**Biological activities of Asteraceae (*Achillea millefolium* and *Calendula officinalis*)
and Lamiaceae (*Melissa officinalis* and *Origanum majorana*) plant extracts**

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Abstract

Asteraceae (*Achillea millefolium* and *Calendula officinalis*) and Lamiaceae (*Melissa officinalis* and *Origanum majorana*) extracts were obtained by applying two successive procedures: supercritical fluid extraction with carbon dioxide, followed by ultrasonic assisted extraction using green solvents (ethanol and ethanol:water 50:50). The extracts were analyzed in terms of total phenolic compounds and flavonoid contents; the volatile oil composition of supercritical extracts was analyzed by gas chromatography and the antioxidant capacity and cell viability was determined.

Lamiaceae plant extracts presented higher content of phenolics (and flavonoids) than Asteraceae extracts. Regardless of the species studied, the supercritical extracts presented the lowest antioxidant activity and the ethanol:water extract offered the largest, following the order *Origanum majorana* > *Melissa officinalis* ≈ *Achillea millefolium* > *Calendula officinalis*. However, concerning the effect on cell viability, Asteraceae (especially *Achillea millefolium*) supercritical extracts were significantly more efficient despite being

the less active as an antioxidant agent. These results indicate that the effect on cell viability is not related to the antioxidant activity of the extracts.

Keywords: *Origanum majorana*; *Melissa officinalis*; *Achillea millefolium*; *Calendula officinalis*; Antioxidant; Antiproliferative.

Abbreviations:

ABTS	2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid)
DMSO	Dimethylsulfoxid
DPPH	2, 2- Diphenil-1-pyrcil hydrazyl hydrate
GC-MS	Gas chromatography–mass spectrometry
MTT	3-(4, 5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide
SFE	Supercritical Fluid Extraction
UAE	Ultrasonic Assisted Extraction

1. Introduction

In recent decades, interest in the use of plants in medicine research has leaned towards finding therapies such as herbal alternatives to synthetic drugs. Plants are used to flavor beverages and food goods, but also as antioxidants [1] and strong ability to stabilize vegetable fats [2]. Particularly, flavonoids and other phenolic compounds, wildy present in plant extracts [3] possess a chemical structure that has shown a high capacity as free radical scavengers (antioxidants) and therefore, to act against free radical damage [4, 5]. It is well known that oxidative stress and free radicals are involved in triggering cell damage and in carcinogenesis process [6, 7]. In this sense, antioxidants were initially proposed as an alternative to struggle against cancer [8]. However, clinical trials in humans and recent *in vivo* experiments determine a lack of success [9] and even tumorogenic promotion [10], in part because they do not induce oxidative damage reduction *in vivo* [11]. In this regard, it is still necessary to determine if both activities are

complementary, or, on the contrary, if anticancer potential of plant extracts and phytochemicals is due to other mechanisms independent of their effect on free radicals. Thus, plant extracts are part of the current trend towards functional foods and therapies, and a source of natural bioactive compounds proposed against cells derived from human tumors, such as colon or glioblastoma [12, 13].

In the present work we propose the study and compare extracts derived from different family plants: two from Asteraceae (*Achillea millefolium* and *Calendula officinalis*) and two plants from Lamiaceae (*Melissa officinalis* and *Origanum majorana*), by using Supercritical Fluid Extraction (SFE) and Ultrasonic Assisted Extraction (UAE). SFE uses supercritical carbon dioxide (SCCO₂), resulting in the extraction of thermolabile and lipid fraction of the plant; while UAE when performed with ethanol and/or water would extract polar compounds such as flavonoids and phenols. Thus, these two techniques may be complemented, by first applying SFE to extract the plant volatile oil, and performing on the residual plant matrix a UAE second step to recover the polar compounds. This two-step extraction scheme was applied to obtain extracts from *Achillea millefolium* L. (Yarrow) *Calendula officinalis* (Marigold) *Melissa officinalis* (Balm) and *Origanum majorana* (Majorana), and to analyze their antioxidant capacity and their antiproliferative effect in a human cancer cell line, in order to determine whether these two biological activities are related, or by contrast, to identify within these herbal species the most appropriate to be studied as an antioxidant agent or an anticancer compound.

