Original article

Extraction of flavonoids from *Tagetes patula*: process optimization and screening for biological activity

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**ABSTRACT**

The flowers of *Tagetes patula* L., Asteraceae, commonly known as French marigold, are used in folk medicine as an antiseptic, diuretic, blood purifier and insect repellent. This study was conducted to optimize the extraction process through the biomonitoring of flavonoids, using a statistical mixture simplex-centroid design, to evaluate the effect of the solvents water, ethanol and acetone, as well as mixtures of these solvents, assessed by the total flavonoid content. The extracts were tested for dry residue, radical scavenging activity, chromatographic profile, and larvicidal activity. The acetone extract had the highest total flavonoid content, 25.13 ± 1.02% (4.07%); and the best radical scavenging activity, with IC50 of 15.74 μg/ml ± 1.09 (6.92%), but with lower dry residue, 6.62 ± 1.33% (20.10%). The water extracts showed higher levels of dry residue, but lower total flavonoid content and radical scavenging activity than the acetone extract. The positive correlation between the total flavonoid content and radical scavenging activity of the extracts showed that flavonoids contribute significantly to the antioxidant capacity. The statistical mixture design allowed us to optimize the extraction of flavonoids from flowers of *T. patula*, with acetone as the best extraction solvent. Preliminary studies on the biological activity of the optimized extracts demonstrated a larvicidal effect of the acetone extract on *Aedes aegypti* mosquitoes.

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**Introduction**

*Tagetes patula* L., Asteraceae, popularly known as French marigold, originated in Mexico. It is widely used as an ornamental plant and is sold freely in open markets and garden shops (Vasudevan et al., 1997). In folk medicine the flowers and leaves have been used for their antiseptic, diuretic, depurative and insect repellent activities (Chadha, 1976). Chemical studies with flowers and leaves of *T. patula* identified terpenes (Prakash et al., 2012), alkaloids (Faizi and Naz, 2002), carotenoids (Piccaglia et al., 1998), thiophenes (Szarka et al., 2006), fatty acids (Deineka et al., 2007), and flavonoids (Guinot et al., 2008; Faizi et al., 2011a) as constituents, some of which...
may elicit the biological activities reported to date; these include insecticidal (Wells et al., 1993), nematicidal (Chadha, 1976; Buena et al., 2008), larvicidal (Dharmagadda et al., 2005), antifungal (Faizi et al., 2008), and anti-inflammatory (Yasukawa and Kasahara, 2013) activities. The flowers have been reported to contain up to 5.5% flavonoids (Munhoz et al., 2012).

As Piccaglia and collaborators (1998) found, the flowers of Tagetes patula are a rich source of lutein and its esters. For this reason the genus is widely cultivated in Central America as food coloring, which is approved by the European Union (Vasudevan et al., 1997). However, after carotenoids are extracted, the residue is discarded or only used as animal feed or fertilizer (Gong et al., 2012). Gong et al. (2012) observed that this discarded residue contained various compounds of interest, including flavonoids, a class of secondary metabolites with high therapeutic potential, including cardioprotective (Bandy and Akhlaghi, 2008), anti-inflammatory (Kim et al., 2004), antimicrobial (Lamb and Cushnie, 2005), and antitumor (Harborne and Williams, 2000) activities, among others.

The extraction method and solvent used to obtain a flavonoid-enriched extract can greatly influence the biological activity. A successful extraction technique combines the optimal solvent or mixture of solvents with a convenient technique. In order to select an extraction method it is necessary to evaluate the efficiency, the stability of the extracted substances, the availability of resources and processing costs, leading towards a biological application of the extract (Louzada et al., 2001).

The optimization of the extraction techniques is important for a variety of applications, for example to obtain the selected compounds of pharmaceutical interest, with a minimum of impurities. In recent years, statistical models have been successfully applied to minimize the number of experiments and to identify the effect of the interactions between experimental variables such as the solvent strength, and the interactions that occur between the biological material and the solvents. These models have provided satisfactory results for the optimization of the extraction procedures (Box et al., 2005; Bruns et al., 2006). Li and collaborators (2007) used response-surface methodology to assess the yield and purity of the polysaccharides by ultrasound extraction. Their optimized method increased the yield by approximately 20% over the classic extraction method.

The experimental mixture design has been widely applied in various areas, such as the development of new biofilms (Mali et al., 2010) and for the extraction of secondary metabolites from natural products (Garcia et al., 2010; Lonni et al., 2012; DiCiaula et al., 2014). The main goal of this method is to evaluate how responses are affected by the variation in the proportions of the mixture components (Cornell, 2002). In the data analysis, a mathematical polynomial model is used to link the different compound proportions to the properties of interest, in order to determine the best system (Audi et al., 2001; Campos et al., 2006).

