Establishment of a Rapid Micropropagation System for Kaempferia parviflora Wall. Ex Baker: Phytochemical Analysis of Leaf Extracts and Evaluation of Biological Activities

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Abstract: This study aimed to establish a rapid in vitro plant regeneration method from rhizome buds of Kaempferia parviflora to obtain the valuable secondary metabolites with antioxidant and enzyme inhibition properties. The disinfection effect of silver oxide nanoparticles (AgO NPs) on rhizome and effects of plant growth regulators on shoot multiplication and subsequent rooting were investigated. Surface sterilization of rhizome buds with sodium hypochlorite was insufficient to control contamination. However, immersing rhizome buds in 100 mg L⁻¹ AgO NPs for 60 min eliminated contamination without affecting the survival of explants. The number of shoots (12.2) produced per rhizome bud was higher in Murashige and Skoog (MS) medium containing 8 µM of 6-Benzyladenine (6-BA) and 0.5 µM of Thidiazuron (TDZ) than other treatments. The highest number of roots (24), with a mean root length of 7.8 cm and the maximum shoot length (9.8 cm), were obtained on medium MS with 2 µM of Indole-3-butyric acid (IBA). A survival rate of 98% was attained when plantlets of K. parviflora were acclimatized in a growth room. Liquid chromatography with tandem mass spectrometry (LC-MS/MS) was used to determine the chemical profile of K. parviflora leaf extracts. Results showed that several biologically active flavonoids reported in rhizomes were also present in leaf tissues of both in vitro cultured and ex vitro (greenhouse-grown) plantlets of K. parviflora. We found 40 and 36 compounds in in vitro cultured and ex vitro grown leaf samples, respectively. Greenhouse leaves exhibited more potent antioxidant activities than leaves from in vitro cultures. A higher acetylcholinesterase inhibitory ability was obtained for greenhouse leaves (1.07 mg/mL). However, leaves from in vitro cultures exhibited stronger butyrylcholinesterase inhibitory abilities. These results suggest that leaves of K. parviflora, as major byproducts of black ginger cultivation, could be used as valuable alternative sources for extracting bioactive compounds.

Keywords: micropropagation; silver oxide nanoparticles; flavonoids; antioxidant activity; enzyme inhibition; Kaempferia parviflora

1. Introduction

Black ginger (Kaempferia parviflora Wall. Ex Baker), a medicinal plant of the family Zingiberaceae, is native to Thailand. Black ginger rhizome is used in traditional medicine to cure colic disorder, weakness, lower blood glucose, male impotence, and ulcers [1–3]. It possesses antioxidant [4], anti-allergenic [5], anticancer [6], antimicrobial [1], anticholinesterase [7],...
anti-inflammatory [8], anti-obesity [9], and antimutagenic [10] properties. Phytochemical analysis of black ginger rhizome extracts has confirmed the presence of flavonoids [1,11], methoxyflavones [5,7,10,12–14], phenolic glycosides [15,16], and terpenoids [17]. Leaf extract of *Kaempferia galanga* has been reported to exhibit antinociceptive, anti-inflammatory [18], and sedative [19] properties. However, the biological activity and phytochemical profile of *K. parviflora* leaves have not been reported yet.

Multiple uses of *K. parviflora* have necessitated its mass collection as a raw material for pharmaceuticals purposes, leading to the depletion of this wild resource and generating pressure on *K. parviflora* populations [20]. Therefore, a sustainable cultivation method is needed to prevent the depletion of natural populations of *K. parviflora* and meet the growing demand from the pharmaceutical market. *K. parviflora* can be propagated using its rhizomes [20,21]. However, the availability of its rhizome is limited because of its use for extracting commercial metabolites and for preparing *K. parviflora* products. In addition, time (12 months) is required to obtain mature rhizomes [22]. Moreover, the yield and content of bioactive metabolites in *K. parviflora* are often affected by climatic change, abiotic factors, and biotic factors. In this regard, in vitro propagation technologies have been implemented for the mass propagation of various medicinal plants [22–24]. The establishment of efficient in vitro cell and plant regeneration techniques is essential for genetic improvement and mass production of valuable *K. parviflora* metabolites. In vitro production of biologically active phytochemicals through cell and organ culture as a reliable method is essential for generating cultures within a short period throughout the year.

In vitro plant regeneration [20,21], microrhizome formation [20,25], and cell suspension-based culturing [26] of *K. parviflora* have been reported. Axillary shoot multiplication has been achieved using terminal shoot buds [21] or rhizomatous buds [20]. The authors used 35.52 µM of 6-BA (6-Benzyladenine) to obtain maximal shoot production [20,21]. Cell suspension of *K. parviflora* can be established the best with liquid Murashige and Skoog [27] (MS) nutrient medium containing 1.0 mg L⁻¹ 2,4-D (2,4-dichlorophenoxyacetic acid) [26]. However, the authors did not evaluate phytochemical compositions/contents or biological activities of the shoot, callus, or cell suspension cultures of *K. parviflora* [20,21,26].

The goals of this current study were (1) to establish a rapid in vitro plant regeneration method from rhizome buds of *K. parviflora* and (2) phytochemical analysis, antioxidant studies, and enzyme inhibition studies. The disinfection effect of AgO NPs on rhizome and effects of plant growth regulators (PGRs) on shoot multiplication and subsequent rooting were investigated. Gradient reversed-phase ultra-high-performance liquid chromatography (UHPLC) separations with electrospray tandem mass spectrometry (MS/MS) detection (in both positive and negative ion modes) were used for the identification of compounds in leaf extracts. The results showed that several biologically active flavonoids reported in rhizomes were also present in both in vitro cultured and ex vitro (greenhouse)-grown leaf tissues of plantlets of *K. parviflora*. Its leaf extracts also exhibited significant free radical scavenging and enzyme inhibitory abilities in in vitro assays. Therefore, the leaves of *K. parviflora* as major byproducts of black ginger cultivation could be used as valuable alternative sources for extracting bioactive compounds.
2. Results and Discussion

2.1. In Vitro Micropropagation

2.1.1. Surface Sterilization

In vitro micropropagation is an effective and applicable method for preserving biodiversity and mass production of important plants [29–31]. The surface disinfection of in vivo plant materials is a prerequisite for initiating in vitro plant cultures. The initiation of sterile culture using explants obtained from underground parts is tricky because numerous microbes are attached to the surface of explants [22,28]. Previous studies have shown that surface disinfection of rhizome buds with mercuric chloride can reduce contamination in Kaempferia angustifolia [32], K. galanga [23,24], K. parviflora [22], and Kaempferia rotunda [23]. In our preliminary experiment, surface sterilization of K. parviflora rhizome buds with mercuric chloride yielded about 65% sterile culture. However, it negatively affected the organogenesis and viability of explants. Silver nitrate and antibiotics have been applied to eliminate contamination in a plant in vitro cultures [33]. Several NPs have been reported to have excellent antimicrobial activities [34]. Silver NP is comparatively free of decontaminators adverse effects, with less toxicity profile and good tissue tolerance [35]. It has been reported that Ag NPs can enter microbial cells and induce changes in intracellular structures, nucleic acids, proteins, and lipids, leading to cell death [36]. Recently, it has been shown that Ag NPs can reduce or eliminate microbial contamination in a wide range of plants, such as almond × peach rootstock [35], Araucaria excelsa [37], Capparis decidua [38], and Valeriana officinalis [39]. In this study, rhizome buds of K. parviflora were subjected to surface disinfection using AgO NPs. Rhizome buds treated with sodium hypochlorite served as controls. Decontamination and explant survival rates were significantly (p = 0.001) affected by AgO NP concentration, exposure time, and their interaction (Table 1).

