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Review

Use of zebrafish (Danio rerio) in experimental models for biological assay with natural products

Igor Victor Ferreira dos Santos¹,², Jonatas Lobato Duarte¹, Caio Pinho Fernandes¹,³, Hady Keita¹,², Jesus Rafael Rodríguez Amado¹,², Josué Arturo Velázquez-Moyado⁴, Andrés Navarrete⁴ and José Carlos Tavares Carvalho¹,²*

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Research on natural products is facing significant difficulty. The analysis "high-throughput screening" has limited effectiveness in their evaluation. This report on in vitro screening of pharmacological activity of the drugs candidate molecule by evaluating a single target. The presence of many substances in a natural extract makes this process unpriecise. The zebrafish (Danio rerio, ZF) is well suited to high-throughput applications owing to its high fecundity, rapid extrauterine development and transparency during organogenesis. This fact promotes increase in the relevance of their use in biological assays, which is hard to be matched in vitro. Tests based on ZF can aid in the isolation of bioactive molecules from plant extracts, which was identified in a large-scale screening. This increases the biological relevance of such findings. Another decisive factor for the use of ZF as an experimental model, for widespread testing, is the few extracts or the isolated compounds required for tests. Therefore, the growing number of publications and innovative models created for the research shows a lot of diseases with this species, revealing the importance of ZF as an experimental model for the screening of natural products.

Key words: Danio rerio, experimental models, natural products.

INTRODUCTION

The term natural products refers to a living organism, a complete plant or any part of it, or to any chemical compound which had been extracted from these without any changes in its chemical structure (secondary metabolites) (Miranda and Cuéllar, 2001). Natural products are an excellent source of chemical compounds

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for new drugs discovery. Until now, bioactive components isolated from plants, fungi and bacteria gave rise to a wide range of therapeutic compounds. Also, a lot of these compounds are useful tools in pharmacology, biochemistry and cell biology and molecular (Crawford et al., 2008).

The improvement of computational chemistry in the 90s and the development of new technologies such as "target validation" and "high-throughput screening", caused the decline in drug research from natural products (Crawford et al., 2008). Many pharmaceutical laboratories eliminated the research program with natural products (Schmid and Smith, 2009). The research with natural products has another inherent challenge: the identification of the mechanism of action of the extracts, the synthesis of complex structures of the active compounds and the difficulty of isolating pure compounds from crude extracts, sometimes in such small quantities, that the "high-throughput screening".

Despite massive investments in new technologies for the development of chemical compounds, the number of new drugs reaching the market has not increased proportionately. Thus, the computational and combinatorial chemistry cannot achieve its goal of being a primary source of new bioactive candidate compounds for innovative drugs (Yunes and Cechinel, 2001).

In this context, the natural products have recovered space and importance in pharmaceutical industry. They are an inspiring source of new bioactive molecular patterns. At the same time, there is a growing appreciation of natural products, because they have chemical structures, with the ability of interacting effectively with biological macromolecules (Koehn and Carter, 2005; McChesney et al., 2007). Therefore, the research with natural products will continue to expand since these are a source of inspiration for the development of new drugs.

Research on natural products is facing another major difficulty. The analysis "high-throughput screening" has limited effectiveness of their evaluation. These analyses make possible in vitro screening of pharmacological activity of the drugs candidate molecule, by evaluating a single target. The presence of many substances in a natural extract make this process unprecise. These analyses often generate compounds with little or no effectiveness in vivo (Van der Greff et al., 2005). However, the consequence of this deficiency has stimulated the search for new methods of bioassays for a screening of pharmacological activity that is more efficient in the evaluation and target independent (Crawford et al., 2008).

The use of zebrafish in experimental models, has occupied space in the research with natural products, currently being used by several research groups for screening and confirmation of various pharmacological activities. The biological response obtained in this model is similar to that achieved in mammals, helping researchers in refining and reducing the use of rodents. In the future, the study of natural products in zebrafish model will contribute to greater understanding of the biological activity of a wide range of bioactive compounds because this model requires a very small amount for the tests.

The zebrafish (Danio rerio Hamilton-Buchanan, 1822) is well suited to high-throughput applications owing to its high fecundity, rapid extraterine development and transparency during organogenesis. This fact promotes and increases the relevance of their use in biological assays, which is hard to be matched in vitro (Lin et al., 2013). The objective of this work is to update knowledge on the use of ZF as an experimental model for biological assays with natural products and derived.

ADVANTAGE OF THE ZEBRAFISH AS EXPERIMENTAL MODEL

The zebrafish (ZF) belongs to the Cyprinidae family. It is a small teleost fish of tropical freshwater. It has a body length of three to four centimetres. It is a species well known for its ornamental use (Poon and Brand, 2013). This small fish is being used every day by the international scientific community. In 1955, it was reported, for the first time, the use of ZF as an experimental model. From that date until now, the use of ZF had an exponential growth (Figure 1 and Table 1).

The key to the complete success of ZF, as an experimental model, lies in the fact that it has biological characteristics and for maintenance, it is very favourable. The ZF has a short cycle of life and a rapid development, which takes place outside the female's uterus. The ZF produces a large progeny (hundreds of eggs per mating). Another critical feature in its use is the low cost and reduced space required for its maintenance and care. The ZF embryos are small (1 to 5 mm), depending on the stage of development) and are translucent. This fact makes them suitable for handling and use (Lele and Krone, 1996; Bailey et al., 2013). Most of the citations of ZF as experimental model refers to the use of embryos and larvae.

The determining factors for use of ZF as an experimental model are the physiological and pharmacological responses similar to humans and other higher mammals. This makes it suitable for identifying drugs and bioactive natural products with therapeutic potential. The biological characteristics of the ZF combined with the advantages it offers in maintenance and care, make it an ideal model in vivo for "medium throughput screening" (Crawford et al., 2008).

The key advantage of the bioactivity assay of small molecules, by using ZF embryos and larvae is that the compounds can be diluted with non-sterile water. The little weight molecules are rapidly absorbed by the skin and gills of ZF. This allows an increase in the "throughput
Figure 1. Number of citation in PUBMED for zebrafish as experimental model from 1955-2015. Only zebrafish keywords was used.

Table 1. Use of vegetable extracts or compounds isolated on zebrafish "model"

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<th>Activity assayed in zebrafish model</th>
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<td>Angiogenic/anti-angiogenic</td>
<td>Lam et al., 2008; He et al., 2009a; He et al., 2009b; Hong et al., 2009; He et al., 2010; Alex et al., 2010; KRILL et al., 2010; Zhu et al., 2011; Liu et al., 2011; Crawford et al., 2011; Tse et al., 2012; Zhong et al., 2012; He et al., 2012; Zhang et al., 2012; Han et al., 2012; Yu et al., 2013; Ba et al., 2013; Lee et al., 2013; Yahg et al., 2013; Fan et al., 2013; Yue et al., 2013, Da-Song et al., 2014; Zhou et al., 2014; Germano et al., 2014 Lai et al., 2015</td>
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<td>Antithrombotic activity</td>
<td>Song et al., 2012; Shi et al., 2015</td>
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<td>anticonvulsant</td>
<td>Orellana-Paucar et al., 2012; Buenafe et al., 2013; Challal et al., 2014</td>
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<td>Jin et al., 2011; Kim et al., 2012; Pardal et al., 2014; Littleton et al., 2014</td>
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<td>Melanogenic</td>
<td>Kim et al., 2008; Ding et al., 2011; Park et al., 2013; Kim et al., 2014; Wang et al., 2014</td>
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<td>Wang et al., 2009; Del Valle-Mojica and Ortiz, 2012; Wei et al., 2012a,b; Ferri-Lagneau et al., 2012; Gebruers et al., 2013; Yu et al., 2013; Ulloa et al., 2013; Kang et al., 2014; Holbech et al., 2013</td>
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screening” of hundreds of molecules per day (Zon and Peterson, 2005; Langheinrich, 2003).

Majority of the studies where ZF is used as the experimental model, reported the use of larvae and embryos. One of the big questions in the use of ZF is how far the results could be extrapolated to humans and other higher mammals?

