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Original Article

Effect of fertilisation and harvest period on polar metabolites of *Calendula oficcinalis*

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ABSTRACT

The present study evaluated the chemical profile of polar extracts of *Calendula officinalis* L., Asteraceae, that were grown under different cultivation conditions: chemical fertilisation, organic fertilisation and mulching. Furthermore, we investigated metabolite variations during plant development by comparing the metabolites from harvested plants at 60 and 120 days after planting. We used HPLC-DAD-MS/MS to tentatively identify metabolites. In total, we identified seven known compounds: five flavonoid glycosides and two caffeoylquinic acids derivatives. There were no statistically significant differences in the expression of metabolites from plants grown under the examined soil treatments. However, five substances varied according to harvest time, suggesting that the biosynthesis of polar metabolites of *Calendula officinalis* is not affected by changes in soil composition. Therefore, this plant could represent a source for phytomedicines with a constant content of polar metabolites.

Introduction

Calendula officinalis L., Asteraceae, is a fast-growing herb that originated in southern Europe. It is harvested throughout the world, and its pharmaceutical uses are described in many Pharmacopeias. The phytochemistry of *Calendula officinalis* has been extensively studied, and it mainly consists of triterpenes, flavonoids and phenolic acids (Re et al., 2009). This species exhibits several biological activities, including anti-oxidant, cytotoxic, photoprotective and anti-inflammatory activities (Ukiya et al., 2006; Fonseca et al., 2010).

Although many reports have focused on the bioactivity triterpenic alcohols isolated from apolar extracts (Wilkomirsky, 1985), *C. officinalis* hydroalcoholic extracts resemble folk medicines that are frequently used for wound healing. Phytochemical studies indicate that these polar extracts primarily consist of flavonoid glycosides and chlorogenic acids, such as rutin, narcissin and quercetin (Leach, 2008).

The chemical profile of plants frequently varies in response to genetic factors and changes in the environment, such as seasonality, soil nutrients, harvest conditions, temperature, UV radiation and herbivores (GobboNeto and Lopes, 2007; Pavarini et al., 2012). Among these factors, soil nutrients clearly affect plant development, and nitrogenated fertilisers increase the yield of crop production. However, the influence on the secondary metabolism is controversial. For example, higher nitrogen levels in soil increased indole alkaloid content in *Tabernaemontana pachysiphon*, Apocynaceae, whereas it

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decreased the pyrrolizidine alkaloid content in *Senecio jacobaea,* Asteraceae (Höft, 1996; Hol, 2003).

Soil coverage is another important factor for the harvest of medicinal plants, as it reduces water loss from the soil and stabilises surface temperature, which increases the growth of plants root systems. It has also been suggested that soil coverage could influence the chemical profile of plants chemical. Heiska et al. (2005) observed that the use of plastic mulch increased the yield of salicylates in *Salix myrsinifolia* by suppressing the weed effect and enhancing soil temperature and water retention.

In this study, we assessed the influence of fertilisation and mulching on polar metabolites. *C. officinalis* samples were treated with organic and chemical fertilisers, and we semi-quantitatively evaluated the variances of each compound identified by HPLC-MS/MS. Moreover, we studied the effect of dead grass mulch and the harvest period in relation to flavonoid and chlorogenic acid biosynthesis.

Materials and methods

Harvesting conditions

The experiments were conducted between June and December of 2007 in the experimental cultivation area of the University of Ribeirão Preto, in Ribeirão Preto, state of São Paulo, Brazil. Its coordinates are 21°10'42"S and 42°48'24"W. Its altitude is 535 m above sea level. The field was kept fallow for a year. A voucher species was deposited in the Herbarium of the University of Ribeirão Preto (number HPMU 1326). Seeds of the *Calendula officinalis* L., Asteraceae, cultivar "Bonina Dobrada" (Isla) were placed in a plastic tray containing a mixture of sand:organic substrate:soil in the proportion of 1:1:1. When the seedlings reached 15 cm, they were replanted in the experimental field and covered with Sombrite®. The plants were distributed randomly with 0.3 m between plants and 0.5 m between rows. Individuals were protected from animals and watered daily. During the experiment, the temperature varied between 13 and 33 °C, and the average precipitation was 523 mm.

