Full Length Research Paper

*In vitro* antioxidant and anticholinesterase activities and *in vivo* toxicological assessment (Zebrafish embryo model) of ethanolic extracts of *Capsicum chinense* Jacq.

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The ripe and dried fruits of organic pepper *Capsicum chinense* Jacq., obtained during planting, cultivation and harvesting, were subjected to solid-liquid extraction using a Soxhlet apparatus with hexane to remove non-polar compounds, and then with ethanol to remove the target compounds. The ethanolic extracts were characterized by gas chromatography-mass spectrometry (GC-MS) determining the composition of capsaicin (74.1%), dihydrocapsaicin (8.9%), docosenamide (11.7%), ethyl linoleate (3.2%) and ethyl palmitate (2.1%). Three different bio-screenings: (1) the Trolox® equivalent antioxidant capacity (TEAC); (2) the inhibition of the enzyme acetylcholinesterase (AChE) and; (3) the embryo toxicity and the induction of phenotypic changes in the Zebrafish model were used. It was found that the ethanol extracts possess antioxidant activity (TAA 1800 ± 91 mmol Trolox/kg extract) and AChE inhibition (IC₅₀ 18.8 ± 0.5 µg/ml), as well as a moderate toxicity in zebrafish embryos (LC₅₀ 39.7 ± 2.1 µg/ml), and good dual activity that make them extremely important not only for nutrition, but also as pharmacological substrates.

**Key words:** *Capsicum chinense*, capsaicinoid, acetylcholinesterase inhibitor, Trolox® equivalent antioxidant capacity (TEAC), zebrafish, developmental toxicity.

INTRODUCTION

The use of plants with medicinal properties is as old as human civilization and plant preparations were one of the main options for the treatment of different diseases (Jayaprakasha et al., 2012). Among these plants, chili
peppers (Capsicum spp.) are a rich source of diverse bioactive compounds with potential health-promoting properties, particularly analgesic, anti-inflammatory and antioxidant due to the presence of capsaicinoids, capsinoids, carotenoids, and phenolic compounds (Wahyuni et al., 2013a; Dubey et al., 2015; Galano and Martínez, 2012; Zimmer et al., 2012; Loizzo et al., 2015; Tundis et al., 2013). These biological effects of those bioactive compounds, in addition to the culinary properties of chili peppers (Capsicum spp.), make them extremely important not only for nutrition, but also as pharmacological substrates.

The genus Capsicum comprises more than 200 varieties, and the fruits vary widely in size, shape, flavor and sensory heat due to changeable chemical composition and content (Wahyuni et al., 2013b). Pepper used as a spice for food preparations is usually a single type or a combination of several different varieties. This choice is usually based on individual preferences, without any consideration of health benefits. Among the five most cited species of important economic value, Capsicum annuum, C. baccatum, C. frutescens, C. pubescens and C. chinense, the latter species, C. chinense Jacq. is often known as orange habanero peppers and is a very aromatic variety native that belongs to the Solanaceae family, characteristic of tropical and humid zones of Central and South America. A lipophilic alkaloid called capsaicin (trans-8-methyl-N-vanillyl-6-nonenamide) is the main representative pungent component of these chili peppers. Although excessive exposure to capsaicin can be toxic, causing irritation on the contact area or respiratory problems, as well as some types of cancers due to ingestion of high quantities, capsaicin has been extensively studied via experimental and clinical investigations, for its prominent pharmaceutical, neurological and antioxidant properties (Bae et al., 2012; Dubey et al., 2015; Rosa et al., 2002; Zeyrek and Oguz, 2005). A common use of capsaicin is in topical anti-arthritic and anti-inflammatory ointments.