Genetic and pharmacological tests using ZF proved to have representativeness for other organisms, including humans highly. Additionally, the identified genes in the organogenesis of ZF has been consistently validated in mice and humans. In many cases, ZF and human genes have been discovered side by side. The remarkable pharmacological homologies between humans and ZF can be extended to phenotypes modified disease identified through these assays (Peterson and Macrae, 2012).

The ZF has been used as an experimental model for genetic studies. In 1981, the embryonic development of ZF was reported (Streinsinger, 1981). In the 2000s, there was considerable progress in genetics and genomics of ZF. The mitochondrial genome of ZF was fully sequenced in October 2001 (Broughton et al., 2001). In 2013, the Sanger Institute (UK) reported the complete sequence of
ZF genome. When comparing the sequence of the ZF genome with the human genome, it was observed that some genes as cell cycle, growth and differentiation, tissue functions, oncogenesis and tumour suppressor, were preserved. Some studies showed that the 70% of the ZF genes are similar to human genes (Stern and Zon, 2003; Howe et al., 2013). These data show ZF as a reliable model for the screening of drugs and natural products (Amatruda et al., 2002; Crawford et al., 2008).

Genetic research on ZF produced a range of mutant strains, which can be used to carry out studies in many different pathologies. Some examples of mutant strains defective of ZF are “Transparent Zebrafish” (Casper) (Witte et al., 2008) and rag2E450fs (Qin et al., 2014). Just the Hopkins’ insertional mutant collection contains more than 500 recessive mutants with embryonic morphological phenotypes, which include mutations in 335 identified genes (Kishi et al., 2008). These mutants allow studying pathologies related to cartilage development, hematopoiesis, cardiovascular development, among others. The Casper mutant strain (totally transparent) is used for research related to cancer and stem cells, previously limited only to embryos and larvae (Witte et al., 2008). Other genetic testing using ZF are gastrointestinal function tests, vascular development, epilepsy and diabetes (Crawford et al., 2008; Seth et al., 2013).

The ZF mutant strains have utility in toxicological research to clarify the roles of particular genes and their interactions with signalling pathways. Also, they are useful in the pathogenesis caused by damage induced by toxicants. Furthermore, ZF with double or triple mutations may help to elucidate the interaction of the suite of genes. The ZF mutants can be produced more economically and efficiently than in murine (Spitbergen et al., 2003; Hill et al., 2005).

The ZF has proved a versatile model for reverse genetics studies. “Antisense morpholino oligonucleotides” commonly referred as “morpholinos” is the most widely used technique for knockdown in ZF. This method specifically blocks the function of a gene in a ZF embryo. The morpholinos are available as genes tool, and its microinjection is performed in a dose-dependent manner in the early stages of embryo development (single-cell-stage embryos). Two types of morpholinos are used to interfere with gene expression of proteins. The ATG-morpholino, that blocks translation of ribosomes and leaves out the embryo without one protein and the splice-morpholino that binds and interferes with RNA splicing, results in a truncated protein form that is used to study a particular area of the protein. However, numerous genes have been functionally analysed in this way, including several identified in the context of large-scale genetic screens reverse (Bill et al., 2009; Heasman, 2002; Bill et al., 2009; Doitidou et al., 2002; Esguerra et al., 2007).

In recent years, there have been several advances in the ability to generate transgenic lines of ZF. The transgenic methods are well established for ZF. This allows the direct generation of transgenic lines expressing fluorescence under the control of tissue-specific promoters. This radically reduces the time to generate new strains. Traditionally, transgenic reporters were generated by microinjection of linearized plasmid DNA, containing the coding sequence of a reporter protein immediately downstream of a minimal promoter fragment of the gene of interest (Higashijima et al., 1997). However, this approach suffered from some limitations, in particular, the low efficiency of germline integration (Hammond and Moro, 2012).

Advances in the technology have included the introduction of the gateway system and the production of compatible plasmids that can be used in zebrafish (Kawakami et al., 2004; Villefranc et al., 2007), I-Soel cloning, whereby introduction of meganuclease sites increased the efficiency of germline integration (Grabher and Wittbrodt, 2008). More recently, improvements have been achieved by bacterial artificial chromosome (BAC) recombineering, in which fluorophores and Tol2 transposase sites are introduced into a BAC containing the gene or promoter of interest.

**ZEBRAFISH AS EXPERIMENTAL MODEL FOR THE EVALUATION OF NATURAL PRODUCTS**

The use of ZF as an in vivo model for the discovery of drug candidate molecules was proposed 58 years ago. This study presents the use of embryos and ZF larvae for the screening of synthetic drugs and natural products (Jones and Huffman, 1957). However, only in the year 2000, was described, the first "screening" using multwell plates. Since then, over 60 studies have reported the use of ZF for performing whole projects aimed at drug discovery (Rennekamp and Peterseon, 2015).

Many compounds used by humans as flavonoids, alkaloids and some drugs have been tested for their teratogenic and embryotoxic potential in ZF model (Jones et al., 1964; Thomas, 1975; Kim et al., 2009; Stewart and Kalueff, 2014; Lu et al., 2014). Adults ZF were used to confirm the piscicide property of arylnaphthalide, a kind of lignans extracted from *Phyllanthus piscatorum*, a medicinal plant used by Yanomami (Amazon Indian tribe) as piscicide and antifungal (Gertsch et al., 2003, 2004). It was also used in a recent study to determine the neurotoxic effects of a Chinese Medicinal formulation of *Azadirachta indica*, by using behavioural models (Bernadi et al., 2013).

Adults ZF can be used for screening anti-inflammatory activity, particularly natural products. A short time ago a model that is based on the intraperitoneal administration of λ-carrageenan was established (Huang et al., 2014). The authors reported a significant increase in abdominal swelling and proinflammatory proteins (iNOS and TNF-α) induced by carrageenan in ZF.

**Evaluation of angiogenic activity in Zebrafish model**

There are many reports assessing the angiogenic activity of natural products by using ZF as the experimental model. Lam et al. (2008) used transgenic ZF to characterise the pro-angiogenic properties of *Angelica sinensis* (Dong Quai). This work tested the crude extract, rather report the isolation of any bioactive compound (Lam et al., 2008). Liu et al. (2011) used transgenic ZF to investigate the
angiogenic activity of the crude aqueous extract of *Rehmannia glutinosa*. This work showed the isolation of norviburtinal, a new active compound (Liu et al., 2011).

Subsequently, Yu et al. (2013) demonstrated the anti-angiogenic effect of the hydroalcoholic extract of *Herba epimedihi*, a medicinal plant from East Asia. In this *in vivo* study, transgenic ZF was used (Yu et al., 2013). In 2013, one platform for biomonitoring tests (bioassay-guided) was developed. This platform combines the screening of bioactivity on transgenic ZF embryo with the rapid fractionation by thin layer chromatography and initial structural elucidation by mass spectrometry (Crawford et al., 2013). Using these procedures, the authors identified two compounds angiogenic inhibitors (emodin and coleone) in crude extracts of *Oxygonum sinuatum* and *Plectranthus barbatus* (Crawford et al., 2013).

**Antithrombotic activity evaluation using zebrafish model**

The antithrombotic activity of compounds isolated from plant extracts has been evaluated using ZF model Shi et al. (2015) glycosides, phenyl and propyl aldehydes and biphenyls from the methanolic extract of *Piper wallichii* (Mic.). Next, these compounds were tested for their antithrombotic activity by using ZF model. From them, just a lignane (–) syringaresinol, showed excellent antithrombotic activity. Song (2012) reported four new phenolic compounds extracted from *Crataegus pinnatifida* leaves. These compounds were tested jointly with other known compounds, to evaluate the antithrombotic activity on transgenic ZF. Among the isolated compounds, the eriodictyol is shown to be a potent inhibitor of thrombus formation.

**Anticonvulsant activity in zebrafish model**

ZF model were used to evaluate the anticonvulsant effect of *Curcuma longa* extract for the treatment of epilepsy. Seizures were induced with pentylenetetrazol. This extract showed antiepileptic effect (Raoa et al., 2005). Orellana-Paucar (2012) reported the use of ZF model to evaluate the anticonvulsant activity of isolated compounds from *Curcuma longa* oil. This study used the same pattern of seizures induced by pentylenetetrazol. In conclusion, in this study, the usefulness of a zebrafish seizure model for rapid bioactivity-guided fractionation of natural products and their purified compounds for the identification of novel small molecules with anticonvulsant activity in *vivo* was demonstrated.

