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In Vitro Micropropagation of Endangered *Achillea fragrantissima* Forssk. Combined with Enhancement of Its Antihyperglycemic Activity

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Abstract: *Achillea fragrantissima* Forssk. (Family: Asteraceae) has been used as a natural remedy in the Arabian region for its antihyperglycemic activity. As a result of the intensive demand for this plant in folk medicinal uses, its scarcity has become problematic. This study has explored methods that produce an efficient in vitro culture protocol for the conservation of this plant as well as the enhancement of its hypoglycemic activity. *A. fragrantissima* cultures on Murashige and Skoog (MS) medium supplemented with 3.6 µM/L of 6-benzyl aminopurine (BAP) for a two month period resulted in maximum in vitro shoot proliferation (12.33 shoots/explant) while MS medium supplemented with 2.4 µM/L 1-naphthalene acetic acid (NAA) provided maximum in vitro adventitious root formation (2.46 roots/shoot tip explant). Callus induction was favored by leaf explants cultured on MS medium and supplemented with 3 µM/L BAP and 3 µM/L IAA media in dark conditions. Further in vivo study of some selected feedings determined that the best hypoglycemic activity was obtained in either indole-3-butyric acid (IBA)-fed plants (24%) or NAA-fed plants (22%). Both treatments enhanced insulin-like activity in STZ-treated diabetic Sprague-Dawley rats when compared with the wild plant (10%). Moreover, the IBA-fed plants showed significant antioxidant activity while the NAA-fed plants inhibited salivary alpha amylase. The framework of this study provides in vitro culture methods that can sustain the cultivation of this over-exploited *A. fragrantissima* plant as well as increase its antioxidant and insulin-like activities.

Keywords: *Achillea fragrantissima*; BAP; NAA; IBA; IAA; Sprague-Dawley rats; insulin-like activity; antioxidant; alpha amylase

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1. Introduction

Achillea fragrantissima Forssk. (Family: Asteraceae) is known as Qaisoum in the Arabian region and has been used as a tea-like infusion in traditional medicine to reduce elevated blood glucose levels [1]. The mild antihyperglycemic activity of *A. fragrantissima* has been confirmed through diminished pathological markers resulting from the induction of type 2 diabetes [2]. In addition, there are several reports that discuss other beneficial pharmacological effects of *A. fragrantissima*, e.g., as an antiviral [3], anti-neuroinflammatory [4], anticancer [5], antispasmodic, and antimicrobial agent [6]. Recently, *A. fragrantissima* has exhibited a potential antioxidant and anti-inflammatory effects that plays a crucial protective role in Adriamycin-induced cardiotoxicity in rats [7]. The safety profile of acute and chronic treatments with alcoholic or aqueous extracts of *A. fragrantissima* has been assessed. The study did not show any evidence of infertility or teratogenic signs at higher

doses than those recommended for human use [8]. Additionally, it has been found that the oral median lethal dose (LD_{50}) is higher than the therapeutic dose used for evaluating anti-inflammatory, antinociceptive, and anti-ulcerative effects [9].

Regarding the chemical profiling of *A. fragrantissima*, previous studies have revealed the presence of different classes of bioactive metabolites such as phenolic acids, flavonoids, tannins, terpenes, and carbohydrates [2,10,11]. These metabolites were isolated and identified as piceol [12], veratric acid [12], cirsiliol [12], eupatilin 7-methyl ether [12], cirsimaritin [12], quercetin [13], acacetin-6-C-(6″-acetyl-*β*-D-glucopyranoside)-8-C-*α*-L-arabinopyranoside [13], quercetin-3,6,7-trimethyl ether (chrysosplenol-D) [13], isovitexin-4′-methyl ether [13], isovitexin [13], afroside [2], luteolin [2], apigenin-6-C-glucoside [2], cosmosiin (apigenin 7-glucoside) [2], resorcin [2], phloroglucin [2], methyl phloroglucin [2], pyrocate [2], *α*-pinene [14], *d*-myrcene [14], sabinene [14], *l*-linalool [14], *α*-terpineol [14], eugenol [14], *α*-thujone [15], and camphor [15]. Keep in mind that it was shown that the polysaccharide fraction displayed the highest anti-hyperglycemic activity compared with other hexane, ethanol, and aqueous fractions of *A. fragrantissima* [16].

Although supplies of *A. fragrantissima* are reduced and have become endangered due to the anthropogenic pressures for its collection for folk medicinal uses, few studies on in vitro micropropagation have been performed [8,17,18]. A propagation protocol for Jordan *A. fragrantissima* has been explored, but there has been no assessment of the hypoglycemic activity of the propagated plants when compared with native wild plants [19]. The current study is the first one that provides a deep insight in a micro propagation protocol for *A. fragrantissima* grown in Egypt. Biologically inactive propagated plants are not valuable products, and their propagation would therefore be a non-productive outcome. Thus, there is an immense need to provide in vitro propagated plants and evaluate their anti-hyperglycemic activity.

This study was designed to develop methods to propagate *A. fragrantissima* using different growth regulators, providing in vitro propagated plants with better hypoglycemic activity compared with native wild plants and explaining their mechanism of action through in vitro and in vivo studies.

