Ranking Mediterranean-type shrubs and trees by their allelopathic activity is not independent of how extract concentration is expressed

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Water extracts from 19 Mediterranean-type shrubs and trees were screened for phytoactivity on germination of lettuce. The existing model for the effects of pH and osmotic pressure on germination requires refitting. Extract concentrations were expressed as plant fresh weight, plant dry weight and extract dry weight, and final ranking of the six phytoactive species was found to strongly depend on the way the concentrations are expressed. This methodological issue requires consideration when designing allelopathic bioassays. Extract dry weight is conceptually the most adequate way to express concentration and should be used, despite the increase in time and labour it requires.

Keywords: Allelopathy, extract concentration, germination, osmotic pressure.

ALLELOPATHY refers to all types of effect in one plant produced by chemical compounds synthesized by another or the same plant, the term having been coined in the late 1930s (ref. 1). Research on allelopathy relies heavily on bioassays in which effects of extracts of plant parts on germination or growth are investigated. A number of methodological requirements of bioassays have been examined, including the dependency of functional availability of phytochemicals on seed density[2,3], the relevance of seed morphology[4], selection of target species or cultivars[5], types of solvent used for phytochemicals extraction[6], and combined effects of pH and osmotic pressure of solutions[7].

However, except when compounds are tested individually, single dose or dose–response designs have to deal with the concentration of tested extracts. This also applies to the study and modelling of the putative hormetic, dual actions (stimulation and inhibition) of mixtures of allelochemicals[8] or the study of density-dependent phytotoxicity[2,9].

Therefore questions inevitably arise on how to express concentration, i.e. in relation to the fresh weight of plant parts extracted, to their dry weight or to the dry weight of the compounds extracted. To answer these questions we set out to investigate the effects, corrected for pH and osmotic pressure, of a series of concentrations of water extracts of intact above-ground vegetative parts of Mediterranean-type shrubs or trees. Since the range of pH and osmotic pressure of extracts lay outside the range to which the equations were fitted[7], the original model was extended and a new equation had to be fitted.

Materials and methods

Plant materials and extraction

Nineteen Mediterranean-type species, mostly non-cultivated aromatic shrubs, from ten different families (Supplementary Table 1), were harvested in mid-winter before flowering, near Valverde, Évora, southern Portugal. Seeds of lettuce (*Lactuca sativa* L. cv. 'Rainha de Maio'; Asteraceae) were used in the bioassays.

Vegetative shoots were collected and transported in refrigerated plastic bags to the laboratory, where 30 g of intact leaves (because of the spiny nature of leaves or their replacement by spines complete shoot tops were used in *Calicotome villosa* and *Ulex parviflorus*) weighed to the nearest milligram were soaked in distilled water and extracted for 40 h at 30°C and constant darkness. For *Eucalyptus globulus*, because of its marked leaf heteroblasty young and mature leaves were separately extracted.

Extracts were filtered through Whatman No. 1 filter paper and adjusted to the concentration of 100 mg ml⁻¹ (plant fresh weight volume⁻¹, PFW), which was used as stock solution for the preparation of 50 and 25 mg ml⁻¹ PFW solutions. When not in use, stock solutions were kept at –20°C. After extraction, leaves (or shoot tops) were dried at 60°C in an aerated oven and weighed to the nearest milligram, thus allowing concentrations to be expressed as plant dry weight volume⁻¹ (PDW) after adding the dry weight of extracted material. Finally, for each donor species, four 2 ml aliquots of the highest PFW were dried at 60°C in an aerated oven and the dry residues weighed to the nearest 0.1 mg, thus allowing

concentrations to be expressed as extract dry weight volume^{-1} (EDW).

The pH and osmotic pressure ($\psi\pi$) of the three concentrations of all extracts were determined with a pH-meter Metrohm E-520 (Metrohm AGH, Switzerland) and a semi-micro freezing-point osmometer Knauer type M (Knauer, Wissenschaftliche Geräte GmbH, Germany) respectively.

