Overproduction of valuable methoxylated flavones in induced tetraploid plants of *Dracocephalum kotschyi* Boiss

Ali Akbar Zahedi¹, Bahman Hosseini²*, Mohammad Fattahi², Esmail Dehghan³, Hadi Parastar⁴ and Hadi Madani¹

**Abstract**

**Background:** Ploidy manipulation is considered an efficient method to increase production potential of medicinally important compounds. *Dracocephalum kotschyi* Boiss. is an endangered medicinal plant of Iran. Various concentrations of colchicine (0.05, 0.10, 0.20, and 0.50% w/v) were applied to shoot apical meristems of *D. kotschyi* seedlings in two and four-leaf stages to induce tetraploidy.

**Results:** According to the results, 0.5% (w/v) of colchicine can be effective for polyploidy induction in *D. kotschyi*. Putative tetraploids were selected by morphological and microscopic characteristics and their ploidy level was confirmed by flow cytometry analysis and chromosome counting. The chromosome number of original diploid plant was confirmed to be $2n = 2x = 20$ whereas that of the tetraploid plant was $2n = 4x = 40$. Tetraploid and mixoploid plants showed different morphological, physiological and microscopic characteristics from those of diploid counterparts. The total content of flavonoids was increased from 1583.28 in diploids to 1890.07 (μg/g DW) in stable tetraploids.

**Conclusion:** High-Performance Liquid Chromatography with Diode-Array Detection (HPLC–DAD) confirmed over accumulation of methoxylated hydroxyflavones in solid tetraploid plants of *D. kotschyi*.

**Keywords:** *Dracocephalum kotschyi* Boiss; Chromosome counting; Flow cytometry; Tetraploidy; HPLC-DAD; Xanthomicrol

**Background**

*Dracocephalum kotschyi* Boiss, belonging to Labiateae family, is an endemic herbaceous plant and is known as Badrandjboie-Dennaie and Zarrin-Giah (Fattahi et al. 2011; Ghahreman 1987). Recent pharmacological studies have confirmed presence of several methoxylated flavonoids with anti-cancer properties (Jahaniani et al. 2005; Moghaddam et al. 2012) and inhibitory effects on the lectin-induced cellular immune response in this plant (Faham et al. 2008). Its leaf extract has been reported to have antihyperlipidemic (Ebrahim Sajjadi et al. 1998), immunomodulatory (Amirghofran et al. 2000), antinociceptive (Golshani et al. 2004) and cytotoxic (Jahaniani et al. 2005) effects. Aerial parts of *D. kotschyi* plants are sources of valuable flavonoids and essential oils (Ebrahim Sajjadi et al. 1998; Gohari et al. 2003; Monsef-Esfahani et al. 2007; Saeidnia et al. 2007) and its seeds are rich in linolenic, oleic and linoleic acids (Goli et al. 2013).

Flavonoids include over 4000 structurally related compounds existing in nature either as free aglycones or glycosidic conjugates and are generally classified according to their chemical structures into flavones, flavanones, flavonols, flavonoids and anthocyanidins (Middleton et al. 2000). This diversity of structural patterns has made flavonoids a rich source of compounds with potential anticancer properties. Recent pharmacological studies suggested some methoxylated flavonoids (Xanthomicrol and Calycopterin) of *D. kotschyi* with anti-cancer properties (Jahaniani et al. 2005; Moghaddam et al. 2012).

Polyploidy has played an important role in genetic and phenotype diversity as well as plant evolution and breeding (Xing et al. 2011). Induction of artificial polyploidy has been considered as a method for increasing production potential of plants secondary metabolites (Dhawan et al. 1998; Gohari et al. 2003; Monsef-Esfahani et al. 2007; Saeidnia et al. 2007) and its seeds are rich in linolenic, oleic and linoleic acids (Goli et al. 2013).

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**Keywords:** *Dracocephalum kotschyi* Boiss; Chromosome counting; Flow cytometry; Tetraploidy; HPLC-DAD; Xanthomicrol

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and Lavania 1996; Omidbaigi et al. 2010a). Many polyploid lines of plants were created by application of artificial selective breeding, tissue culture, distant hybridization, physiochemical factors, protoplast culture, and somatic hybridization (Song et al. 2012). However, despite considerable research on artificial polyploidy in plants, very few cases of polyploid medicinal plants have been reported (De Jesus 2003; Dehghan et al. 2012; Lavania and Lavania 2005).

Colchicine (\(C_{22}H_{25}NO_6\)), originally extracted from \textit{Colchicum autumnale} (autumn crocus, meadow saffron) is a poisonous alkaloid that is extensively used for induction of polyploidy (Ade and Kumar Rai 2010). Colchicine-induced autotetraploid plants has been reported in several medicinal plants including \textit{Tanacetum parthenium} L. (Saharkhiz 2007), \textit{Artemisia annua} L. (Banyai et al. 2010), \textit{Dioscorea zingiberensis} (Zhang et al. 2010), \textit{Dracocephalum moldavica} L. (Omidbaigi et al. 2010b) and \textit{Hyoscyamus niger} (Dehghan et al. 2012).