Table 1. Effect of Ag nanoparticles (NPs) on decontamination and survival of rhizome bud explants of K. parviflora after 3 weeks of incubation.

| Ag NPs (mg L⁻¹) | Duration (min) | Decontamination (%) | Explant Survival (%) |
|-----------------|----------------|---------------------|----------------------|
| 0 (Control)     |                | 15.2 ± 3.3 j        | 100 ± 0.0 a          |
| 25              | 30             | 23.7 ± 4.6 i        | 100 ± 0.0 a          |
| 50              |                | 44.2 ± 3.3 f        | 100 ± 0.0 a          |
| 100             |                | 64.6 ± 4.9 c        | 100 ± 0.0 a          |
| 200             | 30             | 94.9 ± 3.1 b        | 73.7 ± 2.6 d         |
| 25              | 60             | 31.1 ± 4.6 h        | 100 ± 0.0 a          |
| 50              |                | 49.7 ± 3.9 e        | 100 ± 0.0 a          |
| 100             |                | 100 ± 0.0 a         | 100 ± 0.0 a          |
| 200             |                | 100 ± 0.0 a         | 65.2 ± 3.6 f         |
| 25              | 90             | 38.1 ± 3.3 g        | 91.7 ± 2.5 b         |
| 50              |                | 56.7 ± 4.8 d        | 85.2 ± 2.9 c         |
| 200             |                | 100 ± 0.0 a         | 67.8 ± 3.1 e         |
| Mean            |                | 62.93               | 86.7                 |

R-Square 0.9896 0.9889

Coefficient of variation 5.34 2.28

F-test 27086.7 1775.4

Duration 251.4 1149.6

Conc * Duration 53.4 89.0

p-value

Means ± SDs, followed by the same letters within a column, were not significantly different p < 0.05 by Duncan’s multiple range test (DMRT).
Surface sterilization of rhizome buds with sodium hypochlorite was insufficient to control the contamination (Figure 1a). However, the soaking of predisinfected rhizome buds in different AgO NPs significantly inhibited contaminants compared to the control. Treating explants with 25 mg L$^{-1}$ or 50 mg L$^{-1}$ AgO NPs was insufficient to control surface contaminants (Figure 1b,c). A high level of AgO NPs (200 mg L$^{-1}$) harmed explants survival (Table 2). It is well known that doses of decontaminators and exposure period can affect the morphogenetic potential and survival of explants [28,38]. Increasing the concentration and duration of exposure of AgO NPs also increased the rate of decontamination. However, the reverse was observed for the survival rate of *K. parviflora* explants (Table 2). With increasing doses, decontamination and survival rates of explants were increased and decreased, respectively. Among the AgO NPs treatments evaluated, immersing rhizome buds for 60 min in 100 mg L$^{-1}$ AgO NPs eliminated contamination without affecting the survival of explants (Table 1). Similar results have been reported for almond × peach rootstock [35], *A. excelsa* [37], *C. decidua* [38], and *V. officinalis* [39].

**Figure 1.** Photograph showing contamination in (a) sodium hypochlorite-treated explants, (b) 25 mg L$^{-1}$ AgO NPs-treated explants, and (c) 50 mg L$^{-1}$ AgO NPs-treated explants. (d) Root initiation after 9 days of culture, (e) shoot induction after 17 days of culture, (f) multiple plantlets regeneration after 42 days of culture.
Table 2. Effect of concentrations of AgO NPs and exposure time on decontamination and survival of *K. parviflora* rhizome bud explants.

| Factors       | Decontamination (%) | Explant Survival (%) |
|---------------|---------------------|----------------------|
| 25 mg L\(^{-1}\) | 30.9 d              | 97.2 a               |
| 50 mg L\(^{-1}\) | 50.2 c              | 95.1 b               |
| 100 mg L\(^{-1}\) | 88.2 b              | 89.3 c               |
| 200 mg L\(^{-1}\) | 98.3 a              | 61.1 d               |
| LSD           | 1.82                | 1.11                 |
| 30 min        | 56.8 c              | 93.4 a               |
| 60 min        | 70.2 b              | 91.3 b               |
| 90 min        | 73.7 a              | 72.3 c               |
| LSD           | 1.58                | 0.96                 |

Means within a column, followed by the same letters within a column, were not significantly different *p* < 0.05 by DMRT.

2.1.2. Shoot Multiplication

Rhizome buds of *K. parviflora* were cultured on MS nutrient medium containing 0–12 µM of cytokinin for shoot multiplication (Table 3). Roots first appeared after 9 days (Figure 1d) and shoots appeared after 17 days (Figure 1e) of cultivation. *K. parviflora* rhizome buds produced multiple plantlets within 42 days (Figure 1f) of cultivation. These rhizome bud explants (22.6%) produced shoots (1.2) and roots (2.3) together after 56 days on a PGR-free medium. The addition of 6-BA, 6-furfuryladenine (6-KN), or Thidiazuron (TDZ) at 1–12 µM increased the rate of regeneration, number of shoots per *K. parviflora* rhizome bud, and number of roots per shoot. Cytokinins are important PGRs that can promote axillary shoot multiplication [29,31,40], somatic embryogenesis [41], and adventitious shoot regeneration [42] in numerous plants. However, the application of cytokinin often adversely affects in vitro rhizogenesis [31]. In this study, simultaneous regeneration of both shoots and roots was attained using medium MS even with a high cytokinin level. Similar results have been disclosed earlier for *K. galanga* [43], *K. parviflora* [21], *Hedychium coronarium* [44,45], *Globba marantina* [46], and *Hosta minor* [40]. Cytokinin, concentration, and cytokinin × concentration interaction had significant effects on the regeneration rate and the number of shoots. Although cytokinin had no significant (*p* = 0.306) effect on the number of roots per shoot, the concentration of cytokinin (*p* = 0.001) and cytokinin × concentration interaction (*p* = 0.028) significantly affected the induction of roots (Table 3).

Table 3. Effect of cytokinins on shoot multiplication from rhizome bud explants of *K. parviflora* after 8 weeks of incubation.

| Cytokinin | Conc (µM) | Response (%) | No. of Shoots/Explant | No. of Roots/Shoot |
|-----------|-----------|--------------|-----------------------|--------------------|
| Control (MS) | 0 | 22.6 ± 2.2 k | 1.2 ± 0.4 h | 2.3 ± 0.9 gh |
| 6-BA      | 1 | 34.0 ± 3.0 i | 1.4 ± 0.5 gh | 4.3 ± 1.3 b-f |
|           | 2 | 53.9 ± 2.7 f | 2.6 ± 0.9 efg | 5.3 ± 1.0 b    |
|           | 4 | 64.1 ± 2.9 c | 4.2 ± 1.6 bc | 3.6 ± 1.0 c-f |
|           | 8 | 78.8 ± 3.1 a | 6.3 ± 1.6 a  | 4.6 ± 1.0 bcde|
|           | 12| 70.2 ± 4.7 b | 3.1 ± 1.1 cdef| 2.9 ± 0.9 fgh |
| 6-KN      | 1 | 23.0 ± 1.8 k | 1.3 ± 0.5 h  | 3.2 ± 1.2 d-f |
|           | 2 | 29.4 ± 2.9 j | 2.8 ± 1.2 def | 6.6 ± 1.6 a    |
|           | 4 | 46.6 ± 3.1 g | 3.7 ± 1.3 bcd | 4.8 ± 1.5 bc   |
|           | 8 | 57.7 ± 2.9 e | 2.3 ± 1.0 fgh | 3.8 ± 1.3 c-f |
|           | 12| 61.1 ± 3.7 d | 3.4 ± 1.1 cdef| 1.9 ± 0.6 h    |
| TDZ       | 1 | 39.7 ± 2.7 h | 2.9 ± 0.8 def | 3.1 ± 1.5 efgh |
|           | 2 | 67.8 ± 2.3 b | 4.7 ± 1.3 b  | 5.0 ± 1.9 bc   |
|           | 4 | 49.2 ± 2.8 g | 3.8 ± 1.0 bcd | 4.7 ± 2.3 bcd  |
|           | 8 | 53.3 ± 2.7 f | 3.0 ± 1.5 def | 3.7 ± 1.5 c-f |
|           | 12| 32.7 ± 1.6 i | 2.6 ± 0.5 efg | 2.1 ± 1.3 h    |
| Mean      | 49.0                       | 3.08                  | 3.87             |
| R-Square  | 0.9741                 | 0.5997               | 0.4839          |
| Coefficient of variation | 5.93 | 35.68 | 35.30 |
| F-value   |                         |                       |                 |
Shoot formation rate, number of shoots per rhizome bud, and number of roots per shoot varied from 34.0% to 78.8%, 1.4 to 6.3, and 2.9 to 5.3, respectively, when the medium MS was added with 1–12 µM of 6-BA. Rhizome buds of *K. parviflora* (78.8%) produced multiple shoots (6.3) and roots (4.6) on medium MS containing 8 µM of 6-BA (Figure 2a, Table 3). Shoots formed on medium MS added with 2 µM of 6-BA developed maximal roots (5.3). Explant response, shoot production, and root production were decreased on medium MS containing 12 µM of 6-BA. In contrast, terminal buds of *K. parviflora* produced a maximum of 7.16 shoots on medium MS added with 35.52 µM of 6-BA [21]. Shooting response, number of shoots per rhizome bud, and number of roots per shoot varied from 23.0% to 61.1%, 1.3 to 3.7, and 1.9 to 6.6, respectively, when the medium MS was added with 1–12 µM of 6-KN. The best shoot formation (61.1%), number of shoots (3.7), and number of roots (6.6) were noticed on medium MS added with 12 µM, 4 µM, and 2 µM of 6-KN, respectively (Table 3). The response of rhizome buds, number of shoots per rhizome bud, and number of roots per shoot varied from 32.7% to 67.8%, 2.6 to 4.7, and 2.1 to 5.0, respectively, when the medium MS was added with 1–12 µM of TDZ. The maximal response (67.8%), number of shoots (4.7), and number of roots (5.0) were noticed for medium MS added with 2 µM of TDZ (Table 3). Regeneration response, shoot production, and root production was found to be meager on medium MS added with 12 µM of TDZ. Detrimental effects of TDZ at a high dose on shoot production have also been disclosed for *K. parviflora* [21]. Among the cytokinins evaluated, 6-BA yielded the best explant response (60.2%), followed by TDZ (48.5%) and 6-KN (43.6%). Advantages of 6-BA on plant regeneration in vitro have been disclosed for *K. parviflora* [20,21] and other Zingiberaceae members such as *Curcuma angustifolia* [47], *H. coronarium* [44], and *K. galanga* [43]. However, a significant difference in the number of roots was not found among cytokinins (Table 4). Among all concentrations (1–12 µM) evaluated, 8 µM produced a higher shooting rate (63.3%) than other levels. However, 6-BA at a concentration of 2 µM, 4 µM, or 8 µM had a similar impact (*p = 0.05*) on *K. parviflora* shoot production (Table 4).