Effects of pH and osmotic pressure

The experiment followed a dose–response design and a rationale similar to that described elsewhere for a 3×4 arrangement of pH (intended at 3.0, 4.5 and 6.0) and $\psi\pi$ (intended at 0, 25, 50 and 100 mOsmol kg^{-1})[7], except that a 4×2 arrangement of pH (intended at 4.0, 5.0, 6.0 and 8.5) and $\psi\pi$ (intended at 0 and 25 mOsmol kg^{-1}) was used. Solutions were prepared with 0.1 M HCl or NaOH and polyethylene glycol PEG-400 (Carbowax, Fisher Chemical, USA average molecular weight 380–420). pH and $\psi\pi$ values were ascertained with a pH-meter and a semi-micro freezing-point osmometer as described above. Four replicated 10 cm glass petri dishes were fitted with Whatman No. 1 filter paper, sown with 25 lettuce seeds, and wetted with 5 ml of the appropriate pH and $\psi\pi$ solution. Seeds were incubated under 20°C and 8 h photoperiod provided by seven fluorescent tubes (Philips TLD 18W/84), considered germinated if the radicle was at least as long as the greater dimension of the seed[10], and regularly counted and discarded during seven days.

Extract phytoactivity

Two replicated 10 cm glass petri dishes per donor species and concentration were fitted with Whatman No. 1 filter paper, sown with 50 lettuce seeds and wetted with 5 ml of the appropriate extract. Controls were prepared similarly with distilled water adjusted with 0.2 M HCl to pH 5.3. Seeds were incubated and considered germinated as described above. Germinated seeds were regularly counted and discarded during 14 days or less.

Statistical analyses

Linear regression analyses, Pearson's correlation coefficient analyses and ANOVA were done with Statgraphics 4.2 (STSC, Inc., Rockville, MD, USA), all other statistics with Excel®2010 (Microsoft Corporation, USA). Coefficients of determination (R^2) of regressions with replication are presented as the proportion of maximum R^2 value possible[11] and data as mean ± SE.

The equation fitted previously[7] to the relationship between pH and $\psi\pi$ and germination was used to generate expected values of germination using pH and $\psi\pi$ values of this experiment. Assessment of the adequacy of equation was done using two-tailed Student's t test for paired comparisons[12].

Given the result of the test we decided to fit a new equation pooling together data for which the equation had been originally fitted with data from this experiment, datasets A and B respectively (Supplementary Figure 1). As done before[7], the relationship between jackknifed values[13] of germination, and pH and $\psi\pi$ was investigated by stepwise least squares regression with replication and an experiment-wise error rate for coefficients of 0.01 calculated by Dunn-Šidák method[14]. Polynomials were used, and candidate models included up to the third power of pH or $\psi\pi$ and all interactions between them. Adequacy of the new equation was assessed as described above.

In germination bioassays, observed data (Y_{obs}) were transformed to observed effects (Y_{OE}) as

$$Y_{OE} = (Y_{obs} - \bar{Y}_C)/\bar{Y}_C, \tag{1}$$

where $\bar{Y}_C$ is the observed mean of control. Thus, the control was normed so that the mean of its effects was zero. Positive values of Y_{OE} corresponded to values of observed germination higher than the control (stimulation), negative values to observed values lower than the control (inhibition). Values of germination to be expected from the combined effects of pH and $\psi\pi$ alone, regardless of the chemical composition of extracts, were calculated from eq. (2) below.

Expected values were transformed to expected effects (Y_{EE}) using eq. (1). Finally, effects corrected (Y_{CE}) for pH and $\psi\pi$ were determined by subtracting expected effects from observed effects ($Y_{CE} = Y_{OE} - Y_{EE}$). Corrected effects of all treatments were compared with those of control by two-tailed Student's t test for unpaired comparisons after checking for homoscedasticity using the two-tailed F distribution, always using a significance level of $P = 0.05$.

Species for which statistically significant corrected effects of extracts were found were further analysed and the relationship between corrected effects and concentrations (PFW, PDW or EDW normed so that the highest concentration is unity) was studied by stepwise least squares regression with replication and an experiment-wise error rate for coefficients of 0.05 calculated by Dunn-Šidák method[14]. Polynomials were used as candidate models, including up to the third power of normed concentrations and were forced through the origin because corrected effects for controls (PFW = PDW = EDW = 0 mg ml^{-1}) are necessarily zero.

Results

Effects of pH and osmotic pressure

In some cases the intended values of pH or $\psi\pi$ in dataset B were not exactly matched (Supplementary Table 2).

