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**Enhancing Growth, Yield, and Phytochemical Characteristics of
Dracocephalum kotschyi Boiss through Optimized Planting Density**

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ABSTRACT

Dracocephalum kotschyi Boiss. is a medicinal plant used in various industries due to its high health benefits, antioxidant activity, attractive flavor and aroma. Commercial yield and phytochemicals can be enhanced by cultivation management like suitable plant density. This study investigates the impact of planting density (50×40, 25×40, 12.5×40 cm caused to 5, 10, and 20 plants m⁻²) on the morpho-physiological traits, yield, trichomes, and phytochemicals profiles of *D. kotschyi*, based on a Randomized Complete Block Design (RCBD) in the Saravarsu region, Guilan province, Iran (2021-2022). The findings reveal a notable fourfold increase in various traits in 20 plants m⁻² (12.5×40 cm). The highest values for parameters such as plant height (34.66 cm), branch number (14), branch length (9.34 cm), leaf number (138.33), leaf area index (105 mm²), fresh weights of leaves, and stems, as well as total fresh weight (105.1, 98.5, and 203.6 g m⁻² respectively), dry weights of leaves, stems, total dry yield (26.1, 24.675, and 50.775 g m⁻² respectively), chlorophyll a, b, total chlorophyll, and carotenoid content (1.038, 0.653, 1.691, and 0.898 mg g⁻¹ FW, respectively), secretory trichomes density (14 mm⁻²), essential oil content and yield (0.633% and 0.322 g m⁻², respectively), PAL activity (10.221 nmol g⁻¹ FW min⁻¹), antioxidant activity (82.85%), total phenol (1.253 mg g⁻¹ DW), and total flavonoid (2.781 mg g⁻¹ DW) were observed in 20 plants m⁻². In conclusion, a planting density of 20 plants m⁻² (with spacing 12.5×40 cm) is recommended for achieving optimal commercial yield, and phytochemical production of *D. kotschyi* in the Saravarsu region.

Keywords: Essential oil, Glandular trichomes, Medicinal plants, Cultivation management, Secondary metabolites.

INTRODUCTION

Medicinal and aromatic plants are rich Secondary Metabolites (SMs) sources, commonly referred as natural products, renowned for their divers' biological activities, and therapeutic effects in pharmaceutical, food, and cosmetic industries (Zamani *et al.*, 2021; Liu *et al.*, 2021).

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32 *Dracocephalum kotschyi* Boiss is an endangered aromatic and medicinal plant belonging to
33 the Lamiaceae family, is renowned for its abundant essential oils (EOs) and flavonoids,
34 predominantly found in its aerial parts (Fattahi *et al.*, 2013). The EO of *D. kotschyi* contains
35 compounds such as limonene, geraniol, citral, and perillaldehyde, while its methanolic extract
36 boasts phenolic compounds like xanthomicrol, luteolin, apigenin, cirsimaritin, and rosmarinic
37 acid (Fattahi *et al.*, 2013; Heydari *et al.*, 2019). Exhibiting a plethora of medicinal properties
38 including antimicrobial, anti-inflammatory, antioxidant, and anticancer effects, *D. kotschyi* is
39 also utilized for enhancing joint health, immune function, and memory (Zamani *et al.*, 2023),
40 in addition to serving as a flavoring agent in food and tea (Zamani *et al.*, 2023).

41 Optimizing planting density is vital for maximizing resource utilization and achieving peak
42 yield, a factor influenced by environmental conditions and plant species (Baloch *et al.*, 2002).
43 Factors such as temperature, solar radiation, humidity, and soil fertility affect plant growth and
44 nutrient requirements. Interspecies competition intensifies at higher densities, leading to
45 increased humidity, inadequate airflow, and reduced light penetration, thereby increasing the
46 risk of disease and reducing yield. Conversely, lower densities may lead to underutilization of
47 resources and decreased yield. Appropriate density enhances plant establishment, competition
48 against weeds, and light utilization efficiency, and ultimately improves yield and phytochemical
49 production (Copes and Scherm, 2005; Koocheki and Sarmadnia, 2012).

50 Numerous studies have examined planting density's effects on medicinal plants. Optimal plant
51 height, dry matter, and Essential Oil (EO) yield for *Thymus daenensis* and *T. kotschyianus* were
52 observed at medium to high densities (6 and 8 plants m⁻²) (Sepahvand *et al.*, 2023). *Satureja*
53 *bachtiarica* Bunge exhibited increased yield but reduced trichome density and EO content with
54 higher planting density (Mirjalili *et al.*, 2022). Higher plant density led to increase in fresh and
55 dry biomass as well as EO yield of *Origanum vulgare* (Gerami *et al.*, 2018). *Calendula*
56 *officinalis* at a high density (57 plants m⁻²) exhibited maximal height, seed weight, chlorophyll,
57 EO content, and yield (Sepehri *et al.*, 2016). *Dracocephalum moldavica* also demonstrated
58 enhanced growth and phytochemical production with increased density (Hashemian Ahmadi
59 and Hadipanah, 2014).

60 Hence, the current study was undertaken to investigate the influence of planting density on
61 the morphological traits, yield, and phytochemical compounds of the *D. kotschyi*.

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65 MATERIALS AND METHODS

66 Plant Cultivation

67 *D. kotschy* plants were cultivated in Saravarsu (50° 13' 26" E, 36° 49' 0" N, at 1,430 m) in
68 Rahimabad District, Rudsar County, Guilan Province, Iran. The climatic details of this region
69 are mentioned in Table 1.

70 Soil samples were obtained from a depth of 0-30 cm; and the physicochemical characteristics
71 are detailed in Table 2. The experiment followed a Randomized Complete Block Design
72 (RCBD) with three densities in three replications. Initially, the designated land was plowed and
73 enriched with 30 ton/ha cow manure. *D. kotschy* seeds, sourced from Pakan Bazr Company in
74 Isfahan province, were germinated in a greenhouse during late winter. In spring, after levelling
75 the ground and creating ridges and furrows at 40 cm intervals, plots measuring 2×2 meters (4
76 m²) were established. Seedlings, at the 6-8 leaf stage were transplanted to the main field.
77 Planting occurred with intervals of 12.5×40, 25×40, and 50×40 cm, equivalent to plant densities
78 of 20, 10, and 5 plants m⁻², respectively. Following planting, irrigation intervals were minimized
79 initially to aid plant establishment and prevent soil compaction. Subsequent irrigation schedules
80 were adjusted based on regional conditions and the plant's water requirements. Throughout the
81 growth phase, physical and manual weed removal practices were employed, and no instances
82 of pests or diseases were noted.

