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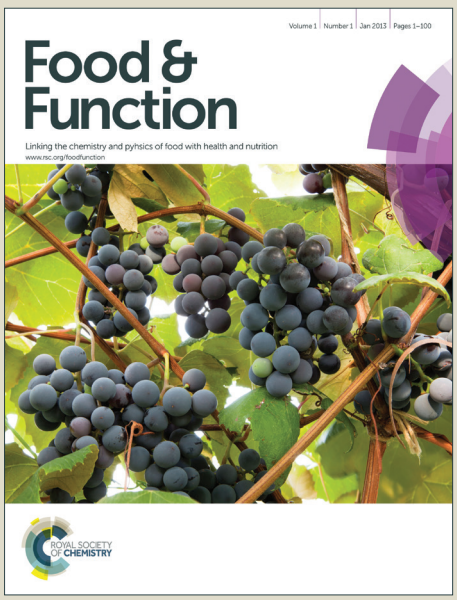

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Effect of five *Taraxacum* species on *in vitro* and *in vivo* antioxidant and antiproliferative activity

Received 00th January 20xx,
Accepted 00th January 20xx

DOI: 10.1039/x0xx00000x

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Plants belonging to *Taraxacum* genus are considered a nutritious food, being consumed raw or cooked. Additionally, these plants have long been used in folk medicine due to their choleretic, diuretic, antitumor, antioxidant, antiinflammatory, and hepatoprotective properties. This genus, with its complex taxonomy, includes several species that are difficult to distinguish. Traditional uses must be related not only to *T. officinale* F.H. Wigg., the most studied species, but also to others. The aim of this work is to compare five different common South European species of *Taraxacum* (*T. obovatum* DC., *T. marginellum* H. Lindb., *T. hispanicum* H. Lindb., *T. lambinonii* Soest and *T. lacistrum* Sahlin), in order to find differences between antioxidant and cytotoxic activities among them. Dissimilarties between species in LC/MS patterns, in *in vitro* and intracellular antioxidant activity and also in the cytotoxicity assay were found. *T. marginellum* was the most efficient extract reducing intracellular ROS levels although in *in vitro* assays, *T. obovatum* was the best free radical sacavenger. A relevant cytotoxic effect was found in *T. lacistrum* extract over Hela and Hep G2 cell lines.

Introduction

Taraxacum belongs to Asteraceae family, widely distributed in the Northern Hemisphere, and in some warm Central and South American regions, as well as in Australia and New Zealand. The genus, with a complex taxonomy, and difficult morphology, includes near 2500 species. Plants belonging to *Taraxacum* genus are biennial or perennial herbaceous plants, and exude milky latex when broken [1]. Leaves and roots of some species are traditionally consumed as food, and are also used in folk medicine due to their choleretic, diuretic, antitumor, antioxidant, antiinflammatory, and hepatoprotective properties [2]. *T. officinale* is the most studied species, and seems to be the most consumed both as food and in traditional medicine. Leaves from this species are considered a good food source with nutritive potential that could improve the nutritional condition of areas of population with poor economic resources [3]. Although this plant is a well-known traditional herbal remedy, there is only limited scientific information available on its pharmacological uses, and results are sometimes contradictory [4].

Recently, some studies have indicated that dandelion presents anti-inflammatory, antioxidant and anti-carcinogenic activities [4]. Sigstedt et al., [5] reported the ability of *T. officinale* aqueous crude leaf extract to decrease MCF-7/AZ breast cancer cell growth. Anticarcinogenic effect of dandelion on Hep G2 via apoptosis has been demonstrated in *in vitro* studies, as well as cytotoxic activities in Caco-2 cell line [6]. In particular, an aqueous extract of dandelion caused a 26 % time and dose dependent reduction of cell viability, inducing apoptosis by the increase of some cytokine levels (TNF-α and IL-1). Dandelion tea caused decreases in induced esophageal tumour multiplication, tumour size and tumour volume in female Wistar rats [7]. Recent results [8] pointed out that natural products, in particular those present in *Taraxacum* root extract, have great potential as non-toxic and effective alternatives to conventional modes of chemotherapy available today.

Studies on anti-oxidative capacity of *T. officinale* showed that both root and leaf extracts were able to diminish lipid peroxidation and reduce cytochrome *c* [9]. In addition, leaf extracts have shown beneficial hepatic effects mainly due to its antioxidant and anti-inflammatory activities, which are attributed to the phenolic compounds contained in the extracts [2, 10]. The elevated content of phenolic compounds in dandelion extracts gave them efficiency in inhibiting both reactive oxygen species and nitric oxide induced damage [11]. The search for effective nontoxic natural antioxidants has been intensified since synthetic antioxidants have been reported to increase risk factors of human to various deadly diseases. In this sense the intake of foods with functional attributes like antioxidants has become a suitable alternative to improve human health [12].