Nevertheless, because the overall design of the experiment is dose–response, the small differences observed do not prevent their use.

Significant differences were found between observed and predicted values of germination for all data (t_{31} = 3.416, P = 0.002) when the equation previously fitted to dataset A[7] was used on dataset B. Differences were essentially due to new combinations of pH and $\psi\pi$ values outside dataset A (t_{15} = 2.992, P = 0.009) while for combinations common to datasets A and B, no significant differences were found (t_{15} = 1.824, P = 0.088). Ordering observed germination data of dataset B by the magnitude
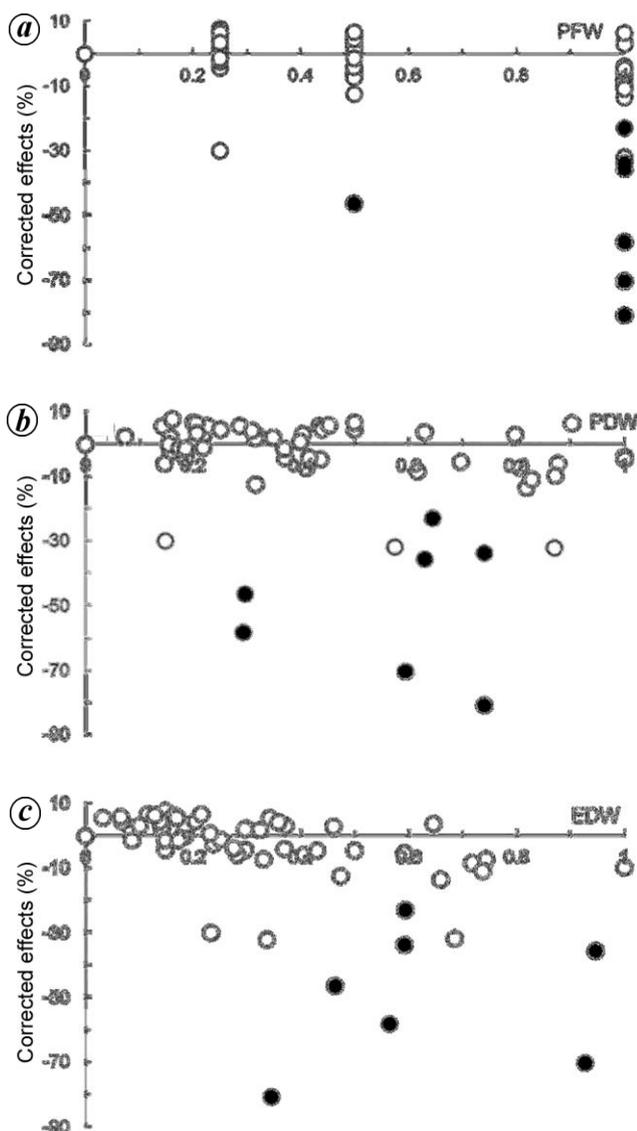

Figure 1. Relationship between effects of extracts corrected for pH and osmotic pressure on germination of *Lactuca sativa* cv. 'Rainha de Maio'. Extract concentrations are expressed as (**a**) plant fresh weight volume^{-1} (PFW), (**b**) plant dry weight volume^{-1} (PDW), and (**c**) extract dry weight volume^{-1} (EDW). All concentrations were normed so that the highest value was unity. Open symbols indicate no significant differences in relation to control and filled symbols show that effects are significantly different from control at P = 0.05.

of errors (observed minus predicted, regardless of sign) and sequentially removing errors starting with the largest, germination under pH 8.5 and $\psi\pi$ 25 mOsmol kg^{-1} was clearly the main cause of extrapolation failure (t_{12} = 1.678, P = 0.121 when the four larger errors, all from that combination, were removed).