### Table 3. Cont.

| Cytokinin | Conc (µM) | Response (%) | No. of Shoots/Explant | No. of Roots/Shoot |
|-----------|-----------|--------------|------------------------|-------------------|
| F-test    | Cyto      | 378.49       | 6.72                   | 1.20              |
|           | Conc      | 405.83       | 14.33                  | 20.83             |
|           | Cyto * Conc | 192.41      | 10.61                  | 2.26              |
|           | p-value   |              |                        |                   |
|           | Cyto      | 0.001        | 0.002                  | 0.306             |
|           | Conc      | 0.001        | 0.001                  | 0.001             |
|           | Cyto * Conc | 0.001       | 0.001                  | 0.028             |

Means ± SDs, followed by the same letters within a column, were not significantly different *p < 0.05* by DMRT.
Figure 2. Micropropagation of *Kaempferia parviflora*. (a) Rhizome buds cultivated on Murashige and Skoog (MS) nutrient medium with 8 µM 6-BA; (b) rhizome buds cultivated on MS nutrient medium with 8 µM 6-BA and 0.5 µM Thidiazuron (TDZ); (c) rhizome buds cultivated on MS nutrient medium with 8 µM 6-BA, 0.5 µM TDZ, and 1 µM Naphthalene-1-acetic acid (NAA); (d) well-developed plantlet cultivated on nutrient medium MS with 2 µM Indole-3-butyric acid (IBA); (e,f) acclimatization.

Table 4. Effect of cytokinin types and their concentration on shoot multiplication from rhizome bud explants of *K. parviflora*.

| Factors | Response (%) | No. of Shoots/Explant | No. of Roots/Shoot |
|---------|--------------|-----------------------|--------------------|
| 6-BA    | 60.2 a       | 3.5 a                 | 4.13 a             |
| 6-KN    | 43.6 b       | 2.7 b                 | 4.07 a             |
| TDZ     | 48.5 c       | 3.4 a                 | 3.71 a             |
| LSD     | 1.23         | 0.47                  | 0.58               |
| 1 µM    | 32.2 d       | 1.9 c                 | 3.6 c              |
| 2 µM    | 50.4 c       | 3.3 ab                | 5.7 a              |
| 4 µM    | 53.3 b       | 3.9 a                 | 4.3 b              |
| 8 µM    | 63.3 a       | 3.9 a                 | 4.0 bc             |
| 12 µM   | 54.7 b       | 3.0 b                 | 2.3 d              |
| LSD     | 1.59         | 0.61                  | 0.75               |

Means within a column, followed by the same letters within a column, were not significantly different *p* < 0.05 by DMRT.

Several works have shown that a combination of PGRs can boost the regeneration of multiple shoots for Zingiberaceae members [43,44,46,47]. Chithra et al. [43] used a combination of 11.4 µM silver nitrate, 8.8 µM 6-BA, and 2.46 µM Indole-3-butyric acid (IBA) to induce maximal axillary buds (8.3) and roots (6.7) for *K. galanga*. Mohanty et al. [44]
used a combination of 8.8 \( \mu M \) 6-BA and 2.7 \( \mu M \) NAA to obtain maximal axillary buds (3.6) and roots (4) for *H. coronarium*. Parida et al. [46] used a combination of 14.1 \( \mu M \) 6-KN and 2.7 \( \mu M \) Naphthalene-1-acetic acid (NAA) to induce 9.5 axillary shoots and 4.5 roots for *G. marantina*. Jena et al. [47] used a combination of 135.7 \( \mu M \) adenine sulfate, 13.3 \( \mu M \) 6-BA, and 5.7 \( \mu M \) Indole-3-acetic acid (IAA) to obtain higher shoots (14.1) and roots (7.6) for *C. angustifolia*. In the present study, rhizome bud explants were placed on OM (optimal medium: MS plus 8 \( \mu M \) 6-BA) combined with other PGRs (Table 5) to produce roots and shoots within 14 days of cultivation. Supplementation of 2 \( \mu M \) and 4 \( \mu M \) of 6-KN to OM enhanced the explant response (89%) and number of shoots (9.2). Similarly, supplementation of 0.5–2 \( \mu M \) TDZ to OM enhanced the explant response (83.6–97.2%). The number of shoots (12.2) was higher in OM added with 0.5 \( \mu M \) TDZ after 56 days (Figure 2b, Table 5). The addition of 1–4 \( \mu M \) of NAA to OM containing 0.5 \( \mu M \) TDZ resulted in the maximum explant response (100%). However, shoot production was decreased (Figure 2c, Table 5). The number of roots increased as NAA level increased from 1–4 \( \mu M \). Prathanturarug et al. [21] also reported that NAA and cytokinin (6-BA, TDZ) in combination cannot increase shoot regeneration for *K. parviflora*.

### Table 5. Effect of combinations of PGRs on shoot multiplication from rhizome bud explants of *K. parviflora* after 8 weeks of incubation.

| PGRs (µM) | Response (%) | No. of Shoots/Explant | No. of Roots/Shoot |
|-----------|--------------|------------------------|-------------------|
| 6-BA  | 6-KN | TDZ | NAA | 6-BA  | 6-KN | TDZ | NAA | 6-BA  | 6-KN | TDZ | NAA |
| 0  | 0  | 0  | 0  | 0  | 22.6 ± 2.2 g | 1.2 ± 0.4 g | 2.3 ± 0.9 e |
| 8  | 2  | 0  | 0  | 89.0 ± 3.7 c | 7.8 ± 1.1 cde | 53 ± 1.4 cd |
| 8  | 4  | 0  | 0  | 86.1 ± 5.2 d | 9.2 ± 1.3 b | 4.2 ± 1.0 d |
| 8  | 6  | 0  | 0  | 76.2 ± 2.2 f | 6.2 ± 1.4 f | 2.8 ± 0.7 e |
| 8  | 0  | 0.5 | 0 | 97.2 ± 1.8 b | 12.2 ± 1.8 a | 4.3 ± 1.2 d |
| 8  | 0  | 1  | 0  | 90.9 ± 2.5 c | 8.1 ± 1.3 bcd | 2.7 ± 1.0 e |
| 8  | 0  | 2  | 0  | 83.6 ± 2.8 e | 6.4 ± 1.7 ef | 3.0 ± 1.1 e |
| 8  | 0  | 0.5 | 1 | 100 ± 0.0 a | 8.8 ± 1.6 bc | 6.3 ± 1.4 c |
| 8  | 0  | 0.5 | 2 | 100 ± 0.0 a | 6.8 ± 1.5 def | 8.1 ± 1.2 b |
| 8  | 0  | 0.5 | 4 | 100 ± 0.0 a | 5.9 ± 1.2 f | 9.7 ± 1.4 a |
| Mean | 84.6 | 7.3 | 4.9 | 0.9878 | 0.8113 | 0.8255 | 3.07 | 18.89 | 23.59 | 719.82 | 38.22 | 42.07 |

Means ± SDs, followed by the same letters within a column, were not significantly different \( p < 0.05 \) by DMRT.

