PROMOTER AND PHYTOTOXIC EFFECTS OF ETHANOLIC EXTRACTS OF CHILTEPIN IN THE ARABIDOPSIS THALIANA MODEL EVALUATED BY ECOEXERGY

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Abstract. Chiltepin (Capsicum annuum var. glabriusculum) is a wild plant resource with a wide geographical distribution in Mexico and it is part of life in rural societies in the state of Puebla. The pungency of chiltepin is due to capsaicinoids, especially capsaicin and dihydrocapsaicin, which are of interest to the agricultural industry due to their insecticidal, bactericidal, and fungicidal effects; however, both a phytotoxic and promoter effects, have not been explored in in-vitro cultures of Arabidopsis thaliana. Therefore, the objective of this research was to evaluate the promoter and phytotoxic effect of ethanolic extracts of chiltepin from the Sierra Norte of Puebla, Mexico, on Arabidopsis thaliana crops based on their energy use evaluated by ecoexergy through neural networks. The ethanolic extract with seed (EECS) and without seed (EESS) was obtained by ultrasound-assisted extraction (UAE) of ripe fruits, using 96% ethanol. Capsaicin content was determined by UV/VIS spectrophotometry. EECS, EESS and EtOH extracts were added in different amounts to the MS medium in which 7-day-old Arabidopsis thaliana seedlings were grown. The concentrations at which a phytotoxic effect was observed after 120h were 28.37 mg/L and 35.62 mg/L, for EECS and EESS respectively. Exposure to a dose 10 times lower than the EC50, in the case of EECS, showed an increase in the calculated energy.

Keywords: neural network, Puebla, CE50, Capsicum annuum var. glabriusculum, seedling

Introduction

Chili (Capsicum) is an important element in Mexican culture and food, its role in pre-Hispanic cultures has been widely documented (Long-Solis, 1986) and it is currently among the seven vegetables with the highest production worldwide (Latournerie et al., 2015). Chiltepin (Capsicum annuum var. glabriusculum) is a wild plant resource with a wide geographical distribution in Mexico (Reyes et al., 2019). In Puebla, Mexico, the cultivation of this food is part of the gastronomy in rural societies (Bañuelos et al., 2008; Morales et al., 2018) who consume it as a seasoning in regional food.

Pungency of chiltepin is due to capsaicinoids, the most abundant being capsaicin (8-methyl-N-vanillyl-6-nonenamide) and dihydrocapsaicin (8-Methyl-N-vanillyl-nonenamide) (Stan et al., 2021). In recent years, these compounds have become of interest to the agricultural industry due to their insecticidal, bactericidal, and fungicidal effects (Fayos et al., 2018).
Because of that, the extracts of carotenoids and phenols present in chiltepin fruits stand out, given that they inhibit the growth of fungi of agricultural importance in Mexico (Rodriguez et al., 2015); however, the effects of ethanolic extracts of chiltepin obtained by ultrasound-assisted extraction (UAE) have not been fully explored, although it is known that some secondary metabolites such as terpenoids, phenolic compounds, phenylpropanoids, stilbenes, alkaloids and saponins present in plant extracts (Dixon, 2001) are easily degraded in soil and do not have toxic effects on mammals, which allows their use in organic and sustainable agriculture systems (Rodriguez et al., 2015).

At the same time, studies are needed that facilitate the estimation and prediction of the effects of the application of said extracts from the energy perspective, which can be quantified with ecoexergy. Ecoexergy is defined as the useful part of the energy (kilojoules) involved in the vital processes of a system and is calculated by multiplying the biomass by its available energy in genetic information (Jørgensen and Mejer, 1977; Jørgensen, 2015). Thus, the exergy reflects the degree of development and growth of a system whose components and configurations always seek to maximize the flow of useful energy and stored exergy (Silow and Mokry, 2010).

Similarly, it can be noted that it is possible to use neural networks as a predictive method that conceives the variations in the input data, which makes it a flexible modeling approach for decision making (Zhang et al., 2020). Thus, the objective of this work is the evaluation of the promoter and phytotoxic effect of ethanolic extracts of chiltepin from the Sierra Norte de Puebla, Mexico, on Arabidopsis thaliana crops based on their energy use evaluated by ecoexergy through neural networks.