Due to genus morphology, determination of the correct *Taraxacum* species is very difficult for a nonexpert. This problem and the fact that *T. officinale* is not present in the South of Europe [1], led us to hypothesize that other common *Taraxacum* species are being used as foods and for traditional medicine in these areas. In the present work antiproliferative, protective and antioxidant activities were analyzed in five common South European *Taraxacum* species. The

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objective of the study was to understand differences among species that may be related with their different traditional uses, and with contradictory nutritious potential and pharmacological effects reported for *Taraxacum* Genus.

Materials and methods

Plant material and extracts preparation

Five *Taraxacum* species, commonly named dandelion, were collected in different locations of the Iberian Peninsula. *T. obovatum* (Willd.) DC. and *T. marginellumm* H. Lindb. in Burgos (Noth of the Iberan Peninsula); *T. hispanicum* H. Lindb. and *T. lambinonii* Soest in Madrid (Center of the Iberian Peninsula) and *T. lacistrum* Sahlin in Leon (North of the Iberian Peninsula). All plants were harvested with flowers to enable their botanical determination (March-May). Plant material was identified and authenticated by the Botanical Department of Universidad CEU–San Pablo, and voucher specimens were kept in the USP-CEU University herbarium.

Aerial part crude extracts were obtained using a Soxhlet system (Buchi V-811) with 80 % methanol as solvent for 4 h. Extracts were then concentrated until dried under reduced pressure at 40–45 °C, and stored at 4° C until use. Extract yield appears in Table 1.

Extracts characterization

Taraxacum extracts were analyzed by liquid chromatography coupled to mass spectrometry (LC-MS). For the chromatographic separation an Agilent 1100 HPLC system was used (Agilent, USA) equipped with a Zorbax Eclips XDB-C_{18} (150 mm × 4.6 mm, 5 µm) column, maintained at 30 °C. Injection volume was 20 µL. The mobile phase consisted of 0.4% (v/v) acetic acid solution (A) and methanol (containing 0.4% (v/v) acetic acid) (B) at a flow rate of 0.8 mL min^{-1}. A gradient program was used as follows: 0 min, 5% B; 7-13 min, 30% B; 25-30 min, 60% B; 38-43 min, 95% B; 45 min, 5% B. Mass spectral analyses were performed with a Esquire 3000 (Bruker) equipped with an electrospray ionization source (ESI). ESI conditions were as follows: temperature: 350°C; nebulizer pressure: 50 psi; drying gas flow: 12 L/min; capillary voltage for positive mode -3000 V; capillary voltage for negative mode: 4000 V. Full mass scan spectra were recorded in negative ion mode over an *m/z* range of 70–1000 dalton.

Determination of total phenolic content

Total phenolic content in the *Taraxacum* extracts samples was determined using Folin–Cicolteau reagent [13]. To 200 µL of the extract (1.3 mg/mL), 1.0 mL of 10% Folin–Cicolteau reagent and 800 µL of 7% Na_2CO_3 were added and mixed. After 2 hours, the absorbance was measured at 750 nm (Shimadzu UV-1601 spectrophotometer). A gallic acid standard curve was used for quantification. The concentration in the extract was expressed as micrograms of gallic acid equivalents. All tests were performed in triplicate. Data were analysed for statistical significance (P< 0.05) by ANOVA followed by Bonferroni's test.

Table 1. Extract yield in percentage of each *Taraxacum* species (w/w).

T. obovatum	*T. marginellum*	*T. hispanicum*	*T. lambinonii*	*T. lacistrum*
24.59	23.55	24.73	29.97	21.21

Determination of total flavonoid content

Flavonoid content was determined following the method of Zhishen *et al.* [14] using epicathechin as standard. 200 µL of each extract at different concentrations, 60 µL of 5% $NaNO_2$ and 800 µL of H_2O were mixed. 5 minutes later, 60 µL of 10% $AlCl_3$ were added, and after a further minute, 400 µL of 1M NaOH. The absorbance of the mixtures was measured at 510 nm (Shimadzu UV-1601 spectrophotometer). All measurements were performed in triplicate. Flavonoid concentration was expressed as epicathechin mg per gram of extract. Data were analysed for statistical significance (P< 0.05) by ANOVA followed by Bonferroni's test.