2. Materials and Methods

2.1. In Vitro Micropropagation Protocol for Achillea fragrantissima Plantlets

2.1.1. Establishment of In Vitro *Achillea fragrantissima* Plantlets

Ripe fruits of *A. fragrantissima* were collected in June from Wadi-Elarbaeen, South Sinai, Egypt. To sterilize the seeds, the seeds were pulled out from the fruits, disinfected through soaking with a 70% ethanol solution for 30 s, rinsed with 5% sodium hypochlorite solution for 5 min, and finally washed three times with sterile tap water under laminar airflow. The sterilized seeds were cultured on a medium of half-strength Murashige and Skoog (MS, Phyto Technology Laboratories®, Lenexa, KS, USA) supplemented with 3% (*w*/*v*) sucrose and 0.6% (*w*/*v*) of agar (Roko®, Llanera, Asturias, Spain) [20]. One hundred seeds were cultured, with three seeds per 50 mL tube. The cultures were maintained under growth room conditions at 24 ± 2 °C using 16-h photoperiods provided by a white LED plant growth tube with a light intensity of 100 μM/m^2/s. The in vitro germinated plantlets were used throughout the remaining propagation studies.

2.1.2. Shoot Proliferation of *A. fragrantissima* Plantlets

Two-month-old shoot tips with a height of 0.5–1 cm were cultured on MS medium supplemented with 3% (*w*/*v*) sucrose, 0.6% (*w*/*v*) agar, and different concentrations of kinetin (Kin., Bio Basic INC®, New York, NY, USA), 6-benzyl aminopurine (BAP, Bio Basic INC®, New York, NY, USA), and 2-isopentyl adenine (2iP, Phyto Technology Laboratories®, Lenexa, KS, USA) at concentrations of 0.3–3.6 μM/L. Each treatment consisted of 10 replicates with samples containing two micro shoots/replicate. These concentrations of growth regulators were determined based on a pre-multiplication step by application of BAP at concentrations of 3 μM/L, 6 μM/L, and 12 μM/L. Data were collected

for the number of new shoots per initial micro shoots, plant weight, and maximum shoot height at one-month intervals for 3 months.

2.1.3. In Vitro Adventitious Root Formation and Ex Vitro Acclimatization

Shoot tips (0.5–1 cm height) were cultured on MS medium containing 0.3–2.4 mM/L of either indole-3-acetic acid (IAA, Oxford®, Lab Fine Chem®, Maharashtra, India), 1-naphthalene acetic acid (NAA, Bio Basic INC®, New York, NY, USA), or indole-3-butyric acid (IBA, Bio Basic INC®, New York, NY, USA). Ten replicate tubes with two micro shoots were prepared. The number of roots per initial micro shoot and maximum root length were recorded over a period of one month.

Three-month-old in vitro rooted plantlets were washed, immersed in 1% rizolex® fungicide for 10 min, and then transplanted to plastic pots with moist sand covered with polyethylene sheets in a greenhouse maintained at 40 °C [21]. The plants were irrigated regularly once every three days. The polyethylene sheets were gradually removed during the day and returned at night for 2 weeks, then removed altogether [22]. The survival percentage of the ex vitro acclimatized plants was recorded after a period of 7 months.

2.1.4. Callus Induction

Two different types of explants, leaf and root segments, were used for callus induction. The MS medium was incorporated with BAP and IAA at different concentrations (Table 1).

Table 1. Different concentrations of BAP and IAA in callus media.

	Growth Media	BAP (µM/L)	IAA (µM/L)
1	MS medium + 3% (*w*/*v*) sucrose + 0.6% (*w*/*v*) agar	0	0
2		0.6	0.6
3		1.2	0.6
4		1.2	0.3
5		2.4	0.6
6		3	3
7		6	6

BAP: 6-benzyl aminopurine; IAA: Indole-3-acetic acid.

Each treatment consisted of 12 replicates containing either 5 leaf explants per jar or 4 root explants per jar. Half of these leaf and root samples were kept in the dark at 22 ± 2 °C while the other half were maintained under growth room conditions at 24 ± 2 °C using 16-h photoperiods provided by a white LED plant growth tube with a light intensity of 100 µM/m^2/s. In initial studies, callus fresh weight and color were recorded over a period of 1 month. In subsequent studies, the calli were cultured on MS medium containing 3 µM/L BAP and 3 µM/L IAA in dark conditions (the media that showed the best results) over periods of 1 month, 2 months, 3 months, 4 months, and 6 months.

2.1.5. Plant Regeneration and Organogenesis

Six leaf explants, six root explants, or mashed aerial stems with a weight of 1 g were cultured on MS medium supplemented with 0.6% (*w*/*v*) agar and thiadiazurone (TDZ, Sigma-Aldrich®, Burlington, MA, USA) at concentrations of 0.0 mM/L, 0.5 mM/L, 1 mM/L, and 2 mM/L. TDZ was added after filter sterilization on autoclaved medium and then cooled to 47 °C [23]. Each 30 mL liquor of the medium was dispensed into petri dishes and allowed to gel. The number of regenerated shoots per explant was assessed over a period of 2 weeks.

2.2. In Vitro α-Amylase Inhibition Study of Ethanolic Extracts of Different Treatments

2.2.1. Ethanol Extraction of Bioactive Compounds from Different In Vitro Cultured Plants

All the different in vitro cultures and ex vitro plants, as well as the wild plant, were dried, weighed, and soaked separately in absolute ethanol (1:3 ratios) for 4 days. Sonication

was carried out at 30 °C for 30 min to aid the process of extracting the metabolites. The whole process of soaking, sonication, and filtration was repeated three times and the filtrates were concentrated under reduced pressure using a rotary evaporator. Finally, the weight of extract of each sample was determined.

2.2.2. α-Amylase Inhibition Assay

The α-amylase inhibition assay was performed using the chromogenic method from Sigma-Aldrich [24]. Human salivary α-amylase (Code A1031, type XIII, Sigma), was dissolved in ice-cold distilled water to give a concentration of 4 unit/mL immediately before use. The pre-incubation method was performed as previously described [24]. The α-amylase activity was determined by measuring the absorbance of the mixture at 540 nm with a UV-Vis spectrophotometer. Control incubations representing 100% enzyme activity were conducted in an identical manner, but replacing different ethanolic extracts with DMSO (0.4 mL). For blank incubations, the enzyme solution was replaced with distilled water. The absorbance (A) due to the generated maltose was calculated as follows: A_{540} nm control or plant extract = A_{540nm} Test − A_{540nm} Blank. From the net absorbance obtained, the % (w/v) of maltose generated was calculated from the equation obtained from the maltose standard calibration curve (0–0.1%, w/v, maltose). The α-amylase inhibition activity was expressed by % inhibition as follows: % inhibition = 100 − % reaction at t = 3 min as the % reaction = (mean maltose in sample/mean maltose in control) × 100.