#### 2.1.3. Rooting and Acclimatization

Although rhizome buds of *K. parviflora* developed both shoots and roots on OM alone or in combination with 0.5 \( \mu M \) TDZ, adventitious roots failed to develop lateral roots even after 56 days of cultivation (Figure 2a,b). Several studies have shown that cytokinins have detrimental effects on lateral root induction (reviewed by Jing and Strader [48]). In general, auxin is often included in a rooting medium to induce rhizogenesis of cultured shoots. IBA is a notable auxin that can stimulate rhizogenesis of diverse plant species [29,31,40]. Therefore, shoot buds (4 weeks old) were transferred to basal medium MS added with IBA (0–12 \( \mu M \)) to induce and develop roots. The addition of IBA to rooting medium MS improved the rooting quality (Figure 2d). Medium MS added with 2 \( \mu M \) of IBA resulted in the highest number of roots (24) with a mean root length of 7.8 cm and the maximum shoot length (9.8 cm) (Table 6). Shoot buds of *K. parviflora* on medium MS added with 4 \( \mu M \) of IBA developed longer roots (8.9 cm) than those in other treatments. Plantlets of *K. parviflora* were acclimatized well in a growth room, having a survival rate of 98% (Figure 2e,f).
Table 6. Effect of IBA on in vitro rooting of *K. parviflora* after 6 weeks of cultivation.

| IBA (µM) | Number of Roots/Shoot | Root Length (cm) | Shoot Length (cm) |
|----------|-----------------------|------------------|-------------------|
| 0        | 6.9 ± 1.5 f           | 4.7 ± 1.3 d      | 5.8 ± 0.5 c       |
| 1        | 11.0 ± 1.7 d          | 5.6 ± 0.4 c      | 6.1 ± 0.4 c       |
| 2        | 24.0 ± 2.5 a          | 7.8 ± 0.5 b      | 9.8 ± 0.5 a       |
| 4        | 15.8 ± 2.4 b          | 8.9 ± 0.5 a      | 6.6 ± 0.4 b       |
| 8        | 13.2 ± 1.5 c          | 5.8 ± 0.5 c      | 4.4 ± 0.3 e       |
| 12       | 8.9 ± 1.1 e           | 3.2 ± 0.7 e      | 5.3 ± 0.4 d       |
| Mean     | 13.29                 | 5.99             | 6.3               |
| R-Square | 0.9096                | 0.8911           | 0.9499            |
| Coefficient of variation | 14.02 | 11.69 | 6.45 |
| F-Value  | 96.55                 | 78.53            | 182.02            |

Means ± SDs, followed by the same letters within a column, were not significantly different *p* < 0.05 by DMRT.

2.2. Phytochemical Compositions

Phenolic compounds are considered as leading contributors to the biological activities of plant extracts. In the present study, we determined total amounts of phenolics and flavonoids in *K. parviflora* extracts. Results are presented in Table 7. Leaves from the greenhouse (18.28 mg GAE/g extract) contained higher phenolics levels than leaves from in vitro cultures (14.07 mg GAE/g extract). However, levels of total flavonoids in leaves from in vitro cultures (1.55 mg RE/g extract) were higher than those from greenhouse ones (0.96 mg RE/g extract). These results indicate that in vitro culture conditions could enhance levels of flavonoids. Previously published papers have also indicated that levels of total flavonoids are changed under in vitro culture conditions [49–51]. Krongrawa et al. [52] reported that levels of total phenolics and flavonoids in *K. parviflora* are 17.88–19.07 mg GAE/g and 15.90–16.68 mg QE/g, respectively, after gamma radiation. In addition, Choi et al. [53] reported that the contents of total phenolics in different fractions of *K. parviflora* are 19.48–92.26 mg GAE/g extract. These different levels of total phenolics could be due to different factors, including in vitro culture conditions, extraction methods, and solvents. On the other hand, spectrophotometric measurements have some drawbacks. Most phytochemists do not use them to perform content analysis. For example, recent papers have shown that the Folin–Ciocalteu assay measures reducing power instead of total phenolics content [54]. To obtain more accurate levels of total phenolics, at least one chromatographic technique has been suggested recently [55–57]. In this sense, the chemical profile of *K. parviflora* extracts was identified by LC-MS/MS.

Table 7. Total phenolic and flavonoid contents in the tested extracts.

| Sources of Leaves | Total Phenolic Content (mg GAE/g) | Total Flavonoid Content (mg RE/g) |
|------------------|----------------------------------|----------------------------------|
| In vitro cultures| 14.07 ± 0.09                     | 1.55 ± 0.07                      |
| The greenhouse   | 18.28 ± 0.20                     | 0.96 ± 0.06                      |

Samples were analyzed by UHPLC to obtain chromatographic profiles of more polar portions of extracts known to mainly contain phenolic and flavonoid compounds.

All characterized compounds with their chromatographic data, MS data (retention times, protonated or deprotonated molecular ions, fragment ions), and assigned identities are shown in Tables 8 and 9. Compounds were numbered by their elution order in a 56-day-old in vitro sample. These same numbers were used in a 90-day-old ex vitro sample. We found 40 and 36 compounds in in vitro and in vivo samples, respectively. Both samples showed a similar chromatographic profile. A wide range of compounds, mainly flavonoids, were characterized.
| No. | Name                                                                 | Formula      | Rt      | [M + H]^+ | [M − H]^− | Fragment 1          | Fragment 2          | Fragment 3          | Fragment 4          | Fragment 5          | Reference |
|-----|----------------------------------------------------------------------|--------------|---------|-----------|-----------|---------------------|---------------------|---------------------|---------------------|---------------------|----------|
| 1   | Caffeic acid                                                          | C_{9}H_{8}O_{4} | 15.22   | 179.03444 | 135.0438  | 107.0492            |                     |                     |                     |                     |          |
| 2   | Vicenin-2 (Apigenin-6,8-di-C-glucoside)                              | C_{27}H_{36}O_{15} | 19.39   | 595.16630 | 577.1575  | 559.1442            | 457.1131            | 325.0707            | 295.0601            |         |
| 3   | Ferulic acid                                                          | C_{10}H_{10}O_{4} | 19.94   | 193.05099 | 178.0261  | 149.0600            | 137.0226            | 134.0360            | 121.0278            |         |
| 4   | Apigenin-C-hexoside-C-pentoside isomer 1                             | C_{25}H_{28}O_{14} | 20.37   | 565.15574 | 547.1459  | 511.1239            | 427.1029            | 409.0923            | 295.0602            |         |
| 5   | Apigenin-C-hexoside-C-pentoside isomer 2                             | C_{25}H_{28}O_{14} | 20.77   | 565.15574 | 547.1455  | 511.1242            | 427.1030            | 379.0814            | 295.0602            |         |
| 6   | Apigenin-C-hexoside-C-pentoside isomer 3                             | C_{25}H_{28}O_{14} | 21.13   | 565.15574 | 547.1458  | 511.1236            | 469.1133            | 379.0813            | 295.0602            |         |
| 7   | Rutin (Quercetin-3-O-rutinoside)                                      | C_{27}H_{30}O_{16} | 23.54   | 611.16122 | 465.1043  | 303.0499            | 129.0549            | 85.0289             | 71.0497             | [15]     |
| 8   | Lumichrome                                                           | C_{12}H_{10}N_{4}O_{2} | 24.45   | 243.08821 | 216.0769  | 200.0820            | 198.0665            | 172.0870            | 145.0761            |         |
| 9   | Cosmosiiin (Apigenin-7-O-gluconoside)                                | C_{21}H_{20}O_{10} | 24.50   | 433.11347 | 271.0601  | 153.0185            | 119.0493            |                     |                     |         |
| 10  | Isoflammenetin-3-O-rutinoside (Narcissin)                             | C_{28}H_{32}O_{16} | 25.55   | 623.16122 | 315.0513  | 314.0432            | 299.0202            | 271.0243            | 243.0300            | [15]     |
| 11  | Tamarixetin-3-O-rutinoside                                           | C_{28}H_{32}O_{16} | 25.74   | 623.16122 | 315.0513  | 314.0435            | 299.0198            | 271.0250            | 243.0294            | [16]     |
| 12  | Syringetin-3-O-rutinoside                                            | C_{29}H_{34}O_{17} | 25.96   | 653.17178 | 345.0616  | 344.0539            | 329.0303            | 315.0151            | 301.0363            | [16]     |
| 13  | Acacetin-7-O-gluconoside (Tilianin)                                   | C_{22}H_{22}O_{10} | 29.06   | 447.12913 | 285.0757  | 270.0523            | 269.0444            | 242.0579            |                     | [16]     |
| 14  | Methoxy-trihydroxy(iso)flavanone                                      | C_{16}H_{14}O_{6}  | 30.20   | 303.08687 | 193.0499  | 167.0340            | 163.0390            | 145.0285            |                     |          |
| 15  | Apigenin (4',5,7-Trihydroxyflavone)                                  | C_{15}H_{10}O_{5}  | 30.29   | 269.04500 | 225.0547  | 201.0548            | 151.0026            | 149.0233            | 117.0331            |         |
| 16  | Isokaempferide (3-Methoxy-4',5,7-trihydroxyflavone)                   | C_{16}H_{12}O_{6}  | 30.97   | 299.05556 | 284.0329  | 256.0371            | 255.0297            | 227.0342            |                     |         |
| 17  | Undecanedioic acid                                                   | C_{11}H_{20}O_{4}  | 31.36   | 215.12834 | 197.1176  | 153.1272            | 125.0955            | 57.0331             |                     |         |
Table 8. Cont.