Soil analyses (20 cm deep) revealed the following characteristics: Macronutrients: pH = 4.9; p = 45 mg dm^{-3}; Ca = 21 mmolc dm^{-3}; Mg = 6 mmolc dm^{-3}; K = 1.4 mmolc dm^{-3}; Al = 0 mmolc dm^{-3}; H + Al = 52 mmolc dm^{-3}; organic matter = 32 g dm^{-3}; V = 35%; Micronutrients: B = 0.23 mg dm^{-3}; Cu = 4.2 mg dm^{-3}; Fe = 29 mg dm^{-3}; Mn = 6.1 mg dm^{-3}; Zn = 1.5 mg dm^{-3}; Grain size: Total sand = 217 g kg^{-1}; Coarse sand = 140 g kg^{-1}; Fine sand = 77 g kg^{-1}; Silt = 600 g kg^{-1}; and Clay = 184 g kg^{-1}.

Experimental design

We aimed to evaluate the influence of different cultivation methods on the biosynthesis of polar constituents in *C. officinalis*. We used a randomised block design with 6 replicates in a factorial design (3 X 2), resulting in six treatments and 36 samples. Plants were divided in two groups: those harvested between 60 to 120 days of sowing and those harvested between 121 to 180 days after sowing. Each sample was composed of 25 plants that were dried and milled and were 1.5 m X 3.0 m (4.5 m^2) in size. The treatments are summarised in Table 1.

Nitrogen was added as urea in two equal doses at the beginning of planting and 30 days after planting. Phosphorus was applied as P_2O_5 once during planting. Cattle manure was added eight days after replanting in the experimental field. *Paspalum notatum* was previously harvested, dried and milled, and it was applied during replanting. The mulch was applied as a 5 cm layer above the soil.

The effect of seasonality on the expression of different metabolites was also assessed. Eighteen samples were analysed at four different times after sowing: 60-90 days; 91-120 days; 121-150 days and 151-180 days in the same cultivation conditions.

Table 1
Cultivation methods.

Treatment	Chemical Fertilisation (kg.ha^{-1})[a]	Organic Fertilisation (t.ha^{-1})[b]	Mulching[c]
1	100N / 50P	0	-
2	100N / 50P	0	+
3	0	50	-
4	0	50	+
5	0	0	+
6	0	0	-

[a]As urea and P_2O_5.
[b]As cured cattle manure.
[c]As dried and milled *Paspalum notatum*.

Extract preparation

Dried and milled inflorescences were harvested and stored in paper bags at 10 °C until analysis. Forty milligrams of each sample was weighted and extracted in 500 µl of an ethanol:water solution at a 1:1 ratio. Then, 0.1 mg/ml salicylamide was added to this solution as an internal standard. The extraction was carried out in a sonication bath for 10 min. Then, 300 µl of the solvent was mixed with 300 µl of hexane to remove low polarity compounds. The hydroalcoholic fraction was filtered with a 0.45 µm filter and submitted to HPLC analysis.

HPLC-MS/MS analysis

Semiquantitative analysis was performed on a Shimadzu LC-20A HPLC apparatus with a diode array detector (CBM20A, Shimadzu), and the analyses were monitored at 290 nm. A Supelcosil ODS column (5 µm, 4.6/250 mm; Supelco) was used as the stationary phase. As the mobile phase, the following elution gradient was employed with a flow rate of 1.0 ml min^{-1} (solvent A = aq. acetic acid 1% (v/v); solvent B = MeCN-HOAc (99:1)): 0 to 10 min, 12 to 18% B (linear gradient), 2 min 18% B (isocratic); 12 to 25 min, 18 to 34% B (linear gradient); 25 to 42 min, 34 to 65% B (linear gradient).