2. Materials and methods

Chemicals. DPPH (DPPH, 95% purity), Folin-Ciocalteu reactive and analytical standards to GC-MS analysis (quercetin eucalyptol, β -linalool, α -amyrin, lupeol, borneol, α -terpineol, carvacrol, tymol, sabineno, squalen, citronellal, α -curcumen, β -ionone, caryophyllene, caryophyllene oxide, camphor and β -eudesmol) were purchased from

Sigma-Aldrich (Madrid, Spain). Ethanol ($\geq 99\%$ purity) were purchased from Panreac Química S.A.U. (Barcelona, España).

Samples preparation. Yarrow, Marigold, Balm and Marjoram were obtained from an herbalist's producer (Murcia, Spain). Samples were grinded using a knife mill (Grindomix GM200 RETSCH) at 9000 rpm for 60 s.

Extractions. The SFE (see Table 1) were carried out in duplicate using a pilot-plant supercritical fluid extractor (Thar Technology, Pittsburgh, PA, USA, model SF2000) comprising a 2 L cylinder extraction cell. Extraction pressure and temperature were 140 bar and 40°C. CO₂ flow was 70 g/min and extraction time 180 min. The oleoresin resulting in the separator was collected using ethanol. Then, ethanol was removed using a rotavapor at low temperature (30 °C).

The residual vegetal material obtained after the SFE step was re-extracted using an ultrasound probe (Branson Digital Sonifier, Branson Ultrasonics, model 250; Danbury, USA). The UAE were carried out using green polar solvents (ethanol or ethanol: water 50:50). UAE (ethanol: water) have demonstrated in previous works to be more efficient at low ethanol:water ratios (25-50 v/v) than UAE (ethanol) and has shown to improve the obtained amount of phenolic compounds[14, 15]. We compare both UAE extractions in order to find the best conditions.

The solvent/plant ratio was 10 (350 g of solvent and 35 g of vegetal material), the temperature 45°C and the extraction time 30 min. When the extraction finalized, sample was filtered under vacuum and ethanol was removed with a rotavapor. In the case of ethanol-water extracts, water was eliminated by freeze drying.

Chemical characterization. The supercritical extracts were analyzed by GC-MS (Agilent Technologies 7890A). The column used was a HP-5MS (5% Phenyl methyl siloxane, Agilent 19091S-433). Oven temperature programming for Yarrow, Marjoram and Balm

analysis was from 60 °C to 220°C at 9 °C/min and from 220 °C to 300 °C at 5 °C/min. This temperature was kept constant for 2 min. Respect to Marigold, oven temperature was from 60 °C for 1 min, then incremented gradually to 180 °C at 15 °C/min and this temperature was kept constant for 2 min and finally from 180 °C to 300 °C at 40 °C/min for 15 min. injector temperature was of 260°C and MS ion source and interface temperatures were 230°C and 350°C, respectively. The mass spectrometer was used in TIC mode, and samples were scanned from 40 to 500 amu. The components were identified based on the comparison on their relative retention time and mass spectra with the Library data of the GC-MS system (NIST MS 2.0). Standards were used for quantification. The components were identified based on the comparison on their relative retention time and mass spectra with the Library data of the GC-MS system (NIST MS 2.0).

Regarding the total phenolic content (TPC), it was determined using the method Folin-Ciocalteu; results were expressed as mg gallic acid equivalents in 1 g of extract (mg GAE/g extract). The total flavonoid content (TFC) was determined using a colorimetric method proposed by Zhishen et al. [16], and results were expressed as mg quercetin equivalents in 1 g of extract (mg QE/g extract).

Antioxidant activity. Scavenging activity was determined by a spectrophotometric method based on the reduction of an ethanol solution of 1,1-diphenyl-2-picrylhydrazyl (DPPH) [17]. The DPPH concentration in the reaction medium was calculated from a calibration curve determined by linear regression ($y = 0.0303x - 0.0349$; $R^2 = 0.9955$). Furthermore, the ABTS^{•+} assay, as described by Re et al. [18], was also used to measure the antioxidant activity of extracts and results were expressed as TEAC values (mmol TE / g extract).