Low rate of genetic diversity as well as homogenous population with limited ecological niche in the natural habitat are the main problems in plant breeding (Fattahi 2012). Excessive harvesting of wild plants, limited distribution areas, and a lack of cultivation and domestication are the main reasons that \textit{D. kotschyi} is now listed as an endangered plant (Jalali and Jamzad 1999). Therefore, induction of genetic variation is vital for protection and domestication of this plant.

In this study, we have established a protocol for the induction of tetraploidy in \textit{D. kotschyi} L. for the first time. We also aimed to increase its genetic variation and producing new genetic material for selection of plants with higher production potential of important flavonoid compounds.

**Methods**

**Plant material**

Seeds of \textit{D. kotschyi} were collected from Chalus, Gachsar, Iran. Seeds were carefully soaked in 98% sulfuric acid for 10 min to remove the external germination inhibitors, according to Fattahi et al. (2011) and then were cultured in plastic pots (15-cm diameter), in a mixture media containing soil, leaf mold and sand (1: 1: 2) and placed in a greenhouse at 25 ± 2°C (days) and 17°C (nights), under a 16/8 h photoperiod at 65% relative humidity.

**Induction of tetraploidy in \textit{D. kotschyi}**

In the present study, different concentrations of colchicine (0.0, 0.05, 0.10, 0.20, 0.50% w/v) were applied to shoot apical meristems of two and four-leaf stages plantlets for 48 h using cotton ball method (Shahriari et al. 2009). At the 6\textsuperscript{th} or 8\textsuperscript{th} plant-leaf stages, the plantlets were assessed for the presence of different morphological characteristics from those of diploid control plants including the lengths and widths of stomatal guard cells, plant height and number of leaves and side branches.

**Determination of ploidy level**

**Flow cytometry analysis**

In order to determine DNA ploidy of the putative tetraploids, flow cytometry analysis (FCM) was conducted by using a Partec, PA, flow cytometer equipped with a mercury lamp (Partec, Germany). Nuclei suspension was prepared by chopping a small piece of fresh leaves (about 0.5 cm\(^2\)) in 400 μl of nuclei extraction buffer (Partec PA, Germany). After filtration through a Partec 30 μm Cell trice disposable filter (Partec) 1600 μl of staining solution containing the dye 4-6-diamino-2-4- phenylindole (DAPI, Partec PA, Germany) was added and a minimum of at least 5000 nuclei per sample were measured and histograms of relative DNA fluorescence were obtained by Mode Fit LT 3.1 software (Dehghan et al. 2012).

**Chromosome counting**

After removing external germination inhibitors of seed coats, as described by Fattahi et al. (2011), seeds were sterilized with 70% ethanol and sodium hypochlorite for 30 s and 20 min respectively, then washed three times by sterile distilled water for 30 min and were placed on wet filter paper in petri dishes and incubated at 25°C until they germinate. Germinated seeds with minimum root length of 2 cm, were pretreated in a saturated solution of 8-hydroxyquinoline 0.02 M at 4°C for 4 h and then at room temperature in dark for 1 h. The samples were then fixed in cold freshly prepared Carnoy’s fixative (ethanol and glacial acetic acid (3: 1)) at room temperature for 3 h and stored in 70% ethanol at 4°C for 12 h. After hydrolyzing by 1 N HCl at 60°C for 10 min, seeds were incubated in Aceto-Iron-Hematoxylin solution at 60°C for 45 min, and squashed on slides in 45% acetic acid-glycerol (9: 1). The chromosomes were observed with the light Olympus microscope (BH2) at 100X under oil immersion objective lens, and the best metaphase views were photographed with digital camera (Canon, Malaysia).

**Evaluation of anatomical and physiological characteristics**

**Stomata characteristics**

Five 8 month-old diploid, mixo- and tetraploid plants were randomly selected and stomatal measurements were conducted on them by. The nail varnish technique (Hamill et al. 1992). Microscopic studies were done under the light microscope (Olympus BH2) at 40X magnification. To determine their length and width, stomata on 25 randomly chosen microscopic fields were counted for each leaf. Counts were taken twice per leaf at random locations across the surface in the unit of 0.15 mm\(^2\).

**Chlorophyll determinations**

The chlorophyll content (a, b, total) of the leaves was evaluated by previously established extraction method
(Arnon 1949). In this method extraction was done by acetone and optical density was read at 645 and 663 μm using a spectrophotometer and finally pigments quantity were calculated by using the formula reported by Arnon (1949).

**Flowering time and fruit set**

Flowering time was the time between seed sowing and the beginning of full flowering (80% flowering) and fruit set was the time between seed sowing and the beginning of fruit setting time among plants with different ploidy levels.

**Comparison of selected morphological treats**

Ten plants of each ploidy levels including diploids, tetraploids and chimeras (mixoploids) were selected for studying leaf number (L. N.), plant height (P. H.), lateral branches number (L. B. N.), stem diameter (mm) (S. D.), inflorescence length (In. L), No. of flower in inflorescence (N. F.)