Using the same chromatographic conditions, an UltrOTOFq (Bruker Daltonics) ESI-qTOF mass spectrometer detector was

used to establish the chemical identity of the substances based on their mass spectra. The column eluent was split at a ratio of 3:1, the larger flow was processed by the DAD detector and the lower flow by the mass spectrometer. MS TIC chromatograms were recorded between *m/z* 50 and 900 in positive and negative ion mode. The mass spectrometer parameters were as follows: 1000 scans per second; spectrum interval: 2 s; drying gas flow: 5.0 $l.min^{-1}$; drying gas temperature: 180 °C; and nebulising gas pressure: 4 bar. After each HPLC-DAD-MS run, MS and retention time data for each chromatographic peak were used to determine the HPLC-DAD-MS/MS fragmentation parameters (*i.e.*, for online MS/MS, the retention time and *m/z* value of the ion to be fragmented in the collision cell were used in combination as the input for the mass spectrometer software). Collision-induced dissociation (CID) fragmentation was performed using N_2 as the collision gas on each selected ion.

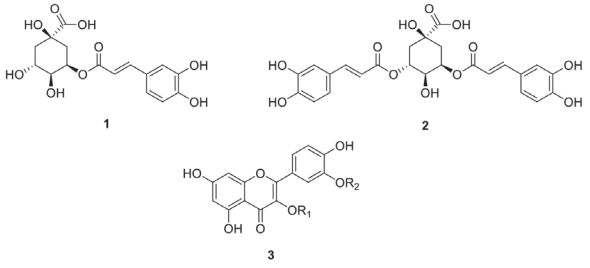
Statistical analysis

The response factor (RF) for each of the identified substances was calculated by dividing its chromatographic area by the internal standard area (salicylamide). As the sample extract preparation and the analytical conditions remained the same for all samples, variations in the RF reflected variations in plant metabolism. However, we did not attempt to quantify the identified compounds.

For comparisons between different soil treatments, the data were tested for normality and homocedasticity and subjected to a two-way ANOVA. For comparisons between different RF according to harvesting period, data were subjected to multivariate analysis (MANOVA), followed by Wilk's lambda test. To identify specific contrasts between periods, the data were also analysed using a two-way ANOVA, followed by a Tukey's *post-hoc test*.

Results and discussion

The identity of the substances was determined by comparing out UV and MS data (Table 3) with that previously reported for *C. oficcinalis* hydroalcoholic extracts (Bilia et al., 2001; Vidal-Olliver et al., 1989; Pietta, et al., 1994; Clifford et al.,2005; GobboNeto & Lopes, 2007; Gouvea et al., 2012). Seven substances were identified: two caffeoylquinic acids, (**1**) 5-*O*-caffeoylquinic acid and (**2**) 3,5-*O*-dicaffeoylquinic acid and five flavonoid glycosides described in Table 2. The structures of substances **1** and **3** were confirmed by comparing their retention times with in-house standards.



Chromatographic analysis of plant extracts cultivated in different soil conditions showed no statistical variations for any of the identified metabolites ($p < 0.05$) between the groups harvested at the beginning of flowering (120 days after sowing) and those harvested at the late period of flowering (between 121 and 180 days of sowing). The same behaviour was observed in all six treatment groups (Table 6)

Table 2
Flavonoid glycosides identified in this study.

Substance	Flavonoid	R_1	R_2
3	Rutin	Rutinose	H
4	Quercetin-3-*O*-rutinosylrhamnoside	Rutinose-rhamnose	H
5	Narcissin (Isorhamnetin -3-*O*-rutinosyl)	Rutinose	CH_3
6	Isorhamnetin-3-*O*-rutinosylrhamnoside	Rutinose-rhamnose	CH_3
7	Isorhamnetin-3-*O*-neohesperidoside	Neohesperidose	CH_3

and for the interaction factors, mulching and fertilisation. These results contrast with the works of Araujo et al. (2009) and Paim et al. (2010), who found that fertilisation increases the total flavonoid content of inflorescences. In agreement with our findings, it was also reported that fertilisation has no influence on the flavonoid contents of *C. oficcinalis*. Król (2011) observed a higher number of inflorescences of the "Orange King" cultivar when fertilisation was used, and he also reported a higher yield of essential oils. However, no variations were found in flavonoid content. Borella et al. (2011) reported similar results using organic and chemical fertilisation. Thus, total flavonoid content in *C. officinalis* might depend on the cultivar, harvest site and time after sowing (Khalid & Silva, 2011.). However, these results do not exclude the possibility that polar metabolites vary over time.