2.3. Quantitative Determination of Polysaccharides in Different Treatments

The phenol/sulfuric acid method was used for the quantitative colorimetric micro determination of polysaccharides in the different ethanolic extracts of the in vitro cultures, ex vitro plants, and the wild plant [25]. Carbohydrates were first hydrolyzed into simple sugars using dilute hydrochloric acid [26]. The phenol/sulfuric acid method was then performed as described in detail elsewhere [25]. The absorbance of the characteristic yellow-orange color was measured at 490 nm using a Shimadzu Model UV-1601 PC, UV-Vis spectrophotometer (Schimadzu®, Kyoto, Japan) and compared with a standard curve constructed using glucose monohydrate.

2.4. Screening of In Vivo Hypoglycemic Activity in Plant Extracts

2.4.1. Experimental Animals

One hundred and twenty-five adult male Sprague-Dawley rats weighing 150 ± 20 g were obtained from the Egyptian Organization for Biological Products and Vaccines. An animal chow diet and water were provided ad libitum. Rats were maintained on a normal light–dark schedule and at a temperature of 25 ± 3 °C throughout the experiment. The experiments were initiated after one week for animal acclimatization. All experimental protocols were approved by the Institutional Animal Care and Use Committee at the Faculty of Pharmacy, Suez Canal University, Egypt (202106RA1).

2.4.2. Preparation of Aqueous Extracts of Different Samples

The polysaccharides of the marc tissues, the tissue remaining after the ethanolic extraction process, were subsequently extracted by tea-like infusion with distilled water and two drops of Tween 80 for 24 h followed by centrifugation at 4000 rpm for 15 min. Supernatant solutions were collected for use in the studies with STZ-diabetic rats described below.

2.4.3. Induction of Hyperglycemia in Sprague-Dawley Rats Using Streptozotocin (STZ)

Hyperglycemia was induced in the Sprague-Dawley rats using a single intraperitoneal (i.p.) injection of 0.5 mL of cold 0.9% NaCl, containing Streptozotocin (STZ, MP Biomedicals, Irvine, CA, USA) at a concentration of 65 mg/kg. After three days, hyperglycemia was established [27] and confirmed using glucose concentration measurements from blood samples obtained via puncture, test strip, and a reflectance meter (BIOSTC blood glucose

monitoring system, Giza, Egypt). In general, more than 90% of the STZ-treated animals had blood glucose concentrations higher than 300 mg/dL, and these animals were used in subsequent studies.

2.4.4. In Vivo Treatment Administration

The tea-like infusion of wild *A. fragrantissima* (200 mg/kg) was administered by intragastric (i.g.) gavage as a single dose in 1 mL vehicle (acute treatment) after overnight fasting of the STZ-treated rats. Metformin was used as a positive control at 500 mg/kg (i.g.), a dose determined to be optimal in preliminary studies [28]. Because i.g. gavage is stressful for mice [29], appropriate vehicle controls were also used in parallel studies with identical experimental protocols. Blood glucose concentrations were estimated at 0 h, 1 h, 2 h, and 4 h after the i.g. administration of different extracts of the in vitro cultures, ex vitro plants, wild plant, and metformin in the vehicle controls [28].

2.4.5. Serum Sample Preparation and Biochemical Estimation of Serum Salivary α-Amylase Enzyme, Malondialdehyde (MDA), Reduced Glutathione (GSH), and Insulin

A blood sample was collected and allowed to clot for 30 min at 25 °C. Centrifugation was performed at 4000 rpm for 15 min at 4 °C and, finally, the top yellow serum layer was pipetted off and stored on ice at −80 °C. The sample would be stable for at least one month for biochemical estimation. The activity of the serum MDA, serum GSH, and salivary α-amylase enzyme was determined using a test reagent kit (Biodiagnostic®, Giza, Egypt) while the serum insulin was determined using a rat insulin enzyme assay kit (Spi-Bio®, Sherbrooke, QC, Canada).

2.5. Statistical Analysis

In vitro culture experiments were set up in a completely randomized design. On the one hand, the data from different in vitro cultures were statistically analyzed using CoStat version 6.303 1998–2004 Cohort software (Monterey, CA, USA), and analysis of variance (ANOVA) was performed to compare the results. A least significance difference (LSD) test at $p = 0.05$ was used to compare the means. On the other hand, SPSS version 16 (Statistical package for the social sciences) was used for the analysis of the biochemical estimation. The quantitative data were presented in the form of mean ± SD using $p = 0.05$. One-way ANOVA was used to compare the means and a p value < 0.05 was significant.

3. Results and Discussion

3.1. In Vitro Micropropagation Protocol for A. fragrantissima

3.1.1. In Vitro Seed Germination and Establishment of Mother Stock

The sterilization procedure was successful and allowed a high percentage of non-contaminant mother stock establishment as the percentage of contamination was less than 5%. The seed culture can therefore be considered as a good starting material for the in vitro establishment of *A. fragrantissima* [19]. At the end of two weeks, seeds cultured on half-strength MS medium showed 95% germination.

Seeds are the most preferred initial step in in vitro mother stock establishment as they guarantee a high yield of non-contaminant in vitro germinated plants of many species [20,21].