| No. | Name                                                                 | Formula       | Rt   | [M + H]<sup>+</sup> | [M − H]<sup>−</sup> | Fragment 1 | Fragment 2 | Fragment 3 | Fragment 4 | Fragment 5 | Reference |
|-----|---------------------------------------------------------------------|---------------|------|---------------------|---------------------|------------|------------|------------|------------|------------|-----------|
| 18  | 3',4',5,7-Tetramethoxyflavone or 3',4',5,7-Tetramethoxyflavone Pinocembrin (5,7-Dihydroxyflavanone) | C<sub>19</sub>H<sub>18</sub>O<sub>6</sub> | 32.44 | 343.11817           | 328.0942           | 327.0862   | 314.0793   | 313.0707   | 285.0765   | [11]       |
| 19  | Dihydroxyflavanone Kaempferide (4'-Methoxy-3,5,7-trihydroxyflavone)  | C<sub>15</sub>H<sub>12</sub>O<sub>4</sub> | 32.77 | 255.06573           | 213.0547           | 151.0023   | 145.0645   | 107.0124   | 83.0122    | [11]       |
| 20  | Pentamethoxyflavone                                                | C<sub>20</sub>H<sub>20</sub>O<sub>7</sub> | 32.92 | 373.12873           | 358.1046           | 357.0968   | 343.0810   | 327.0863   | 312.0990   | [11]       |
| 21  | Dihydroxy-methoxy(iso)flavone-O-acetyhexoside                      | C<sub>24</sub>H<sub>24</sub>O<sub>11</sub>| 33.21 | 489.13969           | 328.0940           | 213.0547   | 151.0023   | 107.0124   | 83.0122    | [11]       |
| 22  | Dimethoxy-trihydroxy(iso)flavone                                   | C<sub>17</sub>H<sub>14</sub>O<sub>7</sub> | 33.35 | 329.06613           | 314.0434           | 299.0198   | 271.0249   | 227.0338   | [11]       |
| 23  | Dodecanedioic acid 5,7-Dimethoxyflavanone                            | C<sub>12</sub>H<sub>22</sub>O<sub>4</sub> | 33.81 | 229.14399           | 211.1334           | 167.1433   | [11]       |
| 24  | Dimethoxyflavone                                                   | C<sub>17</sub>H<sub>16</sub>O<sub>4</sub> | 33.82 | 285.11268           | 181.0497           | 166.0261   | 138.0317   | 131.0494   | 103.0548   | [15]       |
| 25  | 5,7-Dimethoxyflavone Galangin (3,5,7-Trihydroxyflavone)             | C<sub>15</sub>H<sub>10</sub>O<sub>5</sub> | 34.75 | 271.06065           | 242.0572           | 215.0701   | 165.0181   | 153.0184   | 105.0336   | [11]       |
| 26  | 4',5,7-Trimethoxyflavone                                           | C<sub>18</sub>H<sub>16</sub>O<sub>5</sub> | 34.81 | 313.10760           | 298.0837           | 297.0761   | 269.0809   | 255.0649   | 227.0711   | [11]       |
| 27  | 3,5,7-Trimethoxyflavone                                            | C<sub>18</sub>H<sub>16</sub>O<sub>5</sub> | 34.98 | 313.10760           | 298.0836           | 297.0758   | 280.0729   | 279.0652   | 252.0778   | [11]       |
| 28  | Dihydroxymethoxy(iso)flavone Ayanin                                | C<sub>16</sub>H<sub>12</sub>O<sub>5</sub> | 35.05 | 283.06065           | 268.0375           | 267.0294   | 239.0344   | 211.0393   | [11]       |
| 29  | (3',5-Dihydroxy-3,4',7-trimethoxyflavone)                           | C<sub>18</sub>H<sub>16</sub>O<sub>7</sub> | 35.20 | 345.09743           | 330.0733           | 329.0661   | 315.0499   | 287.0551   | 259.0602   | [15]       |
| 30  | Tetramethoxyflavone or 3',4',5,7-Tetramethoxyflavone               | C<sub>19</sub>H<sub>18</sub>O<sub>6</sub> | 35.44 | 343.11817           | 328.0940           | 327.0862   | 310.0837   | 285.0760   | 282.0886   | [11]       |
Table 8. Cont.

| No. | Name                                      | Formula      | Rt  | [M + H]* | [M – H] | Fragment 1 | Fragment 2 | Fragment 3 | Fragment 4 | Fragment 5 | Reference |
|-----|-------------------------------------------|--------------|-----|----------|---------|------------|------------|------------|------------|------------|-----------|
| 34  | Dihydroxydimethoxy(iso)flavone Retusin    | C_{17}H_{14}O_{6} | 35.60 | 315.08686 | 300.0628 | 299.0548  | 272.0680  | 271.0602  | 257.0445  |           |
|     | (5-Hydroxy-3,3',4',7-tetramethoxyflavone) |              |     |          |         |            |            |            |            |            | [11]      |
| 35  | Pinostrobin (5-Hydroxy-7-methoxyflavanone) | C_{19}H_{18}O_{7} | 37.10 | 359.11308 | 344.0890 | 343.0812  | 329.0655  | 301.0706  |           | [11]      |
| 36  | Tectochrysin (5-Hydroxy-7-methoxyflavone) | C_{16}H_{14}O_{4} | 37.14 | 271.09704 | 229.0864 | 173.0599  | 167.0339  | 131.0494  | 103.0547  | [15]      |
| 37  | 4',7-Dimethoxy-5-hydroxyflavone           | C_{16}H_{12}O_{4} | 38.08 | 269.08138 | 254.0573 | 226.0624  | 167.0338  |           |           | [11]      |
| 38  | 5-Hydroxy-3,7-dimethoxyflavone            | C_{17}H_{14}O_{5} | 38.75 | 299.09195 | 284.0678 | 256.0728  |           |           |           | [11]      |
| 39  | 5-Hydroxy-3,4',7-trimethoxyflavone        | C_{18}H_{16}O_{6} | 39.39 | 329.10252 | 314.0784 | 313.0707  | 299.0552  | 285.0756  | 271.0598  | [11]      |
| 40  |                                          |              |     |          |         |            |            |            |            |            |           |

1 Confirmed by standard.

Table 9. Chemical composition of the black ginger leaves from greenhouse.