Significant variation was observed according to seasonality. MANOVA analysis followed by Wilk's lambda test indicated that these variations occurred at the multivariate level (Table 4).

To identify which metabolites were altered, we performed ANOVA followed by a *pos-hoc* Tukey's test. Two of the seven compounds showed no statistically significant variations ($p < 0.05$): rutin and isorhamnetin-3-*O*-neohesperidoside. The remaining 5 metabolites were significantly increased or decreased in at least one period in response to the test factors (Table 5).

These results suggest that chemical variations in *C.officinalis* may correlate with other factors, such as light and plant aging. The observed lack of chemical variation in *C. officinalis* polar extracts may be favourable for cultivation, as it does not require further fertilisation, and as a phytomedicine, as it showed constant metabolite biosynthesis.

Table 3
Spectral data for the seven substances analysed.

t_r (min)	Compound	MS+		MS-		UV
		Parention	Fragments	Parent ion	Fragments	
7.224	5-O-Caffeoylquinic acid	-----		353,191[a]		325,299
15.013	Quercetin-3-O-rutinosylrhamnoside	757	611,465,303	755	300	255,352
16.898	Rutin	611	465,303	609	300,271	255,351
17.974	Isorhamnetina-3-O-rutinosilrhamnosil	771	624,317	769	314	253,352
20.307	Isorhamnetina-3-O-neohesperidoside	625	479,317	623	314	253,352
21.933	3,5-O-Dicaffeoylquinic acid	499,317,241,163[a]		515	353,191	329,299
23.267	Narcissin (Isorhamnetin-3-O-rutinosyl)	625	479,317	623	315	254,352

Table 4
MANOVA analysis of seasonality of the response factors for each substance.

	Wilk's Lambda	F	*p*
Intercept	0.017	419.04	0.00
Periods	0.017	22.19	0.00

Table 5
Seasonality of different metabolites according to time of harvest in *Calendula officinalis*. Means of the response factors with different letters are significantly different. (Tukey, $p < 0.05$).

Substance	60-90 days Average ± SD		91-120 days Average ± SD		121-150 days Average ± SD		151-180 days Average ± SD		F	*p*
5-O-Caffeoylquinic acid	0.75	± 0.21[a]	1.04	± 0.22[b]	1.02	± 0.28[b]	0.95	± 0.29[ab]	4.93	0.004
3,5-O-Dicaffeoylquinic acid	1.20	± 0.30[a]	1.18	± 0.18[a]	0.53	± 0.06[b]	1.52	± 0.35[c]	44.45	0.000
Rutina	0.58	± 0.10[a]	0.58	± 0.10[a]	0.58	± 0.07[a]	0.59	± 0.12[a]	0.03	0.992
Quercetin-3-O-rutinosilrhamnosil	0.42	± 0.08[a]	0.55	± 0.07[b]	0.53	± 0.06[b]	0.51	± 0.09[b]	10.38	0.000
Narcissin	1.26	± 0.41[a]	1.52	± 0.25[a]	1.91	± 0.35[b]	1.33	± 0.32[a]	12.56	0.000
Isorhamnetin-3-O-rutinosilrhamnosil	2.00	± 0.52[a]	2.44	± 0.36[b]	2.25	± 0.29[ab]	1.64	± 0.38[c]	13.08	0.000
Isorhamnetin-3-O-neohesperidoside	1.34	± 0.18[a]	1.24	± 0.17[a]	1.20	± 0.18[a]	1.18	± 0.37[a]	1.60	0.197
Quercetina-3-O-rutinosilrhamnosil										
Narcissina										

Conclusion

Using hyphenated analytical techniques, we identified the chemical identity of seven substances in the polar extracts of *C. officinalis* and evaluated its seasonality and responses to fertilisation and mulching. No variation was observed in response to different soil treatments, but significant changes occurred according to the harvest period.

Authorship

EFAF and JCB wrote the manuscript, EFAF conducted the analytical studies, FM performed the statistical analysis, JCB conducted the field experiments and NPL reviewed the manuscript.

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