Buenafe (2013) reported the evaluation of the ketonic extract of *Salvia miltiorrhiza*, Bunge and four bioactive Tanshinones obtained by fractionation of the extract. Pentylenetetrazol model was also used to induce seizure. The extract showed anticonvulsant activity. One of the active tanshinones, tanshinone IIA, also reduced C-fos expression in the brains of PTZ-exposed ZF larvae.

**Anti-inflammatory activity in zebrafish model**

Kao et al. (2010) showed that grape seed extract reduced the dihydrofolate reductase activity and thus the growth of *S. aureus*. Then, ZF was infected with *S. aureus* pre-incubated with grape seed extract. There was a significant decrease in the inflammatory response and mortality of ZF infected with *S. aureus* (KAO et al., 2010).

Bohni et al. (2013) showed the screening of anti-inflammatory activity of over 80 methanolic extracts of medicinal plants of East Africa, using ZF as an experimental model. The methanolic extract of *Rhynchosia viscosa* (Roth) DC inhibits leukocyte migration in ZF sectional tails (4 dpf). As a result, five bioactive compounds were isolated (rhynchosivasina, genistein, sophorosiflavona A, licoisoflavona and 3-o-methylorobol). It was proven that genistein and sophorosiflavone possess anti-inflammatory and anti-angiogenic effects.

Zebrafish model and cell line RAW 264.7 were used to assess the effects of anti-inflammatory compounds of the ethanol extract of the root of *Gentiana dahurica* (Gentianaceae). The results showed no cytotoxicity of all the tested compounds and an intense inhibitory activity of the roboric acid and liriodendrin (lignans) (Wang et al., 2013).

**Antilipemic activity in zebrafish model**

The zebrafish is becoming an increasingly popular model for automated discovery of drug. It is also used for hypercholesterolemic research. The creation of two algorithms was reported for automated analysis of cardio dynamic data acquired by high-speed confocal microscopy, by using data that was obtained using ZF as the experimental model (Littleton et al., 2013).

The hypolipidemic activity of aqueous extracts of turmeric (*Curcuma longa*) and bay leaves (*Laurus nobilis L.*) was evaluated in ZF model. The results showed that consumption of bay leaf and turmeric extracts produced a hypolipidemic effect and antioxidant activity (Jin et al., 2011). The effect of the aqueous extract of cinnamon (*Cinnamomum verum*) and clove (*Syzygium aromaticum*) on the hypercholesterolemic model in ZF was also studied.

Cinnamon and clove aqueous extracts showed anti-hypolipidemic activity. They showed strongest anti-glycation and antioxidant activity in this study. Cinnamon and clove extracts (at final 10 µg/mL) had the strongest anti-glycation and antioxidant activity in this study. Cinnamon and clove had the strongest inhibition of activity against copper-mediated low-density lipoprotein (LDL) oxidation and LDL phagocytosis by macrophages (Jin, 2011a).

**Melanogenic activity in zebrafish model**

ZF larvae are an ideal model for studies related to melanogenic activity, as they allow an easy observation of phenotypic pigmentation process. Kim (2008) reported on anti-melanogenic activity of hanginine A, an isolated isoflavone from the branches of *Lespedeza cyrtobotrya*. The authors concluded that the hanginine A promotes a similar effect on 1-phenyl-2-thiourea (PTU), an inhibitor of the enzyme tyrosinase in a dose-dependent way.

Park et al. (2013) investigated the melanogenic inhibitory activity of Arctigenin, an isolated compound from the aqueous extract from *Fructus arctii* on zebrafish cell line B16BL6 embryos and Melan-A.

The results obtained proved that the treatment arctigenin (10 uM) produces a moderate decrease of the pigment deposition in ZF, 15 dpf.

In other work, ZF model was used to evaluate the anti-melanogenic effect of rengiolona, a compound obtained from the *Eurya emarginata* extract. The results showed an inhibition of body pigmentation of ZF, besides the reduction of melanin levels and activity of the tyrosinase enzyme (Kim et al., 2014).

**Studies on neurodegenerative diseases in zebrafish model**

The ZF has been used in the study of neurodegenerative diseases such as Parkinson’s disease, Huntington’s and Alzheimer’s diseases. Zhang (2012) reported the use of ZF model and PC-12 cell line for evaluating the neuroprotective effect of ethanolic extract of the fruit of *Alpinia oxyphylla*. This is a plant used in traditional Chinese medicine. The results showed that the extract prevented and restored dopaminergic neurodegeneration induced by 6-OHDA (6-hydroxydopamine) and attenuated deficits in locomotor activity in Parkinson’s disease model in ZF.

Chong et al. (2013) studied the neuroprotective effect of
Danshensu (the hydrophilic component of Radix Salviae miltiorrhiza; Danshen, Chinese Pharmacopoeia) in ZF model and PC-12 cell line. This compound has antioxidant properties, in dopaminergic neurodegeneration, induced by 6-OHDA. The results obtained with treatment with Danshensu on neurodegeneration, suggest that it exerts a neuroprotective effect and can alleviate the loss of cells to the average level (Chong et al., 2013).

Toxicological studies on studies on zebrafish model

The ZF has been widely used for environmental toxicity studies to characterise the damage caused by various pollutants. The ZF is considered the gold standard for evaluating environmental toxicity (Scholz et al., 2008). With the expansion of nanotechnology, the ZF has been gaining ground as an experimental model for environmental health and safety tests of various nanomaterial (Lin et al., 2013; Scholz et al., 2008).

The ZF model has been used for assessing the toxicity of silver nanoparticles (Muth-Köhne et al., 2013), silica (Duan et al., 2013) and other nanomaterials (Jevegeni et al., 2013). Furthermore, ZF has been very useful in evaluating plant extracts and isolated compounds. Thus, the toxic effect of celastrol, one terpenoid isolated from Tripterygium wilfordii Hook F., was assessed. The results showed that celastrol, at micromolar concentrations, affects the healthy development of ZF embryos (Wang et al., 2011).

In 2012, the nephrotoxicity caused by aristolochic acid compound isolated from Aristolochia asarum extract was evaluated. The result showed that aristolochic acid-induced nephropathy, defects in blood circulation and cardiac malformations, were mediated by inflammation process (Ding and Chen, 2012).

Zhang et al. (2014) evaluated the toxicity of 10 compounds isolated from the ketonic extract of Kadsura oblongifolia, over cardiac function and embryonic development of ZF. The results showed that kadsulignane, meso-dihydroguaiaretic acid and kadsufolina produced edema in ZF embryos, a diminution of the heart rate and also interfered with the development of the zebrafish heart (Zhang et al., 2014).

Conclusion

The use of zebrafish is already established as a powerful research platform for the discovery of new drugs. Also, the zebrafish is a useful model in other areas of science. It has become an experimental model for screening extracts and components derived from it, due to the ease in which small molecules and natural products can be studied in this species. A large number of tests that can be performed by using zebrafish are one of the attractions of this model since it is not possible to carry out such a large number of tests in others experimental models. Assays based on zebrafish can aid in the isolation of bioactive molecules from plant extracts, which were identified in a large-scale screening; this increases the biological relevance of such findings. Another decisive factor in using the zebrafish as an experimental model for large-scale screening are a few extracts or the isolated compounds required for tests. Therefore, the growing number of publications and innovative models for research on a lot of diseases with this species, reveal the importance of zebrafish as an experimental model for the screening of natural products.

Conflict of Interests

The authors have not declared any conflict of interests.

ACKNOWLEDGEMENTS

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hancei inhibit advanced glycation end product formation and aldose reductase in vitro and vessel dilation in larval zebrafish in vivo. Planta Med. 79: 1705-1709.