3.1.2. In Vitro Shoot Proliferation

Cytokinins are the crucial growth regulators that induce high shoot induction. Kin, 2iP, and BAP are examples of cytokinins that previously showed a significant shooting response [30,31]. It was noticed that the lower the concentration of BAP, the better the shoot multiplication in the above-mentioned pre-multiplication step. The cytokinins under investigation were therefore applied in a concentration range of 0.6–3.6 µM/L. As the data shown in Table 2 illustrates, the propagation of the shoots was found to be exponential with the culture time, but the in vitro cultured plants that reached an age of 3 months were in an unhealthy state, having wilted leaves. The maximum number of the propagated shoots

(12.33 shoots/explant) was obtained from MS medium accompanied by 3.6 µM/L BAP over a period of 2 months (Figure 1a).

Table 2. Effect of different concentrations of BAP, 2iP, and Kin on multiple shoot induction in cultured *A. fragrantissima*.

Time	Growth Regulator (µM/L)			Length of the Longest Shoot (cm)	No. of Shoots/Explant	Fresh Weight of Explant (g)
	Kin	BAP	2iP			
1 month	0.0	0.0	0.0	2.15 ^{a}	1.41 ^{a}	0.19 ^{a}
	1	0.0	0.0	2.86 ^{ab}	4 ^{ab}	0.55 ^{b}
	2	0.0	0.0	2.72 ^{abc}	3.70 ^{ab}	0.35 ^{bc}
	3	0.0	0.0	2.51 ^{cde}	2.64 ^{bc}	0.35 ^{bc}
	0.0	1.2	0.0	2.17 ^{cde}	5.11 ^{bc}	0.79 ^{bc}
	0.0	2.4	0.0	2.41 ^{cde}	3.64 ^{bcd}	0.48 ^{bcd}
	0.0	3.6	0.0	2.95 ^{cde}	3 ^{bcde}	0.49 ^{bcd}
	0.0	0.0	0.6	2.50 ^{cde}	3.40 ^{cde}	0.45 ^{cd}
	0.0	0.0	1.2	2.61 ^{bc}	2.13 ^{de}	0.31 ^{cd}
	0.0	0.0	1.8	3.06 ^{c}	1.82 ^{e}	0.31 ^{d}
2 months	0.0	0.0	0.0	3.53 ^{a}	5.17 ^{a}	0.53 ^{a}
	1	0.0	0.0	3.44 ^{ab}	10.33 ^{ab}	1.79 ^{a}
	2	0.0	0.0	3.44 ^{ab}	7.50 ^{ab}	0.53 ^{ab}
	3	0.0	0.0	2.36 ^{ab}	6 ^{ab}	1.56 ^{ab}
	0.0	1.2	0.0	2.75 ^{ab}	10.13 ^{bc}	2.05 ^{ab}
	0.0	2.4	0.0	2.99 ^{bc}	10.67 ^{cd}	1.59 ^{abc}
	0.0	3.6	0.0	3.74 ^{bc}	12.33 ^{cde}	2.45 ^{bcd}
	0.0	0.0	0.6	4.29 ^{c}	4.43 ^{cde}	1.65 ^{cd}
	0.0	0.0	1.2	2.88 ^{c}	3.67 ^{de}	0.96 ^{cd}
	0.0	0.0	1.8	3.51 ^{c}	2 ^{e}	0.48 ^{d}

Means with the similar letters are not significantly different at a 0.05 level of probability using an LSD test; n = 20; BAP: 6-benzyl aminopurine; 2iP: 2-isopentyladenine; Kin: kinetin.

(a) **(b)** **(c)** **(d)**

Figure 1. (**a**) Shoot proliferation of *A. fragrantissima* on MS medium containing 3.6 µM/L BAP for 2 months culture time; (**b**) Effect of 2.4 µM/L of NAA on root number; (**c**) Ex vitro acclimated plants in external field conditions; (**d**) Callus induction on MS medium containing 3 µM/L BAP and 3 µM/L IAA in dark conditions.

Basal MS medium is usually supplemented with BAP in concentration between 3 µM/L and 6 µM/L and has been used for the in vitro cultivation of all species from the Asteraceae family [32]. A similar response was observed in *A. millefolium*, where 5.4 µM/L of BAP induced the maximum shoot number [33]. An in vitro study of *Ocimum gratissimum* L. reported that the best proliferation response was obtained by

introducing nodal explants into MS medium combined with 3 µM/L BAP [34]. Among different cytokinins, 3 µM/L BAP was the most effective in inducing multiple shoot formation from nodal explants of *Breynia disticha* [35].

3.1.3. In Vitro Root Formation and Acclimatization

Auxins, e.g., IAA, IBA, and NAA, are essential growth regulators that promote root development. As is shown in Table 3, NAA was the most effective auxin, stimulating adventitious root formation with a high percentage of 40%. The maximum rooting induction (2.46 roots/shoot tip explant) with a 3.4 cm average length was obtained using MS medium supplemented with 2.4 µM/L of NAA (Figure 1b). On the other hand, IAA failed to induce rooting. The in vitro rooted plants had an 88% survival rate when transferred to the soil (Figure 1c).

Table 3. Effects of IAA, IBA, and NAA concentrations on the number of roots, root length, and rooting percentage of *A. fragrantissima*.

