9"). Fresh rhizomes were washed in tap water, dried, cut in a 1-cm thick reel, frozen in liquid nitrogen, lyophilized (5 days, 0.002bar) (Christ Alpha 1-4LSC, Christ, Germany), and ground in a cutting mill (SM 200; Retsch, Germany).

The grain of Tartary buckwheat was obtained from Rangus Mill (Šentjernej, Slovenia, https://www.mlinrangus. si/en/).

Preparation of aqueous extracts

Ground plant material (5 g) was suspended in 100 ml distilled water and placed on a shaker (Laboshake 500, Gerhardt, Germany), where it was shaken at 130 rpm for approximately 24 hours. After aqueous extraction, the suspension was filtered under pressure through filter paper (Whatman filter paper 520A, pore size 15-18 μ m, Ge Healthcare Life Sciences) to remove plant particles and obtain approximately 70 ml of 5% (w/v) aqueous extracts. The extracts were prepared fresh before the experiment and used immediately.

The yield of extract (extractable component) expressed on dry weight basis of pulp was calculated according to the following equation:

Yield (%) = (W1 × 100) / W2

where W1 is the weight of the extract residue obtained after solvent removal, and W2 is the weight of the dry plant material before the extraction.

For the dilution preparation of aqueous extract of *D. graveolens*, a 10% (w/v) aqueous extract was prepared and serially diluted with distilled water to produce 5%, 2.5%, 1.25% and 0.625% aqueous extracts.

Germination and early growth test

For the germination test, sterile covered crystallizing dishes (9 cm diameter, 3 cm height) with two layers of autoclaved filter paper were used, which were moistened with 8 ml test solution (5% aqueous extract of the rhizome of *F. japonica*, the leaf extract of *S. canadensis*, and shoot extract of *D. graveolens*). In addition, for the concentration-dependent experiment, sterile covered crystallizing dishes (9 cm diameter, 3 cm height) with two layers of autoclaved filter paper were used, which were moistened with 8 ml test solution (10%, 5%, 2.5%, 1.25% and 0.625%)

aqueous extracts of D. graveolens). Distilled water was used as a control treatment in both experiments. For each test solution, three replicates with 10 grains in a 2x2 cm arrangement in covered crystallizing dishes were used. The germination test took place in a growth chamber at 22 °C, 60% humidity and a photoperiod of 16 hours. The germination experiment lasted four to five days, with the growth of the seedlings being terminated on the seventh day. The germinated grains were counted and examined every day at intervals of about 24 hours. A grain was considered germinated when its radical had emerged. On the seventh day of the experiment, the root and shoot length were measured, and the number of lateral roots was counted as an indicator of seedling development and growth. The roots were separated from the shoots, and the fresh mass was weighed.

Statistical analysis

For the statistical analyses, the mean values and standard errors were calculated for all treatments. Means were compared between treatments using One-way ANOVA and the Bonferroni-Holm post-hoc test (MS Excel, Daniel's XL Toolbox). The level of significance was set at a P value < 0.05.

Results and Discussion

The germination of Tartary buckwheat grain was not significantly affected by the 5% aqueous extracts of the three invasive plants (Table 1). Similarly, a 5% extract of F. japonica had no effect on radish seed germination on days 5 and 7 (Šoln et al., 2021), while a 10% root extract of F. japonica significantly reduced radish seed germination (Šoln et al., 2023). Both extracts delayed the germination of radish (Šoln et al., 2021), which was confirmed by our experiment in Tartary buckwheat grain (Table 1). The variability of seed germination depends on the plant tested; crop seeds are more sensitive to F. japonica extracts than weed seeds (Kato-Noguchi, 2022). Since Tartary buckwheat has high ecological adaptability and tolerance to nutrient-poor and other unfavourable conditions (Sofi et al., 2023; Zou et al., 2023), it is not surprising that its germination was not affected by the extract of F. japonica. On the other hand, the leaf extract of F. japonica significantly impaired the germination of common wheat (Triticum aestivum), especially at the highest concentration (0.05 g/mL), at which only one-third of the seeds germinated (Levačić et al., 2023).

The aqueous extract of *D. graveolens* had no effect on the germination of Tartary buckwheat grain (Table 1). On the other hand, a 5 % aqueous extract of *D. graveolens* proved to be effective against wheat (*Triticum aestivum*) and common ragweed (*Ambrosia artemisiifolia* L.) germination, as it delayed germination and significantly reduced the germination rate (Grašič et al., 2016).