![Figure 1 Flow cytometric histograms](image)

Figure 1 Flow cytometric histograms [A) 2x, B) 2x-4x, C) 4x], and root tip chromosome number of *D. kotschyi* in metaphase [D) diploid (2n = 2x = 20) and E) tetraploid (2n = 4x = 40)].
In.), flower height (F. H.) (cm), leaf length (L. L.) (cm), leaf width (L. W.) (cm), leaf diameter (L. D.) (mm).

Extraction of leaf surface flavonoids
Dried leaf material (200 mg) from three individuals of each population was extracted in separate vials containing 10 ml diethyl ether for 24 h as described in the literature (Fattahi et al. 2013; Vieira et al. 2001). In order to prevent the evaporation of diethyl ether, the vials were kept closed and the extraction was performed in a cold room. After 24 h, the extracts were poured into clean vials and leaves were rinsed with another 5 ml diethyl ether, which were added to the initial extracts. The diethyl ether was incubated in an extraction cabinet until complete evaporation of solvent. After adding 10 ml of 80% methanol to remain solid material, the extracts were filtered (0.45 μm pore size) into clean vials. 1.5 ml of prepared solution was transferred into a clean injector auto HPLC vials for automatic injection.

HPLC–DAD-ESI-MS conditions
Chromatographic separation was performed using a Knauer HPLC system equipped with a Waters Symmetry Shield column (C8, 3.9 × 150 mm) at a flow rate of 1 ml/min. The column oven temperature was set at 30°C. The mobile phase consisted of an isocratic mixture of 60% of A (2% acetic acid in water) and 40% of B (acetoniitrile) during 32 min (Fattahi et al. 2013). The DAD was set at 215 nm to provide real time chromatograms, and UV spectra from 190 to 400 nm were recorded for plant component identification. An LC/MSD-TOF (2006) (Agilent Technologies) instrument was used for compound mass detection. Electrospray ionization (ESI-MS) was performed in both positive and negative modes, at a fragmentation voltage of 215 V (positive) and −175 V (negative). Drying gas temperature was 300°C and drying gas (N2) flow was 7.01 L/min, with a nebulizer pressure of 15 psi. Capillary voltages were 3.5 kV (negative) and 4 kV (positive).

Statistical analysis
This study was done as factorial base experiment in completely randomized design with two factors (different concentrations of colchicine and growth stages of treated plantlets) and ten replicates. Data analyses were carried out with the SAS 9.1 for windows software package (Statistical). Means were compared using Tukey’s Honestly Significant Difference (HSD) at the 1% and 5% probability levels.

Results and discussion
Determination of ploidy level
Flow cytometry analysis
Flow cytometry analysis of diploid control plants showed a peak at channel 30, related to the G1 of diploid plants (Figure 1a), while the corresponding peak of tetraploids was at about 60 (Figure 1c). Mixoploid plants possess both peaks of 30 and 60 indicating the presence of diploid and tetraploid nuclei (Figure 1b).

Chromosome counting
Chromosome counting is the most direct method of ploidy analysis. In line with the flow cytometric data, chromosome counts in tetraploids demonstrated that these plants doubled their diploid chromosome number to 2n = 4x = 40 (Figure 1). To our knowledge there is no report on chromosome counting of D. kotschyi and this is the first report on chromosome number of this important medicinal plant.

Survival rate and growth of colchicine-treated plantlets
The effect of different colchicine concentrations on the survival rate of plants was assessed 30 days after treatment. The survival rate of treated seedlings decreased with increase of colchicine concentration. The highest number of tetraploid plants (12%) and mortality (56%) were recorded when applying 0.5% colchicine to shoot apical meristems of seedlings (Table 1). Only 47% of colchicine-treated plants survived and continued their growth. Colchicine is an antimitotic agent that inhibits the formation of spindle fibers and effectively arrests mitosis at the metaphase stage leading to polyploidy and, as such, has been widely used to induce polyploidy in plant breeding (Abdoli et al. 2013). It has been reported that colchicine results in low growth rate of Astragalus memberanaceus, possibility due to physiological damage and a reduced rate of cell division (Chen and Gao 2007).

The result showed a lower growth rate of tetraploid plants than their diploid counterparts. Colchicine treatments at concentrations of 0.5% and 0.1% (w/v) resulted in induction of 28% and 12% mixoploids, respectively (Table 1).

| Colchicine (%) | No. of observed plant | Survival rate (%) | Ploidy level (%) |
|---------------|-----------------------|-------------------|------------------|
|               |                       |                   | Diploid Mixoploid Tetraploid |
| Control       | 50                    | 100               | 50 (100) 0 0    |
| 0.05          | 50                    | 76                | 29 (58) 5 (10) 4 (8) |
| 0.1           | 50                    | 68                | 26 (52) 4 (12) 2 (4) |
| 0.2           | 50                    | 64                | 25 (50) 6 (8) 3 (6) |
| 0.5           | 25                    | 44                | 1 (4) 7 (28) 3 (12) |
| Total         | 225                   |                   | 131 22 12       |