Full Length Research Paper

Antinociceptive and antioxidant activity of Calliandra umbellifera Benth

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The genus, Calliandra is popularly used for renal pain, cystitis, prostate inflammation, fever and toothache. This study aimed at investigating the antinociceptive activity of methanolic crude extract (MCE) of Calliandra umbellifera, regarding chemical (acetic acid, formalin and glutamate tests) of nociception (in vivo) and the methanolic extract and hidrobutanolic phase (HBF) antioxidant activity by the 2,2-diphenyl-1-picril-hidrazil (DPPH) method, besides determination of the content of phenolic compounds and flavonoids (in vitro). The pre-treatment with the MCE (100, 200 and 300 mg/kg, p.o) was able to reduce the number of abdominal contortions (p<0.01 or p<0.001), the licking times in the formalin (p<0.001) and the glutamate tests (p<0.01), respectively. In the antioxidant assay, the extract showed optimum EC50 and higher flavonoid content as compared to the hidrobutanolic fraction; however, the content of the obtained phenolic compounds were higher in HBF as compared to MCE. The experimental data showed that C. umbellifera has an antinociceptive activity, a good antioxidant activity and high levels of phenolic compounds, which confirms the popular use of Calliandra, contributing to the scientific knowledge of the species.

Key words: Calliandra umbellifera, antinociceptive activity, antioxidant, total phenol, flavonoids, 2,2-diphenyl-1-picril-hidrazil (DPPH).

INTRODUCTION

Herbal medicine has been used for the treatment of many diseases for a long period of time (Asadbeigi et al., 2014). The drugs commonly used to treat pain have significant adverse effects such as induction of complications such as gastric lesions, addiction, tolerance and sedation, besides presenting limited effectiveness. Thus, it is necessary to seek new therapeutic strategies for the treatment of pain and the reduction of side effects. Preclinical studies using test nociception against mechanical, thermal and chemical stimuli, as well as

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models of inflammatory pain and neuropathic origin in rodents are critical for assessment and development of potential new drugs (Cavendish, 2014).

The chronic oxidative stress is responsible for many degenerative diseases, such as: asthma, autoimmune diseases, gastrointestinal and cardiac diseases and Alzheimer (Lushchak, 2014; Sies, 2015). Hence, it is necessary to assess some mechanisms, as antioxidants, to counteract the damages of oxidation (Wojtunik-Kulesza et al., 2016) and in that respect a large source of antioxidants, such as vegetables have been studied in order to evaluate their antioxidant activity (Boudet, 2007; Rice-Evans et al., 1997).

The genus, Calliandra belong to the family Fabaceae, consisting of approximately 200 species, being spread in Tropical America, India and Madagascar and known as “esponjinha” in Brazil (Mattagajasingh et al., 2006; Lewis and Rico, 2005). The species of Calliandra are used by population for renal pain, cystitis, urethritis, prostate inflammation, fever and toothache (Adesina, 1976; Dimayuga et al., 2006) and some studies showed they are used as anticonvulsivants, analgesic, antiarrhythmic, antispasmodic, antibacterial and antioxidant (Orishadipe et al., 2010; Agunu et al., 2005; Agwu and Lawal, 1988). The principal secondary metabolites find in Calliandra are cassane diterpens, saponins, flavonoids and tannins (Dimayuga et al., 2006; Barbosa et al., 2008; Murillo et al., 2008).

Due to the popular users and the absence of studies on species of Calliandra, this work aims to evaluate the antinociceptive activity of the methanolic extract of the aerial parts of Calliandra umbellifera through nociception induced by chemical stimuli, besides evaluating the total phenolic and flavonoid contents and the antioxidant activity of methanolic extract and hydrobutanolic fraction by DPPH method.

MATERIALS AND METHODS

Animals

Male Swiss mice (Mus musculus) (30 to 40 g), were kept under controlled temperature conditions at 21± 2°C subject to a 12 h light/dark cycle, free access to food and drinking water. The animals were divided into 5 groups (n = 8), being used only once. The experimental procedures were reviewed and approved previously by the CEPA-The Ethics Committee for the Use of Animals CBiotec/UFPB, under certificate No. 0809/12.

Drugs

The chemical substances used in this antinociceptive study were: glacial acetic acid (Synth – U.S.A.), morphine hydrochloride (Merck – U.S.A.), formaldehyde (Vetec – Brazil), DMSO (Sigma – U.S.A) and glutamic acid (Sigma – U.S.A.). The MCE was dissolved in distilled water and DMSO and volume of 0.1 mL/10 g mouse weight was administrated orally (p.o.). For antioxidant study, gallic acid (Sigma-Aldrich), ascorbic acid (Sigma-Aldrich), quercetin (Sigma-Aldrich), as standards, and Folin-Ciocalteu (Sigma-Aldrich), aluminium chloride (Sigma-Aldrich), 1,1-difenil-2-picril-hidrazil – DPPH (Sigma-Aldrich) and sodium carbonate as reagents were used.

Plant

The aerial parts of C. umbellifera, were collected in Matureia - PB, Brazil, identified by the Dra. Maria de Fátima Agra from the Pharmaceutical Technology Laboratory at the Federal University of Paraíba state and catalogued in the Herbarium Professor Lauro Pires Xavier JPB/UFPB, under de code AGRA, 7430.

Extraction

The botanical materials passed through the drying process, followed by a spray in mechanical mill, obtaining 5.0 kg of stem powder which was thoroughly macerate with methanol (MeOH) during 72 h. The extraction solution was concentrated in rot vapor under reduced pressure at a temperature of 35ºC by obtaining the methanolic crude extract - MCE (318 g). The MCE was solubilized in methanol : water (1:1) and subjected to liquid-liquid partition hidrobutanol, by obtaining the hidrobutanolic fraction (HBF).

Acute toxicity

Groups of mice (n = 8) were treated randomly through oral route in doses of 500 and 1000 mg/kg, while the control group received only the vehicle. After the treatment, the pharmacological and/or toxic effects were observed during 4 h. Then, the animals were fed and the number of deaths was observed during 14 days for LD50 determination.

Acetic acid-induced writhing test

This method is based on the fact that the intraperitoneal administration of acetic acid solution at 0.85% in mice causes peritoneal irritation, characterized by abdominal contortions followed by extensions of the hind limbs (Koster et al., 1959). This nociceptive behavior was quantified for 15 min after the administration of the stimulus. The animals were divided into 5 groups (n=8) and it was pre-tread with drug standard (morphine 6 mg/kg, i.p.), vehicle and MCE (100, 200 and 300 mg/kg, p.o.). After 30 min of the initial treatments, the animals were treated with acetic acid at 0.85% (0.1 mL/10 g) via i.p., and subsequently placed into a polyethylene box, in order to observe the number of abdominal contortions presented by each animal.

Formalin test

In the formalin test, mice (N = 8) were pre-treated with the vehicle, MCE (100, 200 and 300 mg/kg, p.o.) and morphine (10 mg/kg, i.p.). After 30 min, 20 µL (2.5% formalin solution) was injected into the sub-plantar region of the hind right paw of the mouse. After the administration of formalin, the animals were placed into observation boxes. The indication of nociception was the total time of paw licking that was counted in 2 phases. The first phase, usually occurs in the first 5 min after the formalin administration (neurogenic response), then there is an interface of approximately 10 min characterized by inhibitory mechanisms of pain. The second phase (15 to 30 min) is known primarily for an inflammatory response (Hunskaar and Hole, 1987).
Glutamate test

The injection of glutamate induces the direct stimulation of nociceptives neurons, causing the liberation of many inflammatory mediators and neuropeptides involved in the pain transmission (Buzzi et al., 2009). For this test, the animals (n = 6–8) were treated with MCE (100, 200 and 300 mg/kg p.o.) or vehicle, 60 min before the intra-planter (i.pl.) glutamate (20 μmol/paw) injection. The time of licking or biting the paw that received the stimulation for 15 min was quantified. MK 801 (0.15 mg/kg i.p.) was administered 30 min before stimulation and used as a positive control.

Determination of phenolic content

The total phenolic content was determined by Folin-Ciocalteu method, which is one of the most used tests for this kind of analysis. For this test, gallic acid was used as standard compound, as described by Gulcin et al. (2004), with some adaptations. 0.5 mL of Folin-Ciocalteu reagent was added to 120 μL of sample (concentration of extract = 450 μg/mL). The reaction was maintained at rest for 5 min and then 400 μL of sodium carbonate (7.5%) was added to neutralize the mixture. Samples were maintained at room temperature, in the dark for 120 min, and then were transferred to 96 well plates and the absorbance was measured at 765 nm on UV-Visible Spectrometer (UV-2550, Shimadzu). Experiments were made in triplicate and phenolic content was determined by linear regression equation from the calibration curve constructed with gallic acid (7.5; 15; 75; 100 and 150 μg/mL). The results were expressed in mg EGA/g of sample.