Radical scavenging activity

DPPH test. Scavenging free radical activity was tested in a DPPH methanol solution [15]. Solution decoloration degree indicates the scavenging efficiency of the sample. Extracts were dissolved in methanol at different concentrations, 100 µL of each dilution was added to 100 µL of DPPH 1 mM in methanol. After 30 minutes, absorbance was measured at 517 nm in a microplate reader (VersaMax, Molecular Devices, Sunnyvale, CA, USA). A blank was prepared with 100 µL of methanol plus 100 µL of DPPH. The inhibition activity percentage was calculated as $[(A_0-A_1)/A_0] \times 100$, where A_0 was the absorbance of the blank and A_1 was the absorbance of the extract. The scavenging activity was expressed as IC_{50}, concentration that reduces 50 % of DPPH radicals, calculated from linear regression analysis. Tests were performed in triplicate. Data were analysed for statistical significance (P< 0.05) by ANOVA followed by Bonferroni's test.

Superoxide anion scavenging assay. Superoxide anion was generated by xanthine-xanthine oxidase system and detected by the NBT reduction. Samples were diluted in phosphate buffer, pH 7.4. The reaction mixture contained 65 µL of buffer (50 nM KH_2PO_4/KOH, pH 7.4), 10 µL of Na2EDTA (15mM), 15 µL of hypoxanthine (3mM), 25 µL of NBT (0.6mM), and 12.5 µL of extracts at different concentrations. The reaction was started by adding 25 µL of xanthine oxidase (1 U/10 mL). Microplates with the reaction mixture were read at 560 nm using a microplate reader (Versa Max) every 5 minutes for 40 minutes. A negative control was prepared without extract. The ability of the extract to scavenge O_2^- was calculated as the inhibition percentage of NBT reduction compared to the control. Results were expressed as IC_{50} concentration required to inhibit 50% of NBT reduction. All experiments were carried out in triplicate. To ensure there was no NBT reduction due to extract compounds, a control was made by mixing the NBT solution with the extract in a phosphate buffer [16]. Data were

analysed for statistical significance (P< 0.05) by ANOVA followed by Bonferroni's test.

Cell culture

Human cervical carcinoma HeLa (ECACC-93021013), human promyelocytic leukemia HL-60 (ECACC-98070106), human hepatocarcinoma Hep G2 (ECACC-85011430) and African green monkey (non tumoral) kidney Vero E6 cell lines, were obtained from the European Collection of Cell Cultures (Health Protection Agency, UK). Cultures were seeded from frozen stocks. Each cell line was maintained in its appropriate medium and incubated at 37 °C in a 5% CO_2 atmosphere.

Cell Viability

The cytotoxic effect of the extracts was evaluated by conventional 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay [17] after 72 h of extract exposure. PBS and doxorubicin were used as negative and positive controls respectively. All experiments were performed at least three times, and the average of the percentage absorbance was plotted against concentration. The concentration of extract required to inhibit 50% of cell growth (IC_{50}) was then calculated for each species.

Intracellular ROS measurement

Measurement of intracellular ROS was based on ROS-mediated conversion of the nonfluorescent 2´,7´-dichlorofluorescein diacetate (DCFHDA) into 2´,7´-dichlorofluorescin (DCFH) [18]. An increase in fluorescence intensity reflects enhanced oxidative stress. Hep G2 were seeded in 96-well plates at a density of 20000 cells per well, with EMEM medium suplemented with 1% fetal bovine serum (HyClone, Logan, UT, USA), 100 U/mL penicillin, 100 µg/mL streptomycin, 1% non-essential amino acids, and 2mM L-glutamine. After 24 h of incubation, cells were washed with PBS (pH 7.4) and then incubated with DCFH-DA (20 µM) in PBS at 37 °C for 30 min. To assess the antioxidant activity, cells were incubated with a growing concentration of extracts, in the presence or absence of 200 µM H_2O_2 (as oxidative stress inducer). Two controls were made, one with PBS, and a second with PBS and H_2O_2. DCFH fluorescence was then measured every 15 min for 90 min, at an emission wavelength of 530 nm and an excitation wavelength of 485 nm, using a Fluostar Optima fluorescence plate reader (BMG Labtech GmbH, Ortenberg, Germany). Results were expressed as percentage of fluorescence intensity related to PBS control (no stressed Hep G2 cells). Data were analysed for statistical significance (P< 0.05) by ANOVA followed by Bonferroni's test.

