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Optimizing Ethanol Concentrations to Enhance Growth and Bioactive Compound Production in *Agastache rugosa* Cultivated in a Plant Factory

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ABSTRACT: *Agastache rugosa*, a medicinal plant known for its bioactive compounds, has gained attention for its pharmacological and commercial potential. This study aimed to optimize ethanol concentration to enhance growth and bioactive compound production in *A. rugosa* cultivated in a controlled plant factory system. Ethanol treatments at 40 and 80 mM significantly promoted both vegetative and reproductive growth. Plants treated with these concentrations exhibited higher net photosynthetic rates (A) and intercellular CO_2 concentration (C_i) compared to the untreated control, whereas stomatal conductance (g_s) and transpiration rate (E) remained unaffected. Chlorophyll and carotenoid concentrations, and SPAD values, significantly increased with ethanol treatment. Total flavonoid and total phenolic contents as well as 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical-scavenging activities were significantly higher in plants treated with ethanol than in the untreated control. Ethanol treatments led to a significant enhancement in the activities of antioxidant enzymes, including superoxide dismutase, peroxidase, and catalase. Furthermore, ethanol treatment elevated rosmarinic acid concentrations in roots and tilianin and acacetin levels in flowers. Collectively, ethanol at 40 and 80 mM effectively enhanced growth, photosynthesis, antioxidant defense, and bioactive compound production in *A. rugosa* cultivated in a plant factory. These findings provide valuable insights for improving cultivation of medicinal plants with high pharmaceutical and nutraceutical value.

KEYWORDS: *Agastache rugosa*; ethanol; plant factory; antioxidant; photosynthetic pigments; bioactive compounds

1 Introduction

The Lamiaceae family, commonly known as the mint family, comprises over 7000 species distributed across tropical, subtropical, and temperate regions worldwide [1]. *Agastache rugosa*, a member of this family, has highly been valued for its essential oil, which has diverse applications in culinary arts, pharmaceuticals, medicine, and cosmetics [2]. This medicinal herb is renowned for its antioxidant [3], antimicrobial [4], analgesic [5], anticancer [6], cardiovascular [7], anti-inflammatory properties [8], as well as its potential to enhance the human immune system response [9]. Plant polyphenols, including hydroxybenzoic acids and simple phenols, contribute to its bioactivity [10]. The primary bioactive compounds in *A. rugosa* are acacetin,

tilianin, and rosmarinic acid (RA) [11]. Acacetin, a flavone, has been used to treat cancers, metabolic disorders, inflammation, and infections [12]. Tilianin, a potent flavonoid glycoside, exhibits anti-inflammatory, antidiabetic, neuroprotective, antihypertensive, cardioprotective, antioxidant, anti-atherogenic, and antidepressant effects [13]. RA, an ester of 3,4-dihydroxyphenyllactic acid and caffeic acid, possesses numerous pharmacological applications, including anti-apoptotic, anti-inflammatory, antioxidant, and antitumor properties [14]. With growing concerns about the nutritional value and bioactive quality of food sources, along with environmental and economic considerations for sustainable crop management, there is an increasing need for profitable, sustainable, and ecologically sound strategies to enhance the quality of *A. rugosa*. Improving growth and bioactive compound production has long been a key objective in medicinal plant research [15]. Biotechnological approaches such as growth regulators, elicitors, and stress inducers have been used to achieve these goals [16,17].

Ethanol has been investigated for its potential to influence plant growth and bioactive compound production [18]. It has shown to act as an elicitor in plants, inducing stress responses that enhance bioactive compound synthesis [19]. Ethanol application has been shown to increase biomass and secondary metabolites levels in several herb species [18]. In oilseed rape seedlings, ethanol at low concentrations $(0.0125–0.05 \text{ mL L}^{-1})$ stimulated root tip growth and enhanced biomass and NPK accumulation, with 0.05 mL L^{-1} being the most effective. However, a higher concentration (0.25 mL L^{-1}) significantly inhibited the growth [20]. Similarly, ethanol at low concentrations increased shoot dry weights in sweet wormwood and sweet basil [18], whereas higher ethanol concentrations reduced leaf area and shoot fresh weight (FW) in both Korean mint and sweet basil compared to untreated control. Total phenolic contents and 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity increased with higher ethanol concentrations in these herbs, but total flavonoid contents showed no clear trend [18].

However, the effects of ethanol on *A. rugosa* remain largely unexplored. Given its medicinal importance, understanding how ethanol influences its growth and bioactive compound production could provide valuable insights for optimizing cultivation practices and enhancing phytochemical yields. This study aims to examine the effects of ethanol spray concentration on the growth, antioxidant enzyme activities, and bioactive compound contents of *A. rugosa*. By analyzing plant morphology, biomass, antioxidant enzyme activities, and secondary metabolite levels under different ethanol concentrations, this research seeks to elucidate the potential benefits and mechanisms underlying ethanol-induced changes. The findings could contribute to the development of improved cultivation techniques for *A. rugosa*, supporting its use in both traditional and modern medicinal applications.

2 Materials and Methods

2.1 Seedling Growing Conditions

A. rugosa seeds (Danong Seed Co., Ltd., Seoul, Republic of Korea) were germinated in trays in a room at $21.2 \pm 4°C$ and $75.5 \pm 9\%$ RH. LED lighting provided a photosynthetic photon flux density (PPFD) of $190 \pm 14 \text{ μmol m}^2 \text{ s}^{-1}$ under a 16-h light/8-h dark cycle. Two weeks after sowing, seedlings were watered with Hoagland solution (pH 6.3, EC 1.15 dS m^{-1}).

2.2 Treatments

Four weeks after sowing, seedlings were moved to a deep flow technique system in a plant factory with controlled conditions: $22.5 \pm 3°C$, $67.5 \pm 12\%$ RH, and a PPFD of $223 \pm 9 \text{ μmol m}^2 \text{ s}^{-1}$ under a 16-h light/8-h dark cycle. Plants were grown for 39 days in Hoagland solution (pH 6.53, EC 2.03 dS m^{-1}). Ethanol (10, 20, 40, 80 mM) was sprayed three times weekly from 7 days after transplanting (DAT), while controls received

distilled water. Samples were collected at 39 DAT for analysis. The experiment was replicated twice at different times under identical conditions and locations.

2.3 Measurement of Plant Growth Parameters

Plant growth parameters included number of flower branches, number of leaves, leaf length, width, and area, stem and root lengths, and flower, leaf, stem, shoot, and root FW. Dry weights (DW) of all plant organs were measured after oven-drying at 70°C for 7 days. Flower DW, leaf area (LA), and leaf DW ratio were calculated relative to total DW, while specific LA was determined by LA/leaf DW.