Determination of flavonoid content

Flavonoid content was determined by spectrophotometric method proposed by Schmidt and Ortega (1983), with some modification, using aluminum chloride (AlCl₃) as reagent and quercetin as standard. The aluminum chloride has the characteristic of forming stable and colorful complexes with flavonoids when dissolved in methanol; it causes a shunt to higher wave lengths and an increasing of absorption (Souza and Giovani, 2005; Petry et al., 1998; Wilczyński et al., 1998). For this test, 0.1 mL of the sample (1mg / mL) was treated with 0.1 mL of aluminum chloride solution (2.5%) in 96-well plates and this reaction was maintained away from light for 30 min and then, the absorbance was measured at 410 nm on UV-Visible spectrometer (UV-2550, Shimadzu) (Marques et al., 2012). The analysis were performed in triplicate and the flavonoid content was determined by the linear regression equation from the calibration curve constructed with quercetin acid (7.5; 15; 75; 100 and 150 μg/mL) and the results were expressed by μg of quercetin/mg of sample.

Antioxidant assay in vitro: DPPH method

Antioxidant activity was determined by the 2,2-diphenyl-1-picril-hidrazil (DPPH) method, using the methodology described by Garcez et al. (2009), with some adaptations. The DPPH method is based on the reduction of this organic radical, which presents its higher absorption at 515 to 520 nm, showing a yellow color. After the abstraction of radical hydrogen from the antioxidant used in the study, it is possible to observe a decrease in absorption and staining solutions, moving from violet to yellow. For this test, 100 μL DPPH solution were added to 100 μL of sample at different concentrations (1.625, 3.125, 6.25, 12.5 and 25 μg/mL) which were determined by initial screening. The mixture was kept at rest and in the dark for 30 min and then the absorbance was measured at 518 nm on UV-Visible spectrometer (UV-2550, Shimadzu). The percentage of scavenging activity (% SA) was calculated according to the equation:

\[
SA(\%) = \frac{(A\text{ negative control} - A\text{ sample})}{A\text{ negative control} \times 100}
\]

Where, A negative control = DPPH and methanolic solution absorbance; A sample = Radical absorbance in presence of extract or standard.

The antioxidant activity was expressed as EC₅₀ (half maximal effective concentration), which means the concentration of the sample that gives half-maximal response, and as positive control was used ascorbic acid. The experiment was performed in triplicate.

Statistical analysis

The data were analyzed through variance analysis (ANOVA) to calculate if there was a significant difference among groups, followed by the Dunnett or Tukey tests. The results were expressed as means ± S.E.M and the p values were considered as significant when less than 0.05. The LD₅₀ was determined using non-linear regression and the EC₅₀ was determined using linear regression.

RESULTS AND DISCUSSION

Acute toxicity of the MCE

The determination of the acute toxicity and the lethal dose 50 (LD₅₀) allows investigating the possible toxic effects of substances and extracts, determining the dose responsible for the death of 50% of the animals (Litchfield and Wilcoxon, 1949), allowing the achievement of pharmacological tests using safe doses. The methanolic extract of C. umbellifera showed no death.

Effects of MCE on the acetic acid-induced writhing test

The writhing test is characterized by its high sensibility, although it does not present any selectivity, being also sensitive to sedative drugs, muscle relaxant, analgesics non-steroidal anti-inflammatory drugs and narcotics (Collier et al., 1968). The nociceptive response to acetic acid may involve a direct stimulation of the nociceptive afferent fibers, due to a reduction of the pH or a synthesis of the inflammatory mediators, such as the arachidonic acid metabolism via COX, with consequent biosynthesis of prostaglandins (Dauerte et al., 1988; Franzotti et al., 2000). In the study, the number of contortions induced by acetic acid was recorded as a significant reduction (p<0.01 or p<0.001) in the experimental doses of 100, 200 and 300 mg/kg p.o. as compared to the control group which was similar to the morphine (positive control) (Figure 1). From these results, it can be seen that increasing the dose caused a reduction in the number of contortions, demonstrating the effectiveness of the MCE in the writhing test. This proposes that this substance showed antinociceptive activity and/or would be inhibiting the release of inflammatory mediators and cytokines.
Effect of oral administration of methanolic extract of *C. umbellifera* (MCE) in the number of abdominal contortions induced by acetic acid in mice (n = 8 per group). **p<0.01, *** p< 0.001 versus the control group. The values are expressed as means ± S.E.M. (ANOVA - Dunett test).

The mice treated with MCE (100, 200 and 300 mg/kg, p. o.) demonstrated no significant reduction in the time of paw licking in the 1st phase, regarding the control group (Figure 2). However, according to the result shown in Figure 3, in the 2nd phase of the test, the animals treated with doses of 100, 200 and 300 mg/kg p.o. of MCE decreased significantly (p < 0.001) the paw licking time, as compared to the control group. Morphine (10 mg/kg, i.p.) reduced the time of licking in both phases. Knowing that drugs which act at central level, such as the analgesic opioids, inhibit both phases of the formalin test; however, drugs of peripheral action like anti-inflammatory are only effective in the second phase. These results indicate a possible peripheral analgesic effect by inhibiting the release of chemical mediators (Adelayemi et al., 2004; Bastos et al., 2006; Ferreira et al., 2006).

**Effect of the MCE on the glutamate test**

The glutamate test was used as evidence for the interaction of the MCE with the glutamate test. The glutamate promotes the activation of NMDA receptors, causing an increased influx of calcium with the activation of neuronal NO synthase and nitric oxide formation. Thus, glutamate participates in the processes involved in the perception of and central sensitization to pain (Bleakman et al., 2006; Beirith et al., 2002).

From the results presented in Figure 4, it was observed that the extract of *C. umbellifera* reduce paw licking when compared with the control group, which means that the MCE could inhibit directly the glutamate action through
the antagonism of its receptors or inhibit the liberation of other inflammatory mediators, like the nitric oxide.

**Determination of phenolic content**

Phenolic compounds are a group of natural substances found in vegetable and are present in roots, leaves, flowers and fruits of many plants (Gilaberte and González, 2010). As examples of secondary metabolites of this group, there are the simple phenols, coumarins, tannins, lignins, naphthoquinones and flavonoids (Cunha, 2009). They exhibit antioxidant properties, anti-inflammatory and immunomodulatory. Studies have demonstrated the effectiveness of natural polyphenols in combating inflammation, oxidative stress, DNA damage and suppression of the immune response induced by UV radiation. These protective effects contribute to anti photo carcinogenic action (Ferdinando et al., 2014; Gregoris et al., 2011).

Plant extracts that are rich in phenolic compounds demonstrate its action in scavenging free radicals. In addition, the plant extracts may also have biological properties such as UV protection, skin hydration, restoring the barrier function and the stimulation of collagen synthesis, which increases the skin’s defenses, but also favors the recovery of the same (Mercurio et al., 2015; Ferdinando et al., 2014). Thus, admitting the importance of phenolic compounds, their concentration was measured, using the method of Folin-Ciocalteu. To perform their quantification, the linear regression equation and the r² of the standard (gallic acid) was obtained and the results are demonstrated in Table 1.

Through the analyzed data in Table 1, it was observed that there was no differences among test days in the 0.05 level of significance being reproducible in the method used in the concentration of 450 µg/ml. It was also observed through this data, that HBF of *C. umbellifera* presents a higher concentration of phenolic compounds (332.1 ± 7.1 mg EGA/g of sample), when compared with MCE (264.21 ± 6.02 mg EGA/g of sample).

**Determination of flavonoid content**

Flavonoids can count on a variable number of hydroxyl groups in its structure and protect the body against damage caused by oxidants, including UV radiation, environmental pollution, and substances in some foods (Gilaberte and González, 2010; Martínez-Flórez et al., 2002). They act as antioxidants, acting in reducing inflammation, and against the harmful effects on the DNA generated by ultraviolet radiation (Nichols and Katiyar, 2010). Because of that, to perform the quantification of the phenolic content of the tested sample, spectrophotometric method was used with aluminum chloride. The linear regression equation and the r² of the standard, quercetin were made for later interpolation of the data and to calculate the flavonoid content in µg quercetin/mg of sample (Table 2).