We used the statistical mixture design with a simplex-centroid model with acetone, ethanol and water as solvents in order to optimize the extraction of flavonoids from the flowers of Tagetes patula. All the extracts obtained were used for a preliminary evaluation of larvicidal activity against the larvae of the mosquito Aedes aegypti.

**Materials and methods**

**Plant material**

Seeds of Tagetes patula L., Asteraceae, were donated by Syngenta Flowers Brazil and were organically grown in the Medicinal Plant Garden of the State University of Londrina, Brazil. The flowers were collected in November 2011, and the material was identified by Professor Dr. Naoki Jimi Nakagima, Federal University of Uberlândia, Brazil. A voucher specimen was deposited in the Herbarium of the State University of Maringá under identification number HUEM 21.907.

The flowers were dried in a kiln with forced-air circulation (Sparrow), heated to 38 ± 2ºC and pulverized in a hammermill (Tigre ASNS).

**Chemicals and reagents**

All solvents and reagents used were analytical grade. Chemicals used were: n-hexane, acetone and methanol (Synth®), ethanol (Cerealcool®), methanol HPLC grade (J.T. Baker®), DPPH (2,2-diphenyl-1-picylhydrazyl) (Sigma Aldrich®), butylated hydroxytoluene (BHT), aluminum chloride and potassium acetate (Synth®), and Quercetin (Acros Organics).

**Extraction of flowers: general equipment and conditions**

To obtain the extract, the plant material, not separated by particle size, was defatted using n-hexane by dynamic maceration for 6 days. After drying, the defatted flowers were macerated for 10 min and the material was extracted in an industrial blender (Skymsen®, LS-04) in a proportion of 2.5% (m/v) (w/v), for a period of 9 min, with 10-min intervals, and using as the extraction liquid, mixtures of three solvents: (\(x_1\) acetone, (\(x_2\) ethanol and (\(x_3\) water, determined through a simplex-centroid design (Fig. 1). The proportions of the solvents used are specified in Table 1.

The extracts were vacuum-filtered, evaporated under reduced pressure (Büchi®, R-200) and lyophilized (Christ®, Alpha 1-4) (CE). All extracts were prepared in triplicate, randomly.

![Figure 1 - Simplex-centroid design with three axial mixture points used to investigate the influence of different solvent proportions on extract preparation.](image-url)
Determination of total flavonoid content (TFC)

The total flavonoids content (TFC) was determined by the aluminum chloride colorimetric method (Chang et al., 2002) with modifications. To 0.5 ml of the sample solution (CEW – 800 μg/ml; other extracts – 400 μg/ml) 1.5 ml ethanol, 0.1 ml of 1 M potassium acetate (w/v), 0.2 ml of 10% aluminum chloride (w/v), and 2.7 ml of distilled water were added. After incubation at room temperature for 30 min, the absorbance of the solution was measured on an OceanOptics USB 2000+ spectrometer at 430 nm. The 10% aluminum chloride was replaced by the same amount of distilled water. Quercetin was used to construct the calibration curve (y = 0.0503x - 0.0128), where y is the value for absorbance of the sample and x is the sample concentration, from 3-15 μg/ml (r² = 0.9944). The total flavonoids content was expressed as a percentage, i.e., flavonoids g/100 g of extract.

Radical-scavenging activity (RSA)

The radical-scavenging ability of the extracts was evaluated using the free radical DPPH scavenging method as described by Amarowicz et al. (2004). The extracts were diluted in methanol (5- 35 μg/ml for CEA and CEAE; 50-140 μg/ml for CEW; and 10-40 μg/ml for other extracts). Next, 375 μl of DPPH solution (1 mmol/l) was added to each of these solutions. The mixture was vortexed for 15 s and left at room temperature for 30 min. The absorbance was read at 517 nm with an OceanOptics USB 2000+ spectrometer. As a blank, a methanol solution of BHT (0.5 mg/ml) added to 500 μl of DPPH solution was used; and the negative control consisted of a solution containing 3 ml of methanol and 375 μl of DPPH solution. The radical-scavenging activity (RSA) was determined as %RSA = [(AbsCN – AbsA) × 100]/AbsCN, where AbsA is the absorbance of the extract solution; and AbsCN is the negative control absorbance. The IC_{50} value, or extract concentration necessary to inhibit 50% of DPPH, was obtained by plotting the %RSA as a function of the sample concentration.