Clinical trials have shown that capsaicin may have potential value in the treatment of rheumatoid arthritis and cluster headaches by producing discharge of a sensory neurotransmitter, causing an insensitivity to pain (Cordell and Araujo, 1993). However, consumption in great amounts of this compound over prolonged period of time can cause chronic gastritis, kidney damage, liver damage and neurotoxic effects (Johnson, 2007). Capsaicin also possesses antimicrobial properties and inhibitory effect on acetylcholinesterase (AChE) activity that open doors for exploring its potential as a natural inhibitor of pathogenic microorganisms in food (Dorantes et al., 2000; Kurita et al., 2002; Xing et al., 2006; Jones et al., 1997; Chatterjee et al., 2010) or as preventive medicine for Alzheimer’s disease (AD) (Orhan et al., 2007). Recent studies indicate that inflammatory processes are directly associated with Alzheimer’s disease (Kepp, 2012). Amyloid peptides in senile plaques found in the brains of Alzheimer’s patients can induce inflammatory processes, in which reactive oxygen species (ROS) are released along with other components. The ROS are responsible for cell damage at the structural level and act as secondary messengers in inflammation. Therefore, the antioxidants can “catch” ROS attenuating inflammation (Vina et al., 2004; Kontush and Schekatolina, 2004). On the other hand, AD is currently treated clinically by the use of agents, which restore the level of acetylcholine mainly through the inhibition of acetylcholinesterase (Rauk, 2009; Citron, 2010; Kar, 2002). Therefore, medicine with dual action (antioxidant and anti-AChE activities) can act as neuroprotective agents and used to prevent or treat this disease.

Capsaicinoid content in peppers increases with maturing and climacteric ripening of the fruit (Grayfed et al., 2001; Estrada et al., 2002). The quality of the extracted compounds as well as their antioxidant activity is affected by processing parameters such as separation, steam blanching, extraction temperature and drying (Ramesh et al., 2001). Solid-liquid extraction with organic solvents such as hexane, chloroform, and ethanol is the most commonly employed method for capsaicin recovery (Tapia et al., 1993). Although it is believed that C. chinense (orange habanero peppers) contains the highest concentrations of capsaicinoids compared to other varieties, the chemical composition and biological properties of C. chinense dried fruits and their extracts are scarce. In particular, there is a lack of information about the potential use of this material and no information exists with respect to its safety and toxicological effects. Recently, it was reported that lipophilic fractions of habanero C. chinense did not exhibit acetylcholinesterase inhibitory activity, but showed weak butyrylcholinesterase inhibitory properties (Menichini et al., 2009).

With these information in mind, we wanted to evaluate ethanolic extracts of C. chinense dried mature fruits as preventive medicine for AD. To this purpose, the antioxidant activity, using the total antioxidant activity (TAA) value, obtained by the ABTS** radical-cation discoloration method, the inhibition of AChE enzyme frequently targeted for the treatment of Alzheimer’s disease were investigated, relating to the chemical composition of C. chinense dried fruits extracts, and their toxicological assessment using for the first time zebrafish embryo model. To the best of our knowledge, this is the first report on three different bioscreenings of C. chinense dried mature fruits, a promising source of valuable capsaicinoids.

MATERIALS AND METHODS

Chemicals and samples

The reagents used were hexane, ethanol, dimethyl sulfoxide
(DMSO), water (JT Baker, analytical grade), sodium hydrogen phosphate, potassium hydrogen phosphate, Tween 20, acetylthiocholine iodide, diethiothreitol, acetylcysteine, sodium azide, acetylcholinesterase from *Electrophorus electricus* (EC 3.1.1.7, Type VI-S), potassium persulfate, 2,2'-azino[3-ethylbenzothiazoline-6-sulfonic acid], BHT, t-butylhydroxytoluene (BHT), t-butylhydroxyanisole (BHA), D-ö-tocopherol (Vitamin E), sodium chloride, potassium chloride, potassium chloride calcium and magnesium sulfate (Sigma-Aldrich).

**Plant**

The plant material was grown organically at a farm in the Municipality of Piedecuesta in Santander, Colombia (geographic coordinates: latitude 7° 4.477' N, longitude 73° 3.205' W and 1020 m altitude). The *Capsicum chinense* fruits were collected at Piedecuesta in the Municipality of Santander, Colombia. Pepper fruits were examined for health and robust appearance. They were devoid of peduncles and seeds as they were cut into small pieces. Samples were dried in an oven at 40°C to constant weight, pulverized and stored at 20°C until they were analyzed.

**Extraction procedure**

Extracts were obtained in triplicate by solid-liquid extraction using a Soxhlet apparatus. In order to remove non-polar compounds, pulverized fruits of pepper were treated first with hexane for 12 h and then, treated fruits were extracted with ethanol for 12 h. The ethanolic extracts were concentrated and dried in vacuum pump (5 mmHg) for four hours to obtain the capsaicinoids.