Therefore we set out to fit a new equation pooling together data of datasets A and B. The equation fitted for the expected values of germination (G_{exp}) of lettuce was significant for all coefficients (P < 0.0002), lack of fit was significant ($F_{11,49}$ = 5.076, P = 3.051 × 10^{-5}) and R^2 = 0.654. The new equation is

$$G_{exp} = 95.52307 - 5.62467 \times 10^{-4} \ \psi\pi^3 + 1.60676$$

$$\times 10^{-4} \ \text{pH} \ \psi\pi^3 - 1.86981 \times 10^{-6} \ \text{pH}^3 \ \psi\pi^3. \qquad (2)$$

The term in $\psi\pi$ alone accounts for 49.3% of the variation of germination explained by the equation, the two terms in pH and $\psi\pi$ for 50.7%. No significant differences were found between observed and expected values for pooled data of datasets A and B ($t_{63} \approx 0$, $P \approx 1$), neither for dataset A alone (t_{31} = 1.692, P = 0.101) nor for dataset B alone (t_{31} = 1.568, P = 0.127).

Extract concentration

Concentrations of undiluted stock solutions (100.0 mg ml^{-1}, PFW) expressed as plant dry weight volume^{-1} (PDW) had a coefficient of variation (CV) of 22.5% ranging from 16.3 mg ml^{-1} in *Foeniculum vulgare*, indicating that more than 80% of its fresh weight was water, to 55.8 mg ml^{-1} in *Quercus coccifera* and *Quercus ilex*, both Fagaceae, indicating that less than half of their fresh weight was water (Supplementary Table 1). Extract concentration expressed as EDW had a larger coefficient of variation (CV = 39.0%) ranging from 1.4 mg ml^{-1} in *Quercus suber*, indicating that less than 3% of biomass of intact leaves could be extracted by water, to 11 mg ml^{-1} in *Phyllirea angustifolia* (Supplementary Table 1). However, in a number of species the fraction of biomass of intact leaves (or shoot tops) that could be extracted by water was greater than that in *P. angustifolia*, the maximum being attained in *F. vulgare* in which almost 40% of biomass was extracted by water.

There was a clear trend of an inverse relationship between PDW and EDW, both expressed as percentage of PFW (Supplementary Figure 2 *a*), but Pearson's correlation coefficient between them was not significant (r_{18} = –0.405, P = 0.077). However, when the data were reanalysed without *F. vulgare*, a significant negative correlation coefficient emerged (r_{17} = –0.546, P = 0.016). Using the ratio EDW/PDW instead of EDW/PFW (Supplementary Figure 2 *b*), a highly significant negative correlation was observed even with *F. vulgare* present (r_{18} = –0.845,

$P < 10^{-4}$ compared with $r_{17} = -0.773$, $P = 10^{-4}$ without *F. vulgare*).

Extract phytoactivity

Germination ranged between 17.6% ± 2.0% (*C. villosa*, higher concentration) and 98.0% ± 0% (*Cistus monspeliensis*, lower concentration). Germination of control was 91.0% ± 3.0%. Effects ranged between –80.6% ± 2.2% and 7.7% ± 0%. Positive effects were found in 47% of bioassays, including all concentrations of *Arbutus*
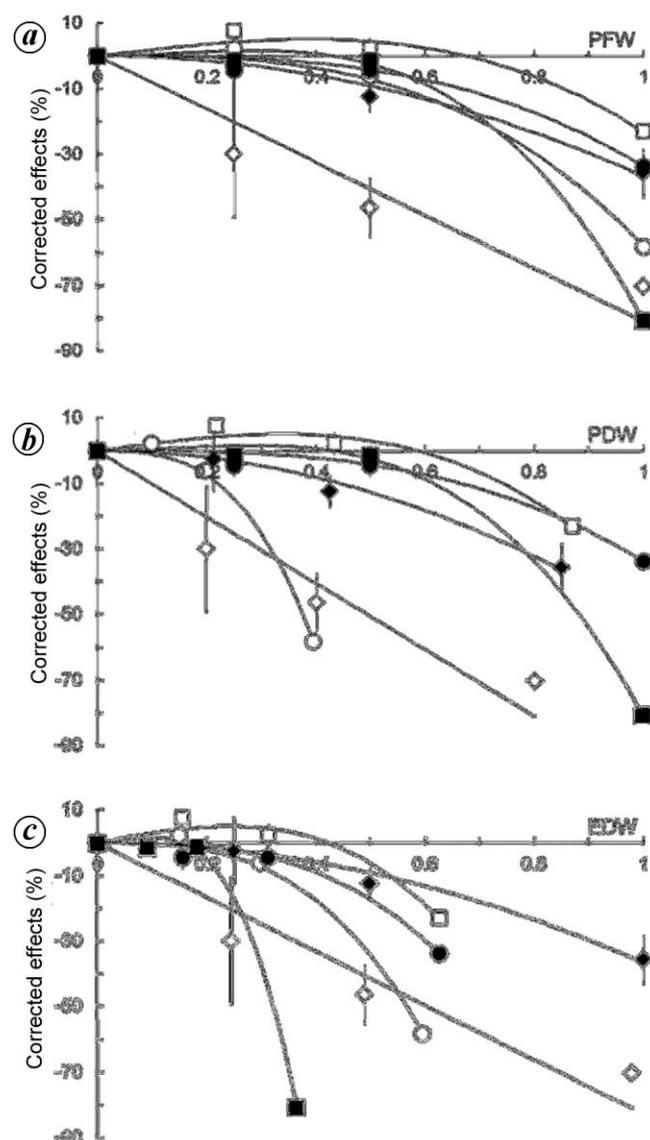

