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Original Article

EFFECTS OF IMPORTANT PLANT SPECIES OF TERUEL (SPAIN) ON THE PRO-INFLAMMATORY SIGNALLING CASCADE OF HUMAN MONOCYTES.

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Abstract

Background: The study of medicinal and edible plants from Teruel (Spain) – one of the biodiverse regions of the Mediterranean has been particularly limited to the write-up of taxonomical databases and lists of protected species. The following analysis and work will be the first for this region and will focus on the anti-inflammatory activity of plant species, based on information gained through knowledge of their traditional use.

Aims: To use a multitarget, *in vitro* approach focusing on the antioxidant and pro-inflammatory signalling cascade. To provide a phytochemical basis to the traditional and oral traditions of ethnographic research.

Methods: Plants extracts (1-50 µg/mL) from Teruel recorded as traditionally used against indications related to inflammation were assessed as potential inhibitors of the release of inflammatory mediators namely interleukin (IL)-6, IL-8, tumour necrosis factor (TNF)-alpha) and prostaglandin (PG) E2 in primary human monocytes challenged with 10 ng/mL of LPS. Their antioxidant activity as the inhibition of thiobarbituric reactive substances (TBARS), total phenolic content (Folin-Ciocalteau) and phenolic fingerprint (HPLC-UV-DAD) were also evaluated.

Results: *Berberis vulgaris*, *Jasonia glutinosa*, *Satureja montana* and *Sideritis hirsuta* ethanol extracts were potent inhibitors of cytokine production but did not exert significant effects on prostaglandins synthesis. This effect was even stronger when monocytes were incubated in the presence of both aqueous and ethanol extracts of *Thymus vulgaris*. *Prunus spinosa* ethanol extract presents an overall strong pro-inflammatory effect instead. *B. vulgaris, J. glutinosa, S. montana* and *T. vulgaris* ethanol extracts were the richest in phenols (*c.a.* 2 mg/mL caffeic acid equivalents) but the antioxidant effect did not correlate with this parameter pointing towards the presence of potent antioxidants from other phytochemical classes.

Conclusion: Overall, this project provides evidence of the efficacy attributed to several species central to the medical folklore in Teruel (Spain). Their anti-inflammatory and antioxidant activity profile will support further phytochemical-pharmacological investigations.

INTRODUCTION

Teruel is a province in the south of the autonomous community of Aragon and has undergone massive depopulation since the second half of the 20th century. In environmental terms, the province is characterised by Carrascales (dry landscapes abundant in *Quercus rotundifolia* Lam.) and the highlands (characterised by species of sabinas (*Juniperus sabina* L. and other junipers). It is thinly populated and has suffered from intense emigration of the younger people for decades, leading to the sociopolitical concept of "the emptied Spain" (García-Moreno Rodríguez, 2019). At the time of the study, it was the least investigated province in Spain concerning local and traditional medicinal knowledge and practice of food-medicine use. In this region, very much like other regions of the Iberian Peninsula, the divide between food/medicine is very tenuous and as such the selection of plants was based on the traditional management of plants (Alarcon, 2010; Benitez, 2011; Tardio, et.al 2018).

One key characteristic of the region, in terms of its ethnobotany and history, is the desire to preserve and rescue the rich historical data and landscape. The key focus of this project, therefore, was to study the generational knowledge of local botanical resources among the people of Teruel, the factors that have affected the perception and usage of the products, as well any documentation of the introduction of new knowledge to the existent local oral floral tradition. Based on this information, the local context was analysed via *in vitro* protocols for the most prominent species for anti-inflammatory protocols in human primary monocytes and antioxidant activities.

The study of medicinal and edible plants from Teruel has been particularly limited to the writing up and analysis of taxonomical databases and lists of protected species. From 2007 to 2011 in various towns of the province of Teruel, Spain, a multidisciplinary research project in ethnobotany and ethnopharmacology took place for the selection of suitable plants, focusing on the plant species traditionally used in medicine and food with the potential to show antioxidant or anti-inflammatory activities.