Growth Regulators (µM/L)			No. of Roots/Explant	Length of the Longest Root (cm)	Rooting %	Fresh Weight of Plant (g)
IAA	IBA	NAA				
0.0	0.0	0.0	0.25 ^{a}	0.5 ^{a}	15%	0.19 ^{a}
0.3	0.0	0.0	0 ^{ab}	0 ^{ab}	0%	0.13 ^{ab}
0.6	0.0	0.0	0 ^{ab}	0 ^{ab}	0%	0.11 ^{abc}
1.2	0.0	0.0	0 ^{ab}	0 ^{ab}	0%	0.10 ^{bc}
0.0	0.4	0.0	0.09 ^{b}	0.46 ^{b}	9.1%	0.16 ^{bc}
0.0	0.8	0.0	0.58 ^{b}	1.35 ^{b}	13%	0.23 ^{bc}
0.0	1.6	0.0	1.7 ^{b}	2.05 ^{b}	22%	0.24 ^{bc}
0.0	0	0.6	1.14 ^{b}	0.75 ^{b}	16.4%	0.17 ^{bc}
0.0	0	1.2	2.33 ^{b}	1.67 ^{b}	21.1%	0.24 ^{bc}
0.0	0	2.4	2.46 ^{b}	3.4 ^{b}	40.3%	0.37 ^{c}

Means followed by the same letter within a column are not significantly different at a 0.05 level of probability according to an LSD test; n = 20; IAA: Indole-3-acetic acid; IBA: Indole-3-butyric acid; NAA: 1-naphthalene acetic acid.

NAA was shown to promote root formation via stimulation of cellular division of radical primordia. These findings are supported by a previous study reporting that NAA favored the highest root number and root length in members of family Asteraceae [36]. An in vitro study of *Broussonetia papyrifera* used 2.4 µM/L of NAA for effective rooting [37]. In addition, IAA failed to promote rooting in Jordan *A. fragrantissima* [13] and delayed rooting in *Acmella calva* (Asteraceae) [38].

3.1.4. Callus Induction

A callus is an undifferentiated tissue of highly active dividing cells. It is formed as a response to injury, so callus induction was initiated in all in vitro cultures by scratching the explants using scalpel. The optimum ratio of auxin and cytokinin encourages callus induction [39]. Calli were efficiently induced on both leaf and root explants in both dark and light conditions (Table 4). Callus induction was optimal for leaf explants on MS medium supplemented with 3 µM/L BAP and 3 µM/L IAA in dark conditions (Figure 1d). For root explants, derived calli obtained under light or dark conditions were of low value (Table 4).

In *Achillea oxyloba*, it was found that callus formation was developed on a medium supplemented with a combination of IAA and BAP [40]. This agrees with previous studies, which have reported that the combination of IAA and BAP promote callus formation and enhanced callus size in *Barleria lupulina* [41], *Physalis minima* [42], and *Celastrus paniculatus* [43].

Table 4. Effects of different concentrations of IAA with BAP on undifferentiated root and leaf callus weight of *A. fragrantissima* under light and dark conditions.

Explant	Treatment	Callus Weight (g)	Rooting	Nature
leaf/light	0.6 µM/L BAP + 0.6 µM/L IAA	0.17 ^{a}	0.0	hard
leaf/light	3 µM/L BAP + 3 µM/L IAA	0.64 ^{ab}	0.0	hard
leaf/light	6 µM/L BAP + 6 µM/L IAA	0.66 ^{b}	0.0	hard
leaf/light	1.2 µM/L BAP + 0.6 µM/L IAA	0.12 ^{b}	0.0	hard
leaf/light	1.2 µM/L BAP + 0.3 µM/L IAA	0.12 ^{c}	0.0	hard
leaf/light	2.4 µM/L BAP + 0.6 µM/L IAA	0.18 ^{c}	0.0	hard
leaf/dark	0.6 µM/L BAP + 0.6 µM/L IAA	0.22 ^{cd}	rooted	hard
leaf/dark	3 µM/L BAP + 3 µM/L IAA	1.06 ^{cd}	0.0	hard
leaf/dark	6 µM/L BAP + 6 µM/L IAA	0.79 ^{cde}	0.0	hard
leaf/dark	1.2 µM/L BAP + 0.6 µM/L IAA	0.32 ^{cde}	rooted	hard
leaf/dark	1.2 µM/L BAP + 0.3 µM/L IAA	0.16 ^{cde}	0.0	hard
leaf/dark	2.4 µM/L BAP + 0.6 µM/L IAA	0.17 ^{cde}	rooted	hard
root/light	0.6 µM/L BAP + 0.6 µM/L IAA	0.26 ^{cde}	0.0	soft
root/light	3 µM/L BAP + 3 µM/L IAA	0.36 ^{cde}	0.0	hard
root/light	1.2 µM/L BAP + 0.6 µM/L IAA	0.21 ^{cde}	rooted	soft
root/light	1.2 µM/L BAP + 0.3 µM/L IAA	0.19 ^{cde}	0.0	soft
root/light	2.4 µM/L BAP + 0.6 µM/L IAA	0.11 ^{de}	0.0	soft
root/dark	0.6 µM/L BAP + 0.6 µM/L IAA	0.14 ^{de}	rooted	soft
root/dark	3 µM/L BAP + 3 µM/L IAA	0.07 ^{de}	0.0	hard
root/dark	6 µM/L BAP + 6 µM/L IAA	0.01 ^{de}	0.0	hard
root/dark	1.2 µM/L BAP + 0.6 µM/L IAA	0.11 ^{de}	rooted	soft
root/dark	1.2 µM/L BAP + 0.3 µM/L IAA	0.04 ^{de}	rooted	soft
root/dark	2.4 µM/L BAP + 0.6 µM/L IAA	0.13 ^{e}	rooted	soft

Means followed by the same letter within a column are not significantly different at a 0.05 level of probability according to an LSD test; *n* = 12–15; AP: 6-benzyl amino purine; IAA: Indole-3-acetic acid.

3.1.5. Plant Regeneration and Organogenesis

Organogenesis is an important way to regenerate plants from the culture. Optimal shoot organogenesis was successfully obtained by mashing the aerial parts of the plant in sterile distilled water and culturing them on different concentrations of TDZ-containing media. As Table 5 shows, the highest number of regenerated plantlets as well as the longest ones were obtained after the treatment of the mashed aerial part with 2 mM/L TDZ. The concentration of TDZ produced concentration-dependent improvements in the obtained results.