Experimental design

Experiments were designed and analyzed using the software Statistica® version 8.0 (StatSoft, Oklahoma, USA). As the experimental design used was simplex-centroid, we used the special cubic model to describe the response of ternary mixture (Eq. (1)) (Barros Neto et al., 2010):

\[
\tilde{y} = b_1x_1 + b_2x_2 + b_3x_3 + b_{12}x_1x_2 + b_{13}x_1x_3 + b_{23}x_2x_3 + b_{123}x_1x_2x_3
\]  

(1)

Where \(\tilde{y}\) is the response of interest predicted by the model; and \(b_1, b_2, b_3, b_{12}, b_{13}, b_{23}, b_{123}\) are the estimated parameters, corresponding to the pure components: acetone (\(x_1\)), ethanol (\(x_2\)) and water (\(x_3\)) and their binary and ternary interactions predicted by the model. Surface charts were constructed.

HPLC analysis

The HPLC analysis was conducted using a liquid chromatograph Thermo Model LC Pump Plus equipped with a Finnigan Surveyor PDA Plus diode array detector and a C18 Gemini Phenomenex HPLC column (250 mm x 4.6 mm), 5 μm particle size, and a guard column (Phenomenex) with a flow rate of 1 ml/min. Each sample was prepared in a 725 μg/ml solution, according to the experimental design, and filtered with a 0.22 μm Millipore® filter. A 10 μl aliquot of this solution was injected into the HPLC column (10 μl loop). The mobile phase contained a mixture of water (phase A), and acetonitrile (phase B), in a gradient system, as follows: 0 min, 5% phase B; 35 min, 58% phase B, followed by a 5-min post-time to re-equilibrate the system with 5% phase B. The temperature was fixed at 25°C. A reference standard (quercetin) was used to identify the major component in the crude extracts. Elution was monitored at 210, 254, and 280 nm.

Bioassay test

The larvicidal activity of the extract was evaluated using laboratory-reared larvae of the dengue vector mosquito Aedes aegypti. Larvae from Ae. aegypti were obtained from a permanent colony maintained at a temperature of 25 ± 2°C and relative humidity of 70 ± 5% kept in the insectary of the Laboratory of General and Medical Entomology, State
The extracts were evaluated in accordance with the protocol of the World Health Organization (WHO) with slight modifications (WHO, 2005). Twenty-five 4th-instar larvae were transferred to transparent polyethylene pots, 11 cm in diameter and 7 cm deep, containing 150 ml of distilled water. Initially, 100 mg of each extract was solubilized in 1 ml of DMSO and 9 ml of water, and then diluted with distilled water to obtain five concentrations (80, 30, 10, 5, and 1 mg/l) for evaluation. Controls were evaluated with DMSO diluted in the same conditions as the samples, but using only distilled water. The number of dead larvae was recorded after 24 and 48 h of exposure, and the percentage of mortality was calculated. Each extract was tested in triplicate and the assay was repeated twice.

Lethal concentration (LC50) values were determined by probit analysis using SPSS version 12 software. Results with p ≥ 0.05 were considered to be statistically significant.

Results and discussion

The experimental results are presented as the mean ± standard deviation and are shown in Table 2.

The dry residue was determined in order to evaluate the strength of the extraction solvent used on flowers of T. patula. The special cubic model gave a satisfactory value for the determination coefficient (r2 = 0.949), and all coefficients were significant at the 95% confidence level (Eq. (2):

\[
DR = 6.62x_1 + 32.22x_3 + 39.25x_5 - 22.00x_2x_5 + 74.52x_2x_3 + 21.84x_2x_3 + 154.69x_1x_2x_3
\]

(2)

The percentage of dried residue was positively and linearly influenced by water (x5) and ethanol (x3), respectively. Of the linear trials, acetone (x1) resulted in the lowest coefficient; the smallest proportion of dried residue (6.62% w/w) was obtained with acetone alone (Table 2). Among the binary interactions, the presence of acetone reduced the power of extraction of ethanol (x1x3), but did not reduce the power of extraction of water (x1x5). The ternary interaction (x1x2x3) displayed a synergistic effect among the components of the mixture.

The extracts produced with solvent mixtures including water contained the highest levels of dry residue, from 39.25 to 41.57% (w/w) (Fig. 2A). However, the Tukey’s test indicated no significant difference between them.