The following analysis and work will be the first of its nature to take place for the province of Teruel (Figure 1) and will help shed light on the understanding of how the culture and history of this province have influenced the informants and their choices for the management of the landscape and the plant species within it.

Figure 1. Geographical position of the zone studied

Table 1. Ethnobiological data of the studied plant species (Viteri, 2013)

Plant Scientific Names	Common Names
Family	Local Traditional Uses
Artemisia herba-alba L.	Spanish: Ascencio, Escoba, Manzanilla de Pastor. English: White Wormwood.
Asteraceae	Whole plants are used as a culinary spice or as tea.
Berberis vulgaris L.	Spanish: Arlos. English: Barberry.
Berberidaceae	Fruits are eaten as they are or prepared as a conserve. Said to aid in the healing of arthritis and general bone illness.
Jasonia glutinosa L.	Spanish: Te de Roca, Te de Pena. English: Rock Tea.
Asteraceae	Whole plants are used as a culinary spice. Infusion is used as a body tonic to provide energy and detoxify.
Ligustrum vulgare L.	Spanish: Aligustre. English: Privet.
Oleaceae	Fruits are eaten as a snack. Fruits and leaves are used in injuries or wounds as a poultice. Leaves are used as infusions
Prunus spinosa L.	Spanish: Endrinos, Pacharanes. English: Blackthorn.
Rosaceae	Fruits are eaten when ripened or macerated in pure ethanol and drunk after heavy meals as a digestive aid. The believed medical properties are various from family to family.
Satureja montana L.	Spanish: Ajedrea. English: Winter savoury.
Lamiaceae	Whole plants are used as a culinary spice. Infusions are used to prevent throat conditions. It is locally used to treat fungal and bacterial infections.
Sideritis hirsuta L.	Spanish: Rabo de gato. Rabogato
Lamiaceae	Whole plants are used as culinary spices. Infusions of flowers are used to treat stomach ulcers and intestinal problems. Used for inflammations from infectious injuries
Sorbus domestica L.	Spanish: Mostajo. English: Service tree
Rosaceae	Fruits are prepared as a beverage by macerating in alcoholic drinks, drunk as juice, or as confectionaries. For inflammation of the digestive system, urinary tract, and aid with pressure problems and general bodily well-being. Immature fruits can cause constipation
Thymus vulgaris L.	Spanish: Tomillo. English: Thyme.
Lamiaceae	Whole plants are used as culinary spices and leaves, and flowers are prepared in tea or macerated in wine and drunk after a meal as digestive aids. Also used for flu, bronchitis and pneumonia, injuries and cuts.
Ziziphus jujuba Mill.	Spanish: Cincole, Jinjole, Azufaifa. English: Chinese jujube
Anacardiaceae	Fruits are eaten as they are when ripened, or they can be macerated in an alcoholic drink. Most people dry mature fruits and preserve them like prunes. Fruits and seeds are reported to be good for asthma and inflammations of the joints

MATERIALS AND METHODS

Collection and preservation

The collection of plants took place during the fieldwork session between the years 2007 to 2011 in various towns of the province of Teruel. The selection criteria for the plants were based on their suitability as edible plants, their relative low toxicity when used appropriately and according to the local traditions and preparation. Some of the selected plants (*Berberis vulgaris and Ligustrum vulgare*) may show some toxicity according to the various documents existent in the pharmacological literature, however what makes them important in the diet of the people, is the dosage and the occasion for their consumption (see Table 1 for plant uses).

The methodology for preservation of these plants consisted in storage in a standard refrigeration unit and air dried in a fresh room. For fleshy fruits, a similar procedure took place; however, the fruits were air dried or sun dried (during the summer collections), and subsequently frozen to kill any insects present. All the plants where packed and sent to the School of Pharmacy herbarium (UCL, London) after which they were frozen in the laboratory units at -20°C or freeze-dried for their subsequent extraction in water or ethanol.