Table 5. Effects of different concentrations of TDZ on the number of regenerated plants of *A. fragrantissima* and their length.

TDZ (mM/L)	No. of Regenerated Plants	Length of Regenerated Plants (cm)
0.5	6.67 ^{a}	0.5 ^{b}
1	7.75 ^{a}	0.83 ^{ab}
2	8 ^{a}	1.5 ^{a}

Means followed by the same letter within a column are not significantly different at a 0.05 level of probability according to an LSD test; *n* = 6: The treatment shown in bold was the most effective treatment tested.

TDZ is the most active cytokinin-like substance that induces shoot regeneration [44]. Our in vitro micropropagation results agreed with a previous study in which TDZ was found to be the most effective substance for the induction of shoot regeneration in various members of the Asteraceae family, such as *Achillea millefolium* L., *Cichorium intybus* L., and *Lythrum salicaria* L. [45]. TDZ is extensively used for regeneration in various plant species, such as *Rauvolfia tetraphylla* L. [46], *Capparis spinosa* L. [21], *Populus alba*, *Populus tremula* L. [47], and *Pterocarpus marsupium* Roxb [44].

3.2. In Vitro α-Amylase Inhibition Assay of Different Treatments

The inhibition of the α-amylase enzyme and consequently the prevention of starch digestion are the targets of some pharmaceutical products. Herein, several treatments, including acclimatization, resulted in a lower maltose concentration than that seen in the negative control with 100% enzymatic activity. The alpha amylase inhibition activity was calculated from the deduced equation y = 5.876x + 0.019 and R^2 = 0.99. The ethanolic extract of wild *A. fragrantissima* only showed an in vitro α-amylase inhibition of about 53%, which confirms its mild antihyperglycemic activity that was previously reported in the literature (Table 6) [2]. Calli produced by root explants subjected to BAP and IAA treatment in light and dark condition showed a similar % inhibition to the reference standard acarbose (Table 6), and for this reason they were selected for further in vivo study.

Table 6. % Inhibition of different sample treatments (α-amylase inhibition assay).

Treatment	% Inhibition (*n* = 3)
Multiplication 1 month	
Control	64 ± 0.03%
Kinetin	14 ± 0.12%
BAP	NA
2iP	NA
Multiplication 2 months	
Control	NA
Kinetin	NA
BAP	NA
2iP	NA
Rooting 1 month	
Control	NA
IAA	NA
IBA	NA
NAA	64 ± 0.21%
Leaf callus on MS medium with 3 µM/L BAP + 3 µM/L IAA at different ages	
1 month	37 ± 0.13%
2 months	NA
3 months	23 ± 0.07%
4 months	NA
6 months	NA
Undifferentiated cells of different explants and light conditions	
LL-control	79 ± 0.09%
LL BAP + IAA	83 ± 0.11%
LD BAP + IAA	85 ± 0.13%
RL BAP + IAA	93 ± 0.32%
RD BAP + IAA	93 ± 0.17%
Wild, Acclimated plants and standard	
Acclimated plant aged 7 months	7 ± 0.04%
Wild	53 ± 0.02%
Acarbose standard	93 ± 0.15%

NA: No activity; LL: leaf/light; LD: leaf/dark; RL: root/light; RD: root/dark.

Growth regulators can affect the accumulation of active metabolites. The literature explains that the aerial part of *A. fragrantissima* is a plentiful source of polyphenols. The total phenols content was shown to be enhanced or increased by using a combination of BAP and auxins [48]. Polyphenols are known for their alpha amylase inhibition activity through structure–activity relationship modeling studies [49]. As a result, the basal media supplemented with IAA and BAP showed a significant inhibition of the alpha amylase enzyme.

3.3. Colorimetric Determination of Polysaccharides in Different Treatments

One of the mechanisms proposed for the anti-hyperglycemic activity of plant extracts is dependent on the presence of polysaccharides [50]. Polysaccharides may alleviate β-cell dysfunction, improve glucose metabolism, enhance insulin action, or inhibit *α*-glucosidases [51]. The polysaccharide fraction of Egyptian *A. fragrantissima* was one of the highest anti-hyperglycemic fractions [16], so the quantification of the polysaccharides in different micro propagated plants was a must. As Table 7 shows, the different feedings and acclimatized plants produced variable concentrations of polysaccharides, which were calculated according to the deduced equation y = 0.015x + 0.05 and R^2 = 0.995. The highest concentrations of polysaccharides were provided by NAA treatment and IBA treatment. In addition to this, 4- and 6-month-old leaf calli showed higher concentrations of polysaccharides than those found in the extracts of native wild plants (Table 6). For these reasons, these treatments were selected for further in vivo study.

Table 7. Concentration of polysaccharides in different sample treatments.

Treatment	Conc. (µg/100 mg Dry Weight)
Multiplication 1 month	
Control	33.45 ± 0.4 *
Kinetin	31.03 ± 1 *
BAP	44.25 ± 0.4 *
2iP	44.39 ± 0.4 *
Multiplication 2 months	
Control	49.46 ± 0.5 *
Kinetin	61.30 ± 0.5 *
BAP	66.55 ± 0.3 *
2iP	79.11 ± 0.6 *
Rooting 1 month	
Control	47.22 ± 0.3 *
IAA	62.59 ± 0.4 *
IBA	94.92 ± 0.5 *
NAA	100.2 ± 0.7 *
Leaf callus on MS medium with 3 µM/L BAP + 3 µM/L IAA at different ages	
1 month	65.54 ± 0.5 *
2 months	73.28 ± 0.5 *
3 months	68.46 ± 0.4 *
4 months	87.53 ± 0.3 *
6 months	88.07 ± 0.2 *
Undifferentiated cells of different explants and light conditions	
LL-control	44.48 ± 0.5 *
LL BAP + IAA	43.5 ± 0.4 *
LD BAP + IAA	51.46 ± 0.5 *
RL BAP + IAA	42.99 ± 0.6 *
RD BAP + IAA	46.61 ± 0.3 *
Wild, Acclimated plants and standard	
Acclimated plant aged 7 months	86.46 ± 0.5 *
Wild	84.22 ± 0.4

Values are expressed by mean ± SD; LL: leaf/light; LD: leaf/dark; RL: root/light; RD: root/dark; * significantly different from the wild plant.