Results and Discussion

Taraxacum extracts contain many pharmacologically active metabolites that display several medicinal properties[19]. Fig. 1 and 2 show the LC/MS fingerprint of all *Taraxacum* species extracts. Each plant species contains several different natural constituents, most of which have not been studied as no global metabolite screen of the diverse *Taraxacum* species has been conducted [20]. Differences in the chemical composition of different *Taraxacum* species remain vague. Tissues of *T. officinale* (the most studied species of the Genus) reportedly contain flavonoids, coumarins, phenolic acids and their derivates, triterpenoids, steroids and sesquiterpene lactones [21]. The most important components of the extract include sesquiterpene lactones and phenilpropanoids, which are believed to have anti-cancer properties leading to the diverse observed effects of dandelion extracts [22]. Several studies have demonstrated further health-promoting properties of either dandelion extracts or individual compounds extracted from dandelion leaves or roots, e.g. anti-inflammatory, anti-carcinogenic and anti-oxidative activities. Although these diverse effects have mainly been attributed to the presence of various polyphenolics and sesquiterpens other components have not been fully characterized and therefore their activities remain unknown [23, 4].

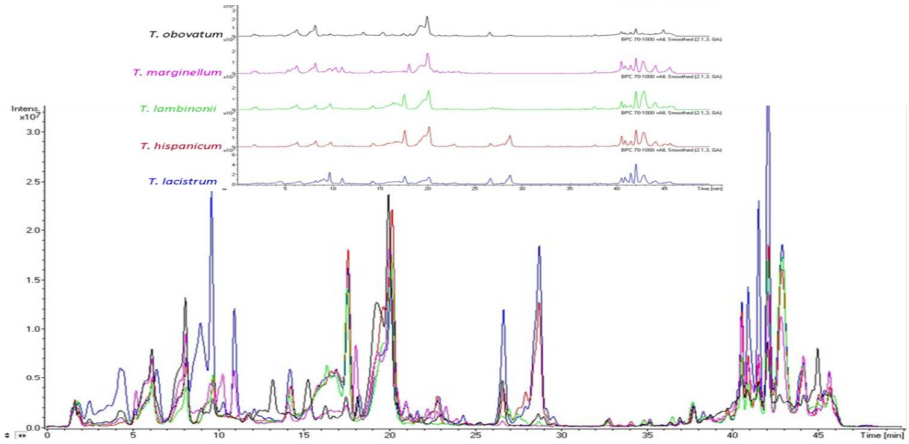

Figure 1. LC/MS fingerprint of *Taraxacum* extracts in positive mode: *T. lacistrum (-) T. lambinonii (-) T. hispanicum (-) T. marginellum (-) T. obovatum (-).*

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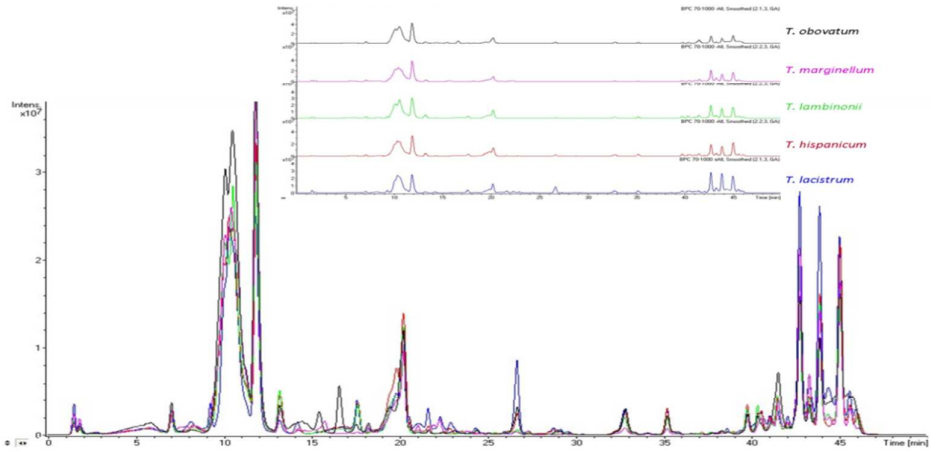

Figure 2. LC/MS fingerprint of *Taraxacum* extracts in negative mode: *T. obovatum (-); T. marginellum (-); T. hispanicum (-);T. lambinonii (-);T. lacistrum (-).*

Compared to roots, *T. officinale* leaves are characterized by higher polyphenol content. The most abundant phenolic compounds in leaves and flowers are hydroxycinnamic acid derivatives, in particular caffeic acid esters such as chlorogenic, dicaffeoyltartaric (chicoric acid) and monocaffeoyltartaric acids [24, 25]. As shown Table 2, although more studies are necessary, our species seem to follow the same phenolic compound pattern as *T. officinale*, with chicoric acid as one of the main phenolic compounds. However, variations in chemical composition may be responsible for some of the differences in pharmacological activities between species that have been found in the present study.

Table 2. Characterization of compounds by LC/MS. *: *T. obovatum (T1); T. marginellum (T2); T. hispanicum (T3); T. lambinonii (T4); T. lacistrum (T5).* Number of + and – signs indicate the presence or absence, and relative amount of each compound.