Extraction

For the aqueous extraction, 20 g of the ground-dried material was extracted with 200 mL of distilled water in an oil bath on a hot plate. The material was constantly stirred for 15 minutes to prevent oxidation of the compounds, after the oil reached a temperature of 100°C the stirring was kept for 15 minutes. The extracts were decanted and cooled to room temperature in a water bath. Further preparations of the extracts were made with any residual materials from the plants. Any other residues were filtered off and the extract freeze-dried for later use.

For the ethanolic extraction, 20 g of ground dried plant samples were extracted with 300mL of EtOH 80%. To prevent oxidation of the compounds from excessive heat for a long-term, the solute was decanted off after it dripped down to a flask once and cooled down to the room temperature in a water bath. The extraction was performed in triplicate with the residual plant samples. If the solute from the third extraction still had strong colour, the fourth extraction was carried out with 200 mL of the solvent. The solvent was removed by rotary evaporator until nothing came out, and the remaining solvent was removed by freeze drier. The dried-up extracts were subsequently dissolved using DMSO.

High performance liquid chromatography

HPLC-UV analysis: Equipment consisted of an Agilent 1200 series HPLC system with UV-VIS PDA detector (Agilent Technologies, UK), Agilent ChemStation software, Phenomenex® C18 column (250 × 4.6 mm id, 5 µm). Solvent A (H_2O + acetic acid 0.2% v/v) and B (methanol + acetic acid 0.2% v/v) were mixed in gradient mode as follows: 0 min 90% A, 0-5 min 80% A, 5-65 min 50% A, 65-75 min 20% A; flow rate 0.8 mL/min. The injection volume and column temperature were set at 10 µL and 40°C, respectively (Giner et al., 1993).

Total phenol quantification by Folin-Ciocalteau

The method was modified from procedures of Waterman and Mole (1994). Readings were made using a UV plate reader, 2 hours from time zero recording the UV absorption at 765nm. For this experiment, a standard of caffeic acid was used for its subsequent comparison of action with the plant extracts. The percentage of phenols present in both experiments was calculated using the following formula:

Phenols % = $(A_{sample} \times C_{standard})/(A_{standard} \times C_{sample}) \times 100\%$, where: A= absorption of sample or standard; C= concentration of sample or standard solution,

Lipid peroxidation

The protocol used for the experiment of lipid peroxidation was based on that of Houghton et.al. (1995) and Burits and Bucar (2000). All solutions and liposomal suspensions were freshly prepared. The reaction mixture contained 50 µl of liposomal suspension (5 mg/ml type VII Folch bovine brain extract, Sigma-Aldrich, UK), 30 µl of either aqueous or methanolic extract solution (5 µl of extract plus 25 µl of PBS), 10 µl $FeCl_3$ (1mM) and 10 µl ascorbate (1 mM). The reaction mixtures were incubated at 37°C; after 20 minutes, 100 µl of TCA, 2.8% 1 ml of TBA (1% in 50mM $NaOH_{aq}$) and 10 µl of 2,6-ditert-butyl-p-kresol (2% in EtOH 98%) were added, and then the solutions were incubated at 90°C for 1 h to allow the formation of TBARS.

Quantitative determination of TBARS was performed by extracting the reaction mixture with 250 µl of *n*-butanol followed by centrifugation and colorimetry of the organic layer with a UV plate reader (535 nm). All plant extracts were assayed at a final concentration of 0.2 mg/ml. The percentage inhibition of the plant extracts was calculated using the following formula: Inhibition % = 100 x [(AS – ASB) / (ASB)], where **I**=is the Inhibition to be calculated, **AS**= the absorbance in the presence of the inhibitor, **ASB =** is the absorbance of blank experiment in presence of inhibitor.