2.4 Determination of Photosynthetic Characteristics

A portable LI-6400 system (Model LI-6400; Li-Cor Co., Inc., Lincoln, NE, USA) was used to measure net photosynthetic rate (A), intercellular CO_2 concentration (C_i), stomatal conductance (g_s), and transpiration rate (E). Leaf chamber conditions were set at 25°C, 60% RH, 1000 $\mu mol\ m^{-2}\ s^{-1}$ PPFD, 400 $\mu mol\ mol^{-1}$ CO_2, and 500 $cm^3\ s^{-1}$ airflow. SPAD values were measured using a Minolta 502 chlorophyll meter (Model 502; Minolta Camera Co., Ltd., Osaka, Japan).

2.5 Determination of Photosynthetic Pigments and 2,2-Diphenyl-1-Picrylhydrazyl (DPPH) Radical Scavenging Activity

Twenty mg of dried powder was extracted in 2 mL of 90% methanol, centrifuged, and the supernatant analyzed for chlorophyll (Chl) a, Chl b, carotenoids, and DPPH activity using an Epoch spectrophotometer at specified wavelengths, following Lam et al. [16,17].

2.6 Determination of Total Flavonoids and Total Phenolics

Total flavonoid and phenolic concentrations were measured colorimetrically as described by Lam et al. [16].

2.7 Antioxidant Enzyme Assays

Enzyme extracts were prepared using an updated protocol based on the nitro blue tetrazolium (NBT) method described by Lam et al. [16]. The SOD (EC 1.15.1.1) assay was prepared with EDTA, NBT, methionine, riboflavin, PBS, and 20 μL of sample extract. The reaction was exposed to 200 $\mu mol\ m^{-2}\ s^{-1}$ LED light for 8 min, and absorbance was recorded at 560 nm. The POD (EC 1.11.1.7) assay consisted of guaiacol, PBS, H_2O_2, and 20 μL of sample extract, with activity assessed based on tetraguaiacol formation at 560 nm. The CAT (EC 1.11.1.6) assay included 3.4 μL of 3% H_2O_2, 193.6 μL PBS, and 3 μL of sample extract, with absorbance measured at 240 nm after 4–5 min.

2.8 Determination of RA, Tilianin, Acacetin, and Acacetin Glycosides

RA, tilianin, acacetin, and acacetin glycosides [acacetin 1, 7-O-(2″-O-acetyl) β-D-glucopyranoside; acacetin 2, 7-O-(6″-O-malonyl) β-D-glucopyranoside; and acacetin 3, 7-O-(2″-O-acetyl-6″-O-malonyl) β-D-glucopyranoside] were analyzed by HPLC following the methods described by Lam et al. [16]. Briefly, 200 mg dried powder was extracted in methanol, sonicated, centrifuged, and filtered. A 10 μL sample was injected into a C18 column (250 × 4.6 mm, 5 μm) at 30°C, using a gradient of formic acid in water (A) and acetonitrile (B) at 0.8 mL/min: 20% B (0–5 min), 20%–50% B (5–10 min), 50% B (10–20 min), 50%–100% B (20–22 min).

2.9 Statistical Analysis

The experiment used a completely randomized design with two independent temporal replicates at the same location. Growth parameters and SPAD values were measured on five plants per replicate, while other measurements were taken from three plants per replicate. Statistical analysis was performed using SPSS 20.0, with one-way ANOVA and Tukey's HSD test ($p < 0.05$) to assess differences among treatments.

3 Results

3.1 Growth Parameters

The growth of *A. rugosa* plants significantly increased with ethanol treatment, particularly at 40 and 80 mM (Fig. 1). Leaf length and width were significantly higher in plants treated with ethanol at 20 and 40 mM and at 20, 40, and 80 mM, respectively, than in the untreated control (Fig. 2A,B). Both the numbers of flower branches and leaves were significantly higher in plants treated with ethanol at 80 mM than in the untreated control (Fig. 2C,D), although the number of flower branches were also higher at 10 or 40 mM (Fig. 2C). Stem length and root DW were highest in plants treated with ethanol at 40 mM (Figs. 2F and 3G), whereas stem FW and DW, as well as root length and FW were highest at 80 mM (Figs. 2I and 3B,C,F). Shoot FW was significantly higher in plants treated with ethanol at 40 and 80 mM than in the untreated control (Fig. 3A). Leaf, shoot, and whole plant DW were also significantly higher in plants treated with ethanol at 20, 40, and 80 mM than in the untreated control (Figs. 3E,H and 4A). However, specific leaf area decreased with ethanol treatment at 10 or 20 mM (Fig. 3I). Leaf and flower weight ratios were highest in plants treated with ethanol at 20 mM (Fig. 4C,D). However, leaf area, flower and leaf FW, flower DW, and leaf area ratio were not significantly affected by ethanol treatment (Figs. 2E,H,G, 3D and 4B).
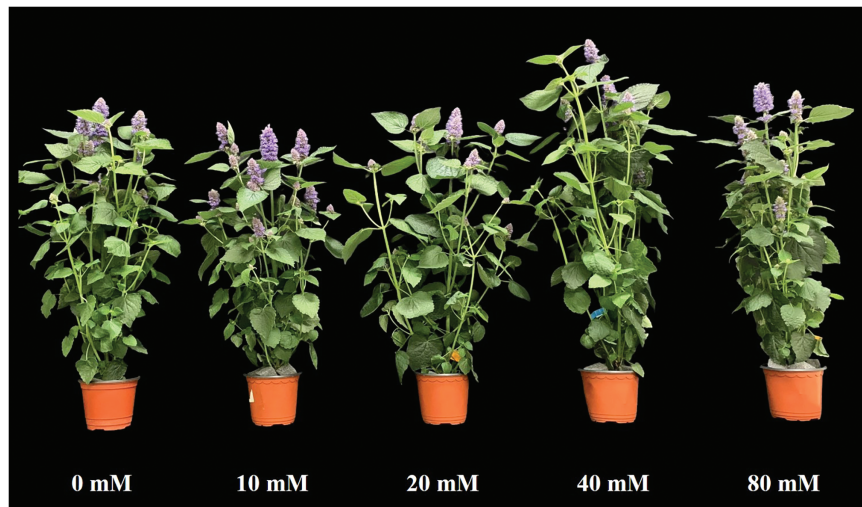