83 84 Morphological Traits Measurement

85 The aerial parts of *D. kotschy* plants were randomly harvested from each plot at the full
86 flowering stage (maximum phytochemicals accumulation). Plant height and branch length were
87 measured using a ruler (cm). The branch and leaf number were quantified numerically. Leaf
88 area index was calculated using grid paper. Yield indices, such as fresh and dry weights of
89 leaves, stems, and total weight, were measured using a digital balance (g m⁻²).

90 91 Chlorophylls and Carotenoids Content

92 To measure chlorophyll and carotenoid content, 0.1 g of the sample was finely ground with 1
93 ml of 80% acetone, and the resulting mixture was refrigerated at 4°C in darkness for 24 hours.
94 Subsequently the absorbance was recorded at wavelengths 470, 645, and 663 nm. The
95 concentrations of chlorophyll a, chlorophyll b, total chlorophyll, and carotenoids were
96 calculated using the formulas and reported as mg g⁻¹ of fresh weight (Lichtenthaler and
97 Buschmann, 2001):

$$98 \text{ Chl } a \text{ (mg g}^{-1} \text{ FW)} = (12.25 A_{663} - 2.79 A_{645}) (V/W 1000) \quad (1)$$

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99 $Chl\ b\ (mg\ g^{-1}\ FW) = (21.5\ A_{645} - 5.10\ A_{663})\ (V/W\ 1000)$ (2)

100 $Total\ Chl\ (mg\ g^{-1}\ FW) = Chl\ a + Chl\ b$ (3)

101 $Carotenoids\ (mg\ g^{-1}\ FW) = [(1000\ A_{470} - 1.82\ Chla - 85.02\ Chlb)/198]\ (V/W\ 1000)$ (4)

102 Which, V= Acetone Volume (ml) and W= Sample Weight (g).

103

Glandular Trichrome Density Measurement

105 Leaf samples measuring 2-3 mm² were cleansed with ethanol and distilled water.
106 Subsequently, the dried samples were fixed on metal bases using conductive glue and coated
107 with a thin layer of gold using a Coater (SBC121, KYKY Company, China). Finally, the
108 samples were examined using a scanning electron microscope (SEM XL 30, Philips,
109 Netherlands). Trichome density was determined by counting the number of trichomes within a
110 1 mm² area.

111

EO Content and Yield (EOC and EOY)

113 For EO extraction, 20 g of dried and powdered plant material was hydro-distilled using a
114 Clevenger apparatus for three hours. The resulted EO samples were stored at 4°C until analysis
115 (Zamani *et al.*, 2023). EO content and yield were determined using Equations 5 and 6,
116 respectively:

117 $EO\ content\ (\%) = (EO\ weight/Dry\ matter\ weight) \times 100$ (5)

118 $EO\ yield\ (g\ m^{-2}) = EO\ content \times Total\ dry\ weight/100$ (6)

119

Phenylalanine Ammonia Lyase (PAL) Enzyme Activity

121 For enzyme extraction, 0.1 g of fresh plant material was homogenized in 1 ml phosphate buffer
122 (pH= 7). Subsequently, 250 µl of the enzyme extract was combined with 250 µl of 10 mM
123 sodium borate buffer (pH= 8.8), 250 µl distilled water, and 250 µl phenylalanine (50 mmol, 250
124 µl) and read at 290 nm using a UV/VIS spectrophotometer (PG Instrument Ltd, Leicester, UK).
125 PAL activity was determined by the Beer-Lambert law with an extinction coefficient of 9630
126 µ⁻¹cm⁻¹ and expressed as nmol g⁻¹ FW min⁻¹ (Saunders and McClure's, 1974).

127

Total Phenol Content (TPC), Total Flavonoid Content (TFC), and Antioxidant Activity (AOA)

130 TPC (mg Gallic acid g⁻¹ DW) was quantified using methanolic extract (1:10) by the Folin-
131 Ciocalteu method at 760 nm (Singleton *et al.*, 1999). TFC (mg Quercetin g⁻¹ DM) was
132 determined using the aluminium chloride method at 506 nm (Du *et al.*, 2009). The antioxidant

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133 activity was assessed using the DPPH (2,2-diphenyl-1-picrylhydrazyl) free radical method at
134 517 nm and determined as the percentage of DPPH inhibition using Equation (7) (Chiou *et al.*,
135 2007; Zamani *et al.*, 2023):

$$136 \text{ Free radical scavenging percentage} = [(A_{\text{control}} - A_{\text{sample}}) / A_{\text{control}}] \times 100 \quad (7)$$

137 A_{control} : Absorbance of DPPH, A_{sample} : Absorbance of plant extract+DPPH.

138

139 **Statistical analysis**

140 The experiment followed a Randomized Complete Block Design (RCBD) with three density
141 treatments replicated three times. Mean data were compared using the LSD test at $P < 0.01$ that
142 analyzed with SAS statistical software (Ver. 9). Correlation and regression analysis were
143 performed using SPSS Ver. 26.

144

145 **RESULTS AND DISCUSSION**

146 **Effect of Plant Density on the Morphological Characteristics of *D. kotschy***

147 The plant density significantly influenced the plant morpho-physiological traits ($P < 0.01$)
148 (Table 3). Increasing the density from 5 to 20 plants m^{-2} resulted in a 2-3fold enhancement in
149 the morpho-physiological characteristics of *D. kotschy*. Specifically, the highest values for
150 plant height (34.66 cm), branch number (14), branch length (9.34 cm), leaf number (138.33),
151 and leaf area index (105 mm^2) were observed in plants cultivated at a density of 20 plants m^{-2} .
152 Conversely, the lowest density (5 plants m^{-2}) exhibited the lowest values for the investigated
153 indices (Figure 1).

154 Increased plant height due to higher plant density is linked to competition for solar radiation.
155 Dense plants populations reduce light penetration, triggering intensified competition for
156 sunlight. Additionally, under shaded conditions with limited light availability, the production
157 of growth hormones, such as auxin increases, resulting in heightened plant height (Arvin and
158 Firouzeh, 2018). Similar to our findings, the positive effects of increasing the cultivation density
159 on the plant height have been reported in ginseng (Liu *et al.*, 2021), stevia (Btru *et al.*, 2017),
160 and peppermint (Mansoori, 2014). In higher planting density, the number of plants per unit area
161 increases, resulting in a greater leaf count per unit area. In our study, an augmentation in plant
162 density resulted in an increased Leaf Area Index (LAI) in *D. kotschy* plants. Optimal density
163 levels caused faster leaf growth and led to an augmented leaf area index. This increase in leaf
164 area positively impacts the photosynthesis rate, influencing various growth parameters,
165 including yield (Li *et al.*, 2019). These findings align with research conducted on sunflowers
166 (Li *et al.*, 2019) and marigolds (Sepehri *et al.*, 2016). While greater spaces between plants (low

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167 densities) lead to an average increase in individual development, but accompany with a decrease
168 in the total potential yield (Moldovan *et al.*, 2022).