Growth regulators were widely applied to enhance the biosynthesis of the active metabolites. Our results are in agreement with a study by Zhong which found that NAA increased the production of ginseng polysaccharide, but the mechanism was not still clear [52]. In *Oplopanax elatus,* the highest content of bioactive polysaccharides was obtained from basal media supplemented with IBA [53].

3.4. Screening of the In Vivo Hypoglycemic Activity of Some Selected Feedings and Estimations of Their Insulin Activity

Egyptian Bedouins traditionally use a tea-like infusion of *A. fragrantissima* for the management of diabetes [2]. The screening of in vivo hypoglycemic activity was performed for some in vitro cultures of high polysaccharide content, in vitro cultures of significant in vitro alpha amylase inhibition activity, ex vitro acclimated plants, and the wild plant. The hypoglycemic activity was assessed after 2 h and 4 h administration to STZ-treated rats. As Table 8 shows, the wild *A. fragrantissima* significantly decreased elevated blood glucose levels in diabetic rats by 10% after 2 h and by 3% after 4 h. This indicates that the wild plant produces a mild and short-lived hypoglycemic effect.

Table 8. Blood glucose level and insulin activity estimation after 2 h and 4 h administration of different in vitro *A. fragrantissima* to STZ diabetic rats.

Groups	Blood Glucose Level (mg/dL)			Insulin Activity (μIU/mL)	
	After 1 h	After 2 h	After 4 h	After 2 h	After 4 h
Normal rats	113 ± 10.1	127.4 ± 9.3	130 ± 14.3	32.2 ± 0.3	31.3 ± 0.3
Diabetic rats	335 ± 25.6 #	373 ± 20 #	381.1 ± 35.5 #	15.8 ± 0.2 #	14.9 ± 0.4 #
Diabetic + metformin	345 ± 31 #	250 ± 29.4 * #	201.5 ± 19.8 * #	25.4 ± 0.2 * #	39 ± 0.5 * #
Diabetic + wild plant	321.9 ± 12.8 #	290.3 ± 7.3 * #	313.1 ± 7.4 * #	16.2 ± 0.1 * #	17.7 ± 0.2 * #
Diabetic + acclimated plant	363 ± 57 #	346.7 ± 55 * #	301.6 ± 57.7 * #	10 ± 0.3 #	8.9 ± 0.3 #
Growth regulators					
Diabetic + IBA-fed plant	373.5 ± 20.2 #	322.3 ± 19.3 * #	285.1 ± 15 * #	24.9 ± 0.4 * #	29.4 ± 0.1 * #
Diabetic + NAA-fed plant	369.5 ± 15.7 #	315.3 ± 18.3 * #	289.5 ± 47.8 * #	22.6 ± 0.1 * #	26.5 ± 0.2 * #
Diabetic + 2iP-fed plant	310.5 ± 12.1 #	285.1 ± 12.8 * #	308.1 ± 24.6 * #	1.8 ± 0.2 #	4.1 ± 0.2 #
Different ages of undifferentiated cells of 3 μM/L BAP + 3 μM/L IAA from leaf explants					
Diabetic + 4-month-old callus	320.1 ± 16.3 #	312.1 ± 16.4 * #	260.8 ± 19.2 * #	11 ± 0.2 #	12.6 ± 0.2 #
Diabetic + 6-month-old callus	349.3 ± 18.8 #	324.8 ± 13.4 * #	296.9 ± 22.5 * #	7.9 ± 0.1 #	9.9 ± 0.1 #
Undifferentiated cells of different explants and light conditions					
Diabetic + root callus of BAP and IAA/dark	383.1 ± 9.5 #	381.4 ± 12.3 #	373.3 ± 10.2 #	1.7 ± 0.2 #	4 ± 0.2 #

Values are expressed as mean ± SD; n = 8 for in vivo hypoglycemic activity; n = 2 for insulin activity estimation; * significantly different from diabetic rats; # significantly different from normal rats; BAP: 6-benzyl aminopurine; IAA: indole-3-acetic acid; IBA: indole-3-butyric acid; 2iP: 2-isopentyladenine; NAA: 1-naphthalene acetic acid.

On the other hand, the ex vitro acclimated plants produced a maximum effect (17%) that was sustained for 4 h with higher activity and a longer life span than that of the wild plant. Extracts from both IBA-fed plants and NAA-fed plants significantly reduced elevated blood glucose levels after 4 h administration (by 24% and 22%, respectively), and significant increased serum insulin levels (by 29.4 μIU/mL and 26.5 μIU/mL, respectively).

The activity of the IBA and NAA extracts may be due to their high content of polysaccharides. As was previously mentioned, over longer time frames, treatment with polysaccharides in *Ganoderma lucidum* and *Panax ginseng* can increase the renewal of beta cells in the pancreas, recover partially destroyed beta cells, or increase pancreatic insulin secretion [50,54].