Figure 1: Representative images of *A. rugosa* grown in a plant factory for 39 days, sprayed three times weekly with ethanol concentrations (0, 10, 20, 40, and 80 mM)
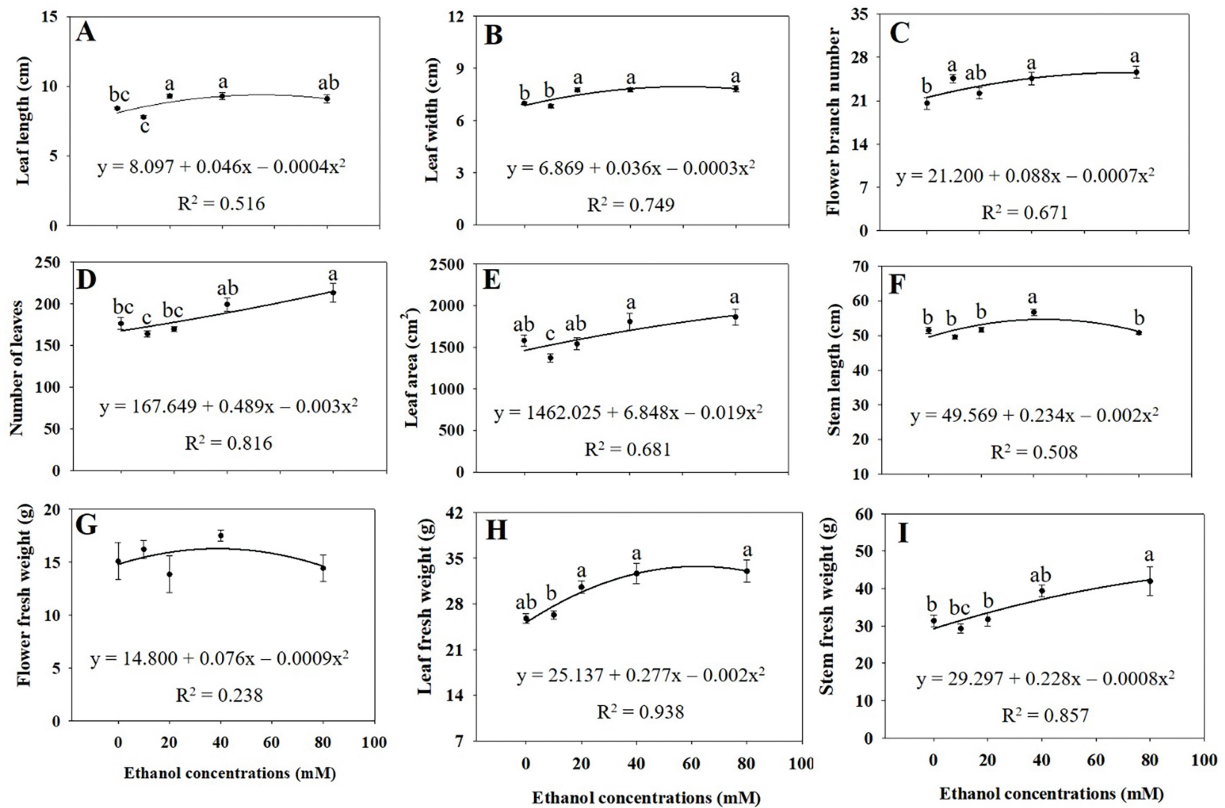
Figure 2: Leaf length (**A**), leaf width (**B**), number of flower branches (**C**), number of leaves (**D**), leaf area (**E**), stem length (**F**), and fresh weights of flower (**G**), leaf (**H**), and stem (**I**) of *A. rugosa*, sprayed with ethanol concentrations (0, 10, 20, 40, and 80 mM) three times a week. Vertical bars indicate standard errors from five samples. Means with the same letter are not significantly different ($p < 0.05$, Tukey's HSD test)
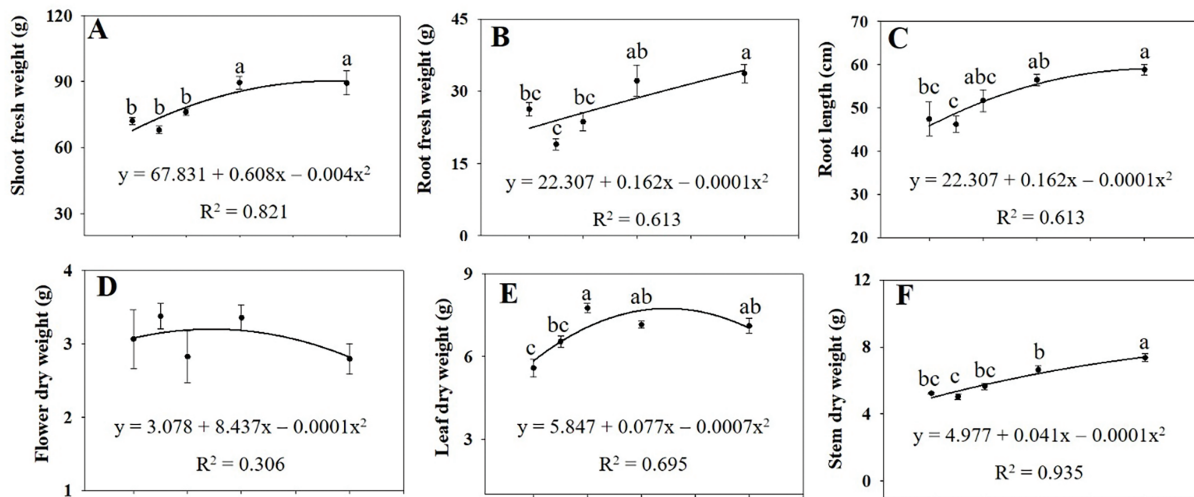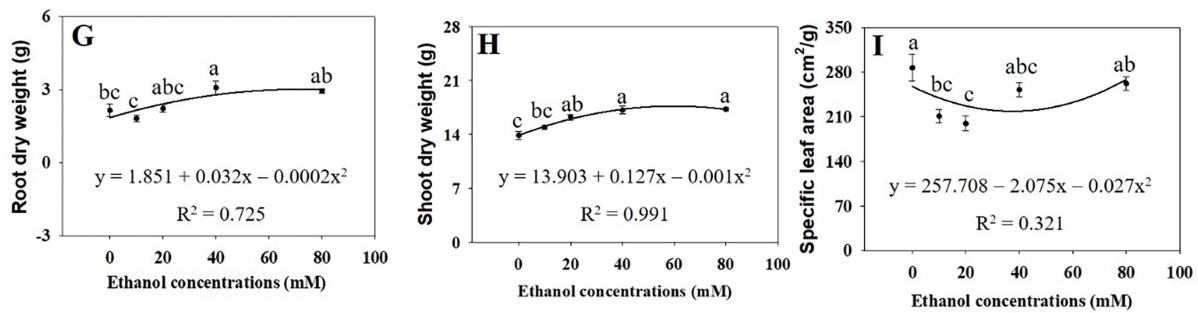