As shown in Table 2, it was observed that there was no difference among test days in the 0.05 significance level, and the method is reproducible in the used concentration (1 mg/ml). It was also possible to observe that the extract presents a higher concentration of flavonoids (1.03 ± 0.08 µg querc/mg of sample), as compared to HBF (0.41 ± 0.05).

**Antioxidant assay in vitro: DPPH method**

Free radicals are physiologic formed in the organism as part of its respiration or metabolism processes and this even includes processes as phagocytosis, prostaglandin synthesis and cytochrome P<sub>450</sub> (Tegeli et al., 2014). Although, the presence of many antioxidant mechanisms in the body to neutralize those reactive species when there is an overproduction of them; sometimes these mechanisms are not enough to eliminate them from the body. This overproduction leading to an imbalance between the production and elimination of free radicals, which is called oxidative stress can cause disturbs in many physiological processes (Cervellati et al., 2014; Kumar, 2011; Lushchak, 2014; Sies, 2015). Because of that, many studies have shown that consuming food rich in antioxidants is very important to reduce the damages caused for reactive species (Rezaire et al., 2014), and many studies with natural products have shown various compounds with antioxidant activity (Almeida et al., 2016), such as phenolic compounds like flavonoids, phenolic acids and anthocyanins (Broinizi et al., 2007).

The DPPH method was the chosen method because it is a simple, fast and sensible technic based on the
Table 1. Total phenolic content in mg EAG/g of sample per day (n = 3) and mean of samples (n = 9) at a concentration of 450 µg/ml. Data are expressed as means ± standard deviation.

<table>
<thead>
<tr>
<th>Phenolic content (mg EGA / g)</th>
<th>Sample</th>
<th>1st day</th>
<th>2nd day</th>
<th>3rd day</th>
<th>Mean</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>MCE</td>
<td>266.26 ± 4.23</td>
<td>267.41 ± 7.67</td>
<td>258.96 ± 2.35</td>
<td>264.21 ± 6.02</td>
<td>0.18</td>
<td></td>
</tr>
<tr>
<td>HBF</td>
<td>334.29 ± 6.71</td>
<td>326.32 ± 5.24</td>
<td>335.70 ± 7.21</td>
<td>332.1 ± 7.1</td>
<td>0.24</td>
<td></td>
</tr>
</tbody>
</table>

Table 2. Content of total flavonoids (µg querc/mg of sample) of the MCE daily (n = 3) and means of the samples (n = 9) at a concentration of 1 mg/ml.

<table>
<thead>
<tr>
<th>Flavonoid content (µg querc / mg of sample)</th>
<th>Sample</th>
<th>1st day</th>
<th>2nd day</th>
<th>3rd day</th>
<th>Mean</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>MCE</td>
<td>1.04 ± 0.03</td>
<td>1.04 ± 0.13</td>
<td>1.01 ± 0.09</td>
<td>1.03 ± 0.08</td>
<td>0.9</td>
<td></td>
</tr>
<tr>
<td>FHB</td>
<td>0.41 ± 0.07</td>
<td>0.41 ± 0.02</td>
<td>0.4 ± 0.06</td>
<td>0.41 ± 0.05</td>
<td>0.98</td>
<td></td>
</tr>
</tbody>
</table>

Data are expressed as means ± standard deviation, in triplicate.

Table 3. Means of EC₅₀ ± SEM of sample analyzed by the DPPH method (n = 3).

<table>
<thead>
<tr>
<th>Mean of EC₅₀ ± SEM (µg/mL)</th>
<th>Sample</th>
<th>1st day</th>
<th>2nd day</th>
<th>3rd day</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>MCE</td>
<td>17.15 ± 2.11</td>
<td>17.83 ± 1.02</td>
<td>17.95 ± 0.90</td>
<td>0.14</td>
<td></td>
</tr>
<tr>
<td>FHB</td>
<td>21.50 ± 0.19</td>
<td>21.78 ± 0.88</td>
<td>21.14 ± 1.01</td>
<td>0.63</td>
<td></td>
</tr>
</tbody>
</table>

Figure 5. Means of EC₅₀ ± SEM of sample for the DPPH radical sequestration test using AA as standard (n = 9).

reduction of this organic radical that allows identifying the antioxidant activity. Through analyzed data presented in Table 3, it was observed that there is no differences among the test days in the 0.05 significance level.

According the results presented in Figure 5, it was possible to observe that the extract of *C. umbellifera* showed the lowest EC₅₀ (17.64 ± 0.43), presenting the optimum antioxidant activity as compared to hydrobutanolic fraction (EC₅₀ 21.47 ± 0.32), though none of them have been effective as the standard (EC₅₀ = 9.39 ± 0.05).

Despite this fact, both samples presented important antioxidant activity, mainly when they are compared with several other studies where the samples showed EC₅₀ > 50 µg/ml. Through all the analyzed data, it was also observed that HBF shows the highest phenolic content, but the antioxidant activity comes from MCE; this fact can be related to the extract presenting the highest flavonoid content, which is one of the most important compounds with this property (Sá et al., 2012; Phang et al., 2011; Degáspari and Waszczynskyj, 2004; Pietta, 2000).

Conclusion

*Calliandra umbellifera* has an antiinociceptive activity, a good antioxidant activity and high levels of phenolic compounds, which confirms the popular use of plants of *Calliandra*, contributing to the scientific knowledge of the species.
Conflict of interests

The authors have not declared any conflicts of interests.

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UFPB and National Council for Scientific and Technological Development – CNPQ.


**Full Length Research Paper**

**Effect of hydroethanolic extract from *Calophyllum brasiliense* Cambess on streptozotocin induced diabetic rats**

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*Calophyllum brasiliense* (Cb) belongs to the Clusiaceae family and it is generally used for diabetes treatment. The aim of this study was to evaluate the anti-diabetic effect of Cb’s hydroethanolic extract (CBE) on diabetic induced rats by streptozotocin and evaluate the content of polyphenols and tannins of the extract. The polyphenols and tannins evaluation in the extract were determined by a spectrophotometer in 760 nm and isocratic HPLC system and reverse phase column (C18). The induction of diabetes was performed by intraperitoneal injection of streptozotocin (55 mg/kg) and was confirmed by a histopathological analysis. The total content of polyphenols and tannins (gallic acid) in CBE was 0.025 ± 0.0028 mg/mL (4.77%) and 8.262 ± 0.417 µg/mL, respectively. According to the oral glucose tolerance test that was performed both in normal and diabetic mice, the treatment with 500 mg/kg of CBE appeared to significantly reduce the blood glucose levels compared to the untreated group (P < 0.001). The treatment of diabetic mice with 500 mg/kg of CBE for 30 days significantly improved diabetes clinical symptoms (polydipsia, polyuria, polyphagia and weight loss) (P < 0.001). After the urinary glucose analysis, it was found that the treatment with 500 mg/kg of CBE significantly decreased the urinary glucose levels at an average of 177.55 ± 17.8 mg/dL (32.40%) (P < 0.001). In relation to the blood glucose measurements, it was shown that the groups treated with 500 mg/kg CBE and 3 mg/kg of Gilbenclamide had significantly lower levels of blood glucose when compared to non-treated group (P < 0.001) 24.09% (143.36 ± 19.6 mg/dL) and 36.04% (200.08 ± 14.9 mg/dL), respectively. The histopathological analysis revealed an increase in the number of endocrine cells in the islets of Langerhans in the groups treated with CBE500 and insulin. Therefore, it was concluded that the treatment with 500 mg/kg of CBE exhibited anti-diabetic activity.

**Key words:** *Calophyllum brasiliense*, polyphenols, diabetes, clinical parameters.
INTRODUCTION

Diabetes mellitus (DM) is a syndrome that interferes directly in the metabolism of carbohydrates, fats and proteins, due to deficiency in the insulin production by the β cells of the pancreas or reduced tissues’ sensitivity to insulin. The DM can be confirmed by symptoms such as hyperglycemia, glycosuria, polyuria, polyphagia, and polydipsia. The chronic hyperglycemia present in DM patients could result in microvascular complications, predominantly retinopathy, nephropathy and neuropathy, but also macrovascular complications, such as stroke and coronary disease. These complications make DM the seventh cause of death in developed countries (Sacks et al., 2002).