Release of inflammatory mediators by human peripheral monocytes

Monocytes were extracted from the whole blood of medically healthy volunteers, who provided written informed consent at the local blood bank (University Hospital of Freiburg, Germany), following a standardized protocol (gradient preparation, lymphocytes separation medium GE Healthcare, Freiburg, Germany) using completely endotoxin-free cultivation as previously described (Schmitter et al., 2018). 25 ml Ficoll gradient preparation was loaded with 25 ml blood of buffy coats from healthy blood donors in a 50 ml tube. Afterwards the cells were seeded in a 24-well plate for EI A/ELI SA measurements and the ethanolic and aqueous extracts were prepared in DMSO. Once the extracts were ready, the cells were incubated with the purified, LPS-free extract for 30 min before stimulation with LPS (10 ng/ml) and then incubated for another 24 hours. Concentrations of IL-1β, IL-6, IL-8, TNF-α, and PGE2 were determined in the monocyte supernatants by enzyme immunoassay for PGE2 (AssayDesign, Germany) or ELISA for all cytokines (Immunotools, Frisoythe, Germany) following the manufacturer's instructions. All experiments were carried out with at least two buffy coats from different blood donors in triplicate.

RESULTS AND DISCUSSION

Phytochemical mapping of phenolic compounds

The extracts were fingerprinted by HPLC, and the presence of key phenolic compounds identified. Table 2 shows the presence of key phytochemical markers in a comparative manner.

Phenolic compounds are a chemically diverse, widely distributed, and physiologically very important group of natural products that are commonly found in many plants used within the various diets and medical traditions around the world. Phenolic compounds are extremely important in the growth and development of the defensive mechanisms of plant species (Maisuthisakul et.al. 2007). Theories and studies abound about the potential benefits of plant antioxidants, from management of degenerative conditions to more cosmetic pursuit. As mentioned previously, significant amounts of phenolics have been reported in vegetables, fruits, and traditional plants in many studies. Antioxidant phenolics may scavenge reactive oxygen and nitrogen species and, therefore, potentially modify mechanisms relevant to oxidative stress related diseases (Alkhatib *et al.,* 2017).

Quantification of total phenolic compounds

The Phenols contents (Table 3) were evaluated by Folin Ciocalteu modified into a micro protocol, based on the reports on wine phenol research by Slinkard (et al. 1977). Overall, the results were as expected, with the highest percentage of phenols found in the ethanolic extracts, particularly among the popularly known herbs: *Thymus vulgaris* (79%), *Satureja montana* (72%), *Jasonia glutinosa* (70%), respectively. This was followed by the unusual fruits of *Berberis vulgaris* (65%), *Artemisia herba-alba* (55%), *Prunus* spinosa (38%), and *Ligustrum vulgare* (34%). The lowest amount s of phenols was detected in *Sideritis hirsuta* (23%), *Sorbus domestica* (23%), and *Ziziphus jujuba* (13%).

In the case of aqueous extracts, *Sorbus domestica* showed the highest percentage concentration of phenols (63%). The same takes place with the aqueous extract of *Ziziphus jujuba* (50%), *Prunus spinosa* (48%), *Artemisia herba-alba* (47%), and *Ligustrum vulgare* (46 %). The lowest phenolic concentrations for the aqueous extracts were found in Sideritis hirsuta (22.90%), *Thymus vulgaris* (20%), *Satureja montana* (17%), *Prunus spinosa* (12%), *Berberis vulgaris* (8%), and *Jasonia glutinosa* (3%).

The contrasting results obtained are interesting, in that fruits seem to yield more phenols in water extracts, (*Sorbus domestic* and *Ziziphus jujube*) whilst phenols of herbs are better extracted in ethanol. It is worth mentioning that many of the plant products for sale today are alcoholic tinctures, because medicinal plants that have been extracted in such way yield far better results in their applications.

Table 2. Determination of key phenolic compounds by HPLC-UV.