**GC–MS analysis of extracts**

The extracts were analyzed by gas chromatography-mass spectrometry (GC-MS). The GC-MS analyses were carried out using a gas chromatograph Agilent Technologies 7890A coupled to a mass selective detector Agilent 5975C with split/splitless inlet (split ratio 1:10) and HP-1MS column (30 m × 0.25 mm ID × 0.25 μm df) with stationary phase 100% poly-(dimethylsiloxane). The heating rate used in the chromatographic oven was 5°C (5 min) @ 5°C/min to 200°C (5 min) and @ 10°C/min to 260°C (20 min). Mass spectra were obtained by electron ionization (70 eV) with a quadrupole analyzer mass range m/z 40 to 400 in full-scan mode. The temperatures of the ionization chamber and the transfer line were maintained at 230 and 285°C, respectively. Chromatographic and spectroscopic data were processed using software MSD ChemStation Agilent Technologies G1701 (EA Version E.02.02.1431). Identifying the components of the extract was performed as follows: the capsaicinoids were cross-referenced with mass spectra and retention times obtained from a registered pattern of capsaicin and dihydrocapsaicin. The other compounds were compared to the spectra obtained with those reported in the databases W8NO8 and NIST05.

**ABTS** assay and TAA values**

In order to determine the Trolox equivalent antioxidant capacity (TEAC), the method of Re et al. (1999), with the modifications proposed by Muñoz-Acevedo et al. (2011) was used. In order to determine the radical cation ABTS**, an aqueous solution of 7 mM concentration ABTS with potassium persulfate (K₂S₂O₈) was allowed to incubate for 16 h at room temperature, in the absence of light. Stock solutions of the extracts at a concentration of 1000 ppm were prepared, which were diluted serially in 96 well plates. Inhibition occurred between 20 and 80% of the blank absorbance after adding 10 μl aliquots of the aqueous solutions to the ABTS** (200 μl). Absorbance measurements were performed on a microplate reader at 734 nm, reading 1 min after the initial mixture up to 30 min. The response-concentration of substances, as a percentage of the absorbance of radical cation ABTS** without inhibition was calculated using Equation (1).

Inhibition of A₇₃₄ (%) = (1 - Ao/A) × 100

(1)

where: Ao: is the absorbance of radical cation without inhibition; Af: is the absorbance measured at 30 min after the addition of the antioxidant agent. The percent inhibition of the absorbance at 734 nm was calculated and plotted as a function of the sample, with the data of the reference substance (Trolox®) and the TAA was determined according to Equation (2) (Muñoz-Acevedo et al., 2011; Arnao et al., 1999):

\[
\text{TAA} = \frac{\text{mmol of Trolox}}{\text{kg of substance evaluated}}
\]

(2)

Solvent blanks were performed on each plate. The experiment control substance was vitamin E, butylated hydroxytoluene (BHT) and butylated hydroxyanisole (BHA) and all assays were performed in triplicate.

**Bioassay for anti-acetylcholinesterase activity**

Acetylcholinesterase (AChE, EC 3.1.1.7, Type VI-S) inhibition was assessed by the Ellman method modifying by scaling microplates (Ellman et al., 1961), which is based on the reaction of released thiocholine to give a coloured product with a chromogenic reagent. Fifty (50 μl) of the extract solution (at serial concentrations from 1000 to 1 ppm), dissolved in phosphate buffered saline pH 7.5 and 50 ml of the AChE (0.25 U/ml) were placed. The plate was incubated at room temperature for 30 min and 100 ml of pH 7.5 substrate solution was added [0.04 M Na₂HPO₄, 2.2'-dinitro-5,5'-dithiobisnitrobenzoic 0.2 mM, 0.24 mM acetylthiocholine iodide]. At five minutes into the reaction, the absorbance at 412 nm in a microplate reader VERSAmax was read. The inhibition rate (%) was calculated by Equation (3):

\[
\text{Inh. A (%)} = 100 \times \frac{(\text{AS}-\text{AB})}{(\text{AS})} \times 100
\]

(3)

Where: AS: is the absorbance of sample; AB is the absorbance of blank; AC is the control absorbance to determine the enzyme activity without inhibitor. Galantamine was used as reference compound and assays were performed in triplicate. The IC₅₀ calculations and graphics were performed using SoftMax Pro 5.2 software from Molecular Devices. The IC₅₀ was defined as the concentration required to achieve 50% of the maximum inhibitory effect.