| No. | Name                                      | Formula      | Rt  | [M + H]* | [M – H] | Fragment 1 | Fragment 2 | Fragment 3 | Fragment 4 | Fragment 5 | Reference |
|-----|-------------------------------------------|--------------|-----|----------|---------|------------|------------|------------|------------|------------|-----------|
| 1   | Caffeic acid                              | C_{9}H_{8}O_{4} | 15.19 | 179.03444 | 135.0438 | 107.0488   |            |            |            |            |           |
| 2   | Vicenin-2 (Apigenin-6,8-di-C-glucoside)   | C_{27}H_{38}O_{15} | 19.38 | 595.16630 | 577.1556 | 559.1453   | 457.1132   | 325.0707   | 295.0602   |           |
| 3   | Ferulic acid                              | C_{10}H_{10}O_{4} | 19.95 | 193.05009 | 178.0261 | 149.0596   | 137.0231   | 134.0360   | 121.0278   |           |
|     | Apigenin-C-hexoside-C-pentoside isomer 1  | C_{26}H_{28}O_{14} | 20.43 | 565.15574 | 547.1460 | 511.1242   | 427.1027   | 409.0920   | 295.0602   |           |
| 4   | Apigenin-C-hexoside-C-pentoside isomer 2  | C_{26}H_{28}O_{14} | 20.78 | 565.15574 | 547.1451 | 511.1239   | 427.1030   | 379.0813   | 295.0601   |           |
|     |                                          |              |     |          |         |            |            |            |            |            |           |
| No. | Name                                      | Formula       | Rt      | [M + H]^+ | [M – H]^- | Fragment 1 | Fragment 2 | Fragment 3 | Fragment 4 | Fragment 5 | Reference |
|-----|------------------------------------------|---------------|---------|-----------|-----------|------------|------------|------------|------------|------------|-----------|
| 6   | Apigenin-C-hexoside-C-pentoside isomer 3 | C_{26}H_{28}O_{14} | 21.14   | 565.15574 | 547.1455  | 511.1245   | 469.1141   | 379.0813   | 295.0602   |            | [15]      |
| 7   | Rutin (Quercetin-3-O-rutinoside)         | C_{27}H_{30}O_{16} | 23.53   | 611.16122 | 465.1033  | 303.0499   | 129.0549   | 85.0290    | 71.0498    | 295.0602   | [15]      |
| 8   | Lumichrome Isorhamnetin-3-O-rutinoside   | C_{12}H_{10}N_{4}O_{2} | 24.46   | 243.08821 | 216.0767  | 200.0819   | 198.0662   | 172.0869   | 145.0760   |            | [15]      |
| 10  | Tamarixetin-3-O-rutinoside               | C_{28}H_{32}O_{16} | 25.74   | 623.16122 | 315.0512  | 314.0440   | 299.0192   | 271.0250   | 243.0288   |            | [16]      |
| 11  | Syringetin-3-O-rutinoside                | C_{28}H_{32}O_{16} | 25.97   | 653.17178 | 345.0618  | 344.0536   | 329.0306   | 315.0142   | 301.0355   |            | [16]      |
| 12  | Acacetin-7-O-glucoside (Tilianin)        | C_{22}H_{22}O_{10} | 29.06   | 447.12913 | 285.0757  | 270.0522   | 269.0443   | 242.0567   |            | [16]      |
| 15  | Apigenin (4',5,7-Trihydroxyflavone)      | C_{15}H_{10}O_{5}  | 30.29   | 269.04500 | 225.0555  | 201.0546   | 151.0019   | 149.0230   | 117.0330   |            | [16]      |
| 41  | Dihydroxy-methoxy(iso)flavone isomer 1  | C_{16}H_{12}O_{5}  | 30.85   | 283.06065 | 268.0377  | 240.0423   | 239.0346   | 211.0394   |            |           |           |
| 16  | Undecanedioic acid 3,4',5,7-Tetramethoxyflavone or 3',4',5,7-Tetramethoxyflavone | C_{19}H_{18}O_{6} | 32.45   | 343.11817 | 328.0943  | 327.0863   | 314.0783   | 313.0707   | 285.0760   |            | [11]      |
| 19  | Pinocembrin (5,7-Dihydroxyflavanone)     | C_{15}H_{12}O_{4}  | 32.78   | 255.06573 | 213.0556  | 151.0024   | 145.0649   | 107.0121   | 83.0123    |            |           |
| 20  | Dihydroxy-methoxy(iso)flavone-O-acetylhexoside | C_{21}H_{20}O_{7} | 32.93   | 373.12873 | 358.1045  | 357.0966   | 343.0811   | 327.0863   | 312.0991   |            | [11]      |
| No. | Name                                                                 | Formula   | Rt     | [M + H]<sup>+</sup> | [M − H]<sup>−</sup> | Fragment 1 | Fragment 2 | Fragment 3 | Fragment 4 | Fragment 5 | Reference |
|-----|---------------------------------------------------------------------|-----------|--------|---------------------|---------------------|------------|------------|------------|------------|------------|-----------|
| 25  | 5,7-Dimethoxyflavanone                                              | C<sub>17</sub>H<sub>16</sub>O<sub>4</sub> | 33.82  | 285.11268          | 181.0496           | 166.0258   | 138.0316   | 131.0493   | 103.0545   |           | [15]      |
| 26  | Chrysine (5,7-Dihydroxyflavone)                                     | C<sub>15</sub>H<sub>10</sub>O<sub>4</sub> | 33.85  | 255.06573          | 209.0596           | 153.0182   | 129.0341   | 103.0546   | 67.0185    |           | [15]      |
| 27  | 5,7-Dimethoxyflavone                                               | C<sub>17</sub>H<sub>14</sub>O<sub>4</sub> | 34.14  | 283.09704          | 268.0730           | 267.0650   | 239.0702   | 238.0626   | 225.0549   |           | [11]      |
| 28  | Galangin (3,5,7-Trihydroxyflavone)                                  | C<sub>15</sub>H<sub>10</sub>O<sub>5</sub> | 34.76  | 271.06065          | 242.0576           | 215.0700   | 165.0183   | 153.0182   | 105.0339   |           | [11]      |
| 29  | 4',5,7-Trimehtoxylavone                                            | C<sub>18</sub>H<sub>16</sub>O<sub>5</sub> | 34.82  | 313.10760          | 298.0836           | 297.0761   | 269.0808   | 255.0662   | 227.0694   |           | [11]      |
| 30  | 3,5,7-Trimehtoxylavone                                              | C<sub>18</sub>H<sub>16</sub>O<sub>5</sub> | 35.00  | 313.10760          | 298.0839           | 297.0757   | 280.0730   | 279.0654   | 252.0782   |           | [11]      |
| 31  | Dihydroxy-methoxy(iso)flavone isomer 2                             | C<sub>16</sub>H<sub>12</sub>O<sub>5</sub> | 35.03  | 283.06065          | 268.0377           | 267.0294   | 239.0345   | 211.0394   |           |           |           |
| 32  | Cyanin (3',5-Dihydroxy-3,4',7-trimethoxyflavone)                   | C<sub>18</sub>H<sub>16</sub>O<sub>7</sub> | 35.21  | 345.09743          | 330.0733           | 329.0653   | 315.0499   | 287.0549   | 259.0601   |           | [15]      |
| 33  | Tetramethoxylavone or 3',4',5,7-Tetramethoxyflavone                 | C<sub>19</sub>H<sub>18</sub>O<sub>6</sub> | 35.45  | 343.11817          | 328.0944           | 327.0863   | 310.0835   | 285.0754   | 282.0886   |           | [11]      |
| 34  | Dihydroxy-dimethoxy(iso)flavone Retusin                           | C<sub>17</sub>H<sub>14</sub>O<sub>6</sub> | 35.60  | 315.08686          | 300.0629           | 299.0552   | 272.0679   | 271.0602   | 257.0436   |           | [11]      |
| 35  | (5-Hydroxy-3,3',4',7-tetramethoxyflavone)                          | C<sub>19</sub>H<sub>18</sub>O<sub>7</sub> | 37.10  | 359.11308          | 344.0890           | 343.0815   | 329.0656   | 301.0706   |           |           | [11]      |
| 36  | (5-Hydroxy-7-methoxyflavanone)                                      | C<sub>16</sub>H<sub>14</sub>O<sub>4</sub> | 37.14  | 271.09704          | 229.0859           | 173.0598   | 167.0339   | 131.0494   | 103.0546   |           | [15]      |
| 37  | Tectochrysin (5-Hydroxy-7-methoxyflavanone)                        | C<sub>16</sub>H<sub>12</sub>O<sub>4</sub> | 38.09  | 269.08138          | 254.0571           | 226.0626   | 167.0342   |           |           |           | [11]      |
| 39  | 5-Hydroxy-3,7-dimethoxyflavone                                      | C<sub>17</sub>H<sub>14</sub>O<sub>5</sub> | 38.94  | 299.09195          | 284.0680           | 283.0620   | 256.0729   | 255.0650   | 241.0494   |           | [11]      |
| 40  | 5-Hydroxy-3,4',7-trimethoxyflavone                                  | C<sub>18</sub>H<sub>16</sub>O<sub>6</sub> | 39.38  | 329.10252          | 314.0784           | 313.0707   | 299.0550   | 285.0757   | 271.0602   |           | [11]      |

<sup>1</sup> Confirmed by standard.
The lowest molecular mass component was caffeic acid (1) (rt: 15.19 min, MW: 180.04226). Compound 12 had the highest molecular mass. It was characterized as syringetin-3-O-rutinoside (MW: 654.17960). Compound 2 at rt: 19.38 min was confirmed as vicenin-2 (apigenin 6,8-di-C-glucoside). Characteristic fragments confirmed the substitution of two C-glucosides at positions 6 and 8 in compound 2. Compounds at rt: 20.46 min, 20.79 min, and 21.15 min were identified as apigenin C-hexoside-C-pentoside isomers (4–6) with [M − H]− at m/z 563.1409, showing ion fragments at m/z 473.1089 corresponding to [M − H-90]−, m/z 443.0974 corresponding to [M − H-120]−, m/z 383.0770 corresponding to [M − H-180]−, and m/z 353.0669 corresponding to [M − H-120-90]− in the MS/MS spectrum (Figure 3a,b). The positive ion mode was a powerful complementary tool of the negative ion mode for compounds’ structural characterization by electrospray ionization (ESI)-MS/MS. In compound 4–6, more fragment ions were detected in the positive mode (Figure 4a,b).