Aqueous extracts	Rutin	Lutein	Catechin	Apigenin	Ferulic Acid	Caffeic Acid	Chlorogenic Acid	Chrysin	Hesperidin	Ellagic Acid	Epicatechin
Artemisia herba-alba				X			X				
Berberis vulgaris	X						X				
Jasonia glutinosa	X				X		X				
Ligustrum vulgare											
Prunus spinosa	X	X		X							
Satureja montana						X	X	X			
Sideritis hirsuta							X			X	
Sorbus domestica						X	X				X
Thymus vulgaris	X			X							
Ziziphus jujuba						X					
Ethanolic extracts											
Artemisia herba-alba				X			X				
Berberis vulgaris	X						X				
Jasonia glutinosa	X				X		X				
Ligustrum vulgare											
Prunus spinosa	X	X		X							
Satureja montana						X	X	X			
Sideritis hirsuta							X			X	
Sorbus domestica						X	X				X
Thymus vulgaris	X			X							
Ziziphus jujuba						X					

Lipid peroxidation

Lipid peroxidation is a well-established mechanism of cellular damage in biological systems, and it is used as an indicator of oxidative stress in cells and tissues. It is a radical-initiated chain reaction with self-propagation in cellular membranes. As a result, isolated oxidative events may have profound effects on membrane function. The reactions of this process involve: initiation, propagation, and termination (Kelly et.al. 1998). Table 3 shows the values of reactive species in the presence of the plant extracts. We could not find any correlation between phenol contents and the inhibition of TBARS, thus other phytochemicals such as terpenoids and alkaloids may have accounted for the results seen.

Table 3. Phenols quantification by Folin-Ciocalteau (equivalents of caffeic acid) and Lipid peroxidation (in TBARS). All results are the Mean ± SD (*n*=3-5).

Plant Species	Phenols (%)		TBARS (mg/ml)	
	EtOH extract	*Aqueous extracts*	*EtOH extract*	*Aqueous extracts*
Artemisia herba-alba	1.60±0.26	0.05±0.024	0.093±0.049	0.04±0.02
Berberis vulgaris	1.90±0.35	0.09±0.033	0.023±0.115	0.088±0.033
Jasonia glutinosa	2.04±0.21	0.16±0.098	0.018±0.0726	0.16±0.097
Ligustrum vulgare	1.00±0.20	0.06±0.010	0.14±0.052	0.062±0.0103
Prunus spinosa	1.12±0.19	0.0052±0.039	0.12±0.062	0.0052±0.038
Satureja montana	2.11±0.70	0.30±0.091	0.086±0.082	0.30±0.09
Sideritis hirsute	0.68±0.14	0.18±0.16	0.275±0.077	0.18±0.16
Sorbus domestica	0.66±0.08	0.1±0.058	0.042 ±1.25	0.18±0.053
Thymus vulgaris	2.31±0.22	0.057±0.023	-0.012±0.17	0.056±0.023
Ziziphus jujuba	0.39±0.10	0.18 ±0.15	0.16 ±0.11	0.18 ±0.15

The inhibition of lipid peroxidation and radical scavenging power of plants might be important in fighting diseases by conferring protection against free radical damage to cellular DNA, lipids, and proteins (Badmus et.al. 2011). Although there has been certain controversy in the literature regarding the specificity of the TBARS method (Siriwatanametanon, 2010), this is a method that is still widely employed. The occurrence of malondialdehyde as the by-product of the process of lipid peroxidation provides with an estimation on the level of action of the plants in the process of stopping or promoting the peroxidation.

Antiinflammatory activity

The results of the plant extracts on the release of inflammatory mediators by human peripheral monocytes are presented in full in Figure 2. The activities of the extracts are summarised in Table 4.

Our results show that ethanol extracts tend to show a stronger anti-inflammatory profile whilst aqueous extracts tend to have an overall weaker action and even a pro-inflammatory effect on one or more mediators.

Berberis vulgaris and *Jasonia glutinosa* ethanol extracts managed to prevent the LPS-induced production of all studied pro-inflammatory mediators in human monocytes at 50 µg/ml. *Satureja montana* and *Sideritis hirsute* ethanol extracts were potent inhibitors of cytokines production but did not exert significant effects on prostaglandins synthesis. This effect was even stronger when monocytes were incubated in the presence of both aqueous and ethanol extracts of *Thymus vulgaris*.