During the last decades, several studies about new hypoglycemic agents have been conducted, with special focus on well-known medicinal plants. A good example is the plant *Galega officinalis*, which led to the development of the oral hypoglycemic drug "Metformin" (Noel et al., 1997).

*Calophyllum brasiliense* Camb. (Cb) species belongs to Clusiaceae family, and can be spontaneously found in Latin America, and mainly in the Amazon and Atlantic Forest. Cb is known by several common names, including “jacareúba”, “guanandi”, “guanandi-carvalho”, “cedro-do-pântano”, and “landim”, among others (Pereira, 1966).

According to phytochemical analyses, Cb contains a variety of substances with biological activities, such as xanthines, triterpenes, benzofurans, polyphenols (coumarins, flavonoids and tannins); at the same time, no presence of alkaloids and quinones was found (Sartori et al., 1999; Carvalho et al., 2013).

A previous study showed that many plant species are used as in alternative therapies for DM control in Brazil, and the Cb is among them (Silva et al., 2015). Cb is also used for the treatment of other diseases, such as bronchitis, liver and gastrointestinal disorders, pain, inflammation, hypertension and rheumatism (Silva et al., 2001).

Pharmacological studies about Cb revealed that it possesses antiretroviral activity (Huerta-Reyes et al., 2004), antiparasitic (against *Trypanosoma cruzi*, the etiological agent of Chagas disease) (Abe et al., 2004), antimicrobial (Cottiglia et al., 2004), gastroprotective and cytoprotective (Sartori et al., 1999) and anti-neoplastic (Ito et al., 2003) properties.

Many studies have already proven the pharmacological activities attributed to Cb, and have identified a wide variety of chemical compounds present in this plant. The objective of this study was to evaluate the anti-diabetic effect in diabetic streptozotocin-induced rats and to determine the total content of polyphenols and tannins.

MATERIALS AND METHODS

Plant material obtainment

Plant material (bark of the stem) of Cb species was collected in Ferreira Gomes City, in the state of Amapá, Brazil. The fertile material was identified in the Herbarium of the Institute of Studies and Research of the State of Amapá - IEPA, Brazil, with 0598AP as voucher specimen number.

Hydroethanolic extract obtainment (CBE)

To obtain the CBE, 2 kg of crushed and milled barks were subjected to a dilution in a percolator (LM20) with 70% hydroethanolic solution at a temperature of 45°C during 4 days in ratio of 1:8 (w/v). The extracted solution was filtered through filter paper and concentrated on rotaevaporator model Q.218.2 (Quimus Ltda., São Paulo, Brazil) at a temperature of 40°C until complete evaporation of the solvent, obtaining a yield of 32%. Finally, it was lyophilized to complete elimination of water, with final yield of 6.95%.

Quantitative analysis of total polyphenols by spectrophotometry and of gallic acid by HPLC

Total polyphenols analysis was performed using 0.750g of lyophilized extract diluted in 250 mL of distilled water and heated for 30 min at 60°C in a water bath. 5 mL of this solution were transferred to a 50 mL volumetric flask, and then 2 mL of acid phosphomolybdotungstic reagent (Sigma Co., São Paulo, Brazil) were added and the volume was completed with sodium carbonate solution of 15% (Sigma Co., São Paulo, Brazil). Later, the absorbance was measured in a 760 nm UV-VIS spectrophotometer (Shimadzu, UVmini-1240 model), according to the methodology described by Carvalho et al. (2013). For the HPLC chromatographic analysis, 10 mg of CBE were weighed and added to 10 mL of methanol:water solution (2:8 v/v); then, filtered through membrane filter with a pore size of 0.45 micrometers (Millipore®) and analyzed by high-performance-HPLC (Shimadzu Corporation) equipped with auto injector, diode array detector scanned from 190 to 500 nm. Chromatographic conditions were: Chromatograms obtained at 350 nm, oven with temperature kept at 30°C, reverse phase column (C18), Shim-pack VP-ODS (150 x 4.6 mm; 5 µm), injection volume of 10 µL using methanol as phase A: water acidified with 0.05% of acetic acid (70:30) and acetonitrile as phase B, in proportions isocratic system of 70% phase A with flow rate of 1 mL/min. The quantification of gallic acid (Sigma Co., São Paulo, Brazil) was accomplished by constructing standard curve with concentrations from 2 to 16 mg/mL.
Animals

Male Wistar rats were used, with an average weight of 210 ± 50 g. During the experiment, animals were placed into individual metabolic cages of stainless steel (60×50×22 cm), in an air-conditioned environment with constant temperature of 25 ± 3°C and humidity of 50 ± 10%. They were subjected to a light regime of 12-hour light / dark, water was offered ad libitum and they were fed with standard rodent chow. The project was approved by the Ethics Commission of the Federal University of Amapá, Amapá, Brazil, under Protocol Number 02A/2014.

Oral glucose tolerance test (OGTT)

To determine glucose tolerance, hyperglycemia was induced in normoglycemic and diabetic rats mice after 16 h of fasting, by the administration (VO) of a glucose solution at a dose of 4 g/kg of body weight. Glucose levels were measured after 0, 30, 60, 90, 120 and 180 min. The animals were randomly divided into 5 groups (n = 5/group). Non-treated group (NDT), group treated (VO) with 3 mg/kg of glibenclamide (GBC), group treated (VO) with 250 mg/kg of CBE (CBE500), group treated (VO) with 500 mg/kg of CBE (CBE500) and the group treated (subcutaneous injection) with 14 IU/kg of NPH insulin (INS).

Diabetes mellitus induction

The DM induction in animals was performed after a 16 h fasting period by peritoneal injection of streptozotocin (Sigma-Aldrich Inc., St. Louis, MO, USA), dissolved in sodium citrate buffer 0.01 M (pH 4.5), with a dose of 55 mg/kg of body weight. 4 days after the injection of streptozotocin, animals showed blood glucose levels greater than 300 mg/dL urine glucose concentration higher than 250 mg/dL, polydipsia and polyuria were considered as diabetic animals (Carvalho et al., 2016).

Development and experimental evaluation

Diabetic animals were maintained in metabolic cages throughout treatment (30 days), where body weight, water intake, feed intake and urine output were daily recorded. Glycemia and glycosuria were evaluated every 5 days, where the blood collection was performed by retro-orbital plexus and glucose levels were estimated by colorimetric glucose oxidase method (Glucox 500, Doles Reagentes and Equip. for Lab., Ltd., Goiânia GO, Brazil).

Histopathological evaluation

The animals were euthanized and the pancreas was removed; then, was inserted in a 10% buffered formaldehyde for 24 h and then dehydration in alcohol, clarification in xylene, impregnation and paraffin embedment at 60°C took place. The histological observations were performed in a semi-automatic rotary microtome model (CUT 5062; SLEE) in 5 µm sections after coloring with hematoxylin-eosin (H/E).

Statistical analysis

For polyphenols and tannins dosage, a linear regression test was used. Anti-diabetic activity was analysed by a variance analysis (ANOVA) followed by Tukey’s test. Results with P < 0.05 significance levels were considered as statistically significant. Statistical software used was GraphPad Instat and Prism (version 5.03).

RESULTS AND DISCUSSION

Polyphenols are chemically characterized by different classes of substances which have at least one aromatic ring with one or more hydroxyl substituents and are derived from the metabolism of shikimic acid and of phenylpropanoids. Polyphenols are widely distributed in the plant kingdom, and serve as essential secondary metabolites for the plant. They are formed during stress conditions, such as infection, injury, ultraviolet radiation with the intention to protect plant molecules (King and Young, 1999; Lee et al., 2005; Naczk and Shahidi, 2004; Swain and Hillis, 1959).

From the quantification method using reduced levels of phosphomolybdotungstic reagent, the linear equation of pyrogallic acid was obtained (y = 10.450x - 0.0118), enabling the determination of the total polyphenols content present in the hydroethanolic extract of Cb, which was 0.025 ± 0.0028 mg/mL (n = 3), corresponding to 4.77%.

As was shown by the HPLC analysis, the tannin retention time (gallic acid) in the Cb extract was 3.827 min. (Figure 1), and from the standard curve (Figure 2) the gallic acid content was equal to 8.262 ± 0.417 (n = 3) µg/mL.