Figure 3: (Continued)

Figure 3: Shoot fresh weight (**A**), root fresh weight (**B**), root length (**C**), dry weights of flower (**D**), leaf (**E**), stem (**F**), root (**G**), and shoot (**H**), and specific leaf area (**I**) of *A. rugosa*, sprayed with ethanol (0, 10, 20, 40, 80 mM) three times weekly. Vertical bars indicate standard errors from five samples. Means with the same letter are not significantly different ($p < 0.05$, Tukey's HSD test)
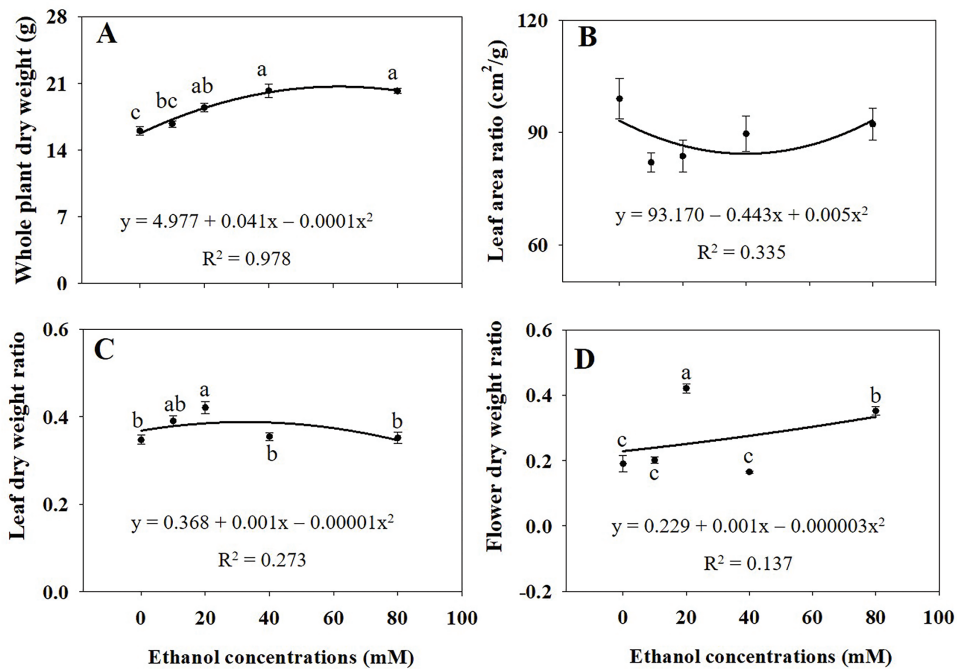

Figure 4: Whole plant dry weight (**A**), leaf area ratio (**B**), leaf dry weight ratio (**C**), and flower dry weight ratio (**D**) of *A. rugosa*, sprayed with ethanol (0, 10, 20, 40, 80 mM) three times weekly. Vertical bars indicate standard errors from five replicates. Means with the same letter are not significantly different ($p < 0.05$, Tukey's HSD test)

3.2 Photosynthetic Characteristics and Pigments

Net photosynthetic rate (A) was significantly higher in plants treated with ethanol at 40 and 80 mM than in the untreated control (Fig. 5A). Intercellular CO_2 concentration (C_i) was highest in plants treated with ethanol at 40 mM (Fig. 5C). However, stomatal conductance (g_s) and transpiration rate (E) were not significantly affected by ethanol treatment (Fig. 5B,D).

Chl a, Chl b, and total Chl concentrations significantly increased with ethanol treatment, except for Chl b concentration at 40 mM (Fig. 6A,B,D). The Chl a/b ratio tended to decrease with increasing ethanol concentration (Fig. 6C). Total carotenoid concentrations were highest in plants treated with ethanol at

40 mM (Fig. 6E). Additionally, SPAD values significantly increased with ethanol treatment at 40 and 80 mM (Fig. 6F).
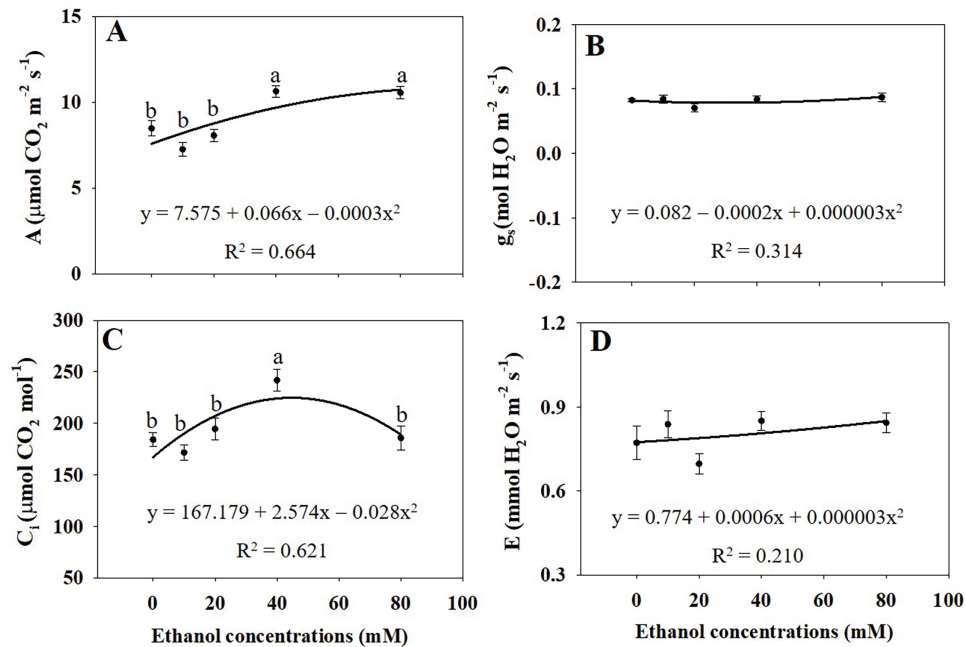