169

Effect of Plant Density on the Fresh and Dry Yield of the *D. kotschy*

171 As indicated in Table 3, the plant density significantly affected the fresh and dry yield of the
172 *D. kotschy* ($P < 0.01$). Reduced planting intervals led to an augmentation in the fresh and dry
173 matter of the *D. kotschy* plant. The highest fresh weights of leaves, stems, and the total (105.1,
174 98.5, and 203.6 g m⁻², respectively) and dry weights of leaves, stems, and total biomass (26.1,
175 24.675, and 50.775 g m⁻², respectively) were observed at a density of 20 plants m⁻², which were
176 3-4 times higher than plants cultivated at a density of 5 plants m⁻² (Figure 2).

177 The correlation between higher plant density and increased biomass production, with peak
178 yield achieved at the highest density (20 plants m⁻²). Alteration in density affects various
179 factors, and in a density with minimized competition between the plants to receive moisture,
180 light and nutrients, maximum photosynthesis, photosynthetic pigments and growth is achieved.
181 This is the optimal density which leads to maximum yield and depends on the plant species and
182 environmental conditions. The increased density more than the optimum usually decreases most
183 growth parameters and photosynthesis (Dai *et al.*, 2022; Sepehri *et al.*, 2016). In *Satureja*
184 *bachtiarica* Bunge, the maximum plant yield was achieved at the highest plant density (80,000
185 plants/ha) (Mirjalili *et al.*, 2022). Similarly, in *Dracocephalum moldavica*, an increase in the
186 plant density enhanced the weight with the highest fresh (8,260.2 kg) and dry yield (2,787.5 kg
187 ha⁻¹) were observed at a cultivation distance of 10 cm (Hashemian Ahmadi and Hadipanah,
188 2014). It was consistent with study on peppermint, where fresh and dry biomass yield and leaves
189 significantly increased with increasing plant density from 8 to 20 plants m⁻² (Mansoori, 2014).
190 Optimum plant density provides favorable condition, leading to increased dry matter
191 accumulation. This phenomenon is observed in medicinal plants such as *Thymus daenensis*
192 (Jasemi *et al.*, 2019) and *Calendula officinalis* (Sepehri *et al.*, 2016).

193

Effect of Plant Density on Photosynthetic Pigments of the *D. kotschy*

195 According to Table 4, the plant density treatment significantly influenced chlorophyll b, total
196 chlorophyll, and carotenoid levels at a 1% significance level, however had no impact on
197 chlorophyll a. The maximum chlorophyll a, b, total chlorophyll, and carotenoid content (1.038,
198 0.653, 1.691, and 0.898 mg g⁻¹ FW, respectively) were observed in the 20 plants m⁻² treatment,
199 representing an increase of up to 3-fold compared to the lowest density. Conversely, the lowest
200 pigment content was noted at 5 plants m⁻² (Figure 3).

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201 Photosynthetic pigments, including chlorophyll a, b, and carotenoids, play direct and indirect
202 roles in photosynthesis, crucial for light absorption and energy regeneration (Tohidi *et al.*,
203 2020). Consistent with our findings, planting density impacted photosynthetic pigments in
204 *Perilla frutescens*. Increased plant density leads to higher content of photosynthetic pigments
205 due to increased leaf number and area (as the source of photosynthetic pigments) (Wu *et al.*,
206 2020). *Thymbra spicata* and *Calendula officinalis* showed higher chlorophyll a, b, and
207 carotenoid with increased row distance (Malekmaleki *et al.*, 2022; Sepehri *et al.*, 2016). On the
208 other hand, increased planting density (especially more than optimum density) reduced plant
209 photosynthetic capacity [net Photosynthetic rate (Pn)], stomatal conductance (Gc), and leaf
210 chlorophyll content (Dai *et al.*, 2022). Additionally, *Levisticum officinale* and *Coriandrum*
211 *sativum* exhibited decreased chlorophyll with increased density (Atteya *et al.*, 2021; Arvin and
212 Firouzeh, 2018). Generally, leaf pigment levels are influenced by factors like density, nutrition,
213 species type, environment conditions, and diseases, impacting photosynthesis and yield (Tohidi
214 *et al.*, 2020; Cui *et al.*, 2018).

215

Effect of Plant Density on Glandular Trichomes of the *D. kotschy*

217 Scanning electron microscope images revealed various types of trichomes in the *D. kotschy*
218 leaves including non-glandular (protective) and glandular (secretory) trichomes. Non-glandular
219 trichomes were unicellular or multicellular, non-branched, sharp-pointed, bent, or erect types.
220 Secretory trichomes were observed in two forms: peltate and capitate. In this experiment, non-
221 glandular and glandular trichomes were observed in all three densities, but with an increase in
222 cultivation density, the number of secretory trichomes increased from 10 numbers mm⁻² in the
223 lowest treatment to 14 mm⁻² in the highest planting density (Figure 4).

224 Glandular trichomes, essential secretory structures in mint plants, are abundant in aerial
225 organs and play a crucial role in producing therapeutic compounds like terpenoids (EOs) and
226 phenolic compounds (Feng *et al.*, 2021; Tozin *et al.*, 2015). Understanding their morphology
227 and response to factors like cultivation density is essential for medicinal plants, as changes in
228 trichome structures and EO accumulation can occur due to environmental adaptations
229 (Tuttolomondao *et al.*, 2016). In our study, an increase in plant density led to a higher number
230 of secretory trichomes on *D. kotschy* leaves, likely due to the increased leaf number (providing
231 more space for trichome accumulation). Similar findings were observed in *Artemisia cina*,
232 where glandular trichome number correlated with leaf number (Herawati *et al.*, 2020). In
233 Oregano, trichome density increased at the middle density (150×50 cm) but no significant
234 difference was noted across density levels (Tuttolomondao *et al.*, 2016). Reports suggest that

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235 high-density conditions, coupled with resource limitations like water, may induce slight stress,
236 affecting trichome numbers. Glandular trichomes density of *Artemisia annua* increased with
237 slight water stress (50% field capacity), while it decreased by 25% of the field capacity (Shi *et*
238 *al.*, 2018). Optimal growth traits and secretory structure development were observed at a density
239 of 20 plants m⁻². Overall, secretory trichome numbers are influenced by various factors,
240 including growth conditions and plant species (Tozin *et al.*, 2015).