Antiproliferative effect. Was carried out on pancreatic human tumor-derived cell line MiaPaca-2 and measured by MTT assay, as described in our previous works [17]. Results

were expressed through the IC₅₀, which describes the concentration value that produces 50% cell viability inhibition. All analyses were done in triplicate.

3. Results and discussion

The extraction approach was designed in order to obtain first a lipophilic fraction by CO₂-SFE, which is expected to contain the typical compounds of the plant volatile oil. Then, the residual vegetal material was extracted by UAE using polar solvents (ethanol or ethanol:water 50:50) to obtain a second fraction containing the representative polar compounds of the plant. The SFE and UAE extraction yields of both Asteraceae and Lamiaceae plants are given in Table 2. Relative deviations between duplicates were lower than 15 %, being the higher deviations for Marjoram yields. Mean relative deviation for SFE and UAE yields were, respectively, 7.0 and 4.4 %.

UAE yields were considerably higher than SFE yields for all plants studied, and the yields obtained with ethanol:water were higher than those obtained with pure ethanol. Significant differences ($p < 0.05$) between SFE and UAE (Ethanol:water 50:50) for Marigold and Balm were found after carrying out an ANOVA two way analysis. In general, despite the method or the solvent used, the higher extractions yields were obtained with Marigold, followed by Marjoram, Balm and Yarrow.

GC-MS analysis. SFE extracts were analyzed by GC-MS to identify these components, mainly monoterpenes and sesquiterpenes. The results obtained are discussed below.

Yarrow. Table 3 present the main compounds identified in Yarrow SFE extracts. In accordance with the literature and our results (Table 3), the most important compounds of *Achillea millefolium* volatile oil include monoterpenes [19] such as terpineol and borneol [20], and sesquiterpenes such as germacrene D caryophyllene, bisabolene, α -bisabolol, δ -cadinen [21, 22], spathuleol and cadinol [20].

Caryophyllene, caryophyllene oxide, β -eudesmol and α -curcumene were identified and

quantified in this work in concentrations of 0.14, 2.21, 5.22 and 8.07 mg/g of extract, respectively. Some authors have reported around 4, 7, 16 and 2 mg/g, respectively, to the same compounds [19], in an extract produced by hydrodistillation. Nevertheless, others studies comprising Yarrow supercritical extracts have not reported the presence of α -curcumene [21].

Marigold. Several sesquiterpenes (γ -muurolen, δ -cadinen, α -muurolol and α -cadinol) were identified in Marigold in different percentages according to sample preparation and SFE parameters [23, 24]. In this work, the sesquiterpenes identified in Marigold SFE extracts are reported in Table 4 and include α - and β -muurolene, α - and β -cadinene, α -cadinol and T cadinol, in agreement with previous works from the literature [25, 26]. Furthermore, also triterpenes, such as α -amyrin (8.67mg/g) and lupeol (7.39 mg/g) were identified in the SFE extract of Marigold (data not shown in Table 3) in accordance with works reporting high content of triterpenes in marigold supercritical CO₂ extracts [23, 27].

Balm. Previous studies indicated that the volatile oil of Balm is rich in monoterpenes such as citral [28], citronellal and α -caryophyllene [29], and sesquiterpenes such as caryophyllene oxide [29] or germacrene D [30]. These compounds were identified in the extracts obtained by steam distillation, infusion and by SFE extractions, and were also identified in the SFE extract obtained in this work. Table 5 present the monoterpenes and sesquiterpenes observed in Balm SFE extract.

Marjoram. Table 6 shows the Marjoram supercritical extract composition. The presence of monoterpenes such as terpineol, and sesquiterpenes like spathulenol or β -caryophyllene as the most abundant compounds, agree with previous works focus to hydrodistillation extracts [31] but also for SFE [32]. In addition, 23 isoprenoids were identified in this work as reported in Table 5.