Compound name	M	tR (min)	m/z traces (-)	m/z traces (+)	T1*	T2*	T3*	T4*	T5*
Chlorogenic Ac.	354	9.8	353	377 , 135	-	-	-	+	++
Caffeic Acid	180	10.5	179, 135	181, 163, 135	+	+	++	++	+
Chicoric Acid	474	12.2	473	497	+++	+++	++++	++	++
Luteolin-7-glucoside	448	20.3	447	449	+++	++	++	++	++
Rutine	610	20.9	609	303	++	++	++	++	++
Hesperidin	610	21.5	609, 301	633, 303	-	+	+	-	-
Quercetine	302	25.7	301	303	+	-	-	-	-
Luteolin	286	26.6	285	287	+++	+	+	++	++++
Apigenin	270	28.6	269	271	+++	++	++	++	+++
β-Sitosterol	414	38.0	413	437	++	+++	++	++	++

Table 3. Total phenol concentration of *Taraxacum* extracts expressed as Gallic acid mg/g of extract. Total flavonoid concentration expressed as epicatechin mg/g of extract. Results are expressed as mean ± S.E. of n=3 .Different letters indicate statistically significance differences between treatments (ANOVA-Bonferroni, P< 0,05).

	T. obovatum	*T. marginellum*	*T. hispanicum*	*T. lambinonii*	*T. lacistrum*
Total phenolic content Gallic acid mg/g of extract	135.87 ± 0.70 *a*	104.90 ± 0.92 *b*	103.50 ± 2.21 *b*	150.09 ± 3.40 *c*	89.70 ± 0.66 *d*
Flavonoid concentraction epicatechin/ g of extract	106.73 ± 0.23 *a*	84.01 ± 1.73 *b*	86.93 ± 0.61 *b*	111.60 ± 0.80 *c*	73.73 ± 1.10 *d*

Total phenol and flavonoid concentrations of each extract appear in Table 3. Extracts had a moderately high amount of these kinds of compounds, *T. lambinonii* and *T. obovatum* being the species with the highest concentrations. There are significant differences between species, except for *T. marginellum* and *T. hispanicum* both in total phenols and flavonoids. The percentage of phenols that correspond with flavonoids is notable, exceeding 74 % of the total phenolic content in all cases. Flavonoids are interesting compounds with the capacity to scavenge free radicals and prevent oxidation of other molecules and therefore, able to control oxidative stress [26]. It has been reported an inverse relationship between the dietary intake of antioxidant-rich food and medicinal plants and incidence of human diseases [12].

All extracts studied had a moderate capacity to scavenge the DPPH radical (Table 4). All of the extracts showed dose dependent radical scavenging activity. *T. lambinonii*, the species with the highest amount of phenolic compounds, showed the best scavenging activity. This result indicates that these kinds of compounds are primarily responsible for the antioxidant activity of the extracts, as has been previously reported [26, 27]. However, quantitative phenolic concentration differences between *T. marginellum* and *T. hispanicum*, does not explain their differing ability to scavenge the DPPH radical. Results suggest that there are marked qualitative differences in the chemical composition of the extracts between the species that justify the data.

Results of superoxide scavenge capacity appears in Table 5. Again, there are differences between the species, those ones with the highest amounts of phenolic compounds, particularly flavonoids being more active. Flavonoids have been described as active principles mainly involved in antioxidation, acting as prophylactic agents for both health and disease management [28]. Polyphenolic compounds are able to efficiently scavenge superoxide radicals. These compounds may react with the superoxide radical, via a one-electron transfer, or by a hydrogen abstraction mechanism. They could also have certain inhibitory activity towards xanthine oxidase[29].

Fig. 3 shows the development of fluorescence measurements of the intracellular ROS assay at the assay end point (90 min). The results are shown after treatment of cells in the presence or absence of an oxidative stress inducer (H_2O_2 200 µM). The response pattern is different for each species, three of them being unable to reverse the ROS concentration increase generated by the oxidizing agent (H_2O_2): *T. obovatum, T. hispanicum* and *T.lacistrum.* However a small fluorescence decrease was detected with the highest concentrations of *T. hispanicum* and *T. lacistrum*. On the other hand, *T. lambinoníi* and *T. marginellum* reduce the oxidative stress significantly, raising levels similar to those of the control with PBS. At the lowest concentration, the percentage of fluorescence is reduced by *T. marginellum* extract from 160 to 120 %. This extract exhibits a high antioxidant potential in culture assays, protecting cells against oxidative stress, although it did not produce the best results in *in vitro* scavenging studies. These kinds of divergences have been reported previously in studies with other phytoextracts, and could be explained in terms of bioavailability and metabolism amongst others [30]. These findings provide pharmacological explanation for some *Taraxacum* uses in folk medicine. The antioxidant activity of the plants can be correlated for their therapeutic effect against cancer, their anti-inflammatory effects and hepatoprotective properties.