241

Effect of Plant Density on EO Content (EOC) and Yield (EOY) of the *D. kotschy*

242 As indicated in Table 4, plant density significantly influenced both the EOC and EOY (P<
243 0.01). With an increase in plant density, both the plant EOC and EOY showed an upward trend.
244 The maximum EOC and EOY (0.633% and 0.322 g m⁻², respectively) were recorded in the 20
245 plants m⁻² treatment (Figure 5).
246

247 EOs valued for their diverse medicinal properties, have extensive applications across the
248 pharmaceutical, food, and cosmetic industries (Masyita *et al.*, 2022). Increasing cultivation
249 density in *Levisticum officinale* led to higher EO content, with optimal yield obtained at a
250 distance of 15 cm (Atteya *et al.*, 2021). *Satureja bachtiarica* showed increased EO yield at
251 higher plant densities (80,000 plants ha⁻¹) (Mirjalili *et al.*, 2022). *Dracocephalum moldavica*
252 achieved the highest EO yield at a cultivation distance of 10 cm (Hashemian Ahmadi and
253 Hadipanah, 2014). EO content in *Thymus daenensis* and *Calendula officinalis* increased with
254 cultivation density (Jasemi *et al.*, 2019; Sepehri *et al.*, 2016). Higher planting density boosts
255 Essential Oil (EO) yield in savory plants due to elevated biomass production in dense
256 conditions. Since EO is mainly stored in leaves and flowering branches, plants cultivated at
257 high density, yielding more leaves and flowering branches, also exhibit higher EO yield
258 (Abbaszadeh *et al.*, 2014). These findings are consistent with our observations. In *Thymbra*
259 *spicata*, a row distance of 20 cm resulted in the highest EO content and yield (Malekmaleki *et*
260 *al.*, 2022). Coriander plants exhibited increased EO content and yield with higher density (Arvin
261 and Firouzeh, 2018). Various physiological and environmental factors influence EO
262 biosynthesis and accumulation, including geographical location, agricultural techniques, plant
263 type, and harvest time (Tuttolomondao *et al.*, 2016). Plant density plays a crucial role in
264 maximizing crop quality, biomass, and EO yield, as observed in our study and others.

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269 **Effect of Plant Density on PAL Enzyme Activity and Antioxidant Compounds of the *D.*** 270 ***kotschy***

271 The plant density exerted a significant impact on antioxidant activity, total phenol, and
272 flavonoid content ($P < 0.05$), but did not influence PAL enzyme activity (Table 4). As illustrated
273 in Figure 5, the highest PAL activity ($10.221 \text{ nmol g}^{-1} \text{ FW min}^{-1}$), antioxidant activity (82.85%),
274 total phenol ($1.253 \text{ mg g}^{-1} \text{ DW}$), and total flavonoid ($2.781 \text{ mg g}^{-1} \text{ DW}$) were observed in the
275 20 plants m^{-2} treatment. Conversely, the lowest the investigated traits were noted at a density of
276 5 plants m^{-2} .

277 Phenylalanine Ammonia-Lyase (PAL), a crucial enzyme, contributes to the formation of
278 antioxidant phenylpropanoid compounds like phenolic and flavonoid compounds which aid
279 plant defense mechanisms (Zamani *et al.*, 2021). These compounds have diverse biological
280 activities including antioxidant, antimicrobial, anticancer, and anti-inflammatory properties
281 (Maina *et al.*, 2021). PAL activity is influenced by various factors including nutrition, light,
282 environmental changes, density, stresses, etc. which can enhance gene expression, enzyme
283 production and phenolic productions (Zamani *et al.*, 2021; Medda *et al.*, 2020). Studies on
284 *Linum usitatissimum* L., have shown the increasing plant density enhances PAL enzyme
285 activity in specific cultivars (Gao *et al.*, 2018). Alterations in planting density impact the
286 availability of growth factors, influencing plant competition for optimal growth and
287 subsequently phytochemicals such as phenolic compounds and antioxidants (Tazeh *et al.*,
288 2016). Consistent with these findings, studies indicated a positive correlation between higher
289 plant density and elevated phenol and flavonoid levels, as well as increased antioxidant activity.
290 For instance, in ginseng, higher and medium planting densities were associated with the highest
291 ginsenoside production (Liu *et al.*, 2021). Increasing planting density also led to an increase in
292 rosmarinic acid content in *Perilla* increases (Wu *et al.*, 2020). In *Levisticum officinale*, total
293 phenol and antioxidant activities increased with higher density (Atteya *et al.*, 2021). High
294 planting density in some studies limits several factors like light, water, and nutrients, and leads
295 to plant competition, and induced stress can stimulate PAL enzyme activity and alter
296 phytochemicals (Maina *et al.*, 2021). However, in our study, it seems that the high density of
297 plants provided the maximum availability of nutrients and the growth requirements of the plant,
298 thus the plant has optimal growth, and it produces high phytochemical compounds.

299 300 **Correlation and Regression Analysis of Measured Traits**

301 Positive and significant correlations were observed among growth traits, yield, photosynthetic
302 pigments, glandular trichomes and phytochemical traits in *D. kotschy* (Table 5), which are