The germination of Tartary buckwheat was not affected by the extract of S. canadensis, as the germination rate on the first day was 63 % (Table 1). This inhibition is comparable to that reported for the aboveground aqueous extract of S. canadensis on Zoysia japonica seeds, which significantly reduced the germination rate to 60% at a concentration of 50 g L-1 (Sun et al., 2021). The 2.5% extracts of S. canadensis leaves also significantly inhibited radish seed germination only at the beginning, while the difference was no longer significant on the fourth day compared to the control, reaching 76% (Anžlovar and Anžlovar, 2019). Tartary buckwheat grain appears to be more resistant to S. canadensis extract compared to the other seeds tested: Raphanus sativus, Lactuca sativa, Triticum aestivum, Setaria viridi (see Kato-Noguchi and Kato 2022), as the germination rate was not significantly reduced across all days (Table 1).

Overall, the invasive plant extracts had only a moderate, non-significant effect on the germination of buckwheat grain, but the results showed that these extracts significantly inhibited the growth of 7-day-old Tartary buckwheat seedlings compared to the control (Figure 1). The early growth of the treated seedlings was slower than that of the control group. Figure 1 shows that all extracts significantly inhibited root and shoot growth compared to the control, with the extract of F. japonica having a significantly lower effect on the growth of Tartary buckwheat than S. canadensis and D. graveolens. Nevertheless, the root extract of F. japonica significantly reduced the length of shoots and roots of Tartay buckwheat seedlings compared to the control (Figure 1). These results are consistent with those of other studies that have shown that the aqueous root extract of F. japonica reduces root length significantly more than shoot length or seed germination (Šoln et al., 2021, 2022, 2023; Kato-Noguchi 2022). Both treatments with S. canadensis and D. graveolens resulted in significantly shorter root and shoot lengths compared to F. japonica extracts, while there was no difference between S. canadensis and D. graveoTable 1. Germination of Tartary buckwheat grain treated with 5% aqueous extracts of *F. japonica*, *S. canadensis*, and *D. graveolens* for four days. Data are means \pm standard error (N = 30). Different letters indicate statistically significant differences within rows (p <0.05).

Tabela 1. Delež kalivosti zrn tatarske ajde tretiranih z vodnimi izvlečki *F. japonica*, *S. canadensis* in *D. graveolens* tekom 4 dni. Podatki so povprečja ± standardna napaka (N = 30). Različne črke prikazujejo statistično značilne razlike med tretmaji (p <0,05).

	Germination rate (%)					
	Control	F. japonica	S. canadensis	D. graveolens		
Day 1	73 ± 9ª	77 ± 3ª	63 ± 9^{a}	77 ± 3^{a}		
Day 2	87 ± 9 ^a	77 ± 3ª	80 ± 10 ^a	77 ± 3ª		
Day 3	87 ± 9^{a}	80 ± 6^{a}	80 ± 10^{a}	83 ± 3ª		
Day 4	90 ± 6^{a}	$90 \pm 0^{\rm a}$	80 ± 10^{a}	83 ± 3ª		



Figure 1. Shoot and root length of Tartary buckwheat seedlings after 7 days of growth and treatment with 5% aqueous extracts of *F. japonica*, *S. canadensis* and *D. graveolens*. Shown are means \pm standard error is shown (N=30). Different letters indicate statistically significant difference between treatments for each seelding part separately (One-way ANOVA p <0.05).

Slika 1. Dolžina poganjka in korenine kalic tatarske ajde po 7 dneh rasti in tretmaju z vodnimi izvlečki *F. japonica*, *S. canadensis* in *D. grave*olens Prikazana so povprečja ± standardne napake (N = 30). Različne črke prikazujejo statistično značilne razlike (p <0,05).

lens treatments (Figure 1). The roots of seedlings treated with *S. canadensis* and *D. graveolens* extracts were 94% and 96 % shorter, respectively, compared to the control. Similar growth inhibition was observed in *Raphanus sativus* and *Lactuca sativa* treated with *S. canadensis* (Butcko and Jensen 2002) and *D. graveolens* (Omezzine et al., 2011), while seed germination was not affected (Butcko and Jensen 2002), comparable to our results.