**Toxicity assay, analysis and phenotypic changes of the ethanol extracts using the zebrafish embryonic model**

The toxicity studies of ethanolic extracts using zebrafish embryos (*Danio rerio*, wild type) were performed based on the protocol developed by Ali et al. (2011) and adapted in our laboratories (Puerto and Kouznetsov, 2013). The embryos were collected, washed twice with E3 medium (5 mM NaCl, 0.17 mM KCl, 0.33 mM CaCl₂, and 0.33 mM MgSO₄, pH 6.8 - 6.9), and transferred to a Petri dish. The embryos were examined periodically through a...
dissecting microscope in order to remove dead, unfertilized, malformed or developmentally delayed embryos. During this period, the embryos were kept in an incubator at 28 ± 2°C under natural light-dark photoperiod. The selected embryos were carefully transferred from the Petri dish to a 96-well microplate, placing one embryo with E3 medium in 200 µl per well. The care and use of fish were performed according to international guidelines of the National Institute of Health in the United States for the care and use of laboratory animals, keeping them healthy and free of any sign of disease. The research ethics committee of the Heart Institute of Bucaramanga, approved this protocol in its meeting on May 26, 2012 (No.050).

**RESULTS AND DISCUSSION**

**Determination of LC₅₀ in zebrafish embryos**

Three independent experiments in three different plates, where the extract was tested three times in each plate were carried out with embryos obtained from different matings. In total, 72 embryos were employed. geometric series of concentrations, starting at 300 µg/ml and ending at 4.68 µg/ml were calculated on each plate. Determining the LC₅₀ (expressed as µg of extract/ml of solution) was based on the cumulative mortality, which was monitored in a dissecting microscope (trinocular stereomicroscope OPTIKA, model version SZM-1) at 72 h after chemical exposure (96 hpf).

**Analysis of phenotypic changes using embryonic zebrafish model**

In order to correlate this experiment with the determined LC₅₀ values, the embryos selected for the phenotypic study were exposed to the established geometric series after 24 hpf. Each extract was diluted in the medium E3 with 2% v/v of dimethyl sulphoxide (DMSO) and aliquots of 200 µl were prepared in a 96 well plate at concentrations of 100, 50, 25, 12.5, 6.25, 3.12, 1.56, 0.78 and 0.39 µg/ml and the appropriate aliquot of the ethanolic extract (200 µl) was transferred into the corresponding well of the embryonic plate. Two controls wells were used peer plate, each containing E3 medium with 2% v/v of DMSO. The embryonic plates were incubated at 28°C and photographed at 48, 72 and 96 h post-fertilization (hpf) using an OPTIKA zoom stereo microscope (trinocular version of model SZM-1).

**Statistical analysis**

All experiments were carried out in triplicate. Data were expressed as means ± standard deviation (SD). The concentration giving 50% inhibition (IC) was calculated by nonlinear regression with the use of Prism Graphpad Prism version 4.0 for Windows (GraphPad Software, San Diego, CA, USA). The dose-response curve was obtained by plotting the percentage inhibition versus concentration. The lethal concentration (LC₅₀) is expressed as the standard error of the mean (SEM) of three different experiments in triplicate, the analysis was made using regression probit analysis with SPSS for windows version 19.0.