Table 1 The effect of different concentrations of colchicine treatment on survival rate and ploidy induction of D. kotschyi
Figure 2  Comparison of morphological characteristics among diploid, mixoploid and tetraploid plants of *D. kotschyi*. plant stomata [A) 2×, B) 2×-4×, C) 4×], leaf [D) 2×, E) 2×-4×, F) 4×], Plant morphology [G) 2×, H) 2×-4×, I) 4×], glandular trichome [J) 4×, K) 2×, L) 2×-4×], and plant flower [1) diploid, 2) mixoploid, 3) tetraploid].
Table 2 Some selected anatomical and physiological characteristics of diploid, mixoploid and tetraploid plants of *D. kotschyi*.

| Density (no./mm²) | Stomata | Chlorophyll (mg/g FW) | Trichome density | Planting to flowering time (month) | Fruit set |
|-------------------|----------|-----------------------|------------------|-----------------------------------|-----------|
|                   | Density  | Length (µm) | Width (µm) | a | b | Total |                      |                        |                      |
|                   | (no./mm²) |            |            |   |   |       |                      |                        |                      |
| 2x                | 202.8 ± 28.8 a | 20.01 ± 2.42 b | 5.63 ± 1.06 b | 0.55 ± 0.01 c | 0.246 ± 0.03 c | 0.80 ± 0.03 c | Low | 6 | High |
| 2x + 4x           | 103.7 ± 13.3 b | 33.02 ± 3.18 a | 11.64 ± 1.77 a | 1.01 ± 0.05 b | 0.337 ± 0.03 b | 1.35 ± 0.07 b | Medium to high | 7 | Low |
| 4x                | 68.8 ± 14.0 c  | 36.42 ± 5.21 a | 12.27 ± 1.64 a | 1.15 ± 0.05 a | 0.658 ± 0.06 a | 1.81 ± 0.11 a | High | 8 | Low |

Fertility and seed set was lower in induced autotetraploids than their parental diploid plants (Table 2). Similar anatomical and structural changes were also reported in other plants such as *Zinger officinalis* Roscoe (Adaniya and Shirai 2001) and *Dracocephalum moldavica* L. (Omidbaigi et al. 2010b).

**Comparison of selected morphological treats**

Leaf length (L. L.) and thickness (L. D.) were significantly affected by induction of tetraploidy (P < 0.01), however no significant difference was observed between leaf width (L. W.) of different ploidy levels (Figure 2; Table 3). Tetraploid and mixoploid plants showed a lower L. L. than that of diploid plants (Table 3). Similar results have been reported in other studies (Roy et al. 2001; Viehmannová et al. 2012).

Plant growth indices including plant height (P. H.), leaf number (L. N.), lateral branch numbers (L. B. N.) and stem diameter (S. D.) were significantly affected by ploidy level (P < 0.01). While P. H., L. N. and L. B. N. decreased, the S. D. increased in mixoploid and tetraploid plants (Table 3).

Analysis of variance showed a significant increase in corolla length caused by induction of tetraploidy (P < 0.01). The highest corolla length (3.33 cm) was recorded in tetraploids while it was 2.33 cm for diploid plants (Figure 2; Table 3). Similar results have been previously reported for *Gerbera jamesonii* Bolus cv. Sciella (Abd El-Naby et al. 2012; Gantait et al. 2011). It seems that higher DNA content and larger cell size in tetraploid plants is associated with the late flowering time (Gantait et al. 2011; Stebbins 1984). There was also a significant difference

Table 3 Morphological characteristics of diploid, mixoploid and tetraploid plants of *D. kotschyi*.

| Ploidy | Vegetative stage | Flower stage |
|--------|------------------|--------------|
|        | L. N. | P. H. | L. B. N. | S. D. (mm) | In. L. (cm) | N. F. In. | F. H. (cm) | L. L. (cm) | L. W. (cm) | L. D. (mm) |
| 2x     | 247.8 ± 48.89 a | 63.90 ± 10.99 a | 55.10 ± 10.24 a | 1.376 ± 0.23 b | 8.33 ± 3.05 b | 13.33 ± 1.52 b | 2.33 ± 0.057 b | 2.10 ± 0.26 a | 1.73 ± 0.24 a | 0.302 ± 0.016 c |
| 2x + 4x| 91.3 ± 47.47 b | 26.20 ± 11.04 b | 12.90 ± 9.20 b | 1.718 ± 0.18 a | 17.50 ± 0.5 a | 14.5 ± 0.5 b | 3.30 ± 0.435 ab | 1.52 ± 0.48 b | 1.62 ± 0.38 a | 0.412 ± 0.042 b |
| 4x     | 70.3 ± 36.90 b | 20.55 ± 11.09 b | 8.50 ± 5.44 b | 1.879 ± 0.16 a | 17.83 ± 0.76 a | 19 ± 1 a | 3.33 ± 0.208 a | 1.50 ± 0.46 b | 1.70 ± 0.33 a | 0.481 ± 0.053 a |

Different letters within the column indicate a highly significant difference of mean (±SD) tested by Tukey's Studentized Range (HSD) at p ≤ 0.01. The data were analyzed from 10 replications of each treatment. L. N.: Leaf number; P. H.: Plant height; L. B. N.: lateral branches number; S. D.: Stem diameter (mm); In. L.: Inflorescence length; N. F. In.: No. of Flower in inflorescence; F. H.: Flower height (cm); L. L.: Leaf length (cm); L. W.: Leaf width (cm); L. D.: Leaf diameter (mm).
in Inflorescence length (In. L.) and number of flowers in inflorescence (N. F. In.) between diploid and tetraploid plants (Table 3).