Target

The objective of this study was to evaluate the promoter and phytotoxic effect of ethanolic extracts from ripe chiltepin fruits in the Arabidopsis thaliana model by quantifying ecoexergy.

Materials and methods

Collection of samples and obtaining extracts

Ripe chiltepin fruits were collected and subsequently dried in a conventional oven at 60 °C for 4 h. Then, the material was separated into two samples: one with the fruit and its seeds (EECS) and the other with the fruit but no seeds (EESS). Both samples were crushed in a blender at medium speed until fine powder was obtained, to which 96% ethanol was added. The compounds of both samples were extracted with an ultrasonic bath during a period of 25 minutes at 55 °C and 40 kHz (Barbero et al., 2008). Finally, the obtained extract was filtered twice, the first time to remove larger solids and the second one with the objective of eliminating viruses or bacteria through a 0.22 μm membrane (Lu et al., 2017).

Capsaicin quantification

The capsaicinoid content in the ethanolic extracts of ripe chiltepin fruits, EESS and EECS, was determined by using the standard curve of the pure compound 8-Methyl-N-vanillyl-trans-6-nonenamide (Sigma-Aldrich – 12084) at concentrations of 25, 50, 75 and 100 mg/L by UV absorption spectroscopy at a wavelength of 266 nm (Genesis 10uv-ThermoScientific). This was done from the identification of the maximum absorbance
through spectral scanning of the extracts in the region between 230 to 280 nm (Davis et al., 2007; González et al., 2015). The absorbances were processed by logistic regression with the software RCommander (adjusted $R^2 = 0.9762$).

**Acute test of extracts on A. thaliana seedlings**

7-day-old seedlings grown in MS medium (Phytotechnology with added vitamins) were exposed to concentrations of 27.20, 30.60 and 34.00 mg/L of capsaicin from EESS and 26.17, 29.43 and 32.71 mg/L from EECS in a final volume of 20 mL of culture medium. The reference groups included cultures in the absence of the extracts and one with the same volume of 96% ethanol applied along with the EESS and EECS treatments. All treatments were carried out in triplicate, each with four seedlings and incubating at 25 °C with monitoring at 72 and 120 h.

The mean lethal concentrations for both extracts were established referring to the loss of coloration of the seedlings exposed over 5 days. The data were approached using probit analysis to calculate the EC$_{50}$ from the mortality percentage with respect to the color of the control seedlings (Table 1). Similarly, the ethanol extract was included to rule out the possible effects of 96% ethanol, which was used as a mean of extracting the compounds in the fruits.

**Chronic test of extracts on A. thaliana seedlings**

Chronic exposure was carried out for a period of 20 days at concentrations of 3.40 mg/L and 3.27 mg/L of capsaicin from EESS and EECS, respectively, in a volume of 20 mL of MS culture medium, four seedlings were used in each replicate with 7 days old. Again, the control groups consisted of culture media without ethanolic extracts, as well as the treatment that considered only 96% ethanol. An analysis of variance (ANOVA) type 1 was performed as well as a Tukey post-hoc test to determine significant differences between groups in the R commander program (ver. 4.2.2).

**Arabidopsis thaliana ecoexergy calculation**

The ecoexergy calculation for the seedlings exposed to both extracts, EECS and EESS, was carried out by recording the changes in fresh weight (Eq.1) per seedling considering the measurements of the width and length of the leaf, as well as the length of the stem in mm. The above from the use of the following Equation 2 (Jørgensen, 2005):

\[
\text{Fresh weight} = \text{Leaf width mm} (-6.5538) \\
+ \text{Leaf length mm} (2.86677) \\
+ \text{Stem length} (3.7163) - 13.4245 \ *
\]

(Eq.1)

where:
- $\beta_i$ = The weighting factor of the organism according to the amount of bp contained in its genome,
- $c_i$ = Concentration of the Carbon component of the dry biomass,
- $\beta$ value for the *Arabidopsis thaliana* model was obtained by using the following equation:
\[ \beta_i = \frac{\ln \left( 20^{-\left( \frac{C_i}{T} \right)^{\frac{bp}{T^2}}} \right)}{Ex_{det}} \]  
\text{(Eq.3)}

where:
\[ C_i = C \]
\[ bp = 9.8 \times 10^8 \]
\[ Ex_{det} = 7.43 \times 10^5 \]