3.5. Biochemical Estimation of Antioxidant Activity and In Vivo α-Amylase Inhibition Assay

Many mechanisms are involved in lowering high blood glucose levels. One of them is scavenging the reactive oxygen species that destroy β-cells. Oxidative stress plays a vital role in the deterioration of all biological functions [55]. Therefore, it is important to assess the antioxidant capacity of the serum samples of treated, untreated, and normal rats. As is shown in Table 9, the wild plant, the acclimated plant, and IBA-fed plants produced significant antioxidant activity through decreasing MDA and increasing GSH in the serum sample of the treated rats. GSH is an intracellular antioxidant that can prevent oxidative stress and MDA is a highly reactive marker of oxidative stress [55].

Table 9. Biochemical estimation of antioxidant activity by GSH and MDA determination as well as in vivo α-amylase inhibition activity of different in vitro *A. fragrantissima* administered to STZ diabetic rats.

Groups	Salivary α-Amylase Activity (U/L)		GSH (mg/dL)		MDA (nM/mL)	
	After 2 h	After 4 h	After 2 h	After 4 h	After 2 h	After 4 h
Normal rats	900 ± 15.3	910 ± 15.3	3.3± 0.2	3.2 ± 0.3	10.5 ± 0.3	11.2 ± 0.3
Diabetic rats	975.7 ± 5 #	994 ± 9.5 #	2.3 ± 0.19 #	2 ± 0.15 #	65.3 ± 0.3 #	72.2 ± 0.2 #
Diabetic + metformin	972 ± 8 #	987 ± 7 #	2.2 ± 0.18 #	2.3 ± 0.2 * #	62.5 ± 0.4 #	70.5 ± 0.5 #
Diabetic + wild plant	960 ± 9 * #	974 ± 24 * #	2.2 ± 0.17 #	2.6 ± 0.2 * #	17.5 ± 0.1 * #	20.5 ± 0.1 * #
Diabetic + acclimated plant	920 ± 7 * #	864 ± 10.5 * #	2.2 ± 0.2 * #	2.7 ± 0.1 * #	23.8 ± 0.3 * #	18.5 ± 0.2 * #
Growth regulators						
Diabetic + IBA-fed plant	940 ± 2.9 * #	960 ± 3 * #	2.7 ± 0.3 * #	2.9 ± 0.2 * #	52.2 ± 0.2 * #	48.8 ± 0.3 * #
Diabetic + NAA-fed plant	526 ± 6 * #	672 ± 12 * #	2.1 ± 0.15 #	1.8 ± 0.3 #	72.3 ± 0.3 #	76.1 ± 0.3 #
Diabetic + 2iP-fed plant	861 ± 11.5 * #	804 ± 13 * #	0.8 ± 0.13 #	2.1 ± 0.1 #	60.2 ± 0.1 #	65.6 ± 0.1 #
Different ages of undifferentiated cells of 3 μM/L BAP + 3 μM/L IAA from leaf explants						
Diabetic + 4-month-old callus	731 ± 10 * #	648 ± 10.5 * #	4.4 ± 0.2 * #	5.6 ± 0.1 * #	8.2 ± 0.1 * #	9.4 ± 0.1 * #
Diabetic + 6-month-old callus	658 ± 18.5 * #	757 ± 25 * #	3.2 ± 0.2 *	4.6 ± 0.1 * #	9.7 ± 0.1 * #	10.7 ± 0.1 * #
Undifferentiated cells of different explants and light conditions						
Diabetic + root callus of BAP and IAA/dark	980 ± 18#	997 ± 15 #	0.2 ± 0.13 #	0.2 ± 0.1 #	75.1 ± 0.1 #	71.3 ± 0.3 #

Values are expressed as mean ± SD; n = 3 for biochemical estimation of α-amylase, GSH, and MDA; * significantly different from diabetic rats; # significantly different from normal rats; BAP: 6-benzyl aminopurine; GSH: glutathione reduced; IAA: indole-3-acetic acid; IBA: indole-3-butyric acid; 2iP: 2-isopentyladenine; MDA: malondialdehyde; NAA: 1-naphthalene acetic acid.

Concerning the salivary alpha amylase activity, the NAA-fed plants also showed salivary alpha amylase enzyme inhibition, a result that is in agreement with other previous reports [56]. The undifferentiated callus cells from the leaf explants fed with a combination of BAP and IAA over periods of 4 months and 6 months exhibited both salivary alpha amylase and antioxidant effects.

These preliminary findings agree with a previous report in which the histopathological examination of islets of Langerhans cells in rats with type 2 diabetes showed improvements in the inhibition of pro-inflammatory markers and a significant antioxidant effect after chronic treatment with *A. fragrantissima* extract for 3 weeks [2]. In summary, the best in vivo antihyperglycemic screening was obtained from the IBA-fed plants and the NAA-fed plants after 4 h. Their potent activity was provided by insulin activity enhancement in both cases, and by the antioxidant activity of the IBA-fed plants and the in vivo salivary alpha amylase inhibition of the NAA-fed plants. In each case, these treatments provided improved antihyperglycemic activity when compared with the native wild plant.

4. Conclusions

The mild antihyperglycemic activity of the threatened *Achillea fragrantissima* can be enhanced by using IBA or NAA growth regulators through improvement of insulin secretion, antioxidant activity, and α-amylase inhibition. The IBA-fed plants and NAA-fed plants exhibited two-fold higher in vivo hypoglycemic activity than extracts from the wild plant. This threatened plant can be also conserved by in vitro culture on MS medium supplemented with 3.6 μM/L BAP for shooting, 2.4 μM/L NAA for rooting, or 2 mM/L TDZ for organogenesis. Herein, the study introduced an in vitro culture protocol for the conservation of *A. fragrantissima* with better hypoglycemic activity than the wild plant. Further studies handling the nano formulation of the IBA- and NAA-fed plants are recommended.

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