Prunus spinosa extract present an overall strong pro-inflammatory effect. Therefore, their use should be cautionarily discouraged until their *in vivo* effects are ascertained. Pro-inflammatory effects of plant extracts have been previously described for several plant extracts. For example, extracts of *Ranunuculus sceleratus* show both pro- and anti-inflammatory effects depending on the nature of the pro-inflammatory challenge used in *in vitro* models (Prieto et al., 2008). This is not a negative effect as ethnopharmacological uses of such plant extracts with “dual effects” are exploited by ancient cultures, in this case the inhabitants of the Vancouver region in Canada using them as “rubefacient” or “counter irritants” (Turner, 1984). The aqueous extract of *Sideritis hirsuta* shows a specific pro-inflammatory effect on the arachidonic acid pathway. It would be important to ascertain if this effect is restricted to the COX pathway or if it also enhances the production of leukotrienes as they have an important role in chronic skin inflammation (Sharma & Mohammed, 2006).

Figure 2. Release of inflammatory mediators (pg/ml) by human monocytes treated with increasing concentrations (1, 10 or 50 µg/ml) of the herbal drug extract in the presence of LPS. Bars are Mean ± SD (*n*=3). Solvent (DMSO) negative controls are in represented by the blue bars; vertical axis shows normalised % of control.