Figure 2. Expected and observed effects of extracts corrected for pH and osmotic pressure on germination of *L. sativa* cv. 'Rainha de Maio' (means ± SE). Extract concentrations are expressed as (***a***) PFW, (***b***) PDW and (***c***) EDW. Concentrations were normed so that the highest value was unity. Open circles, *Foeniculum vulgare*; filled circles, *Cistus ladanifer*; open squares, *Cistus monspeliensis*; filled squares, *Calicotome villosa*; open diamonds, *Lavandula stoechas* and filled diamonds, *Daphne gnidium*.

unedo and *Q. suber*, while negative effects were found in 53% of bioassays, including all concentrations of *Cistus ladanifer*, *C. villosa*, *Lavandula stoechas*, *P. angustifolia* and *Daphne gnidium*.

After being corrected, pH and $\psi\pi$ effects ranged between −80.7% ± 2.2% (*C. villosa*, higher concentration) and 7.7% ± 0% (*C. monspeliensis*, lower concentration). Positive corrected effects were found in 44% of bioassays, including all concentrations of *A. unedo*, *Q. suber* and *Cistus crispus*, while negative corrected effects were found in 56% of bioassays, including all concentrations of the five species found in uncorrected effects.

Significant differences between corrected effects of extracts and control were found in the intermediate concentration of *L. stoechas* ($t_2 = 4.856$, $P = 0.040$) and in the higher concentrations of *F. vulgare* ($t_2 = 15.435$, $P = 0.004$), *C. ladanifer* ($t_2 = 9.236$, $P = 0.012$), *C. monspeliensis* ($t_2 = 6.915$, $P = 0.020$), *C. villosa* ($t_2 = 20.502$, $P = 0.002$), *L. stoechas* ($t_2 = 12.315$, $P = 0.007$), and *D. gnidium* ($t_2 = 4.432$, $P = 0.047$).

When corrected effects were plotted against PFW (Figure 1 *a*), there was a clear increase in the variability of responses with concentration, with almost all significant differences at the higher end of the range of concentrations. Conversely, when corrected effects were plotted against PDW (Figure 1 *b*), the larger variability of responses and significant differences were located mostly in the middle of the range of concentrations, with the lower and higher ends showing less variability and no significant differences. Finally, when corrected effects were plotted against EDW (Figure 1 *c*), the larger variability of responses and significant differences were located mostly in the upper half of the range of concentrations.

Regression equations could always be fitted to the relationship between corrected effects on germination of lettuce and concentration of extracts of the six species found to significantly affect germination, with coefficients of determination always greater than 0.966 (Supplementary Table 3). Relationships were always curvilinear, except in *L. stoechas* for which a straight line was found (Figure 2).

Discussion

Effects of pH and osmotic pressure

The possible contribution of pH and $\psi\pi$ to the effects of allelochemicals on germination has long been recognized but only rarely accounted for[15,16], being assumed without evidences that pH and $\psi\pi$ act independently on seed germination.