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303 crucial for plant production and breeding programs. Elevated plant density led to increased
304 growth traits, positively correlated with yield, indicating the contribution of vegetative organs
305 to overall yield enhancement. Morpho-physiological factors showed a positive correlation with
306 photosynthetic pigments, influenced by increased leaf number and area (as the source of
307 photosynthetic pigments) due to density increment, leading to higher fresh and dry matter
308 production. A study on *Levisticum officinale* found a significant positive correlation between
309 growth characteristics, fresh and dry weight, and chlorophyll (Atteya *et al.*, 2021). Additionally,
310 positive correlations were noted between fresh weight, dry matter weight, photosynthetic
311 pigment, and active substances (Table 5), suggesting enhanced pigment levels contribute to
312 increased biomass, enzyme activity, and phytochemical production. Similar findings were
313 observed in studies on *Mentha piperita* and *Satureja sahendica* Bornm, emphasizing the
314 importance of growth traits in Essential Oil (EO) production (Ostadi *et al.*, 2023; Abbaszadeh
315 *et al.*, 2014). Positive correlations between EO yield and overall fresh and dry yield were
316 reported in thyme (Sepahvand *et al.*, 2016). Similar positive correlations between growth traits
317 and plant yield were also observed in *Stevia Rebaudiana* (Btru *et al.*, 2017). Since secretory
318 trichomes are biosynthesis and storage places of essential oil, their density is positively
319 correlated with essential oil and is affected by several factors including planting density
320 (Esmaeili *et al.*, 2019). In *Origanum majorana*, more and larger secretory trichomes with higher
321 EO content were observed in high light intensity (Shafiee-Hajiabad *et al.*, 2015). Table 5 reveals
322 a positive correlation between the PAL enzyme and antioxidant compounds, emphasizing its
323 role in phenylpropanoid and antioxidant synthesis. Similar findings were reported by Zamani
324 *et al.* (2021) for *Cynara scolymus*, highlighting PAL's influence on phenylpropanoid
325 compounds. Atteya *et al.* (2021) found a strong positive correlation between phenol and
326 antioxidant activity in *Levisticum officinale* across various cultivation densities. Increased
327 density likely enhances antioxidant activity through elevated phenolic compounds, which
328 inhibit free radicals directly and enhance hydrogen donation potential (Ghimire *et al.*, 2021).

329 The multivariate regression analysis was employed to explore the relationship between EO
330 Yield (EOY), as a dependent variable (Y), and its affecting traits, treated as independent
331 variables (X). The regression findings indicated that the Total Dry Weight (TDW) of the entire
332 plant, with an explanation coefficient of 98%, and the EO Content (EOC), with an explanation
333 coefficient of 94.8%, emerged as the most influential determinants of EOY (Equation 8):

$$334 \quad Y = -0.232 + 0.005X_1 + 0.468X_2 \quad (8)$$

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335 Where, Y = EO yield (g m^{-2}), X_1 = Total dry yield of the whole plant (g m^{-2}), X_2 = EO content
336 (%).

337 Given that EO yield is affected by both plant dry weight and EO content, augmenting dry
338 weight and enhancing the EO content due to plant density has consistently resulted in
339 heightened EO yield, as shown in various studies (Ostadi *et al.*, 2023; Sepahvand *et al.*, 2016;
340 Abbaszadeh *et al.*, 2014).

341

342 CONCLUSIONS

343 Based on the results, plant density significantly affected the most measured traits and the
344 highest plant height, branch number, branch length, leaf area index, fresh and dry yield,
345 photosynthetic pigments, secretory trichomes density, EO content and yield, PAL activity,
346 antioxidant activity, total phenol, and total flavonoid were observed in high density (20 plants
347 m^{-2}). Generally, *D. kotschyi* plant cultivation with distances of 12.5×40 cm (a density of 20
348 plants m^{-2}) due to the optimal growth conditions (nutrients, water, light, etc.), causes maximum
349 growth and phytochemicals production. Therefore, this cultivation interval is recommendable
350 for the economic production of the *D. kotschyi* plant in similar ecological regions.

351

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546 **Table 1.** Climatic characteristics of Saravarsu Region during growth season in 2021-2022.

Months	Minimum temperature (°C)	Mean temperature (°C)	Maximum temperature (°C)	Average relative humidity (%)	Total rainfall (mm)
April	6.09	12.92	19.75	71	37.7
May	9.14	15.02	20.91	77.5	46.9
June	12.96	18.92	24.88	78	28.1
July	16.08	21.37	26.67	80	57.4
August	16.04	20.89	25.74	79	45.4
September	14.7	19.79	24.88	77.5	53.3
Annual mean	7.68	13.32	18.96	72	680.5

547

548 **Table 2.** Soil physicochemical characteristics of experimental field.

OM (%)	pH	EC (ds/m)	Clay (%)	Silt (%)	Sand (%)	Texture	N (%)	P (ppm)	K (ppm)
1.91	7	0.36	22.8	21.2	56	Sandy clay loam	0.2	255	6.4

549

550 **Table 3.** ANOVA of plant density effect on morpho-physiological and yield of *D. kotschyi*.

		Mean Square										
SOV	df	PLH	BN	BL	LN	LAI	LFW	SFW	TFW	LDW	SDW	TDW
Block	2	0.44 ^{n.s}	3.11 ^{n.s}	1.3 ^{n.s}	108.33 ^{n.s}	47.44 ^{n.s}	124.3 ^{n.s}	104.64 ^{n.s}	428.24 ^{n.s}	11.29 ^{n.s}	9.15 ^{n.s}	25.18 ^{n.s}
Density	2	308.77 ^{**}	45.44 ^{**}	23.7 ^{**}	7033.33 ^{**}	1596.77 ^{**}	3970.5 ^{**}	4136.82 ^{**}	16155.9 ^{**}	289.99 ^{**}	318.62 ^{**}	1215.89 ^{**}
Error	4	7.27	0.61	0.60	66.66	23.44	191.7	78.29	452.91	12.9	5.643	30.91
CV	-	11.61	7.99	12.65	9.24	6.08	21.46	15.79	17.65	20.12	18.49	20.04

551 *, **: Significant differences at 5 and 1% ; ^{n.s}: no significant difference

552 SOV: Source Of Variances; df: Degree of freedom; CV: Coefficient of Variation; PLH: Plant Height; BN: Branch
553 Number; BL: Branch Length; LN: Leaf Number; LAI: Leaf Area Index; LFW: Leaf Fresh Weight; SFW: Stem
554 Fresh Weight; TFW: Total Fresh Weight; LDW: Leaf Dry Weight; SDW: Stem Dry Weight; TDW: Total Dry
555 Weight.

556

557 **Table 4.** ANOVA of plant density effect on biochemical and phytochemical traits of *D. kotschyi*.

		Mean Square										
SOV	df	Chl a	Chl b	Chl t	Car	EOC	EOY	PAL	TPC	TFC	AOA	
Block	2	0.01 ^{n.s}	0.001 ^{n.s}	0.017 ^{n.s}	0.001 ^{n.s}	0.003 ^{n.s}	0.001 ^{n.s}	0.017 ^{n.s}	0.013 ^{n.s}	0.005 ^{n.s}	0.046 ^{n.s}	
Density	2	0.109 ^{n.s}	0.151 ^{**}	0.518 ^{**}	0.225 ^{**}	0.016 ^{**}	0.057 ^{**}	0.246 ^{n.s}	0.213 [*]	1.186 [*]	8.208 [*]	
Error	4	0.024	0.001	0.024	0.003	0.0002	0.001	0.038	0.022	0.077	0.585	
CV	-	19.087	10.464	12.893	9.299	3	21.86	1.983	14.81	12.8	0.948	

558 *, **: Significant differences at 5 and 1% ; ^{n.s}: no significant difference.