**RESULTS AND DISCUSSION**

**Planting, growing and harvesting of plant material and extraction procedure**

Seeds of selected native species were collected in the municipality of San Gil, Santander, Colombia (location 33° N, 78° 8’ W). No chemical weed or control pest were used during the planting of the organic chili peppers in order to ensure that the plant material was free from pesticide residues that could result in false positive tests in AChE inhibition. After planting, growing and harvesting, selected mature seedless fruits were washed and disinfected with 3% sodium hypochlorite. The material was dried in an oven at 40°C to constant weight for 96 h (humidity at 80.1 ± 0.4%), and pulverized to facilitate and streamline the process of extraction. Extraction was performed using a Soxhlet apparatus. 10 g of pepper were weighed and were added to 300 ml of hexane and extracted for 12 h, the hexane was removed and added to 300 ml of ethanol extractor and extracted for 12 h. The extractions were performed in triplicate. The dry weight yield was 7.9 ± 0.5%. The GC-MS analysis of the chemical composition (Figure 1) and the relative amount of components in the extract are shown in Table 1. Capsaicin (74.1%) was determined as a main constituent followed by docosanamide (11.7%), dihydrocapsaicin (8.9%), and two esters of fatty acids: ethyl linoleate (3.2%) and ethyl palmitate (2.1%).

**Determination of the Trolox Equivalent Antioxidant Capacity-TEAC**

The TEAC is widely applied to assess the amount of radicals that can be trapped by an antioxidant (Re et al., 1999). In this research, the extract solution had a preformed radical cation ABTS⁺ and after 30 min, the remaining radical cation ABTS⁺ was quantified spectrophotometrically at 734 nm. The TAA values for controls substances: vitamin E, BHA and BHT were 2659 ± 74, 6819 ± 46 and 4272 ± 38, respectively, while the value of TAA (mmol Trolox/kg of extract) for the extract was 1800 ± 91. These results showed that C. chinense extract had a moderate antioxidant capacity.

**Study to determine the inhibitory activity of the extract against the acetylcholinesterase enzyme**

The profiles of the results revealed interesting numbers, where the extract was able to inhibit the enzyme with IC₅₀ 18.8 ± 0.5 µg/ml. The value for vitamin E was 42.0 ± 6 µg/ml. It should be noted that vitamin E is one of most studied molecules for use in neurodegenerative disorders (Singh et al., 2008; Kontush and Schekatolina, 2004; Sen and Chakraborty, 2011). While the inhibitory concentration for the control drug, galantamine was 0.30 ± 0.01 µg/ml.

**Determination of LC₅₀ in zebrafish embryos**

In order to study the toxicity (lethal concentration - LC₅₀, µg/ml), zebrafish embryos were treated with the obtained
Figure 1. Chromatographic profile of the ethanolic extract of *Capsicum chinense*.

Table 1. Secondary metabolites obtained by Soxhlet extraction and analyzed by GC-MS, present in mature fruits of *C. chinense*.

| Order elution | Components         | Structure | \(T_R\) (min) | Relative amount (%) |
|---------------|--------------------|-----------|---------------|---------------------|
| 1             | Ethyl palmitate    |           | 51.60         | 2.1                 |
| 2             | Ethyl linoleate    |           | 54.95         | 3.2                 |
| 3             | Capsaicin          | ![Structure](capsaicin.png) | 63.84         | 74.1                |
| 4             | Dihydrocapsaicin   | ![Structure](dihydrocapsaicin.png) | 64.41         | 8.9                 |
| 5             | Docosenamide       | ![Structure](docosenamide.png) | 69.74         | 11.7                |
|               | **Total**          |           |               | 100                 |
extracts followed by the Ali protocol (Ali et al., 2011) with some modifications (Puerto and Kouznetsov, 2013). Data collected from three independent experiments were analyzed statistically and the extract of C. chinense Jacq. determined that LC$_{50}$ value was 39.7 ± 2.1 µg/ml. From these results it can be seen that the toxicity of C. chinense extract is comparable to that of other bioactive substances or drugs, such as salicylic acid (LC$_{50}$ = 46.7 µg/ml) (Ali et al., 2011).

Analysis of phenotypic changes using embryonic zebrafish model

Because the optical clarity of the embryo, a wide variety of functional and morphological changes may be observed without killing, dissecting or manipulating the embryos (Goldsmith, 2004; Eggert, 2013; Puerto and Kouznetsov, 2013; Ali et al., 2011; Peterson et al., 2000). Once the LC$_{50}$ was determined for the extract of C. chinense, a range of concentrations below this value was established to evaluate the phenotypic changes induced by the ethanolic extract using the zebrafish embryo model, considering that every fertilized embryo can become a macroscopically representation of a cell. For this experiment the embryos, at 24 hpf to correlate both assays, were exposed to a geometric series of concentrations below the LC$_{50}$ in order to observe a detailed development of each embryo until the end of the experiment.