The quantification of carbon biomass per seedling was established based on the data collected through the dry weight of the seedlings at 105 °C in an oven (BLUE M) and the use of the value reported by Küstner et al. (2019) for the calculation of carbon content:

\[ \text{Dry weight} = (0.0534 \times \text{Fresh weight g}) \]
\[ + (0.000092885 \times \text{Water loss percentage}) \]
\[ + 0.00877 \times \]  
\text{eq.4}

* Linear regression model, \( R^2 = 0.999 \), P-value: 0.00707, Reference this work.

\[ C_i = \text{Dry weight [mg]} \times 0.45 \]  
\text{eq.5}

The echoexergy data calculated in each treatment were processed in R commander (ver. 4.2.2) using a type 1 analysis of variance (ANOVA) as well as a Tukey test to determine significant differences between groups.

**Design of a neural network for the calculation of echoexergy**

The neural network used for the echoexergy calculation was built in the Rstudio ID, the "neuralnet", "MASS", "ROCR", "gmodels", "caret" and "lattice" libraries were used in the network programming whose architecture consisted of a perceptron of 4 information inputs with 3 hidden layers and an output with the average echoexergy recorded at concentrations of 3.27 and 3.40 mg/L in the treatments with EECS, EESS and 7970 mg/L of EtOH. The "Resilient Backpropagation" algorithm, which updates the weights considering the sign of change, was used to activate the network with a total of 1000 iterations.

**Results and discussion**

**Capsaicin quantification in the ethanolic extracts**

The wavelength of maximum absorbance for the extracts corresponded to 266 nm (Figure 1), it was identified from a spectral scan between 230 and 280 nm, which was used in the quantification of the ethanolic extracts of chiltepin, EESS and EECS.

Thus, the quantification of the capsaicin standard was established at 266 nm and was corroborated with a scan of the standard within the aforementioned interval (Figure 2).

It was observed that most of the capsaicinoids presented some absorption in the UV region of the spectrum, between 200 and 350 nm. Therefore, by using the Beer-Lambert law (González et al., 2015) it was possible to estimate the quantity of capsaicin contained in the extracts: 340.06 mg/L for EESS and 327.15 mg/L for EECS.
Figure 1. UV spectra of chiltepin (Capsicum annuum L. var glabriusculum) extracted with ethanol. Spectra obtained at wavelengths between 230 and 280 nm for the EESS and EECS extracts.

Figure 2. UV spectra at different concentrations of the capsaicin standard (a). Calibration curve of each capsaicin (b). The equation of the regression line for capsaicin was \( y = 0.0072x + 0.2185 \).

**EC_{50} determination**

Table 1 summarizes the phytotoxic effect of exposure to ethanolic extracts of chiltepin for 5 days, estimating the mean effective concentration at 72 and 120 h using the probit method. 50% mortality was observed in 7-day-old seedlings in a time of 72 h at a concentration of 39.84 mg/L for EECS and 35.78 mg/L for EESS. It should be noted that prolonging the exposure period (48 additional hours) increased the effectiveness for EECS as compared to EESS. Although there is no information about the long-term effect of high doses of chiltepin extracts on *Arabidopsis thaliana*, capsaicinoids isolated from *Capsicum annuum* L. have been evaluated in *Lactuca sativa* seedlings, concluding that the inhibition is dose-dependent (Wang et al., 2020). Regarding EtOH, concentrations higher than \(10^6\) mg/L are required to cause 50% mortality.

Table 1. Herbicidal effect of ethanolic extracts of chiltepin

<table>
<thead>
<tr>
<th>Treatment</th>
<th>CE_{50} 72 h (3rd day)</th>
<th>CE_{50} 120 h (5th day)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ethanolic extract with seed (EECS)</td>
<td>39.84 mg/L ****</td>
<td>28.37 mg/L ***</td>
</tr>
<tr>
<td>Ethanolic extract without seed (EESS)</td>
<td>35.78 mg/L ***</td>
<td>35.62 mg/L ***</td>
</tr>
<tr>
<td>Ethanol (EtOH)</td>
<td>(6.23 \times 10^7) mg/L</td>
<td>(3.30 \times 10^7) mg/L</td>
</tr>
</tbody>
</table>

Significance code: 0 '****' 0.001 '***' 0.01 '**' 0.05 '*' 0.1
Such a precedent implies that the effective dose for EECS when presented with prolonged exposure is lower compared to EESS (Figure 3), which can be attributed to the presence of saponins in chiltepin seeds; it is to be noted that Osorio et al. (2009) associate a phytotoxic effect with these compounds.