559 SOV: Source of Variances; df: Degree of freedom; CV: Coefficient of Variation; Chl a: Chlorophyll a; Chl b:
560 Chlorophyll b; Chl t: Chlorophyll total; Car: Carotenoid; EOC: EO Content; EOY: EO Yield; PAL: Phenylalanine
561 Ammonia Lyase enzyme; TPC: Total Phenol Content; TFC: Total Flavonoid Content; AOA: Antioxidant Activity.

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Table 5. Correlation between morpho-physiological and phytochemical traits of *D. kotschyi*.

	PLH	BN	BL	LN	LAI	LFW	SFW	TFW	LDW	SDW	TDW	Chl a	Chl b	Chl t	Car	GTs	EOC	EOY	PAL	TPC	TFC	AOA	
PLH	1																						
BN	0.935**	1																					
BL	0.866**	0.905**	1																				
LN	0.907**	0.961**	0.902**	1																			
LAI	0.944**	0.955**	0.954**	0.954**	1																		
LFW	0.901**	0.832**	0.862**	0.911**	0.92**	1																	
SFW	0.93**	0.915**	0.782*	0.944**	0.88**	0.887**	1																
TFW	0.909**	0.904**	0.8**	0.948**	0.88**	0.892**	0.988**	1															
LDW	0.912**	0.843**	0.842**	0.889**	0.93**	0.984**	0.86**	0.848**	1														
SDW	0.819**	0.818**	0.723*	0.867**	0.82**	0.844**	0.878**	0.928**	0.806**	1													
TDW	0.904**	0.937**	0.856**	0.962**	0.941**	0.898**	0.951**	0.963**	0.88**	0.903**	1												
Chl a	0.855**	0.825**	0.742*	0.704*	0.816*	0.641	0.749*	0.721*	0.682*	0.632	0.763*	1											
Chl b	0.951**	0.928**	0.887**	0.939**	0.938**	0.907**	0.926**	0.948**	0.884**	0.935**	0.931**	0.794*	1										
Chl t	0.969**	0.924**	0.858**	0.865**	0.924**	0.815**	0.883**	0.879**	0.824**	0.824**	0.893**	0.949**	0.945**	1									
Car	0.906**	0.924**	0.862**	0.942**	0.921**	0.844**	0.946**	0.953**	0.814**	0.862**	0.968**	0.823**	0.925**	0.922**	1								
GTs	0.93**	0.941**	0.898**	0.982**	0.948**	0.909**	0.963**	0.963**	0.88**	0.864**	0.963**	0.768*	0.946**	0.904**	0.979**	1							
EOC	0.839*	0.761*	0.78*	0.83*	0.793*	0.813**	0.855**	0.881**	0.748*	0.854**	0.801**	0.687*	0.908**	0.841**	0.875**	0.894**	1						
EOY	0.887**	0.909**	0.828**	0.957**	0.914**	0.893**	0.946**	0.969**	0.862**	0.953**	0.973**	0.736*	0.952**	0.881**	0.963**	0.967**	0.887**	1					
PAL	0.894**	0.909**	0.942**	0.929**	0.96**	0.866**	0.722*	0.824**	0.869**	0.731*	0.872**	0.562	0.88**	0.872**	0.905**	0.943**	0.826**	0.876**	1				
TPC	0.803*	0.809**	0.707*	0.833*	0.735*	0.681*	0.726*	0.88**	0.619	0.792*	0.784*	0.69*	0.856**	0.816**	0.878**	0.883**	0.93**	0.856**	0.783*	1			
TFC	0.804*	0.832**	0.724*	0.9**	0.834**	0.834**	0.819**	0.94**	0.798**	0.938**	0.943**	0.648	0.882**	0.806**	0.935**	0.920**	0.85**	0.981**	0.803**	0.823**	1		
AOA	0.914**	0.9**	0.797*	0.9**	0.927**	0.873**	0.894**	0.902**	0.891**	0.859**	0.955**	0.833**	0.894**	0.911**	0.943**	0.928**	0.79*	0.944**	0.882**	0.744*	0.927**	1	

*, **: Significant differences at 5 and 1%.

PLH: Plant Height; BN: Branch Number; BL: Branch Length; LN: Leaf Number; LAI: Leaf Area Index; LFW: Leaf Fresh Weight; SFW: Stem Fresh Weight; TFW: Total Fresh Weight; LDW: Leaf Dry Weight; SDW: Stem Dry Weight; TDW: Total Dry Weight; Chl a: Chlorophyll a; Chl b: Chlorophyll b; Chl t: Chlorophyll total; Car: Carotenoid; GTs: Glandular Trichomes; EOC: EO Content; EOY: EO Yield; PAL: Phenylalanine Ammonia Lyase enzyme; TPC: Total Phenol Content; TFC: Total Flavonoid Content; AOA: Antioxidant Activity.

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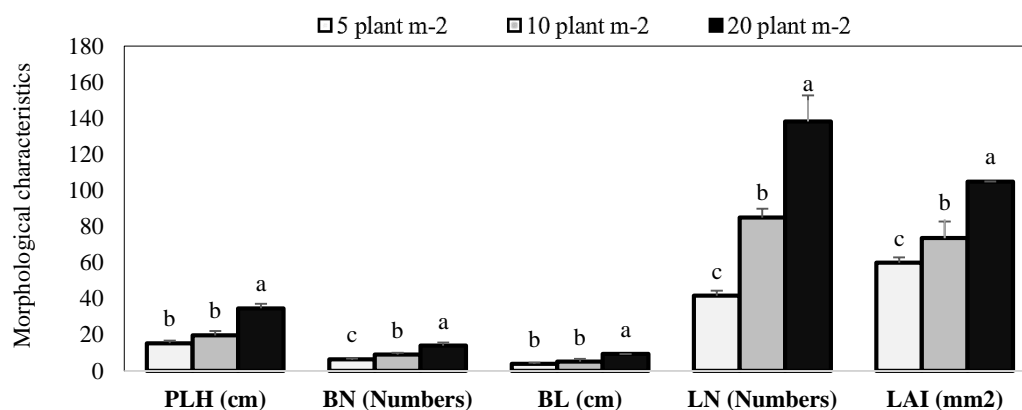


Figure 1. Morpho-physiological characteristics of *D. kotschyi* under different plant densities. PLH: Plant Height; BN: Branch Number; BL: Branch Length; LN: Leaf Number; LAI: Leaf Area Index. Means with same letters had no significant different at $P < 0.01$.