Identification and quantification of flavonoids by HPLC-DAD-ESI-MS

In order to identification first of all, molecular ion in [M-H] ions and UV spectra were provided for all compounds (Table 4). According to this table eleven compounds including luteolin-7-O-β-D-glucopyranoside, apigenin 7-O-glucoside (cosmosisin), rosmarinic acid, luteolin 3′-O-β-D-glucuronide, luteolin, apigenin, cirsimaritin, isokaempferide, penduletin, xanthomicrol and calycopterin (Additional file 1) identified by a comparison of the TR, UV λ<sub>max</sub> and [M-H] ions of the <i>D. kotschyi</i> peaks with those of known standards (compounds 3, 5, 6, 7, 8, 10 and 11) and quantified by its standard calibration curve and for the others (compounds 1, 2, 4 and 9) identification was done by comparing spectral data reported in previous work (Greenham et al. 2003; Fattahi et al. 2013) and quantified by xanthomicrol calibration curve.

The analytical method applied in this study lending to chromatographic peaks separation shown in Figure 3. In this study quantity of phenolic and flavonoid compounds shown in Table 4. Although the content of hydroxyflavones (apigenin and cosmosisin) was not significantly changed by tetraploidy induction, the results showed that the content of methoxylated flavones (penduletin, xanthomicrol and calycopterin) were increased in tetraploid plants (Table 4). As the ploidy level increased, the percentage of calycopterin, xanthomicrol and calycopterin compared with their diploid parents, isokaempferide decreased. The tetraploid plants could produce about 2 and 21 times higher xanthomicrol and calycopterin compared with their diploid parents, respectively.

Artificial polyploidy generally enhances the concentration of secondary metabolites (Lavania 2013). The mechanism to explain these changes relies on the assumption that chromosome doubling induces an increase in cell size the amount of genetic substance within the nuclei and the nuclear membrane decreases the chromatin is in contact with the nuclear membranes, thereby enhancing gene activity and photosynthetic rate on a per cell basis (Lavania and Lavania 2005; Levin 2002). It may be that with more chromatin coming into contact with the nuclear membrane due to the lower ratio of nuclear membrane to gene dosage after polyploidization, gene activity is elevated (Levin 2002). Changing the metabolic profile in autopolyploid plants by a simple duplication of the basic genome was interpreted as a cause of an alteration in the mechanism(s) which regulates the biosynthesis of individual compounds (Dehghan et al. 2012). Lipophilic flavonoid aglycones have a limited distribution in plants compared to their water-soluble glycosides. As they usually accumulate on the leaf surface, and are found in glandular trichomes or are extruded through the cuticle, they are known as surface or external flavonoids. Aglycone flavonoids, especially in the highly methylated form, accumulate in the Lamiaceae family (Tomás-Barberán and Wollenweber 1990). Therefore another possible reason for flavonoid methoxy enhancement can be because of high glandular trichomes structure in tetraploid plants. To our knowledge there is only a few reports related to the effects of ploidy level on accumulation of medicinally important flavonoids. Griesbach and Kamo (1996) reported increasing of the major flavonols

Table 4 Retention time, maximum UV absorption, and molecular weight, flavonoid contents (μg/g DW) in the di-, mix- and tetraploid plants and identification methods of phenolic and flavonoid compounds of <i>D. kotschyi</i>

| Peak | Compound name | RT (min) | UV (nm) | (m/z) [M-H]/ [M-H]<sup>+</sup> | Diploid | Mixploid | Tetraploid | Identification methods |
|------|--------------|----------|---------|--------------------------------|---------|----------|-----------|------------------------|
| 1    | Luteolin-7-O-β-D-glucopyranoside | 1.66 | 205, 255–266, 348 | 447.09/- | 64.92 | 16.63 | 93.82 | UV, MS, Ref. |
| 2    | Apigenin 7-O-glucoside (cosmosisin) | 9.20 | 233, 269 | 431.10/- | 32.72 | 28.59 | 26.63 | UV, MS, Ref. |
| 3    | Rosmarinic acid | 16.65 | 234, 290, 329 | 359.07/- | 938.82 | 963.96 | 952.33 | UV, MS, Ref., St. |
| 4    | Luteolin 3′-O-β-D-glucuronide | 17.11 | 236, 267, 340 | 461.07/- | 246.48 | 246.27 | 252.05 | UV, MS, Ref. |
| 5    | Luteolin | 20.13 | 232, 267, 344 | 285.04/- | 20.96 | 15.78 | 24.97 | UV, MS, Ref., St. |
| 6    | Apigenin | 22.46 | 232,268,337 | 269.04/- | 43.15 | 42.26 | 35.86 | UV, MS, Ref., St. |
| 7    | Cirsimaritin | 23.2 | 276, 232,234 | 313.07/- | 45.87 | 42.38 | 36.48 | UV, MS, Ref., St. |
| 8    | Isokaempferide | 24.68 | 232, 256, 350 | 299.05/- | 54.86 | 31.57 | 54.35 | UV, MS, Ref., St. |
| 9    | Penduletin | 26.36 | 234, 272, 340 | 343.08/345.09 | 44.69 | 16.33 | 80.21 | UV, MS, Ref., St. |
| 10   | Xanthomicrol | 27.7 | 232, 282, 333 | 343.08/345.09 | 81.75 | 31.09 | 140.17 | UV, MS, Ref., St. |
| 11   | Calycopterin | 30.24 | 233, 278, 337 | 373.09/375.10 | 9.06 | 61.41 | 193.20 | UV, MS, Ref., St. |