The results clearly indicate that the extraction is affected by the polarity of the solvents. Prewashing the flowers with n-hexane decreased the dry mass of the plant by approximately 14%. Using GC-MS (gas chromatography-mass spectroscopy), Faizi et al. (2011b) identified 63 compounds in the nonpolar fractions of T. patula flowers, with long-chain fatty acids, hydrocarbons and thiophenes being the predominant classes of compounds in the petroleum ether fraction. This suggests that the lower efficiency of the acetone is related to the fact that some medium- and low-polarity substances were initially extracted with n-hexane. The predominant positive effect of water and ethanol may be due to the presence of polar substances such as phenolic glycosides.

The TFC ranged between 4.63 and 25.13% (w/w) (Table 2). In contrast with the results for the dried residue, the CEA showed the largest TFC, 25.13% (w/w). This was confirmed through Equation 3, obtained by the special cubic model (r2 = 0.979), which showed that acetone (x1) had a strong effect on the TFC when used either pure or in combination with ethanol (x1x2). The linear effect of acetone (x1) was positive, while water (x5) was less effective (TFC = 4.63% (w/w)). The presence of water in the ternary mixture (x1x2x3) decreased the extractive capacity compared to the pure solvents acetone (x1) and ethanol (x2), as shown by a negative coefficient and a TFC of 12.94% (w/w). The binary interaction (x1x2) was not significant (p ≥ 0.05).

Purification with n-hexane increased the selectivity of the solvent, especially acetone, for flavonoids. Thus, acetone was the most appropriate solvent for extracting these compounds from French marigold flowers, possibly because of the predominance of free and/or methoxylated aglycones in the defatted plant material, such as kaempferol, quercetin (Ivanccheva and Zdravkova, 1993), patuletin (Tarpo, 1967; Guinot et al., 2008), quercetin (Tarpo, 1969), luteolin and quercetagetin 5-methyl ether (Bhardwaj et al., 1980), which are less soluble in water.

### Table 2

<table>
<thead>
<tr>
<th>Extracts</th>
<th>Dry residue (%) w/w</th>
<th>Total flavonoids (%) w/w</th>
<th>Radical scavenging activity (IC50 μg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 (CEA)</td>
<td>6.62 ± 1.33</td>
<td>25.13 ± 1.02</td>
<td>15.74 ± 1.09</td>
</tr>
<tr>
<td>2 (CEE)</td>
<td>32.22 ± 3.98</td>
<td>16.77 ± 0.77</td>
<td>24.92 ± 1.68</td>
</tr>
<tr>
<td>3 (CEW)</td>
<td>39.25 ± 3.89</td>
<td>4.63 ± 0.47</td>
<td>114.76 ± 3.07</td>
</tr>
<tr>
<td>4 (CEAE)</td>
<td>13.92 ± 2.90</td>
<td>23.44 ± 0.96</td>
<td>17.77 ± 2.06</td>
</tr>
<tr>
<td>5 (CEAW)</td>
<td>41.57 ± 2.07</td>
<td>14.43 ± 1.28</td>
<td>23.37 ± 2.49</td>
</tr>
<tr>
<td>6 (CEEW)</td>
<td>41.20 ± 3.68</td>
<td>13.19 ± 0.86</td>
<td>25.46 ± 1.35</td>
</tr>
<tr>
<td>7 (CEAEW)</td>
<td>40.02 ± 2.62</td>
<td>12.94 ± 1.00</td>
<td>21.87 ± 1.18</td>
</tr>
</tbody>
</table>

CEA, acetone extract; CEE, ethanol extract; CEW, aqueous extract; CEA, acetone:ethanol extract; CEAW, acetone:water extract; CEEW, ethanol:water extract; CEAEW, acetone:ethanol:water extract.
Chebil et al., 2007). This is probably the reason that the TFC aqueous extract (CEW) gave the lowest result.

Previous reports indicate that water is not an efficient solvent for extracting phenolic compounds, which are generally more soluble in organic solvents less polar than water (Kim and Lee, 2001). Several studies have reported that the extraction efficiency of phenolic compounds, including flavonoids, is increased by the use of a mixture of water and organic solvents such as acetone, methanol and ethanol; whereas the use of pure solvents can reduce the power of extraction (Gong et al., 2012; Meneses et al., 2013). As it can be seen in Figure 2B, the addition of ethanol or acetone to water increased the TFC. However, pure acetone gave the best results, probably due to the chemical composition of French marigolds.

Munhoz et al. (2012) used HPLC to generate a chemical profile for T. patula, and found that quercetin was the major substance in the plant drug. Malwade et al. (2013) evaluated the solubility of quercetin in four common solvents, and reported that quercetin is soluble in: acetone > ethanol > methanol > acetonitrile, in decreasing order. Those reports are in accordance with the results presented here for TFC.