Figure 5: Net photosynthetic rate (**A**), stomatal conductance (**B**), intercellular CO_2 concentration (**C**), and transpiration rate (**D**) of *A. rugosa*, sprayed with ethanol (0, 10, 20, 40, 80 mM) three times weekly. Vertical bars indicate standard errors from three replicates. Means with the same letter are not significantly different ($p < 0.05$, Tukey's HSD test)
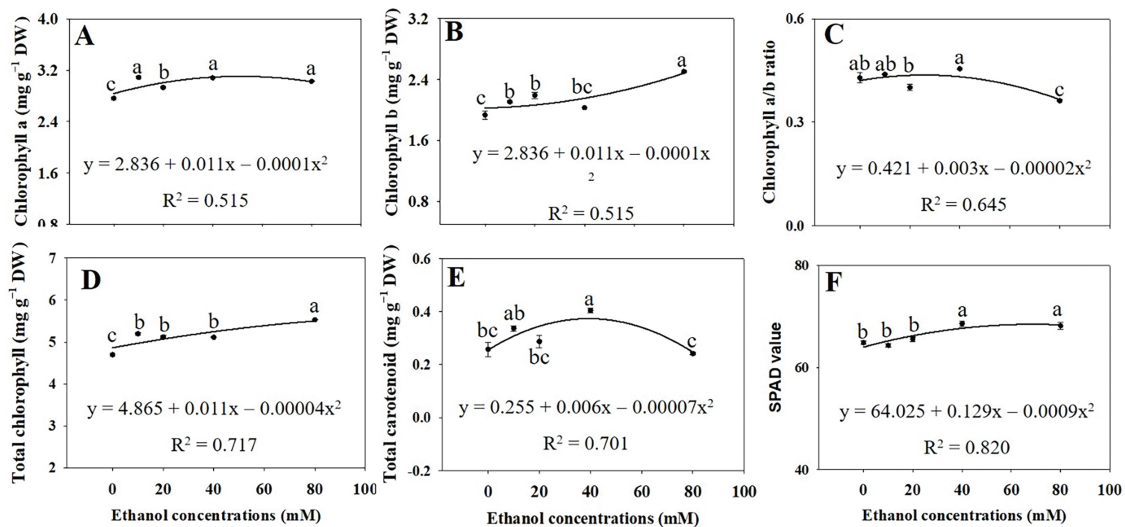

Figure 6: Chlorophyll a (**A**), chlorophyll b (**B**), chlorophyll a/b ratio (**C**), total chlorophyll (**D**), carotenoids (**E**), and SPAD (**F**) in *A. rugosa*, sprayed with ethanol (0, 10, 20, 40, and 80 mM) three times weekly. Bars show standard errors ($n = 5$). Means with the same letter are not significantly different ($p < 0.05$, Tukey's HSD test)

3.3 Antioxidant Capacity and Total Flavonoid and Total Phenolic Concentrations

Total flavonoid concentrations significantly increased by 17.2% and 14.2% with ethanol treatment at 40 and 80 mM, respectively, compared to the control (Fig. 7B). Total phenolic concentrations increased by 15.6%, 18.2%, and 21.1% at 20, 40, and 80 mM, respectively (Fig. 7C). Additionally, DPPH radical-scavenging activity increased by 55.3% at 80 mM (Fig. 7A).
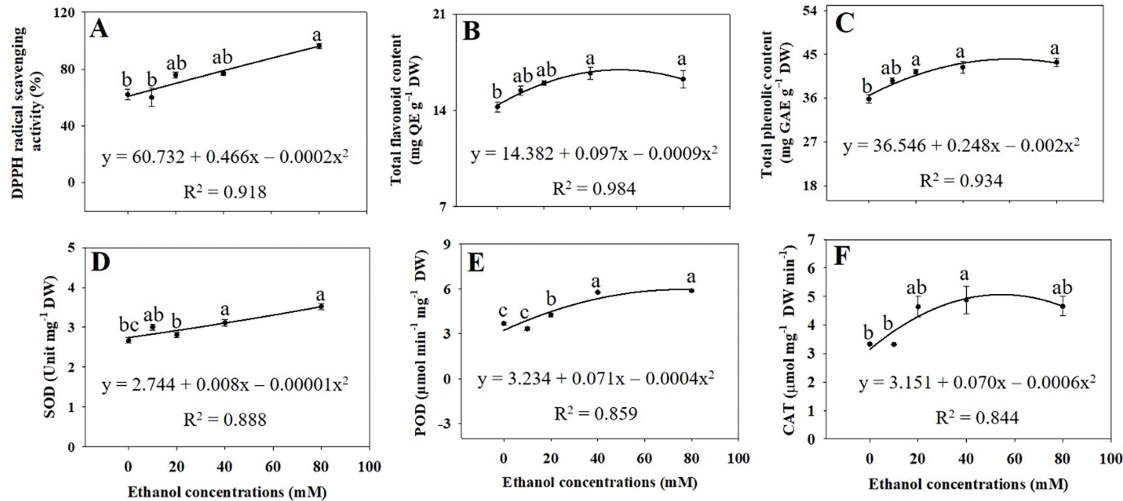

Figure 7: DPPH radical scavenging (**A**), total flavonoids (**B**), total phenolics (**C**), SOD (**D**), POD (**E**), and CAT (**F**) activity in *A. rugosa*, sprayed with ethanol (0, 10, 20, 40, and 80 mM) three times weekly. Bars show standard errors ($n = 3$). Means with the same letter are not significantly different ($p < 0.05$, Tukey's HSD test)

SOD activity increased by 16.5% and 31.8% with the ethanol treatment at 40 and 80 mM, respectively, compared to the control (Fig. 7D). POD activity increased by 1.15-, 1.56-, and 3.26-fold with ethanol treatment at 20, 40, and 80 mM, respectively (Fig. 7E). CAT activity was highest at 40 mM, showing a 1.46-fold increase compared to the control (Fig. 7F).

3.4 RA, Tilianin, and Acacetin Concentrations and Contents

RA, tilianin, and acacetin concentrations varied across *A. rugosa* organs, with roots having the highest RA and flowers the highest tilianin and acacetin. Ethanol treatment at 80 mM maximized RA in roots and tilianin and acacetin in flowers (Fig. 8A–C). In contrast, RA content in leaves and tilianin contents in flowers were highest with ethanol treatment at 20 and 40 mM, respectively (Fig. 8D,E). With ethanol treatments, acacetin content decreased in flowers but increased in stems (Fig. 8F).