**Figure 3. Phytotoxicity of the ethanolic extracts of chiltepin and solvent used at 120 h on Arabidopsis thaliana**

**Calculation of ecoexergy for Arabidopsis thaliana**

The effects of the ethanolic extracts of chiltepin were modeled through ecoexergy of *Arabidopsis thaliana*. The quantification of ecoexergy allowed us to know the energy flow and its orientation by combining structural and functional components of *A. thaliana*. The calculation considered the carbon biomass in the seedlings, the βi and the value of the detritus as energy reference (*Table 2*).

**Table 2. Information used for mathematical modeling**

<table>
<thead>
<tr>
<th>Variable</th>
<th>Formula</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Carbon biomass (g)</td>
<td>Dry weight g * 0.45</td>
<td>Küstner et al., 2019</td>
</tr>
<tr>
<td>βi coefficient A. thaliana</td>
<td>81.00 g/m²</td>
<td>Calculated according to Libralato et al., 2006; Jørgensen et al., 2005; Álvarez et al., 2015.</td>
</tr>
<tr>
<td>Detritus reference value</td>
<td>18.7 kJ/g</td>
<td>Jørgensen et al., 1995; Álvarez et al., 2015, Jørgensen &amp; Fath 2004.</td>
</tr>
</tbody>
</table>

**Ecoexergy of acute exposure in the A. thaliana model**

Acute exposure to EECS and EESS caused loss of color in the leaves of the seedlings due to the high concentrations of compounds with phytotoxic potential, which also had negative effects on their growth and energy (Jorgensen, 1995; Libralato et al., 2006). The reduction in the energy use of the seedlings in the presence of capsaicin (*Figure 4*) was more notable with EECS at the concentration of 35.98 mg/L (p<0.05), but not for the treatments with EtOH and EESS (p > 0.05); however, a decrease in ecoexergy was observed with respect to the control. The energy decrease is proportional to the loss of biomass derived from the presence of compounds in chiltepin extracts such as tannins, glycosides, terpenoids, flavonoids, saponins, reducing sugars, steroids and resins that are responsible for the inhibitory effects (Rosulu et al., 2022) on seedlings.

Energy loss became evident when comparing the groups subjected to EESS, EECS and EtOH regarding the average ecoexergy recorded in the control group at 72 h.
A decrease in ecoexergy was observed in the EESS treatment, which contrasts with the assumption of an increase in capsaicin (p < 0.05), the same effect was recorded for the EtOH group.

Figure 4. Ecoexergy of Arabidopsis thaliana exposed to EC50 concentrations for 72h. From the fresh weight of seedlings exposed to EESS, EECS and EtOH.

On the contrary, mortality of 50% of the seedlings was observed in the groups with EECS in a capsaicin interval of 32.71 to 35.98 mg/L, which entails an evident decrease in ecoexergy (p < 0.05) regarding the EtOH and EESS treatments. It is noteworthy how the phytotoxic effect considers not only the presence of capsaicin, but also other compounds that are present in the seed of chili peppers (Cervantes-Hernández et al., 2019; Rosulu et al., 2022).

Ecoexergy of A. thaliana on chronic exposure

Exposure to low concentrations of capsaicin in ethanolic extracts of chiltepin on models such as Arabidopsis thaliana has not been fully explored, therefore, it was essential to analyze the energy changes that could occur in exposed seedlings based on...
the calculated fresh weight of the parameters, width and length of the leaf, as well as the length of the stem in mm during 20 days at EESS, EECS and EtOH with four-time cuts at days 5, 10, 15 and 20 (Figure 6).