Plant roots show morphological plasticity and play an important role in tolerance to various stress factors (Karlova

et al., 2021). The reduction of lateral roots in buckwheat seedlings was significantly affected by both extracts, *S. canadensis* and *D. graveolens*, compared to the control, as 90% of the treated seedlings did not develop lateral roots (Figure 2). The *F. japonica* extract also significantly affected the formation of lateral roots, as the number of roots per seedling decreased by almost 50 % compared to the control. Šoln et al. (2021) reported that the reduction of lateral roots in radish seedlings was differentially affected by the concentration of *F. japonica* extract; lower

concentrations (0.5%) stimulated lateral root formation with up to 25% higher root number per seedling than in control, while higher concentrations (5%, 10%) reduced lateral root formation by up to 54%, which is similar to the result in buckwheat seedlings (Figure 2). Zhang et al. (2023) investigated the effects of salt stress on the growth of Tartary buckwheat and found that low salt stress treatment promoted root growth and improved seedling root activity, while medium and high salt stress treatment reduced root growth compared to the control. In several crops, such as rice, wheat and *Arabidopsis*, the root elongation rate decreased under salt stress, while increased elongation of lateral roots was observed in *Silene vulgaris* and *Brassica napus* (Arif et al., 2019). Root elongation is the result of cell division and cell expansion in the root apical meristem, and salt stress can alter root elongation by both promoting and reducing cell division and expansion (West et al., 2004).

Figures (1 and 2) show that the extract of *F. japonica* was significantly less effective than that of *S. canadensis* and *D. graveolens*, which is also a consequence of the low extraction yield (Table 2). The extract of *F. japonica* contained less dry matter and had the lowest yield compared to the extracts of *S. canadensis* and *D. graveolens* (Table 2). Despite the highest yield of the *S. canadensis* extract, the effects on buckwheat morphology were greater when treated with the *D. graveolens* extract, so we decided to conduct a concentration-dependent experiment with *D. graveolens*.



Figure 2. Number of lateral root of Tartary buckwheat seedlings after 7 days of growth and treatment with aqueous extracts of *F. japonica*, *S. canadensis* and *D. graveolens*. Mean value \pm SE is shown (N=30). Different letters indicate statistically significant difference among treatments (p <0.05).

Slika 2. Število stranskih korenin kalic tatarske ajde po 7 dneh rasti in tretmaju z vodnimi izvlečki *F. japonica*, *S. canadensis* in *D. graveolens* Prikazana so povprečja ± standardne napake (N = 30). Različne črke prikazujejo statistično značilne razlike (p <0,05).

Table 2. Yield of aqueous extracts. The yield was calculated as the percentage of extract dry mass according to the starting material. Data are means (N = 3). Tabela 2. Izkoristek vodnih izvlečkov. Izkoristek je delež suhe snovi, glede na maso začetnega materiala, izražen v odstotkih. Podatki so povprečja (N = 3).

Extract	Yield (%)
F. japonica	13.27±0.61
S. canadensis	38.00±0.20
D. graveolens	25.87±0.76

Aqueous extracts obtained from a 2-fold serial dilution of a 10% extract of *D. graveolens* moderately affected the germination of Tartary buckwheat grain (Table 3). After 24 hours, 73% of the control grain had germinated, and a significant difference in germination rate was observed between the control and the treatment at 1.25% to 10%, while the lowest treatment was comparable to the control. After 48 hours, the germination rate was comparable to the control in all treatments (Table 3).

Despite the small effect on the germination rate of Dittrichia-treated grain, further growth of Tartary buckwheat seedlings was significantly reduced (Figure 3). The only exception was the 0.625% extract, which actually promoted the growth (Figure 3). Almhemed et al. (2021) reported that the aqueous extract of *D. graveolens* at concentrations of 2%, 6%, and 10% inhibited the germination and growth of some weed species. The germination rate and growth varied depending on the weed species, with greater effects on germination than on growth. Delayed germination and significantly reduced germination rate were also observed in wheat (Triticum aestivum) and common ragweed (Ambrosia artemisiifolia L.) treated with 5% aqueous extracts of D. graveolens (Grašič et al., 2016). In contrast, the results of Abu Irmaileh et al. (2015) showed that the ethanolic extract of D. graveolens at 200 ppm reduced root length significantly more than shoot length or seed germination, which is similar to our results (Figure 3, Table 3).

The shoots were less affected by the aqueous extracts of *D. graveolens* than the roots (Figure 3). Here, the different concentrations appear to inhibit shoot growth equally, except for the weakest concentration, which appears to be at the same level as the control and has no significant effect on shoot growth. The inhibition of root growth is dose-dependent (Figure 3). Similarly, volatile monoterpenoids of Salvia leucophylla inhibited root growth of B. campestris in a dose-dependent manner, while hypocotyl growth remained largely unaffected (Nishida et al., 2005). The morphological and biochemical analyses showed that the size of mature cells was not altered in either hypocotyls or roots but that the monoterpenoids specifically decreased the mitotic index in the root apical meristem, while they did not reduce the mitotic index in the shoot apical region (Nishida et al., 2005). These results suggest that the allelochemicals may affect the growth of other plants in their vicinity by inhibiting cell proliferation in the root apical meristem. This is consistent with the finding that the sensitivity of root growth is the best indicator of the phytotoxicity of allelochemicals, as the root is highly permeable to chemicals (Ponticelli et al., 2022).