Table 4. DPPH free radical scavenging activity IC_{50} of *Taraxacum* species (reference substance) (mg/mL). Results are expressed as mean ± S.E. of n=3. Different letters indicate statistically significance differences between treatments (ANOVA-Bonferroni, P< 0,05).

T. obovatum	*T.marginellum*	*T. hispanicum*	*T. lambinonii*	*T. lacistrum*
0.087 ± 0.006 *a*	0.161 ± 0.008 *b*	0.086 ± 0.007 *a*	0.083 ± 0.006 *a*	0.170 ± 0.008 *c*

Table 5. Superoxide scavenging capacity IC_{50} of *Taraxacum* species (reference substance) (mg/mL). Results are expressed as mean ± S.E. of n=3. Different letters indicate statistically significance differences between treatments (ANOVA-Bonferroni, P< 0,05).

T. obovatum	*T. marginellum*	*T. hispanicum*	*T. lambinonii*	*T. lacistrum*
0.199 ± 0.015 *a*	0.421 ± 0.010 *b*	> 0.50	0.387 ± 0.019 *c*	> 0.50

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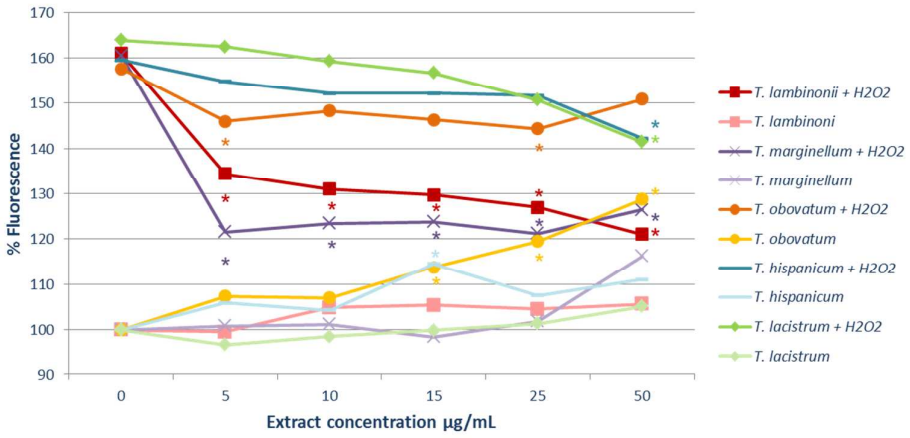

Figure 3. Intracellular ROS concentration after 90 minutes of treatment. Results appear as percentage of Fluorescence respect to control with PBS (without H_2O2) (100 %). (*) indicates statistically significance difference between control (0 µg/mL with or without H_2O_2) and treatment (ANOVA-Bonferroni, P< 0,05).

The species did not affect the production of ROS in normal conditions (without oxidative stress). However, at the highest concentrations, cell treatment with *T. obovatum* extract increased fluorescence percentage significantly. The prooxidant effect of high concentrations of phenolic compounds has been previously reported in the literature [31].

The results of MTT assay appear in Table 6. The cytotoxic ability of these species do not seem to be relevant, except for *T. lacistrum* over Hela and Hep G2 cell lines. The selectivity index (SI), which represents IC_{50} for Vero E6 cell line/IC_{50} for cancerous cell line, was calculated for HeLa and Hep G2 treated with *T. lacistrum* aerial crude extract.

The criteria of cytotoxic activity for crude extracts according to the US National Cancer Institute (NCI) guidelines, is an IC_{50} value below 30 µg/mL [32]. As is shown in Table 6, the *T. lacistrum* extract over Hep G2, presents an interesting IC_{50} (15 µg/mL). In addition, the lack of cytotoxic activity against the non tumoral cell line Vero E6 in this extract, suggest good potential, as it leads to selectivity index values of 16.7 and 10.7 for Hep G2 and HeLa respectively. Samples with SI value greater than 3 were considered to have high selectivity [33]. Results show an interesting cytotoxic activity of *T. lacistrum* over two of the tumoral cell lines with relevant Selectivity Indices (SI), higher than 10 in both cases. Dandelions have been used for centuries for nourishment and therapy, with few reports of little to no toxicity, due to the lack of toxins and alkaloids in this plant. High doses have been reported to cause allergic contact dermatitis, although this was shown in studies using dandelion extracts as a topical treatment [8]. *T. lacistrum* results suggest that this plant is non-toxic to non–tumoral cells, making it selective to HeLa and Hep G2 cells. *T. officinale* toxicity over Hep G2 has been already reported, suggesting that the extract induced cytotoxicity through TNF-α and IL-1α secretion [6]. Further studies are needed to determine the extract mechanism of action.