Figure 3. (a) Extracted ion chromatogram of apigenin C-hexoside-C-pentoside isomers (m/z: 563.14087) in negative ion mode (56-day-old in vitro sample). (b) MS2 spectrum of apigenin C-hexoside-C-pentoside isomer 1 in negative mode at a retention time of 20.52 min (56-day-old in vitro sample).
Figure 4. (a) Extracted ion chromatogram of apigenin C-hexoside-C-pentoside isomers ($m/z$: 565.15574) in positive ion mode (56-day-old in vitro sample). (b) MS2 spectrum of apigenin C-hexoside-C-pentoside isomer 1 in positive mode at a retention time of 20.41 min (56-day-old in vitro sample).

2.3. Antioxidant Ability

To detect the antioxidant potential of *K. parviflora* in the present study, we used six assays. Antioxidant compounds are closely linked to positive effects on human health. They can minimize the negative effects of free radicals that are instable and very active. Scavenging of free radicals (3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) and 2,2-diphenyl-1-picrylhydrazyl (DPPH) was tested. Results are given as IC$_{50}$ values. As shown in Table 10, *K. parviflora* leaf extracts exhibited low DPPH scavenging abilities, with IC$_{50}$ values $>$3 mg/mL. Regarding ABTS scavenging abilities, greenhouse leaves exhibited more potent activities than leaves from in vitro cultures. However, these tested extracts had weaker scavenging abilities compared to Trolox (IC$_{50}$: 0.06 mg/mL for DPPH and 0.09 mg/mL for ABTS). Their reducing
abilities were evaluated by cupric reducing antioxidant capacity (CUPRAC) and ferric reducing antioxidant power (FRAP) assays. In both assays, greenhouse leaves possessed higher abilities (CUPRAC and FRAP: 2.07 mg/mL and 1.52 mg/mL, respectively). Reducing power assays reflect the electron-donating abilities of plant extracts and antioxidant compounds. Phosphomolybdenum assay is based on Mo (VI) transformation to Mo (V) by antioxidant compounds at an acidic condition. Both leaves samples had weak ability in phosphomolybdenum assays. Their IC_{50} values were higher than 3 mg/mL. In the last assay, the metal chelating abilities of leaf extracts were determined. Results showed that leaves from in vitro cultures had higher metal-chelating abilities, with an average IC_{50} value of 0.59 mg/mL. However, ethylenediaminetetraacetic acid was a better chelator. Several studies have reported antioxidant properties of *K. parviflora* extracts or fractions. For example, Krongrawa et al. [52] demonstrated antioxidant properties of *K. parviflora* extracts using DPPH and FRAP assays. In their study, IC_{50} values ranged from 129.08 µg/mL to 165.26 µg/mL in the DPPH assay. The reducing power in FRAP assay was found to be 11.96–12.48 mg ascorbic acid equivalent/g extract. Thao et al. [4] reported antioxidant abilities for peroxyl radicals and cupric reducing power of *K. parviflora* rhizomes. Choi et al. [53] disclosed that the ethyl acetate fraction of *K. parviflora* has the strongest DPPH, ABTS, and ferric reducing power. As can be seen in earlier papers, few reports are available on the antioxidant properties of *K. parviflora*. Thus, the results of the present study provide valuable scientific knowledge of *K. parviflora*.

Table 10. Antioxidant properties of the tested samples (IC_{50} (mg/mL)).

| Sources of Leaves and Standards | DPPH | ABTS | CUPRAC | FRAP | PBD | Chelating |
|---------------------------------|------|------|--------|------|-----|----------|
| In vitro cultures               |     |      |        |      |     |          |
| >3 b                            |     |      |        |      |     |          |
| The greenhouse                  | >3 b| 2.20 | 2.07   | 1.52 | >3  | 0.70     |
| Trolox                          | 0.06| 0.09 | 0.11   | 0.04 | 0.52|          |
| EDTA                            | nt  | nt   | nt     | nt   | nt  | 0.02     |
| nt: Not tested. PBD: Phosphomolybdenum. Means ± SDs, followed by the same letters within a column, were not significantly different p < 0.05 by DMRT.

2.4. Enzyme Inhibitory Effects

Although the world is a healthier place today, humanity still faces global health problems. Several infectious diseases, including polio, Ebola, and smallpox, have been eliminated by some effective treatment strategies over the centuries. However, the prevalence of some noncommunicable diseases is almost epidemic all over the world. For example, about 500 million people are affected by diabetes mellitus [58]. In this sense, we need effective therapeutic tools to overcome the burden. Many studies have demonstrated that enzymes are effective drug targets [59]. According to this approach, inhibition of some clinical enzymes is linked to the alleviated symptoms observed. For example, inhibition of amylase and glucosidase as main hydrolyzing enzymes of carbohydrates can control blood glucose levels after a carbohydrate-rich diet [60]. Thus, enzyme inhibitors are among the most common topics in medical and pharmaceutical areas. Researchers have attempted to use several chemicals for this purpose. However, these chemicals have produced unpleasant effects, including toxicity and gastrointestinal disturbances [61,62]. Taken together, these findings suggest that we need to replace synthetics with safe and effective ones from natural resources.

In the current paper, we tested the enzyme inhibiting properties of *K. parviflora* leaves. Results are given as IC_{50} values (Table 11). The best AChE inhibitory ability was obtained for leaves from greenhouse-grown *K. parviflora* (1.07 mg/mL). However, leaves from in vitro cultures exhibited stronger BChE inhibitory abilities than leaves from the greenhouse. Regarding tyrosinase inhibitory activity, both leaves had some potential, with an average IC_{50} value of 0.71 mg/mL. Finally, the best amylase inhibition ability was obtained for leaves from the greenhouse, with an IC_{50} value of 1.37 mg/mL. However, inhibitor standards were more active than tested extracts in all assays performed.
enzyme inhibitory abilities might be explained by the presence of some compounds in these leaf extracts. For example, some phenolic acids such as caffeic [63–65] and ferulic acids [66–68] have been shown to possess significant inhibitor properties in earlier studies. Again, flavonoids including rutin [69,70] and quercetin [69,71] have been reported as effective enzyme inhibitors. Thus, K. parviflora leaves could be useful as sources of natural enzyme inhibitors for pharmaceutical and cosmetic applications.

Table 11. Enzyme inhibitory properties of tested samples (IC\textsubscript{50} (mg/mL)).

| Sources of Leaves and Standards | AChE    | BChE    | Tyrosinase | Amylase |
|-------------------------------|---------|---------|------------|---------|
| In vitro cultures             | 1.15 ± 0.04 b | 0.95 ± 0.10 c | 0.71 ± 0.01 b | 1.41 ± 0.01 b |
| The greenhouse                | 1.07 ± 0.10 b | 1.67 ± 0.11 b | 0.71 ± 0.01 b | 1.37 ± 0.05 b |
| Galantamine                   | 0.003 ± 0.001 a | 0.007 ± 0.002 a | nt         | nt      |
| Kojic acid                    | nt      | nt      | 0.08 ± 0.001 a | nt      |
| Acarbose                      | nt      | nt      | nt         | 0.68 ± 0.01 a |

nt: Not tested. Means ± SDs, followed by the same letters within a column, were not significantly different \( p < 0.05 \) by DMRT.

3. Materials and Methods

3.1. In Vitro Micropropagation

3.1.1. Synthesis and Characterization of Silver Oxide Nanoparticles (AgO NPs)

Silver oxide nanoparticles were synthesized with the hydrothermal method using polyethylene glycol and silver nitrates purchased from Sigma-Aldrich. For the synthesis of AgO nanoparticles, 25 g of polyethylene glycol (PEG) was dissolved in 1 L of deionized (DI) water and stirred unceasingly for 1 h at 60 °C. After complete dissolution as a homogeneous solution, 1 g of silver nitrate salt was added into the aqueous PEG solution under constant stirring for another 1 h. After a set period, formed AgO nanoparticles were filtered using a membrane filter (0.2 \( \mu \)m, Millipore). These filtered particles were washed several times with DI water. After washing with ethanol, they were then dried in an oven at 60 °C overnight [72]. These dried AgO nanoparticles were characterized by a field-emission scanning electron microscope (FESEM) attached with an energy-dispersive X-ray analysis (EDAX) setup to determine their morphological and composition properties. A Hitachi Ultrahigh Resolution SEM (S-4800) attached with an EDAX module was used for morphological and compositional characterization of silver oxide nanoparticles. For SEM characterization, synthesized particles were spread onto adhesive conductive carbon tapes. The platinum metal was then used to coat these particles.