After the chemical exposure, the morphology and development of embryos exposed to seven concentrations below the LC$_{50}$ were observed at different stages from 48 to 96 hpf using a dissecting microscope. In general, all embryos treated with the extract, at two or three levels below the respective LC$_{50}$, showed no visible phenotypic change during the early hours of chemical exposure. However, at one or two concentrations close to the LC$_{50}$ of the ethanolic extract, the embryos showed a delayed development characterized by a lack of pigmentation in the tail and the abnormal development of the somites, evidenced by the pronounced tail curvature and its wide end, that are responsible for giving rise to most of the axial skeleton and the muscles around the cord during the first stages of development. Figure 2 shows photographs of embryos with observed normal development and the curvature that suggest the abnormal development of the somites.

After 96 hpf, the embryos treated with the ethanolic extract at concentrations above the LC$_{50}$ and those exhibited delayed development during the first 48 hpf eventually died, confirming the toxicity of the extract. However, those embryos that showed no visual phenotypic changes during the first hours after the chemical exposure, reached later stages of development after 96 hpf with no any visual evidence of malformations, defects or injuries in comparison with the control, suggesting that their morphology and development did not differ from the embryogenesis of the control embryos, as seen in Figure 3.

The obtained biological data of ethanolic extracts of C. chinense Jacq are summarized in the following table (Table 2). It should be noted that obtained ethanolic fraction contains capsaicin as the main phytoconstituent (74.1%) (Table 1). Therefore, tested biological properties of this fraction can be attributed to the capsaicin.

Conclusion

It was found that the TAA value (1800 ± 91 mmol Trolox/kg of extract) and the AChE inhibitory activity of the ethanolic extract (IC$_{50}$ 18.80 ± 0.5 µg/ml) were below its lethal concentration (LC$_{50}$ 39.7 ± 2.1 µg/ml), suggesting that antioxidant and/or the inhibition of AChE activity is not being lethal for the embryos and it does not induce phenotypic changes, teratogenic injury or developmental malformations. Toxicity tests of C.
Table 2. Bioevaluation of ethanolic extracts of *Capsicum chinense* Jacq.

| Parameter                  | Antioxidant activity, TAA (mmol Trolox/kg of extract) | Anticholinesterase activity, IC₅₀ (µg/ml) | Toxicity on zebrafish embryos, LC₅₀ (µg/ml) |
|----------------------------|------------------------------------------------------|----------------------------------------|------------------------------------------|
| Ethanol extract            | 1800 ± 91                                             | 18.8 ± 0.5                              | 39.7 ± 2.1                               |
| Vitamin E                  | 2659 ± 74                                             | 42.0 ± 0.6                              | nt                                       |
| BHA                       | 6819 ± 46                                             | nt                                      | nt                                       |
| BHT                       | 4272 ± 38                                             | nt                                      | nt                                       |
| Galantamine                | nt*                                                  | 0.30 ± 0.01                             | nt                                       |
| Salicylic acid             | nt                                                   | nt                                      | 46.7± 1.1                                |

*nt – not tested

Figure 3. Photographs of zebrafish embryos treated at 96 hpf. (A) Control. (B) Embryo treated with *Capsicum chinense* Jacq. extract.

*Capsicum chinense* using zebrafish embryo model were realized for the first time and consistent with previous studies (Wahyuni et al., 2013a). The capsaicinoids like capsaicin and dihydrocapsaicin present in plants of the genus *Capsicum* have been associated with biochemical and pharmacological effects, including antioxidant and anti-inflammatory activities. However, there are few reports on the AChE inhibitory activity, key enzyme associated with AD. It was found that the ethanolic extract of *C. chinense*, an important species with economic value, showed a high inhibitory activity of AChE.

From this work it can be concluded that moderate antioxidant capacities and high inhibitory activity of AChE of *C. chinense* extract, along with its moderate toxicity, makes the consideration of the ethanolic extract *C. chinense*, and its fruits, as a promising, recoverable source with high content of capsaicinoids, and consolidate the model as a tool with chilli high usability for the development of phyto-pharmaceutical and ethnopharmacology in Colombia.

Conflicts of interest

The authors have not declared any conflict of interest.

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