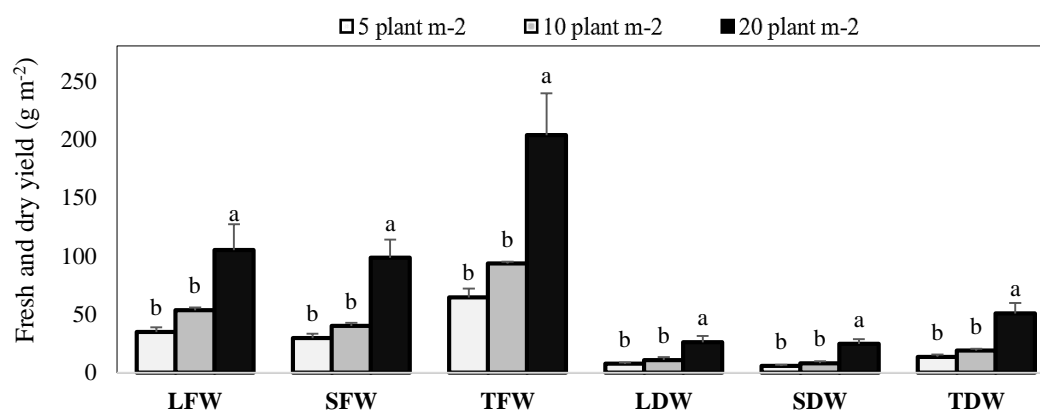


Figure 2. Fresh and dry yield of *D. kotschyi* under different plant densities. LFW: Leaf Fresh Weight; SFW: Stem Fresh Weight; TFW: Total Fresh Weight; LDW: Leaf Dry Weight; SDW: Stem Dry Weight; TDW: Total Dry Weight. Means with same letters had no significant different at $P < 0.01$.

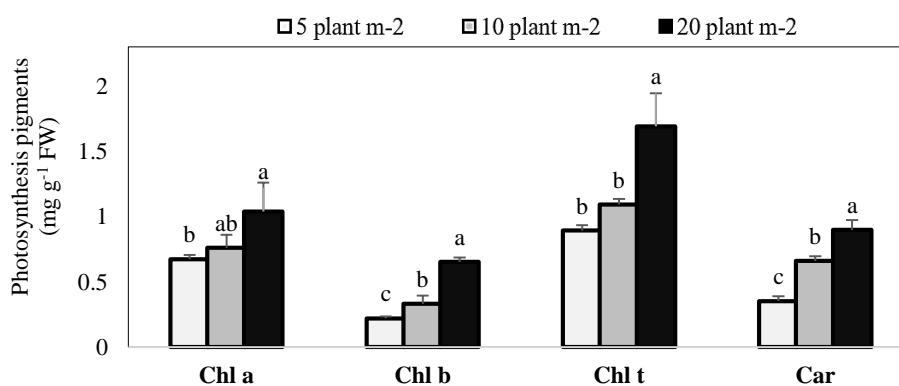


Figure 3. Chlorophyll and Carotenoid pigments of *D. kotschyi* under different plant densities. Chl a: Chlorophyll a; Chl b: Chlorophyll b; Chl t: Chlorophyll total; Car: Carotenoid. Means with same letters had no significant different at $P < 0.01$.