Table 6. Extracts cytotoxic activity expressed as IC_{50} values (mg/mL). Selectivity Index (SI) of active extracts.

	HepG2	HeLa	HL60	Vero E6
T. lacistrum	0.015 ± 0.001	0.023 ± 0.002	>0.25	>0.25
	SI 16.67	SI 10.87	>0,25	>0,25
T. obovatum; T. lambinonii; T. hispanicum T. marginellum	>0.25	>0.25	>0.25	>0.25

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Conclusions

According to the WHO (World Health Organization), traditional medicine is an important and often underestimated part of health care. Dandelion has been empirically used due to its health-promoting properties as an anti-carcinogenic, anti-inflammatory and anti-oxidative [4]. However some studies trying to demonstrate traditional dandelion application are contradictory. The incomplete or inadequate description of the species and the difficulty in determining the appropriate species are some of the principal factors that could explain this controversy. In this study, different species morphologically very similar, have demonstrated several differences chemical composition. These differences agree with pharmacological activity differences, which provide each species with particular health abilities. We found *T. marginellum* to be the most promising species with anti-oxidative capacity and only *T. lacistrum* to present reliable cytotoxicity over HeLa and Hep G2, with an interesting SI. A proper species determination, using its distribution or deep botanical description is required with plants of *Taraxacum* genus as pharmacological abilities mainly vary between species.

Acknowledgements

A. Plaza acknowledges Universidad CEU San Pablo for his predoctoral grant. Authors wish to gratefully acknowledge Brian Crilly for linguistic assistance.

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T. obovatum (1)

T. marginellum (2)

T. hispanicum (3)

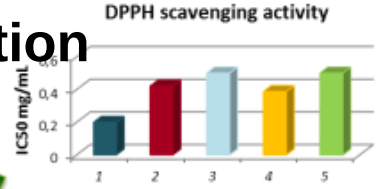
T. lambinonii (4)

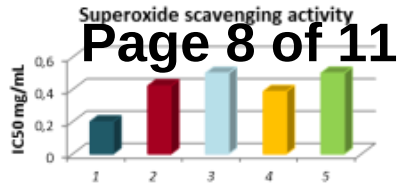
T. lacistrum (5)