From the chromatographic profiles of the extracts obtained by the simplex centroid design, it was possible to determine the retention time of quercetin (21.05 min) using the spectroscopic data for the standard, and to analyze the area under the curve. Fig. 3 illustrates the chromatograms obtained for the seven extracts. As can be seen, the chromatographic profiles were not affected by the different solvents used to extract the flavonoids. The evaluation of the area under the quercetin peak revealed the positive effect of acetone and ethanol on the extraction (Fig. 4).

The evaluation of the radical scavenger activity of the extracts generally revealed a similar behavior to that obtained for flavonoids. The results, described in Table 2, are expressed as IC50. CEA had the lowest IC50, in contrast to CEW. In Equation 4 (special cubic model; \( r^2 = 0.997 \)), among the linear effects, water \( (x_3) \) had a significant negative effect on the extraction of compounds with antioxidant activity; however, combining water with acetone \( (x_1 x_3) \) or ethanol \( (x_2 x_3) \) increased the extraction efficiency. As seen in Fig. 2C, the lowest IC50 values were obtained with acetone and its binary mixtures, as well as the mixture of ethanol: water (75:25 v/v).

\[
\text{RSA} = 15.74 x_1 + 24.93 x_2 + 114.76 x_3 - 10.25 x_1 x_2 - 163.53 x_1 x_3 - 177.55 x_2 x_3 + 245.54 x_1 x_2 x_3 \\
(4)
\]

The phenolic compounds present in plants are known for their antioxidant activity. Flavonoids have this capability mainly because their hydroxyls donate an electron \((H^+\) to radicals as hydroxyl \((\text{HO}•\)), superoxide \((\text{O}_2•^-\)), and peroxyl \((\text{ROO}•\)), neutralizing them (Harborne and Williams, 2000). Previous studies have shown that the antioxidant properties of plants are highly correlated with the content of total phenols and flavonoids (Gong et al., 2012; Meneses et al., 2013), as it was further confirmed in this study (Fig. 5).

The results suggest that flavonoids contribute significantly to the antioxidant activity of the extracts obtained with acetone and acetone:ethanol. However, the extracts derived from binary mixtures containing water also inhibited the DPPH radical, possibly due to the presence of phenolic acids such as gallic, chlorogenic, caffeic, vanillic, p-coumaric, and ferulic

![Figure 2](image-url)
acid, among others, which have been found in members of the genus *Tagetes* (Kaisoon et al., 2012).

The mosquito *Aedes aegypti* is the principal vector of dengue. To date there is no vaccine for dengue fever, and the best procedure to combat the disease is to attack the vector, mainly by eliminating the sites of oviposition and larval development. Currently, mosquito populations are controlled with the use of organophosphate insecticides, in increasingly larger doses, a process that has allowed the selection of resistant mosquito populations (Fontoura et al., 2012). In view of the increasing interest in insecticide development of plant origin as an alternative to chemical insecticides (Cappiello et al., 2012), this study was undertaken to assess the larvicidal potential of the flavonoid-enriched extracts, obtained by the simplex-centroid statistical model. The bioassay test revealed larvicidal activity in only two extracts. No larval mortality was observed in controls. Of the active extracts, the acetone extract showed a higher larvicidal activity (100%) at a concentration of 1 ppm, followed by the 50% ethanol extract (100%) in a concentration of 10 ppm, both after 48 h. In view of the promising response of the acetone extract, further studies will be conducted towards chemical fractionation, in search of a new bioactive molecule.

### Conclusions

The statistical mixture design was successfully applied to obtain an optimized set of extraction conditions to maximize the extraction of flavonoids with antioxidant activity from *T. patula* flowers. The favorable effects of acetone on the extraction results were maximized to select for quercetin. Values less than 18 μg/ml (IC₅₀) were obtained with pure acetone and a binary mixture of acetone: ethanol, to evaluate antioxidant capacity. This study demonstrated the potential of *T. patula* flowers as a source of flavonoids with antioxidant and larvicidal activities, using acetone and a binary mixture of acetone as solvents.

### Authors’ contributions

VMM (MSc student) collected the plant samples, confection of herbarium, performed the laboratory work, analyzed the data and drafted the paper. RL performed the chromatographic analysis. JRPS grew the plants and collected and dried the flowers. JACZ contributed to larvicidal activity analysis. EVSLM contributed to designing the study, discussed the results, and critically read the manuscript. GCL contributed to designing the study, supervised the laboratory work, and critically read the manuscript. JCPM contributed to designing the study, supervised the laboratory work, and helped write the manuscript. All the authors have read the final manuscript and approved the submission.

### Conflicts of interest

The authors declare no conflicts of interest.

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