The same reduction pattern was observed in the fresh mass of the shoots and roots of the buckwheat seedlings (Figure 4). After seven days, the inhibitory effect of the 10% *D. graveolens* extract was significant and reduced the root mass by 95%. The 5% and 2.5% extracts showed similar effects: Root mass was significantly smaller, while shoot mass did not decrease significantly. The shoot and root mass of the 0.625% treatment corresponded to the control level.

The morphological observations showed that the allelopathic effects of the aqueous extracts of *D. graveolens* on Tartary buckwheat were mainly observed on the roots of the seedlings (Figure 5). They were more severely damaged than the shoots. The roots of Tartary buckwheat seedlings treated with the 10% *D. graveolens* extracts were

Table 3. The germination rate of Tartary buckwheat grain was treated with different concentrations of *D. graveolens* aqueous extracts over five days. Data are means \pm standard error (N = 30). Different letters indicate statistically significant differences within rows (p <0.05).

Tabela 3. Delež kalivosti zrn tatarske ajde tretiranih z različnimi koncentracijami vodnega izvlečka *D. graveolens* tekom 5 dni. Podatki so povprečja ± standardna napaka (N = 30). Različne črke prikazujejo statistično značilne razlike med tretmaji (p <0,05).

	Germination rate (%)							
	Control	0.625%	1.25%	2.5%	5.0%	10.0%		
Day 1	73 ± 7a	60 ± 6a	37 ± 7ab	43 ± 9ab	33 ± 7ab	$7\pm3b$		
Day 2	90 ± 6a	90 ± 6a	80 ± 6a	90 ± 10a	77 ± 9a	87 ± 3a		
Day 3	90 ± 6a	90 ± 6a	80 ± 6a	90 ± 10a	80 ± 6a	87 ± 3a		
Day 4	97 ± 3a	97 ± 3a	83 ± 9a	90 ± 10a	90 ± 0a	87 ± 3a		
Day 5	97 ± 3ab	97 ± 3ab	100 ± 0a	97 ± 3ab	$90\pm0b$	$87\pm 3ab$		



Figure 3. Shoot and root length of Tartary buckwheat seedlings after 7 days of growth treated with different concentrations of *D. graveolens* aqueous extracts. Mean value ± SE is shown (N=30). Different letters indicate statistically significant difference among treatments (p <0.05).

Slika 3. Dolžina poganjka in korenine kalic tatarske ajde tretiranih z različnimi koncentracijami vodnega izvlečka *D. graveolens* po 7 dneh rasti. Prikazana so povprečja ± standardne napake (N = 30). Različne črke prikazujejo statistično značilne razlike (p <0,05).



Figure 4. Fresh mass of Tartary buckwheat seedlings after 7 days of growth and treatment with different concentration of *D. graveolens*. Mean value \pm SE is shown (N=30). Different letters indicate statistically significant difference among treatments (p <0.05).

Slika 4. Sveža masa kalic tatarske ajde tretiranih z različnimi koncentracijami vodnega izvlečka *D. graveolens* po 7 dneh rasti. Prikazana so povprečja ± standardne napake (N = 30). Različne črke prikazujejo statistično značilne razlike (p <0,05).

not only shorter but also thicker and darker in colour. The same observation was made in radish roots treated with rhizome extracts of *F. japonica* (Šoln et al., 2021). The roots of radish seedlings treated with a 10% extract were shorter and thicker due to the shorter and wider shape of their cortex cells (Šoln et al., 2022) and the reduced cell length in the root cap (Šoln et al., 2023). In addition, these cells exhibited various ultrastructural and biochemical changes, which could be the reason for the more than 60% shorter root length of the treated radish seedlings compared to the control (Šoln et al., 2023). The morphological adaptation, in which the roots become shorter and thicker, was also observed in plants that respond to water stress (Yetgin 2024).

The invasive nature of *D. graveolens* appears to be related to its ability to produce allelochemicals, which significantly inhibit the growth of Tartary buckwheat seedlings in a dose-dependent manner. The results showed that the root length of Tartary buckwheat seedlings was reduced significantly more than shoot length, while germination remained largely unaffected.

Author Contributions

Conceptualization, A.M.A.; methodology, A.M.A.; software, A.M.A.; validation, A.M.A.; formal analysis, A.M.A.; investigation, A.M.A.; data curation, A.M.A.; writing—original draft preparation, A.M.A.; writing—review and editing, S.A. All authors have read and agreed to the published version of the manuscript.

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Conflicts of Interest

The authors declare no conflict of interest.

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