Total Phenolic Compounds (TPC). The assessment of the content of TPC is of great interest due to the recognized capacity of these substances to act as scavenging agents and thus, they are closely connected with the antioxidant activity of the plant extracts. Main phenolic compounds reported in Yarrow were flavonoids, highlighting rutin, vicenin-2, or apigenin and luteolin and their glycosylic derivatives [33, 34]. In addition, caffeic acid derivatives and ascorbic acids were also found in Yarrow [35] ultrasonic extraction. Regarding to Marigold, the other Asteraceae plant investigated in this work, triterpenoids with several hydroxyl substitutions were described previously [27], such as α - and β -amyrin, lupeol and faradiol. Concerning Lamiaceae plants, both Balm and Marjoram were reported to contain phenolic acids and flavonoids, mainly apigenin, luteolin and quercetin [36, 37].

The TPC of plant extracts investigated in this work is show in Figure 1(A). The SFE extracts presented values of 12.6 to 56.2 mg GAE /g extract, compared with those obtained by UAE extractions with values of 65.0 to 232.7 mg GAE /g extract, around statistically 4-7 times higher than those obtained in SFE extracts for Marigold and Marjoram, and 13-15 times higher for Yarrow and Balm. Although all SFE extracts exhibit low content of TPC in comparison with UAE extracts, Marjoram extract showed considerably higher amounts than the supercritical extracts of the other plants. In the case of UAE extracts, despite the solvent used, Marjoram resulted with the highest content of TPC, followed by Balm, Yarrow and Marigold (Figure 1A, Table S1).

Total Flavonoid Compounds (TFC). Content of TFC is shown in Figure 1(B). As in the case of phenolic compounds, low amounts of flavonoids were determined in the SFE extracts in comparison with UAE extracts. Marjoram extracts were those with highest significant TFC content, despite the method or the solvent used in the extraction (Figure 1B, Table S2). In general, TFC follow the same tendency observed in TPC.

A lineal connection between TPC and TFC can be established for all the extracts, as illustrated in Figure S1, although somewhat more scattering relationship is observed in the case of Balm extracts.

Antioxidant activity. The antioxidant activity of all extracts produced was determined by to different methods: the DPPH radical method, stating the antioxidant capacity in terms of the EC50 value, and the ABTS radical method, by means of the TEAC value. Table 7 shows the results obtained for all plant extracts produced. As can be observed, both methods indicate the same tendency for all plant extracts: UAE ethanol:water extracts presented the highest antioxidant activity (lowest EC50 values and highest TEAC values), followed by UAE ethanol extracts. Additionally, for all plants studied, SFE extracts exhibit significant lower antioxidant activity than UAE extracts.

Marjoram extracts presented the highest antioxidant activity, despite the type of extraction or solvent used. Results were rather similar for Balm and Yarrow, and definitively Marigold extracts presented the lowest antioxidant capacity. Particularly, the EC50 value resulted for Marjoram ethanol:water extract (13.74 µg/ml) are in the same order of magnitude that the values reported for Rosemary extracts, which is a commercial antioxidant for foodstuffs (E392) [17]. As can be observed in Figure S2(A), high correlation resulted between the EC50 values and TPC was obtained, being the regression coefficient $R^2 = 0.985$.

Nevertheless, although the ABTS test show the same tendency regarding the antioxidant capacity of the extracts (Marjoram > Balm $\approx$ Yarrow > Marigold) the TEAC values do not show a linear correlation with TPC Figure S2(B). The same conclusions can be established regarding the connection between the total content of flavonoid compounds (TFC) and the antioxidant activity of the UAE extracts, although in this case the regression coefficient of the EC50 vs. TFC correlation is somewhat lower ($R^2 = 0.858$).

Antiproliferative activity. Cells were treated with different concentrations (from 10 to 100 $\mu\text{g/mL}$) of each extract during 48 h. Extracts from Lamiaceae family (Balm and Marjoram) do not demonstrate any effect on the growth of MiaPaca-2 cells: the IC₅₀ was higher than 100 $\mu\text{g/mL}$, showing the absence of a significant effect on cell proliferation in the range of concentrations tested. By contrast, Yarrow and Marigold extracts (Asteraceae family) show an effect on these cells (Table 8). The IC₅₀ for Yarrow SFE and UAE extracts is in a range between 30 and 65 $\mu\text{g/mL}$, similar to Rosemary SFE extract tested in other tumor types [38, 39], which can lead to suggest Yarrow and Marigold extracts as potential antitumorogenic agents.