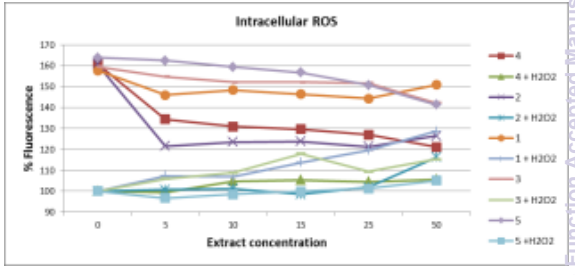
SIMILAR MORPHOLOGY

DIFFERENT COMPOSITION

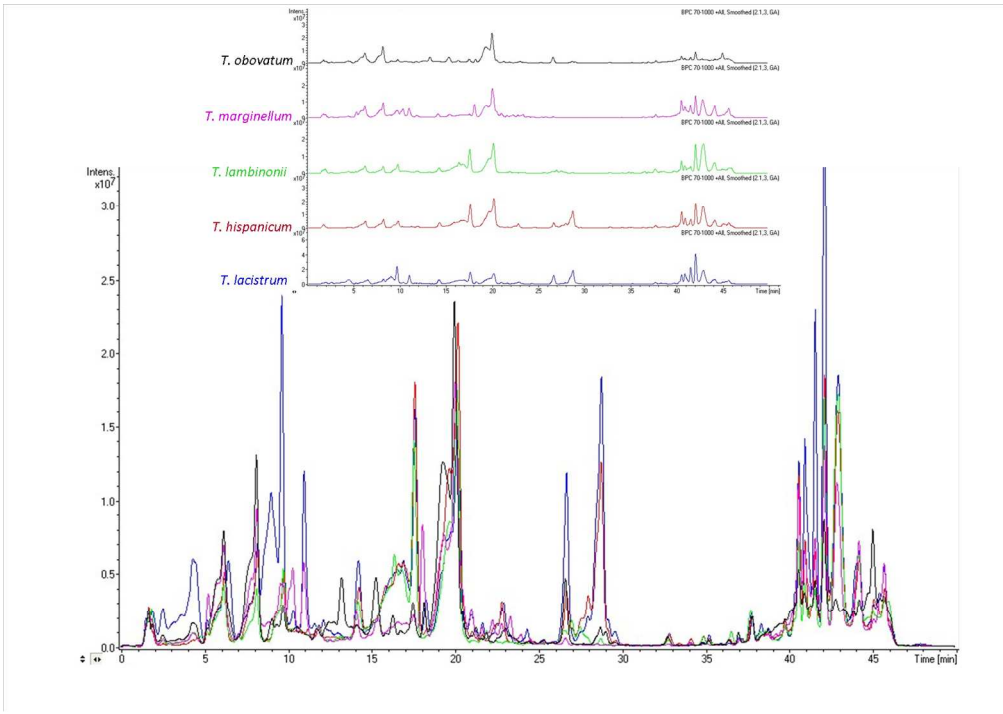
DIFFERENT PHARMACOLOGICAL ACTIVITIES







CYTOTOXICITY	*T. lacistrum*	*T. obovatum* *T. hispanicum* *T. lambinonii* *T. marginellum*
HepG2	0,015 ± 0,001 SI 16,67	>0,25
HeLa	0,023 ± 0,002 SI 10,87	>0,25



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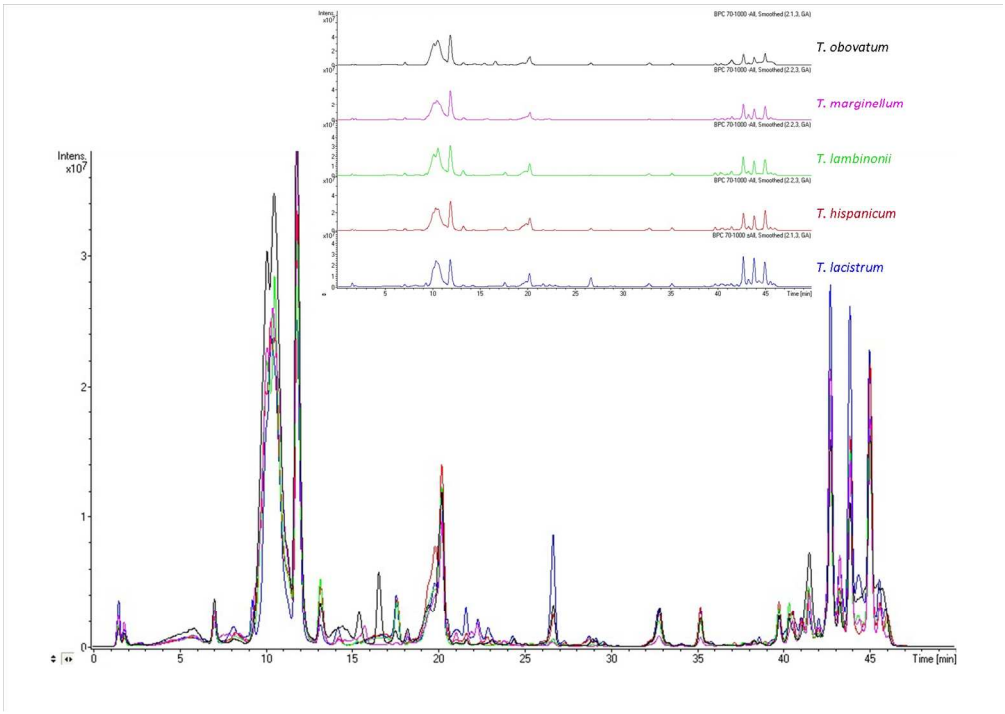

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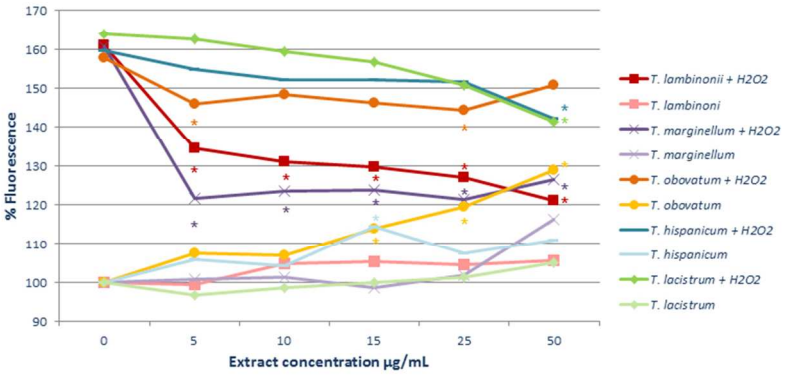

254x190mm (96 x 96 DPI)