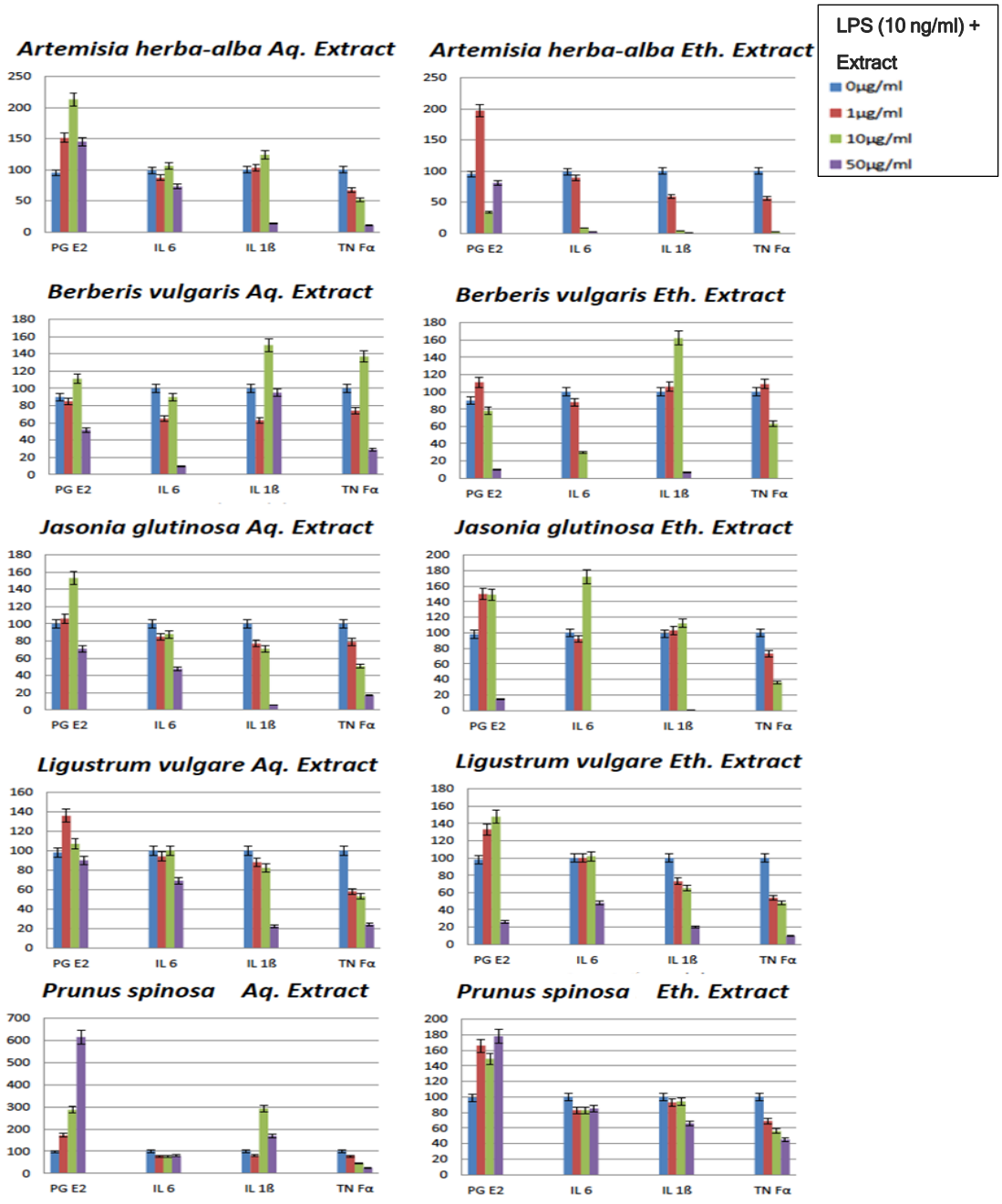

Figure 2 (Continuation). Release of inflammatory mediators (pg/ml) by human monocytes treated with increasing concentrations (1, 10 or 50 µg/ml) of the herbal drug extract in the presence of LPS. Bars are Mean ± SD (*n*=3). Solvent (DMSO) negative controls are in represented by the blue bars; vertical axis shows normalised % of control.