Conclusions

Lamiaceae plant family seems to be the most appropriate source of antioxidant compounds and the use of polar solvents the best extraction method, and Asteraceae family shows the most promising results as a reservoir of potential antiproliferative agents and SFE the most efficient method for this purpose. These results support previous studies suggesting a potential anticancer activity of plant extracts independent of their antioxidant activity.

Related to the composition, the GC-MS analysis of supercritical extracts demonstrates differences in the volatile oil composition of Lamiaceae and Asteraceae extracts, especially in the type of alcohols content. Lamiaceae species are particularly rich in phenolic monoterpenes and sesquiterpenes (around 32% Balm and 53% Marjoram), with 7-12% of cyclic alcohols. In the contrary, the volatile oil content in Asteraceae plants, comprise high amounts of bicyclic and tricyclic alcohols (52% Yarrow and 32% Marigold) and almost no phenolic alcohols were identified. This result, encourage further studies to elucidate a possible effect of bicyclic and tricyclic monoterpene and

sesquiterpene alcohols as responsible of the antiproliferative effect of these Asteraceae extracts.

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359 **Tables and Figures**

Table 1. Supercritical CO₂ extraction of Asteraceae and Lamiaceae plants.

	Yarrow	Marigold	Marjoram	Balm
Plant part	Inflorescences; upper leaves	Flowers	Leaves	Leaves
Water content (%)	<5	<5	<5	<5
Particle size (µm)	1000	1000	1000	1000
Mass loaded for SFE (g)	383	500	550	713
CO ₂ /plant ratio (Kg/Kg)	33	25	23	18

Table 2. Extraction yield (mass of extract / mass of plant material).

	Yarrow	Marigold	Marjoram	Balm
SFE	0.79 ± 0.03	2.78 ± 0.21	1.51 ± 0.16	0.65 ± 0.04
UAE (Ethanol)	2.82 ± 0.01	6.83 ± 0.49	6.17 ± 0.90	4.35 ± 0.06
UAE (Ethanol:water 50:50)	10.82 ± 0.42	29.13 ± 1.41	12.83 ± 0.15	22.99 ± 0.37

Table 3. Volatile oil compounds identified by GC-MS in the SFE extract of Yarrow.

Retention time	Compound	% Area	Type
4589	Yomogi alcohol	2.56	M (acyclic alcohol)
5115	Eucalyptol	2.23	M (cyclic ether)
5471	Artemisia ketone	2.12	M (acyclic ketone)
5823	Sabinene	2.80	M (bicyclic hydrocarbon)
6078	Linalool	1.31	M (acyclic alcohol)
6224	Thujone	1.84	M (cyclic ketone)
6853	Citronellal	5.63	M (acyclic aldehyde)
7203	Borneol	16.28	M (bicyclic alcohol)
7482	Terpineol	2.56	M (propan-2-ol)
9287	Carvacrol	3.52	M (phenol)
10411	Verbenol	1.76	M (bicyclic alcohol)
11234	Caryophyllene	0.95	S (hydrocarbon)
11356	Nerolidol acetate	0.90	S (acyclic ester)
12074	α-Curcumene	3.61	S (cyclic hydrocarbon)
13077	Elemol	1.37	S (propan-2-ol)
13469	Cubenol	5.43	S (bicyclic alcohol)
13541	Spathulenol	5.55	S (tricyclic alcohol)
13637	Caryophyllene oxide	4.90	S (oxide)
13765	Viridiflorol	5.12	S (tricyclic alcohol)
14300	α-Cadinol	8.22	S (bicyclic alcohol)
14555	Eudesmol	11.19	S (propan-2-ol)
14900	Bisabolol	2.73	S (cyclic alcohol)
16833	Corymbolone	7.44	S (bicyclic keto-alcohol)

M: monoterpene; S: sesquiterpene

Table 4. Volatile oil compounds identified by GC-MS in the SFE extract of Marigold.