RT, retention time; Ref or reference, means that compound previously reported by other researchers.
(quercetin-3-sophoroside) and decreasing of the minor flavonols (quercetin-3,7-diglucoside) in colchicine treated Petunia 'Mitchell'. In another study Abdoli et al. (2013) reported a 71% and 45% increase in cholorgenic acid content and cichoric acid compounds of tetraploid leaves of Echinacea purpurea L., respectively. Similar results were also reported for different secondary compounds including apigenin (Švehlíková and Repčák 2000), artemisinin (Banyai et al. 2010), Phenylpropanoid content (Caruso et al. 2011) and scopolamine (Dehghan et al. 2012).

**Conclusion**

This study is the first report on induction and establishment of autotetraploid plants of *D. kotschyi*. The highest applied concentration (0.5% v/w) of colchicine, results in the induction of highest number of stable tetraploid plants. Induction of tetraploidy significantly affected different morphological, microscopic, physiological and biochemical characteristics of *D. kotschyi*. These changes suggested ploidy manipulation as a rapid and effective method for enhancing genetic diversity and metabolite production of *D. kotschyi*.

**Additional file**

Additional file 1: Chemical structure of compounds in *Dracocephalum kotschyi* L. corresponding to 1) Luteolin-7-O-β-D-glucopyranoside; 2) Apigenin 7-O-glucoside (cosmosin); 3) Rosmarinic acid; 4) Luteolin 3′-O-β-D-glucuronide; 5) Luteolin; 6) Apigenin; 7) Cirsimaritin; 8) Isokaempferide; 9) Penduletin; 10) Xanthomicrol; 11) Calycopterin.
Abbreviations
HPLC–DAD: High-performance liquid chromatography with diode-array detection; FCM: Flow cytometry; LN: Leaf number; PH: Plant height; LBN: Lateral branches number; SD: Stem diameter; InL: Inflorescence length; FH: Flower height; LL: Leaf length; LW: Leaf width; LD: Leaf diameter.

Competing interests
The authors declare that they have no competing interests.

Authors’ contributions
AAZ, HM and BH responsible for D. kotschyi cultivation, sampling, colchicines treatments, Chromosome counting and Statistical analysis presented in this paper. MF and HP responsible for Extraction of flavonoids and HPLC-DAD-ESI-MS analysis. ED is responsible for Flow cytometry analysis. All authors read and approved the final manuscript.