The equation that was previously fitted to predict the response of germination of lettuce cv. 'Rainha de Maio'[7] was based on a range of pH and $\psi\pi$ values that only

partly coincided with the values measured in this study. That equation clearly had good interpolation properties but failed when used for extrapolation, especially for the combination of pH 8.5 and $\psi\pi$ 25 mOsmol kg^{-1}. Therefore, its use outside the range for which it was originally fitted should be avoided. Conversely eq. (2) is clearly an adequate predictor of the combined effects of pH and $\psi\pi$ measured in extracts bioassayed in this experiment.

Extract concentration

Given the experimental design adopted, the concentration of all stock solutions expressed as PFW was necessarily the same. Naturally a completely different picture emerges when extracts concentration are expressed as PDW or EDW, with the variation in concentration extending by almost one order of magnitude in the latter.

Also, there is a clear inverse trend between PDW and EDW, stronger when the ratio EDW/PDW is used instead of EDW/PFW. This implies that as the amount of water in leaves (or shoot tops) decreases and consequently as PDW increases, there is a decrease in the amount of leaf or shoot top materials extractable by water in a gradient of diminishing water extractability from *F. vulgare* to the highly sclerophyllous *Quercus*.

Extract phytoactivity

Most parts of the extracts tested were ineffective, with less than one-third of species able to reduce germination of lettuce, a species known to be highly sensitive to exogenous phytochemicals[5]. This prevalence of ineffectiveness is somehow surprising because phytoactivity was reported in some of those species, namely *Rosmarinus officinalis*[17] and *E. globulus*[18], while the known phytochemical composition of others would make them strong candidates to affect germination, namely *Cistus salviifolius*[19], *A. unedo*[20] or *Myrtus communis*[21].

Chaparral shrubs and trees as those tested here produce high amounts of terpenoids[22] known to possess a large spectrum of biological activities, notably phytoactivity and allelopathy[23], their production increasing strongly, at least in isoprene and monoterpenes, as temperature rises[24]. Therefore, explanation for the small number of phytoactive species found might be that the collection of materials for extraction was done in mid-winter, the rainy and colder season, while in the above-mentioned studies collection of plant materials was done in dry, hotter periods, when higher amounts of allelochemicals were probably present.

Conversely, to our knowledge there are no previous reports of allelochemicals phytoactivity for three of the six species effective on germination of lettuce, namely *C. monspeliensis*, *C. villosa* and *D. gnidium*.

When effective, significant inhibition of germination of lettuce after correction for pH and osmotic pressure extended from moderate to very intense, ranging from −23% to –81%.

Ranking species by corrected effects, the least effective was *C. monspeliensis* and the most effective was *L. stoechas* followed by *C. villosa* and *F. vulgare* when extract concentration was expressed as PFW.

Ranking by PDW, the least effective was either *D. gnidium* or *C. monspeliensis* and the most effective was clearly *F. vulgare* because even if corrected effects were slightly less intense than those by *L. stoechas* and *C. villosa*, they resulted from a much lesser concentration in terms of plant dry weight.

Finally, ranking by EDW the least effective was again *D. gnidium* and the most effective was *C. villosa*, because not only its corrected effects were the largest but also because they were obtained from a much smaller amount of extracted allelochemicals.

Therefore, the way concentration of extracts is expressed has important consequences in ranking species by their effectiveness. Considering only the most effective species, expressing concentration by the usual plant fresh weight or plant dry weight, *L. stoechas* or *F. vulgare* respectively, two species known for their phytoactivity, top the list. Conversely, expressing concentration in terms of extract dry weight, the pioneer, spiny but digestible legume *C. villosa*[25,26], known to produce a number of alkaloids, flavones, isoflavones, and phenolics[27–29] is undoubtedly the most effective species. This is noticeable because shoot tops instead of leaves had to be extracted, while a similar thorny legume, *U. parviflorus*, in which shoot tops had also to be extracted, had approximately the same dry/fresh weight ratio and twice the amount of extract dry weight, but was clearly ineffective.

Determining the weight of extracts is time-consuming and labour-intensive in relation to just weighing fresh plant parts or dry plant parts. However, it is clearly the most appropriate way to express extract concentration because it is the extracted materials alone and, not the extracted and non-extracted materials (as in PDW), or the latter and water (as in PFW) that are actually being assayed. Given the differences associated with the way concentration is expressed, determining extract weight is worth the additional effort, especially when implicit or explicit comparisons between sources of allelochemicals are intended.

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