Retention time	Compound	% Area	Type
9795	β -Muurolene	13.86	S (hydrocarbon)
9984	α -Cadinene	23.36	S (hydrocarbon)
10051	β -Cadinene	21.26	S (hydrocarbon)
10236	α -Muurolene	9.79	S (hydrocarbon)
11497	T-Cadinol	13.76	S (bicyclic alcohol)
11636	α -Cadinol	17.96	S (bicyclic alcohol)

S; sesquiterpene; T: triterpene

Table 5. Volatile oil compounds identified by GC-MS in the SFE extract of Balm.

Retention time	Compound	% Area	Type
6078	Linalool	3.70	M (acyclic alcohol)
6853	Citronellal	23.87	M (acyclic aldehyde)
7203	Borneol	4.37	M (bicyclic alcohol)
7482	Terpineol	4.79	M (propan-2-ol)
9125	Thymol	6.13	M (phenol)
9287	Carvacrol	25.44	M (phenol)
11234	Caryophyllene	4.79	S (hydrocarbon)
13077	Elemol	2.90	S (propan-2-ol)
13637	Caryophyllene oxide	17.73	S (oxide)
13,763	Epiglobulol	2.34	S (bicyclic alcohol)
14555	Eudesmol	3.94	S (propan-ol alcohol)

M: monoterpene; S: sesquiterpene; D: diterpene; T: triterpene

Table 6. Volatile oil compounds identified by GC-MS in the SFE extract of Marjoram.

Retention time	Compound	% Area	Type
5115	Eucalyptol	11.48	M (cyclic ether)
6078	Linalool	9.84	M (acyclic alcohol)
6865	Camphor	0.55	M (ketone)
7203	Borneol	4.16	M (bicyclic alcohol)
7482	Terpineol	0.38	M (propan-2-ol)
9125	Thymol	2.52	M (phenol)
9287	Carvacrol	50.72	M (phenol)
9927	γ -Elemen	0.94	S (hydrocarbon)
10077	α -Terpineol acetate	1.71	M (ester)
105075	Neryl acetate	0.34	M (ester)
11234	Caryophyllene	2.57	S (hydrocarbon)
12076	Longiborneol	0.46	S (bicyclic alcohol)
12373	α -Elemen	0.76	S (hydrocarbon)
12456	α -Himachalen	0.48	S (hydrocarbon)
126115	γ -Cadinene	0.34	S (hydrocarbon)
12701	Epiglobulol	1.30	S (tricyclic alcohol)
127785	Globulol	0.57	S (tricyclic alcohol)
13541	Ent-spathulenol	3.13	S (tricyclic alcohol)
13637	Caryophyllene oxide	1.70	S (oxide)
13778	Ledol	2.02	S (tricyclic alcohol)
14276	γ -Eudesmol	0.76	S (propan-2-ol)
143665	β -Guaiene	0.83	S (hydrocarbon)
14555	β -Eudesmol	2.44	S (propan-2-ol)

M: monoterpene; S: sesquiterpene; D: diterpene; T: triterpene

Table 7. Antioxidant activity of extracts: EC₅₀ (mg / ml) and TEAC (mmol / g) values.

	Yarrow		Marigold		Balm		Marjoram	
	EC50	TEAC	EC50	TEAC	EC50	TEAC	EC50	TEAC
SFE	367.15	0.077	442.40	0.046	318.38	0.033	229.84	0.642
UAE (ethanol)	43.44	0.327	154.77	0.062	38.04	0.238	22.17	0.967
UAE (ethanol:water)	40.27	0.644	123.35	0.331	22.67	0.697	13.74	1550

Table 8. Antiproliferative effect in MiaPaCa-2 cell line: IC50 (µg/mL) values after 48 h of exposure (mean ± SEM of three independent experiments performed in quadruplicate).

	SFE	UAE Ethanol	UAE Ethanol:water
Marjoram	> 100	> 100	> 100
Balm	> 100	> 100	> 100
Yarrow	31.45 ± 8.56	65.04 ± 2.55	> 100
Marigold	39.84 ± 4.57	> 100	> 100

Figure 1. (A) TPC and (B) TFC in SFE, ethanol-UAE and ethanol:water-UAE extracts. Data represent means ± S.E.M of at least two independent experiments. Asterisks indicate statistical differences between treatments after one-way Anova comparison. *p<0.05, **p<0.01; ***p<0.001.