Whole plant RA concentrations significantly increased with ethanol treatments at all concentrations examined (Fig. 9A). Whole plant tilianin and acacetin concentrations were highest at 80 mM (Fig. 9B,C). Additionally, whole plant RA contents significantly increased with ethanol treatments at all concentrations examined (Fig. 9D). In contrast, the increases in both whole plant tilianin and acacetin contents were significant only at 40 and 80 mM (Fig. 9E,F).
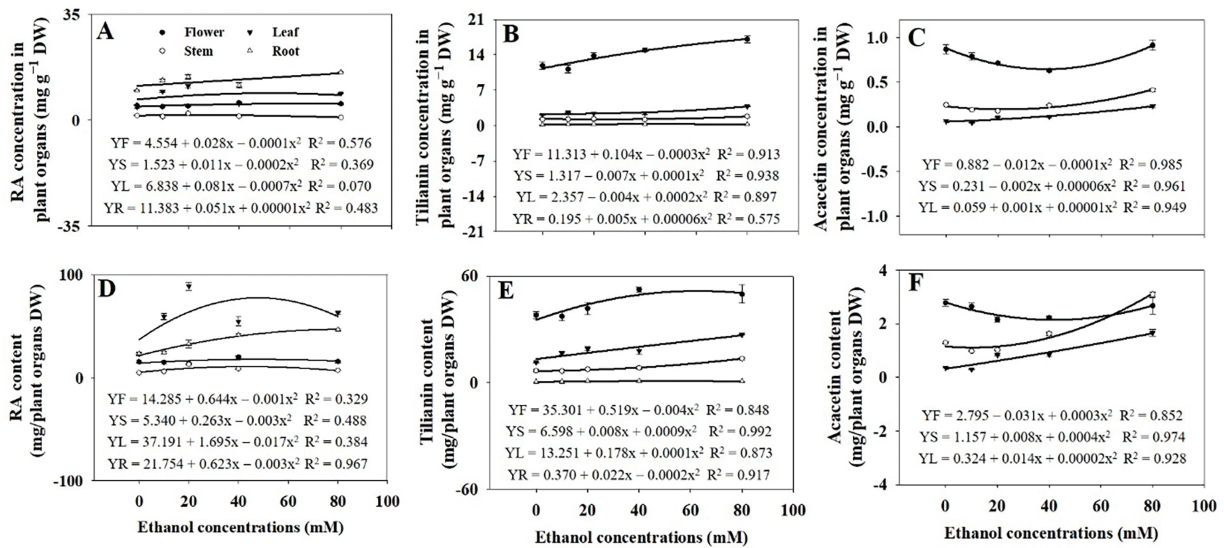
Figure 8: Rosmarinic acid (RA) (**A,D**), tilianin (**B,E**), and acacetin (**C,F**) concentrations and contents in *A. rugosa* organs (F: flower, S: stem, L: leaf, R: root), sprayed with ethanol (0, 10, 20, 40, and 80 mM) three times weekly. Bars show standard errors ($n = 3$). Means with the same letter are not significantly different ($p < 0.05$, Tukey's HSD test)

Figure 9: RA (**A,D**), tilianin (**B,E**), and acacetin (**C,F**) concentrations and contents in whole *A. rugosa* plants, sprayed with ethanol (0, 10, 20, 40, and 80 mM) three times weekly. Bars show standard errors ($n = 3$). Means with the same letter are not significantly different ($p < 0.05$, Tukey's HSD test)
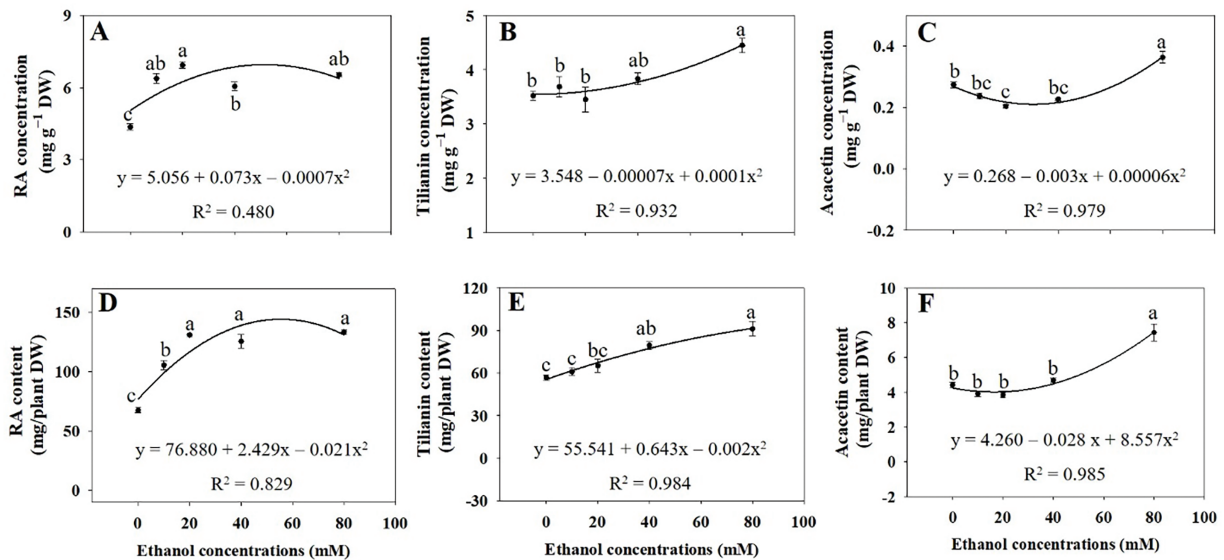
3.5 Acacetin Glycoside Concentrations and Contents

Flowers exhibited the highest concentrations of the three acacetin glycosides (acacetin 1, 2, and 3) compared to other organs of *A. rugosa* (Fig. 10). The concentrations and contents of the three acacetin glycosides increased with ethanol treatments. Acacetin 1 concentration and content increased by 20.3% and 54.6%, respectively, with ethanol treatment at 40 mM, compared to the respective controls (Fig. 11A,D).