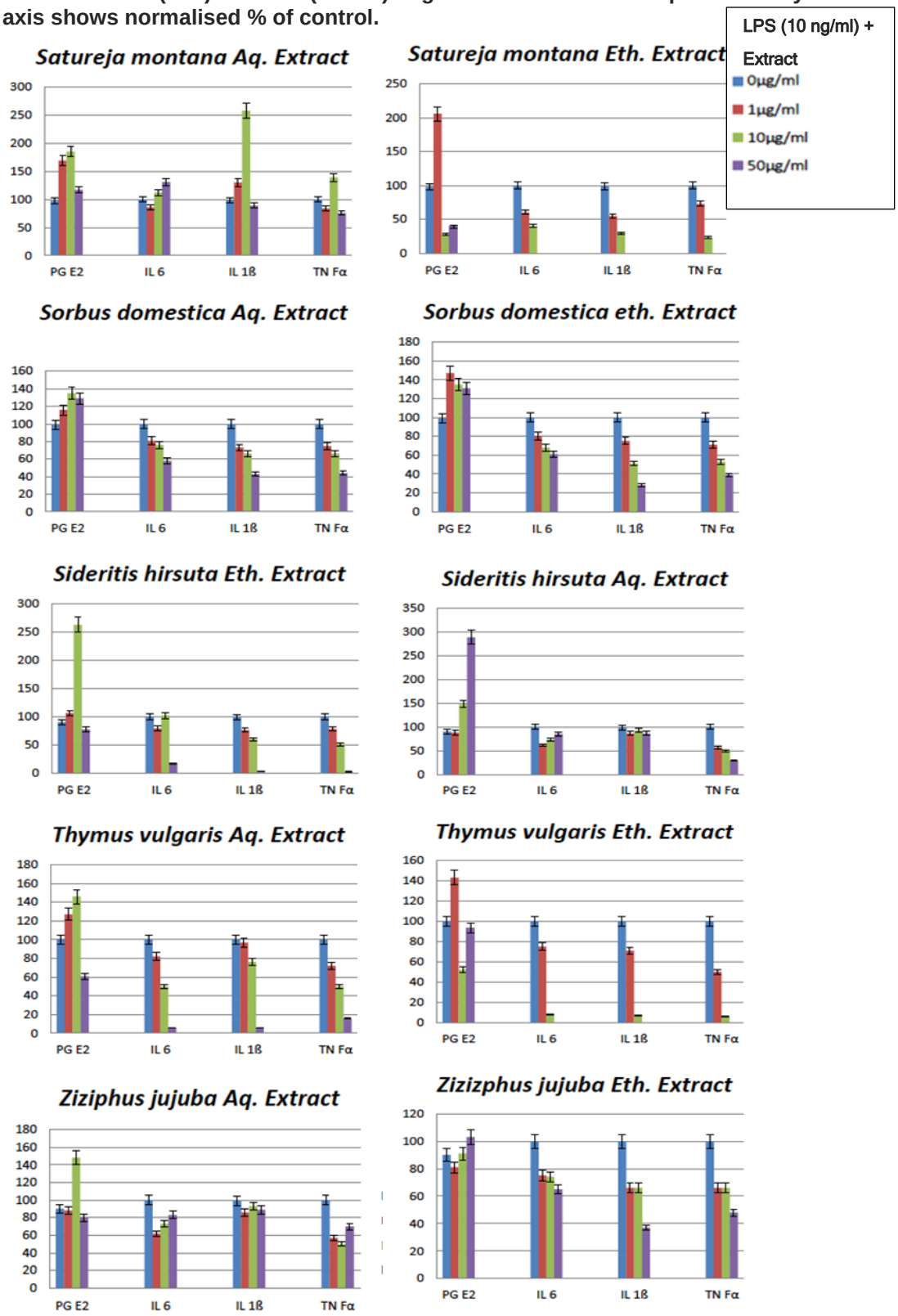

Table 4. Relative *in vitro* anti-inflammatory effect of the herbal drug extracts at their maximum concentration (50 µg/ml).

Plant species	Extract	PGE_2	IL-1β	IL-6	TNFα
Artemisia herba-alba	Aqueous	-50%	26%	87%	89%
	Ethanol	14%	97%	99%	100%
Berberis vulgaris	Aqueous	38%	90%	5%	71%
	Ethanol	80%	100%	93%	100%
Jasonia glutinosa	Aqueous	29%	52%	94%	83%
	Ethanol	84%	100%	98%	100%
Ligustrum vulgare	Aqueous	8%	31%	78%	76%
	Ethanol	72%	52%	80%	81%
Prunus spinosa	Aqueous	-79%	15%	34%	55%
	Ethanol	-516%	20%	-70%	74%
Satureja montana	Aqueous	-19%	-31%	10%	24%
	Ethanol	59%	100%	99%	100%
Sideritis hirsuta	Aqueous	-199%	15%	13%	71%
	Ethanol	13%	83%	96%	98%
Sorbus domestica	Aqueous	-30%	42%	57%	56%
	Ethanol	-32%	39%	72%	61%
Thymus vulgaris	Aqueous	39%	94%	94%	84%
	Ethanol	7%	100%	100%	100%
Ziziphus jujuba	Aqueous	17%	34%	62%	38%
	Ethanol	-13%	35%	63%	52%

CONCLUSIONS

Berberis vulgaris, *Jasonia glutinosa*, *Satureja montana* and *Sideritis hirsuta* ethanol extracts were potent inhibitors of cytokines production but did not exert significant effects on prostaglandins synthesis. This effect was even stronger when monocytes were incubated in the presence of both aqueous and ethanol extracts of *Thymus vulgaris*. *Prunus spinosa* ethanol extract presents an overall strong pro-inflammatory effect instead.

Berberis vulgaris, Jasonia glutinosa, Satureja montana and *Thymus vulgaris* ethanol extracts were the richest in phenols (*c.a.* 2 mg/mL caffeic acid eq) but the antioxidant effect did not correlate with this parameter pointing towards the presence of potent antioxidants from other phytochemical classes.

Overall, this project provides evidence of the efficacy attributed to several species central to the medical folklore in Teruel (Spain). Their anti-inflammatory and antioxidant activity profiles will support further phytochemical-pharmacological investigations.

Conflicts of Interest

The authors declare no personal or financial conflict of interest related to this work.

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Authors contribution

Investigation (M.V.); Resources (M.V. & M.H.); Writing-Original Draft (M.V., J.M.P.); Writing - Review & Editing (M.H., B.F.). Visualization (J.M.P.); Supervision (M.H. & J.M.P.); Funding acquisition (M.H.).

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