SEM images of hydrothermally synthesized silver oxide nanoparticles are presented in Figure 5, showing that these AgO particles were spherical in shape with different sizes due to the highly agglomerated nano-crystalline grains of AgO. The surface of these agglomerated particles clearly showed nanocrystalline grains, confirming the nano-nature of the synthesized AgO. The compositions of these synthesized AgO particles are presented in Figure 6, showing the EDX mapping (Figure 6a) and EDX spectrum (Figure 6b) of the product. EDX mapping displayed a uniform distribution for both Ag and O elements. The composition levels are shown in Figure 6b. The results confirmed a stoichiometric formation of AgO nanoparticles through hydrothermal synthesis.
Figure 5. Field-emission scanning electron microscope (FESEM) image of synthesized AgO nanoparticles.

Figure 6. (a) Energy-dispersive X-ray (EDX) mapping and (b) spectrum of AgO nanoparticles.

3.1.2. Plant Materials and Surface Decontamination

Rhizomes of *K. parviflora* harvested from field-grown plants were cleaned under running tap water, planted in plastic trays containing a mixture of autoclaved perlite/peat moss (1:1, v/v), and kept in a growth room in darkness at 24 ± 1 °C. After 3 weeks, rhizome developing buds were isolated, soaked in detergent solution (0.01%, v/v) for 5 min, and then thoroughly washed under tap water for 45 min. These rhizome buds were sterilized in sodium hypochlorite (2.5% v/v) for 12 min and rinsed several times with sterilized distilled water. These rhizome buds were again immersed in 0–200 mg L^{-1} AgO NPs suspension for 30 min, 60 min, or 90 min and rinsed 6 times with sterilized distilled water. These buds were excised from sterilized rhizomes and placed on MS nutrient medium containing 2.0 μM Thidiazuron...
Plants 2021, 10, 698

(TDZ), 3% sucrose, and 0.8% plant agar (pH 5.6–5.8). Cultures were kept at 24 ± 1 °C for 21 days with a 16h/8h light/dark photoperiod (40–45 µmol m⁻² s⁻¹) provided by cool white fluorescent tubes. Experiments were conducted with as a completely randomized design (CRD). In each treatment, 20 rhizome buds were used with 3 replications. All experiments were performed twice. The decontamination rate was recorded at 7 days after incubation, and the survival rate of explants was determined at 3 weeks after incubation.

3.1.3. Shoot Multiplication

Buds were excised from 100 mg L⁻¹ Ag₂O NPs treated K. parviflora rhizomes cultured on MS nutrient medium containing 0–12 µM TDZ, 6-furfuryladenine (6-KN), or 6-BA and 8 µM 6-BA. The basal medium was then combined with 2 µM, 4 µM, and 6 µM 6-KN; 0.5 µM, 1 µM, and 2 µM TDZ; or 0.5 µM TDZ and 1 µM, 2 µM, or 4 µM Naphthalene-1-acetic acid (NAA) to induce multiple shoots. These cultures were kept for 8 weeks at 24 ± 1 °C with a 16 h/8 h light/dark photoperiod (40–45 µmol m⁻² s⁻¹) provided by cool white fluorescent tubes. Experiments were conducted as a CRD (20 rhizome buds were used in each treatment, 3 replications). All experiments were performed twice. Regeneration rate, number of shoots per rhizome bud, and number of roots per induced shoot were assessed after 8 weeks of incubation.

3.1.4. Rooting and Acclimatization

Four-week-old in vitro-induced shoots (≥2–3 cm in height) were obtained from shoot clusters and cultivated on nutrient medium MS containing 0–12 µM Indole-3-butyric acid (IBA) to induce the growth of shoots and roots. After 6 weeks, the number of roots, lengths of roots, and lengths of shoots were recorded. Plantlets were removed from the medium MS containing 2 µM IBA, cleaned with tap water, and transplanted into plastic trays containing sterile soilless substrates composed of 40% peat moss, 30% perlite, and 30% vermiculite based on volume. They were kept in a growth room at 24 ± 1 °C with a 16 h/8 h light/dark photoperiod (90 µmol m⁻² s⁻¹) and irrigated at 3-day intervals. The survival of plants was recorded after 5 weeks. Experiments were conducted as a CRD (50 shoots or plantlets for each treatment with three replications). All experiments were performed twice.

3.2. Phytochemical Analysis

3.2.1. Extract Preparation

Leaves of K. parviflora were collected from in vitro cultured plantlets (56 days old) and greenhouse-grown plants (90 days old), minced, stored at −70 °C for 12 h, and lyophilized. Dried leaf powder samples of K. parviflora (50 mg) were extracted with 80% methanol using an Ultraturrax at 6000 g for 30 min. After filtration, extracts were dried using a rotary vacuum evaporator and kept at 4 °C until further investigation.

3.2.2. Estimation of Total Phenolics Content (TPC) and Flavonoid Content (TFC)

TPC of K. parviflora leaf extract was determined using the Folin–Ciocalteu assay described by Slinkard and Singleton [73] and calculated as mg of gallic acid equivalent (GAE). TFC of K. parviflora leaf extract was determined using the aluminum chloride (AlCl₃) technique according to Zengin et al. [74] and expressed as mg of rutin equivalent (RE).

3.2.3. Chemical Characterization

Chromatographic separation was accomplished with a Dionex Ultimate 3000RS UH-PLC instrument equipped with a Thermo Accucore C18 (100 mm × 2.1 mm i. d., 2.6 µm) analytical column for separation of compounds. Water (A) and methanol (B) containing 0.1% formic acid were employed as mobile phases. The total run time was 70 min. The elution profile and exact analytical conditions have been published [75]. Electrospray ionization (ESI) was performed in both negative and positive ion modes to obtain more data. Mass spectra were recorded between m/z 100 and 1500 atomic mass units using a Q-Exactive (Thermo Fisher Scientific, Waltham, MA, USA) Orbitrap mass spectrometer.
Chemical constituents were identified by comparison with authentic standards and their MS/MS spectra as well as fragmentation patterns. Peaks and spectra were processed using the TraceFinder software and tentatively identified by comparing their (Rt) retention time and mass spectrum based on reported data and library search.

3.3. Biological Activities of K. parviflora Leaf Extracts

3.3.1. Antioxidant Assay

Several antioxidant assays, such as 2,2-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid) (ABTS), 2,2-diphenyl-1-picrylhydrazyl (DPPH), cupric reducing antioxidant capacity (CUPRAC), ferric reducing antioxidant power (FRAP), metal chelating ability (MCA), and phosphomolybdenum (PBD), were carried out to determine the antioxidant potential of K. parviflora leaf extract using published methods [76]. Assays were performed in triplicate.

3.3.2. Enzyme Inhibition Assay

Acetylcholinesterase (AChE), amylase, and butyrylcholinesterase (BChE), and tyrosinase inhibitory activities of K. parviflora leaf extract were conducted in triplicates according to procedures described by Uysal et al. [76].

3.4. Statistical Analysis

Data were analyzed using SAS version 9.4 (SAS Institute, NC, USA). Significant difference among means was determined by analysis of variance and Duncan’s multiple range test (DMRT).

4. Conclusions

Treatment of K. parviflora rhizome buds with AgO NPs solution resulted in excellent surface sterilization. Among all cytokinins (6-BA, 6-KN, TDZ) and their concentrations (1–12 µM) evaluated, 8 µM of 6-BA yielded the best explant response. The supplementation of 6-KN (2 µM and 4 µM) or TDZ (0.5–2 µM) to medium MS containing 8 µM 6-BA enhanced the explant response and the number of shoots. The addition of IBA to rooting medium MS improved rooting quality. Higher levels of phenolics and flavonoids were found in leaves from the greenhouse and in vitro cultures, respectively. Leaf extracts exhibited free radical scavenging and enzyme inhibitory activities in in vitro assays. Phytochemical and biological activities of K. parviflora leaf extracts are reported in this study for the first time. Further studies are needed to quantify individual flavonoids in leaf tissues. The micropropagation protocol optimized in the current study can be used for mass-clonal propagation of K. parviflora.

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