![Figure 6. Ecoexergy of chronic exposure with EESS, EECS and EtOH in an Arabidopsis thaliana model](image)

It should be noted that, in the case of EECS, exposure to a dose 10 times lower than that determined in the EC50 showed an increase in the specific energy on days 5, 10 and 20 in the exposed seedlings, at the same time, a positive evolution of the same (Figure 7), not so for day 15, which would indicate a process of adaptation of the seedlings to the presence of saponins and terpenoids (López-Muñoz et al., 2019). However, the effects of the treatment with EESS reveal a slight increase on days 5 and 10 (p > 0.05), with significance in the energy decrease on days 15 and 20, which can be attributed to the absence of flavonoids, which are responsible for of the antioxidant activity in plants (Vásquez, 2015), these compounds are present in the placenta of Capsicum fruits (Cervantes-Hernández, 2019), a section discarded in the preparation of the EESS extract. Regarding the exposure that only considers the EtOH solvent, a decrease in Jg/m² was noted on days 5 and 15 with increases on days 10 and 20, which could indicate adaptation of the seedlings during those periods of time (Chen et al., 2015; Bui et al., 2019).

![Figure 7. Difference in ecoexergy for chronic exposure of Arabidopsis thaliana with EESS, EECS and EtOH](image)
Ecoexergy prediction in A. thaliana using neural networks

The construction of a deep learning neural network with a multilayer structure (Figure 8) and supervised learning algorithms (Johnson et al., 2017; Horie, 2019) facilitated the admission of input variables as signals between the hidden layers, which improved the prediction of ecoexergy (Geetha and Thilagam, 2021). Thus, the variables used in this study were: Exposure time, Extract concentration, Fresh weight (g), Biomass (g) and, as output layer, the Ecoexergy value (Jg/m2).

The prediction of the ecoexergy values (at 98% precision) for Arabidopsis thaliana in treatments at low concentrations of ethanolic extracts of chiltepin was calculated using "R-prop+" as an activation equation, which is available in the neuralnet library at Rstudio.

The ecoexergy values predicted by means of the neural network were close to those quantified from the fresh weight of the seedlings (Figure 9), which highlights the importance of prior training of the network in the face of changes in the input variables (Exposure time, Concentration and Fresh Weight), in order for the predictions to improve substantially.

Figure 8. Architecture of the neural network for the prediction of ecoexergy in A. thaliana that was chronically exposed to EESS, EECS and EtOH

Figure 9. Actual ecoexergy vs Calculated ecoexergy by the neural network for A. thaliana chronically exposed to EESS, EECS and EtOH
Ecoexergy values produced by the neural network were weighted for their analysis, positive evolution was coded with the numerical value 1, while negative evolutions were characterized with 0. Ecoexergy data for the treatment with EECS on day 20 registered an output of 1 with the consequent activation of the nodes of the first layer, allowing the data to be passed to the subsequent layer in the network. Values above 300 Jg/m² presented a positive development for treatment with EECS, which indicated the hierarchy of variables in the ecoexergy calculation (Figure 10): Biomass > Fresh weight > Exposure time > Concentration in the treatments (EECS, EESS and EtOH) and control.

Thus, the contribution of the variable Biomass in the total value of ecoexergy stood out, given that its importance was greater than that of the remaining variables since the exergy residing in each treated seedling was obtained from the aforementioned value. Therefore, the design of this neural network preserved the ecoexergy estimation principle by considering biomass and fresh weight as important parts of the information transferred through the nodes in the hidden layers.

Conclusions

Ethanolic extracts obtained from ripe chiltepin fruits from the Sierra Norte de Puebla showed a phytotoxic and promoter effect. The phytotoxicity evaluated in *Arabidopsis thaliana* in the short term was better with EESS treatments, while the EECS reflected a decrease in ecoexergy as a result of greater phytotoxicity in the long term.

By the same token, the decrease in the volume of the extracts had a beneficial effect on *A. thaliana* by registering increases in ecoexergy for culture media with EECS. In-vitro tests for EECS and EESS elucidate a panorama of benefits that can be obtained from mixing organic compounds extracted from ripe chiltepin fruits, which evaluated with holistic parameters, such as ecoexergy, clarify the possible effects in a microsystem which in turn can be complemented by using modeling tools, such as neural networks, to predict long-term effects.
REFERENCES