Acacetin 2 concentrations were not significant affected by the ethanol treatment (Fig. 11B), but its contents increased by 35.5% and 36.7% with ethanol treatment at 40 and 80 mM, respectively, compared to the control (Fig. 11E). Both acacetin 3 concentration and content were significantly higher in plants treated with ethanol at 40 and 80 mM than in the untreated control (Fig. 11C,F).
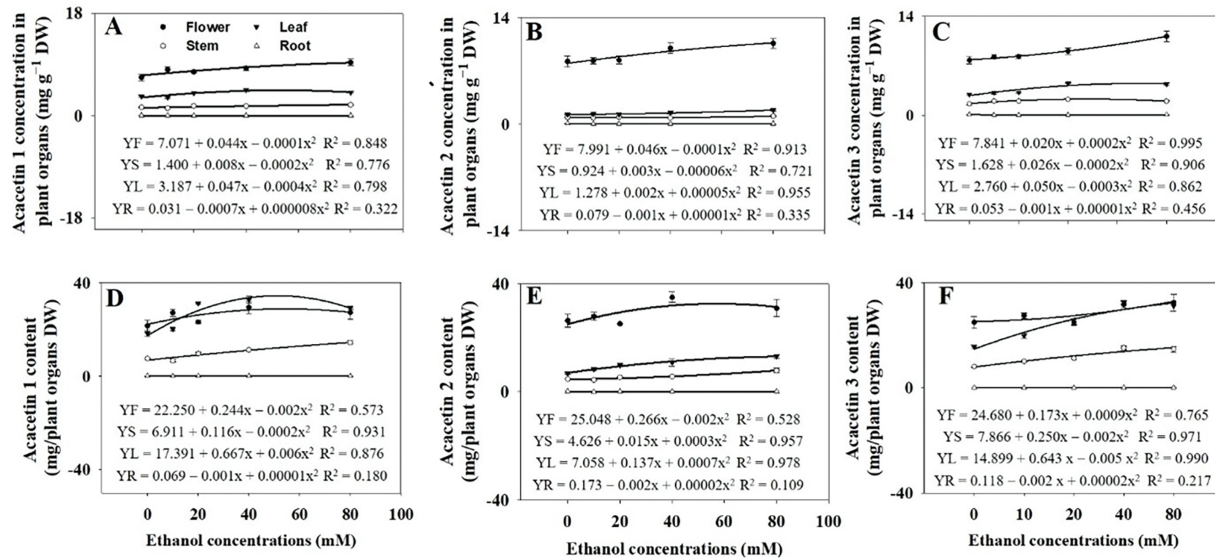

Figure 10: Acacetin 1 (**A,D**), acacetin 2 (**B,E**), and acacetin 3 (**C,F**) concentrations and contents in *A. rugosa* organs (F: flower, S: stem, L: leaf, R: root), sprayed with ethanol (0, 10, 20, 40, and 80 mM) three times weekly. Bars show standard errors ($n = 3$). Means with the same letter are not significantly different ($p < 0.05$, Tukey's HSD test)

Figure 11: Acacetin 1 (**A,D**), acacetin 2 (**B,E**), and acacetin 3 (**C,F**) concentrations and contents in whole *A. rugosa* plants, sprayed with ethanol (0–80 mM) three times weekly. Bars show standard errors ($n = 3$). Means with the same letter are not significantly different ($p < 0.05$, Tukey's HSD test)
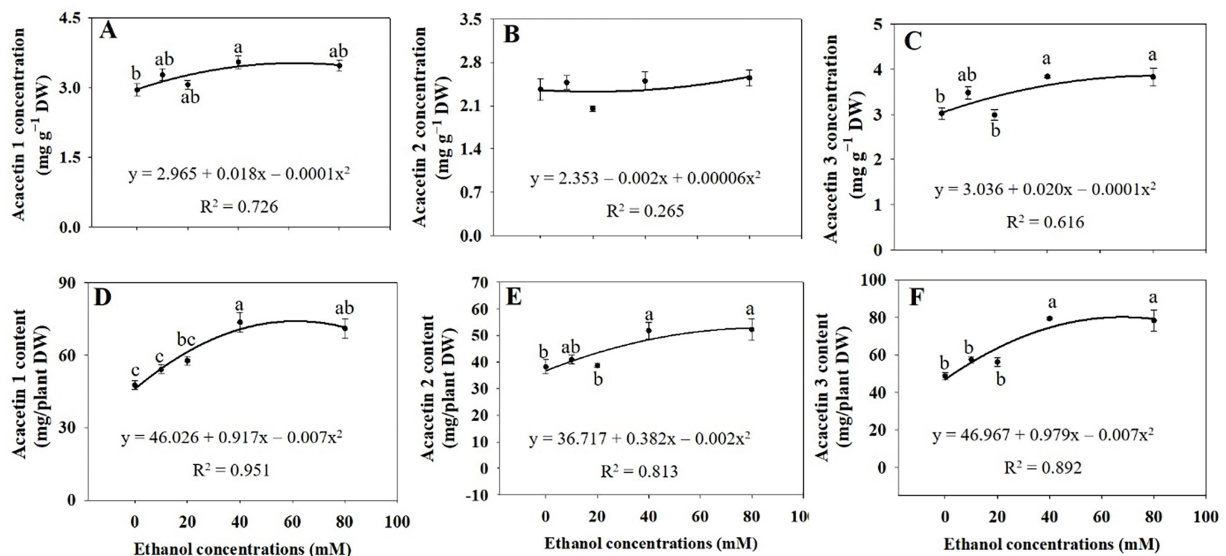
4 Discussion

4.1 Growth Parameters

Ethanol treatments significantly enhanced the growth of *A. rugosa*. Particularly in terms of leaf length and width (Fig. 2A,B), indicating that ethanol promoted leaf expansion. Additionally, both the number of leaves and flower branches increased with ethanol treatment (Figs. 2C,D), suggesting that ethanol plays a dural role in promoting both vegetative and reproductive growth. This effect may be attributed to ethanol's influence on hormonal balances or stress responses which promote branching and leaf production under certain conditions [20,21]. Both stem and root growth also increased with ethanol treatment (Figs. 2F,I and 3B,C,F,G), suggesting that ethanol enhances nutrient and water uptake, possibly by promoting below-ground growth, which in turn supports greater above-ground growth [20]. The highest stem length and root DW at 40 mM, coupled with the maximum stem and root FW, root length, and stem DW at 80 mM, further illustrate ethanol's role in promoting both above-ground and below-ground growth. Root architectural traits, which contribute to plant productivity, are strongly influenced by growth conditions [22].

However, leaf area, flower and leaf FW, flower DW, and leaf area ratio were not significantly affected by ethanol treatment (Figs. 2E,H,G, 3D and 4B). These results indicated that the effects of ethanol treatment on growth parameters were not uniform, but rather concentration-dependent and selective. Leaf, shoot, and whole plant DW were significantly higher in plants treated with ethanol at 20, 40, and 80 mM than in the untreated control (Figs. 3E,H and 4A), whereas shoot FW was higher at 40 and 80 mM (Fig. 3A). Specific LA decreased with ethanol treatment at 10 and 20 mM (Fig. 3I), suggesting thicker, possibly more stress-resistant leaves. The leaf and flower weight ratios, which indicate the balance between vegetative and reproductive growth, were highest in plants treated with the ethanol treatment at 20 mM (Fig. 4C,D). Similarly to our study, leaf and stem growth in tomato were significantly higher when treated with 15% or 20% alcohol (methanol or ethanol), compared to the untreated control [23]. Both foliar and soil application of ethanol also increased fruit weight and yield in strawberry [24]. Ethanol treatment has also been reported to enhance the growth of soybean plants, including shoot height, root length, root DW, shoot DW, total LA per trifoliate, and leaf succulence [25]. These findings demonstrate that exogenous ethanol can promote plant growth.