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Abd El-Naby ZM, Mohamed NA, Radwan KH, El-Kishnawy DA (2012) Colchicine induction of tetraploidy in Egyptian clover genotypes. J Am Sci 8(10):221–227
Abdol M, Moenkin A, Badi HN (2013) Morphological, physiological, cytological and phytochemical studies in diploid and colchicine-induced tetraploid plants of Erinacea purpurea L. Acta Physiol Plant 1:9–10. doi:10.1117/1378:013-1242-9
Adaniyi S, Shari D (2001) In vitro induction of tetraploid ginger (Zingiber officinale Roscoe) and its pollen fertility and germinability. Sci Hortic 88(4):277–287
Ade R, Kumar Rai M (2010) Review. colchicine, current advances and future prospects. Nusant Biosci 2(9):90–96
Aina O, Quesenbery K, Gallow M (2012) In vitro induction of tetraploids in Arachis pasquiniana. Plant Cell Tiss Organ Cult 1:8–9. doi:10.1007/s11240-012-0191-0
Aminizadeghooz Z, Azadbakht M, Karimi MH (2008) Evaluation of the immunomodulatory effects of five herbal plants. J ethnopharmacol 72 (1):167–172
Amiri S, Kazemitabar S, Ranjbar G, Azadbakht M (2010) The effect of trifluralin and colchicine treatments on morphological characteristics of Jinnsoweed (Daturus Standingum L). Trakia J Sci 8(4):47–61
Amon DJ (1949) Copper enzymes in isolated chloroplasts. polyphenoloxidase in Beta vulgaris. Plant physiol 24(1):1–15
Banyai W, Sangthong R, Karaket N, Inthima P, Mili M, Supapubwatavat K (2010) Overproduction of artemisinin in tetraploid Artemisia annua L. Plant biotechnol 27(5):427–433
Caruso I, Lepore L, De Tommasi N, Dal Piaz F, Frusciante L, Aversano R, Gagliardi F (2011) Secondary metabolite profile in induced tetraploids of wild Salvia communis L and Salvia officinalis L. Z. Naturfor 82(12):2226–2237
Chen L-L, Gao S-L (2007) In vitro induction of tetraploid induction and generation of tetraploids from mixoploids in Astragalus membranaceus (Bge.) Bunge. J. Plant Cell Tiss Organ Cult 110(1):35–44
Dhawan O, Lavinia U (1996) Enhancing the productivity of secondary metabolites via induced polyploidy: a review. Euphytica 87(2):81–89
Ebrahìm Sajjadi S, Movahedian A, Atar A, Yekaïa A (1998) Antihyperlipidemic effect of hydroalcoholic extract, and polyphenolic fraction from Dracocephalum kotschyi Boiss. Pharm Acta Helv 73(3):167–170
Faham N, Javidinia K, Bahmani M, Amrighofian Z (2008) Calycoperitin, an immunoinhibitory compound from the extract of Dracocephalum kotschyi. Plant Physiol Res 22(9):1154–1158
Fattahi M (2012) Evaluation of morphological, phytochemical diversity and hairy root production in Dracocephalum kotschyi Boiss. University of Tehran, Iran, Dissertation
Fattahi M, Nazeri V, Sefidkon F, Zamani Z, Palazon J (2011) The effect of pre-sowing treatments and light on seed germination of Dracocephalum kotschyi Boiss: An endangered medicinal plant in Iran. Hort Environ Biotechnol 52(6):559–566
Fattahi M, Nazeri V, Torras-Claveria L, Sefidkon F, Cusido RM, Zamani Z, Palazon J (2013) Identification and quantification of leaf surface flavonoids in wild-growing populations of Dracocephalum kotschyi by LC–DAD–ESI-MS. Food Chem 141(1):139–146, http://dx.doi.org/10.1016/j.foodchem.2013.03.019
Gantatt S, Mandal N, Bhattacharyya S, Das PK (2011) Induction and identification of tetraploids using in vitro colchicine treatment of Gerbera jamesonii Bolus cv. Scilla. Plant Cell Tiss Organ Cult 106(3):485–493
Ghahreman A (1987) Flore de iranica en couleur naturelle, faculty of science. University of Tehran, p 432
Gohari AR, Saeidinia S, Matsuo K, Uchiyama N, Yagura T, Ito M, Kuchi F, Honda G (2003) Flavonoid constituents of Dracocephalum kotschyi growing in Iran and their trypanocidal activity. Nat Med 57(6):250–252
Goli SAH, Sahafi SM, Rashidi B, Rahimmalek M (2013) Novel oilseed of Dracocephalum kotschyi with high n-3 to n-6 polysaturated fatty acid ratio. Ind Crop Prod 43:188–193
Golshani S, Karmakhi F, Monsef-Esfahani HR, Abdollahi M (2004) Anticoagulant effects of the essential oil of Dracocephalum kotschyi in the mouse bleeding test. J Pharm Pharmac 71(7):76–79
Greenham J, Harborne JB, Williams CA (2003) Identification of lipophilic flavones and flavonols by comparative HPLC, TLC and UV spectral analysis. Phytochem Anal 14(2):100–118
Griesbach R, Kamo K (1996) The effect of induced polyploidy on the flavonoids of Petunia ‘Mitchell’. Phytochem 42(2):361–363
Hamil S, Smith M, Dodd W (1992) In vitro induction of banana autotetraploids by colchicine treatment of micropropagated diploids. Aust J Bot 40(6):887–896
Hodgson J, Sarraf M, Jali A, Diaz S, Montserrat-Marti G, Palmer C, Gerabolini B, Pierce S, Hamzeeh B, Asiryi A (2010) Stomatol vs. genome size in angiosperms: the somatic tail wagging the genomic dog? Ann Bot 105(4):573–584
Jahanian F, Ebahish Ni, Rahbar-Roshandel N, Mahmoudian M (2005) Xanthocrolim is the main cytoxic component of Dracocephalum kotschyi and a potential anti-cancer agent. Phytomed 12(1):1581–1592
Jalali A, Izadmard Z (1999) Red data book of Iran: research institute of forests and rangelands Iran Tehran., p 748
Lavinia UC (2013) Polyploidy, body size, and opportunities for genetic enhancement and fixation of heterozygosity in plants. Nuclear 56(1):1–6
Lavinia U, Lavinia U (2005) Genomic and ploidy manipulation for enhanced production of phyto-pharmaceuticals. Plant Genet Resour 3(2):170–177
Levin DA (2002) The role of chromosomal change in plant evolution. Oxford University Press, USA
Li W, Sxiang Z, Zhijian G (2002) In vitro culture of tetraploids of Aloe vera L. Acta Hortic 592(1):76–178
Lin X, Zhou Y, Zhang J, Lu X, Zhang F, Shen Q, Wu S, Chen Y, Wang T, Tang K (2011) Enhancement of artemisinin content in tetraploid Artemisia annua plants by modulating the expression of genes in artemisinin biosynthetic pathway: Biotechnol and Appl Biochem 58(5):50–57
Majdy M, Karimzadeh G, Malboobbi MA, Omidbaki R, Mirzaghaderi G (2010) Induction of tetraploidy to feverfew (Tanacetum parthenium Schulz-Bip): morphological, physiological, cytological, and phytochemical changes. HortSci 45(1):16–21
Middleton E, Kandaswamy C, Theoharides TC (2000) The effects of plant flavonoids on mammalian cells: implications for inflammation, heart disease, and cancer. Pharmacol Rev 52(4):675–753
Moghadam G, Ebrahim SA, Rahbar-Roshandel N, Forouamadi A (2012) Antiproliferative activity of flavonoids: influence of the sequential methoxylation state of the flavonoid structure. Phytother Res 26(10):1302–1028
Monsef-Esfahani H, Karmakhi F, Nickavar B, Abd K, Faramarzi M (2007) The volatile constituents of Dracocephalum kotschyi oils. Chem Nat Compd 43(1):40–43