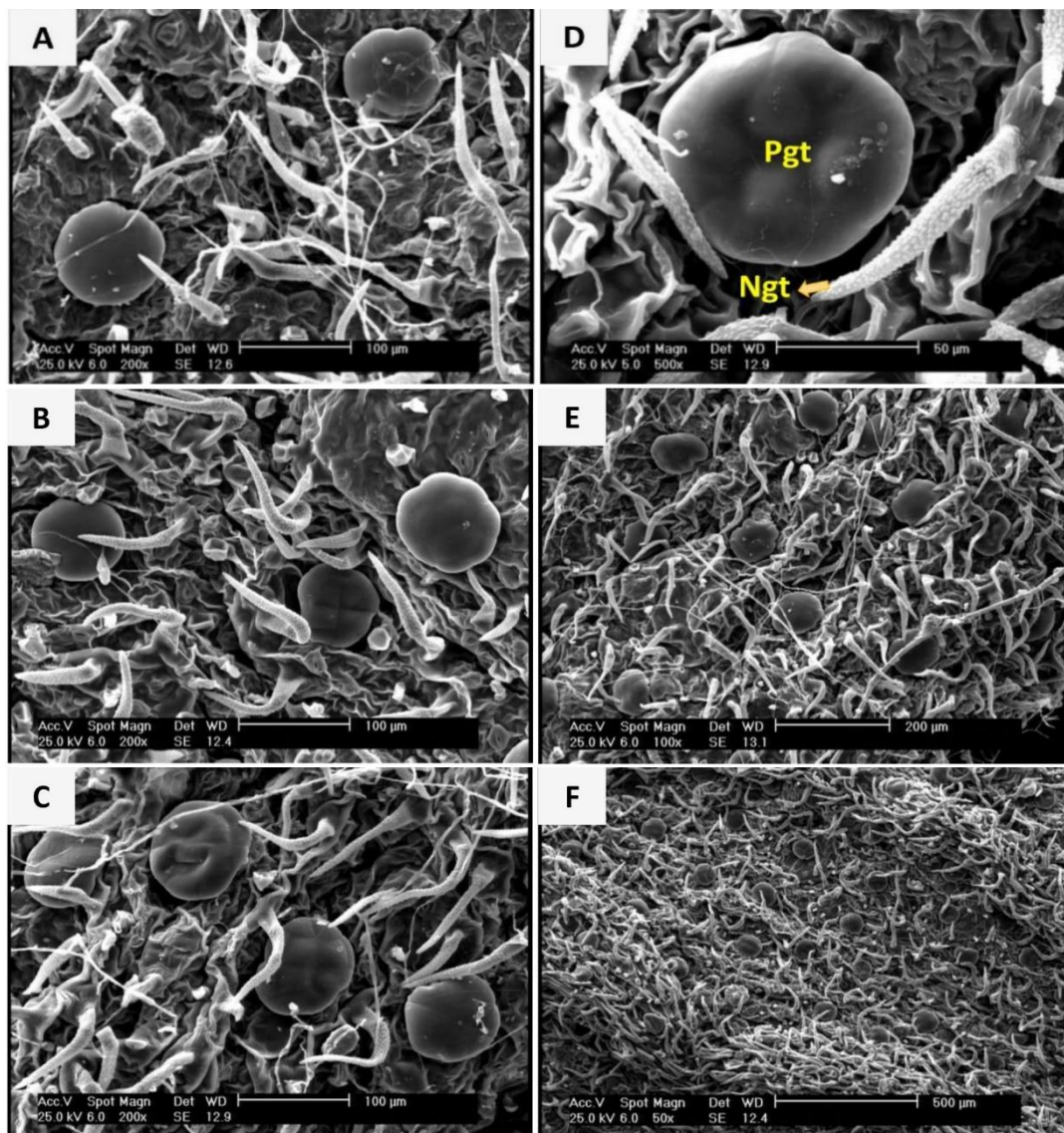


Figure 4. SEM images of *D. kotschy* leaf under different plant densities. Distribution of Peltate glandular trichomes (Pgt), and Non-glandular trichomes (Ngt) of plants cultivated with densities at 5 (A, D), 10 (B, E), and 20 plant m⁻² (C, F); Scale: A, B, C: 100 μm; D: 50 μm; E: 200 μm; F: 500 μm.

Growth, Yield, Phytochemical under Planting Density

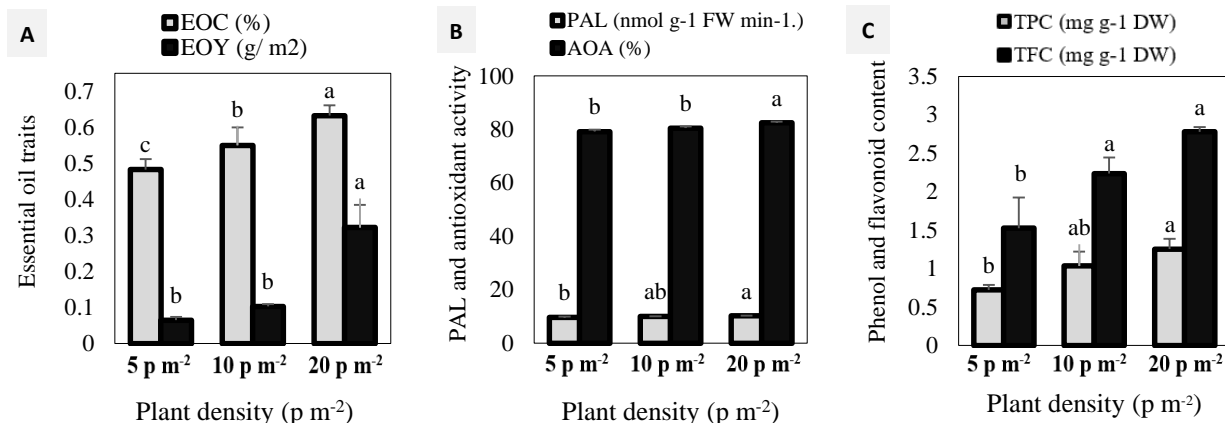


Figure 5. EOC (EO Content); EOY: EO yield (A); PAL: Phenylalanine ammonia lyase enzyme; AOA; Antioxidant activity (B); and TPC: Total Phenol Content; TFC: Total flavonoid content (C) of *D. kotschyi* under different plant densities. p m⁻²: Plant per m². Means with same letters had no significant different at P < 0.05.

افزایش رشد، عملکرد و ویژگی‌های فیتوشیمیایی زین گیاه (*Dracocephalum kotschyi* Boiss) حاصل از تراکم کشت بهینه

سحر زمانی، داوود بخشی، محمدتقی عبادی، امیر صحرارو، و مهران فتحی

چکیده

زین گیاه (*Dracocephalum kotschyi* Boiss.) گیاه دارویی است که به دلیل داشتن خواص درمانی، فعالیت آنتی اکسیدانی، طعم و عطر جذاب در صنایع مختلف مورد استفاده قرار می‌گیرد. عملکرد تجاری و تولید ترکیبات فیتوشیمیایی را می‌توان با مدیریت کشت مانند انتخاب تراکم کشت مناسب افزایش داد. این مطالعه، با هدف بررسی تراکم بوته (فواصل 40×50، 40×25 و 40×12/5 سانتی‌متر به ترتیب با تراکم 5، 10 و 20 بوته در متر مربع) بر صفات مورفولوژیکی، عملکرد، کرک‌های ترش‌حی و ترکیبات فیتوشیمیایی زین گیاه، بر اساس طرح بلوک‌های کامل تصادفی (RCBD) در منطقه سرورسو استان گیلان در سال‌های 01-1400 انجام شد. نتایج نشان داد که تراکم 20 بوته در مترمربع (40×12/5 سانتی‌متر) سبب افزایش 4 برابری صفات مورد بررسی شد و بالاترین مقادیر ارتفاع بوته (34/66 سانتی‌متر)، تعداد شاخه (14)، طول شاخه (9/34 سانتی‌متر)، تعداد برگ (138/33)، شاخص سطح برگ (105 میلی‌مترمربع)، وزن تر برگ‌ها و ساقه‌ها و کل (105/1، 98/5 و 203/6 گرم بر متر مربع)، وزن خشک برگ‌ها و ساقه‌ها و کل (26/1، 24/675 و 50/775 گرم در متر مربع) کلروفیل a، b، کلروفیل کل و محتوای کاروتنوئید (به ترتیب 1/038، 0/653، 1/691 و 0/898 میلی‌گرم در گرم وزن تر)، تراکم کرک‌های ترش‌حی (14 عدد در میلی‌مترمربع)، محتوای اسانس و عملکرد اسانس (به ترتیب 0/633٪ و 0/322 گرم بر مترمربع)، فعالیت آنزیم PAL (10/221 نانومول بر گرم وزن خشک در دقیقه)، فعالیت آنتی اکسیدانی (82/85 درصد)، فنل کل (1/253 میلی‌گرم بر گرم وزن خشک) و فلاونوئید کل (2/781 میلی‌گرم بر گرم وزن خشک) در 20 بوته در مترمربع مشاهده شد. در نتیجه، تراکم 20 بوته در متر مربع (40×12/5 سانتی‌متر) به منظور افزایش عملکرد و تولید تجاری ترکیبات فیتوشیمیایی زین گیاه در منطقه سرورسو توصیه می‌شود.