4.2 Photosynthetic Characteristics and Pigments

Ethanol, particularly at 40 and 80 mM, significantly enhanced photosynthetic activity and pigment content. This suggests a mild stress response that optimizes photosynthesis, improving energy use and efficiency. The net photosynthetic rate (A) showed a tendency to increase as ethanol concentrations rose, with a significant enhancement observed at 40 and 80 mM ethanol compared to the controls (Fig. 5A). Interestingly, despite this increase in photosynthesis, there were no significant differences in stomatal conductance (g_s) or transpiration rate (E) between ethanol-treated plants and the control group (Fig. 5B,D). Because the photosynthetic rate was determined by stomatal or no stomatal limitations [26]. The intercellular CO_2 concentration (Ci) reached its maximum under the 40 mM ethanol treatment (Fig. 5C). The increased C_i at 40 mM indicates enhanced CO_2 diffusion, contributing to a higher net photosynthetic rate, possibly due to a hormesis effect. Since g_s and E remained unchanged, the increase in Ci may reflect greater CO_2 assimilation efficiency due to improved internal CO_2 fixation capacity or adjustments in photosynthetic enzymes. Typically, an increase in net photosynthesis is accompanied by higher g_s, facilitating CO_2 uptake. However, the stable g_s observed here implies that ethanol might be improving photosynthesis through non-stomatal mechanisms. The increased Ci at 40 mM suggests enhanced internal CO_2 diffusion and fixation efficiency, likely due to improved enzymatic activity or structural adjustments in the photosynthetic apparatus.

Chl a, Chl b, and total Chl concentrations significantly increased with ethanol treatment (Fig. 6A,B,D), whereas Chl a/b ratio tended to decrease with increasing ethanol concentration (Fig. 6C). These observations suggest that ethanol treatment improve the light absorption capacity of the photosynthetic machinery, consistent with the growth promoting effects of ethanol (Fig. 1), The increased total carotenoid concentrations (Fig. 6E) and SPAD values (Fig. 6F) could further contribute to enhanced photoprotection as well as light absorption.

In soybean, ethanol treatment at 20 mM enhanced photosynthetic capacity as evidenced by increased CO_2 fixation and energy metabolism, pigment production, water retention, and oxidative stress protection [27]. Ethanol treatment also significantly increased Chl a, Chl b, total Chl, and total carotenoid contents in both drought-stressed and well-watered plants compared to their respective controls [27]. Similar increases in Chl contents have been observed with ethanol treatment in *Arabidopsis thaliana* [28] and strawberry [24]. Together with our results, these findings suggest that ethanol, particularly at moderate concentrations, exerts positive hormetic effects on photosynthesis and consequently growth.

4.3 Antioxidant Capacity and Total Flavonoid and Total Phenolic Contents

DPPH radical-scavenging activities significantly increased with ethanol treatment, particularly at 80 mM (Fig. 7A). This increase in DPPH radical-scavenging activities was consistent with the higher total flavonoid (Fig. 7B) and total phenolic contents (Fig. 7C), suggesting that ethanol enhances the antioxidant defense system. Both flavonoids and phenolics are well known for their role in combating oxidative stresses and their contents have been positively correlated with antioxidant capacity in various plant species [29,30]. Therefore, our results support that both flavonoids and phenolics are key contributors to overall defense system in plants [27–29].

Activities of SOD, POD, and CAT significantly increased with ethanol treatments (Fig. 7D–F), suggesting that ethanol upregulates these three antioxidant enzymes [31]. Since SOD, POD, and CAT detoxify superoxide, peroxide, and H_2O_2, respectively, converting them into less harmful molecules [31] enhanced protection against oxidative stresses. POD plays a critical role in scavenging hydrogen peroxide and other peroxides, preventing their accumulation and subsequent cellular damage [32]. The significant rise in POD activity, particularly under higher ethanol concentrations, indicates a robust activation of the plant's peroxidase system, likely contributing to enhanced stress tolerance [33]. CAT is essential for decomposing hydrogen peroxide into water and oxygen, thus preventing the harmful effects of this reactive oxygen species [34].

4.4 RA, Tilianin, Acacetin, and Acacetin Glycoside Concentrations and Contents

RA was found to be most concentrated in roots, whereas tilianin and acacetin were primarily found in flowers (Fig. 8), consistent with the findings of Tuan et al. [35]. Ethanol treatment increased RA in roots and tilianin and acacetin in flowers, especially at 80 mM (Fig. 8A–C). RA and tilianin were highest in leaves and flowers (Fig. 8D,E), while acacetin decreased in flowers but increased in stems (Fig. 8F), indicating metabolic shifts between organs. Additionally, the ethanol treatment significantly increased both concentrations and contents of the three acacetin glycosides, except for acacetin 2 concentrations (Figs. 10 and 11). Given that ethanol acts as an abiotic stressor [31], these increases in bioactive compounds could be a result of the defense responses triggered by ethanol exposure [36]. In response to ethanol, specific metabolic pathways leading to the production of secondary metabolites [18], such as RA, tilianin, and acacetin, may be activated through the upregulation of genes involved in their biosynthesis [37]. For example, the phenylpropanoid pathway, responsible for flavonoid production like RA, could be more active with ethanol treatment, leading to increased synthesis of these bioactive compounds. Ethanol treatments may enhance enzyme activity and

cell membrane permeability, aiding the accumulation of bioactive compounds, though further research is needed to confirm these mechanisms.

5 Conclusions

Ethanol treatment at 40 and 80 mM significantly enhanced plant growth and the biosynthesis of key secondary metabolites in *A. rugosa*, particularly RA, tilianin, and acacetin, with the highest accumulation in roots and flowers. It also improved photosynthetic pigments, antioxidant activity, and total flavonoid and phenolic content. These findings suggest ethanol as a potential enhancer for both growth and metabolite production. Future studies should investigate the molecular mechanisms and key genes involved in this process.

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Availability of Data and Materials: The datasets generated and/or analyzed during the current study are available from the corresponding author upon reasonable request.

Ethics Approval: Not applicable.

Conflicts of Interest: The authors declare no conflicts of interest to report regarding the present study.

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