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Omidbaigi R, Mirzaee M, Hassani M, Moghadam M (2010a) Induction and identification of polyploidy in basil (Ocimum basilicum L.) medicinal plant by colchicine treatment. Int J Plant Prod 4(2):87–98
Omidbaigi R, Yavari S, Hassani ME, Yavari S (2010b) Induction of autotetraploidy in dragonhead (Dracocephalum moldavica L.) by colchicine treatment. J Fruit Ornam Plant Res 18(1):23–35
Rêgo M, Rêgo E, Bruckner C, Finger F, Otoni W (2011) In vitro induction of autotetraploids from diploid yellow passion fruit mediated by colchicine and oryzalin. Plant Cell Tiss Organ Cult 107(3):451–459
Roy A, Leggett G, Koutoulis A (2001) In vitro tetraploid induction and generation of tetraploids from mixoploids in hop (Humulus lupulus L.). Plant Cell Rep 20(6):499–495
Saeidnia S, Gohari A, Hadjikhoondi A, Shafiee A (2007) Bioactive compounds of the volatile oil of Dracocephalum kotschyi: Zeitschrift für Naturforschung C, A. J biosci 62(11):793–796
Saharkhiz MJ (2007) The effects of some environmental factors and ploidy level on morphological and physiological characteristics of feverfew (Tanacetum parthenium L.) medicinal ornamental plant. Dissertation, Tarbiat Modares University, Iran
Shahriari F, Dehghan E, Farsi M (2009) Tetraploid induction of Hyoscyamus muticus L. using colchicine treatment. In: Proceedings of the 14th Australasian plant breeding, 11th SABRAO conference., Australia
Song C, Liu S, Xiao J, He W, Zhou Y, Qin Q, Zhang C, Liu Y (2012) Polyploid organisms. Sci China Life Sci 55(4):301–311
Stebbins G (1984) Polyploidy and the distribution of the arctic-alpine flora: new evidence and a new approach. Bot Helv 94(1):1–13
Švehlíková V, Repčák M (2000) Variation of apigenin quantity in diploid and tetraploid Chamomilla recutita (L.) Rauschert. Plant Biol 2(4):403–407
Tang Z-Q, Chen D-L, Song Z-J, He Y-C, Cai D-T (2010) In vitro induction and identification of tetraploid plants of Paulownia tomentosa. Plant Cell Tiss Organ Cult 102(2):213–220
Tomás-Barberán FA, Wollenweber E (1990) Flavonoid aglycones from the leaf surfaces of some Labiatae species. Plant Syst Evol 173(3):109–118. doi:10.1007/bf00940856
Urwin NA, Horsnell J, Moon T (2007) Generation and characterisation of colchicine-induced autotetraploid Lavandula angustifolia. Euphytica 156(1–2):257–266
Viehmannová I, Trávníčková M, Špatenková E, Črná M, Trávníček P (2012) Induced polyploidization and its influence on yield, morphological, and qualitative characteristics of microtubers in Ullucus tuberosus. Plant Cell Tiss Organ Cult 108: doi:10.1007/s11240-011-0076-7
Vieira RF, Grayer RJ, Paton A, Simon JE (2001) Genetic diversity of Ocimum gratissimum L. based on volatile oil constituents, flavonoids and RAPD markers. Biochem Syst Ecol 29(3):287–304. doi:10.1016/s0305-1978(00)0062-4
Xing S-H, Guo X-B, Wang Q, Pan Q-F, Tian Y-S, Liu P, Zhao J-Y, Wang G-F, Sun X-F, Tang K-X (2011) Induction and flow cytometry identification of tetraploids from seed-derived explants through colchicine treatments in Catharanthus roseus (L.) G. Don. J Biomed Biotechnol 1:10, doi:10.1155/2011/793198
Zhang X-Y, Hu C-G, Yao J-L (2010) Tetraploidization of diploid Dioscorea results in activation of the antioxidant defense system and increased heat tolerance. J plant physiol 167(2):88–94

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