In a study conducted by Carvalho et al. (2013), the Cb extract showed high levels of polyphenolic compounds, and these natural antioxidants have great potential for the treatment of DM, as several studies have proven the hypoglycemic activity attributed to these compounds (Hou et al., 2007; Jia et al., 2009; Panda and Kar, 2007). The oral glucose tolerance test (OGTT) is characterized as a clinical test used to assess the ability of pancreas’ β-cells to secrete insulin and to evaluate the tissue sensitivity to insulin. This allows to check the carbohydrate metabolism behaviour (Bhuiyan et al., 2011), as through this test it is possible to evaluate the ability of probable anti-diabetic drugs to reduce the postprandial blood glucose and to define the dose responsible for such activity.

After the implementation of the OGTT in normal rats (Figures 3), it was observed that blood glucose levels were not significantly reduced in the ECB250 compared to the NTD group, but ECB500 and GBC groups had significantly lower values (P < 0.001). According to these findings, we selected the dose of 500 mg/kg of CBE in the present study for diabetic rats (Figure 4), and it was observed that the treatment with 500 mg/kg of CBE (CBE500) significantly reduced blood glucose levels. At the end of the analysis time, blood glucose levels in CBE500 group were still quite high, as well as in the
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Figure 1. Chromatographic profile of the Cb extract and standard substance (gallic acid), obtained at 350 nm with a reversed phase C18 column (Shim-pack VP-ODS (150 × 4.6 mm; 5 µm), with retention time peak at 3.827 min.

Figure 2. Standard curve of gallic acid by HPLC, concentrations from 2 to 16 µg/mL. Linear equation: \( y = 39842x - 13364 \), correlation coefficient, \( R^2 = 0.9961 \).

Figure 3. Acute effect of CBE250, CBE500 and GBC on normal rats’s blood glucose levels. Where significance: *** \( P < 0.001 \) compared to non-treated group (NTD). Values express the mean ± S.D \((n = 5/group)\).

In a study by Panda and Kar (2007), the crude extract and fractions of *Gentiana olivieri* (Gentianaceae) and its isolated polyphenols reduced blood glucose in hyperglycemic, normal and diabetic rats as shown by the OGTT, findings that are similar with that obtained in this study for the Cb species.

As it is shown in Table 1, NTD group displayed the clinical characteristic of diabetes, such as increased water intake (polydipsia), increased excreted urine volume (polyuria), increased feed intake (polyphagia) and body weight reduction.

The increase in feed intake is due to the lack or the reduction of insulin release, since insulin stimulates the release of leptin from fat cells entering the central

group treated with glibenclamide with levels of 443 ± 13.5 and 343 ± 19.6 mg/dL, respectively. These high values can be justified by the fact that there are few functional β cells due to the deleterious effect of diabetes drugs induction.

Considering the results obtained in the OGTT with normal and diabetic rats, we can conclude that the CBE at the dose of 500 mg/kg had an acute hypoglycemic activity. Similar results were found in the study of Jia et al. (2009), where doses of 100, 200 and 300 mg/kg of *C. parthenoxygen* extract were offered and the highest dose showed the greatest hypoglycemic activity in the OGTT.

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nervous system, which may decrease food intake by affecting the actions of neuropeptide Y (NPY) of neurons in the arched nucleus of the hypothalamus (Cambraia, 2004; Halpern, 2002). With the reduction of insulin levels in diabetic as well as in the fasted rats, there was a reduction of leptin and therefore an increased feed intake. This data can be confirmed by results obtained from GBC and INS groups, where feed intake was reduced when compared with NTD group (P < 0.05).

Moreover, it was observed that the CBE500 and GBC groups had similar values for body weight; however, these values were significantly higher compared to the NTD group (P < 0.001). Finally, INS and NDC groups had the highest values for body weight (P < 0.001).

Weight reduction that occurs in individuals with DM is due to lack of insulin and absence of glucose uptake by cells; as a result, body seeks for new sources of energy, causing intense process of structural proteins catabolism and β-oxidation of fatty acids, which, therefore, reduce the body mass of the individual with untreated DM (Molina et al., 1989; Queiroz et al., 2009).

Water intake and urine excretion were higher in the NTD compared to the other groups (P < 0.001). GBC and CBE500 appeared to have decreased values and INS and NDC groups had the lowest levels of water intake and urine (P < 0.001).

Polydipsia present in diabetic animals is due to the blood hyperosmolarity, which makes the water to pass from intracellular to extracellular space in order to maintain the osmotic balance. The intracellular dehydration is recognized by osmoreceptors in brain that generate a response triggering intense thirst, characteristic of DM. Increased water consumption causes an osmotic imbalance in different cellular compartments, and to maintain the balance, the diabetic people excrete large amounts of urine (Jacobson, 1996; Lerco et al., 2003).

Glucose and amino acids are the most important

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**Figure 4.** Acute effect CBE500, GBC and INS on diabetic rats’ blood glucose levels. Significance: **P < 0.01, ***P < 0.001 (a) compared with the untreated group (NTD), (b) compared to the treated group and GBC (c) compared to all groups. Values express the mean ± S.D (n = 5/group).

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**Table 1.** Effect of CBE500, GBC and INS 30d treatment on body weight, feed intake, water intake and urine production

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Groups</th>
<th>NDC</th>
<th>NTD</th>
<th>GBC</th>
<th>INS</th>
<th>CBE500</th>
</tr>
</thead>
<tbody>
<tr>
<td>Weight (g)</td>
<td></td>
<td>292±9.2</td>
<td>241±7.1</td>
<td>259±5.4***</td>
<td>279±6.9***</td>
<td>257±6.9***</td>
</tr>
<tr>
<td>Feed (g)</td>
<td></td>
<td>33±4.4</td>
<td>44±4.7</td>
<td>34±5.4*</td>
<td>30±3.8**</td>
<td>37±6.3</td>
</tr>
<tr>
<td>Water (mL)</td>
<td></td>
<td>43±4.9</td>
<td>149±11.4</td>
<td>91±13.4***</td>
<td>47±7.5***</td>
<td>108±9.5***</td>
</tr>
<tr>
<td>Urine (mL)</td>
<td></td>
<td>10±3.5</td>
<td>118±6.8</td>
<td>72±5.5***</td>
<td>20±5.1***</td>
<td>58±7.2***</td>
</tr>
</tbody>
</table>

The data represent the mean ± standard deviation (n = 5/30 days), where **p < 0.01 and ***p < 0.001 represents statistically significant results compared with the untreated group diabetic (NTD).
substances which are absorbed specifically due a secondary active transport in the kidney’s proximal tubules, as happens for almost all substances that undergo an active reabsorption. There is a maximum level of reabsorption and substances that are not absorbed are excreted in the urine. In normal individuals the level of glucose in urine is very close to 0. Glucose levels exceeding the level of 220 mg/minute in urine, and 180 mg/dL in blood are indicative of a diabetic patient (Mather and Pollock, 2011).

According to the results obtained from the evaluation of glucose excretion levels in urine (Figure 5), the NTD group presented a large amount of glucose in the urine during the treatment, with a mean of 547.91 ± 23.9 mg/dL. The CBE500 group compared to the NTD group, showed a significant decrease in glucose excretion levels from the 15th day of treatment (p < 0.01) and at the end of treatment it was reduced by 32.40% (177.55 ± 17 8 mg/dL). The GBC group had decreased values from the 10th day of treatment (p < 0.001) and at the end of the treatment showed a reduction in urine glucose of 49.36% (270.48 mg/dL). The INS group showed significantly lower levels of urine glucose (p < 0.001) compared to all treatment groups and this reduction in excretion of glucose levels was approximately 94.32% (516.79 ± 28.6 mg/dL) in comparison to the NTD group.

The blood glucose levels evaluation (Figure 6) showed that the NTD group had high levels throughout the analysis period, with an average of 555.08 ± 16.90 mg/dL, and when this value is compared to the NDC group, there was an increase of 387.13 mg/dL. The CBE500 group showed statistical significance from the 10th day of treatment (P < 0.05), with progressive reductions in blood glucose levels, and at the end of the treatment, it was reduced by 24.09% (143.36 ± 19 6 mg/dL) when compared to NTD (P < 0.001). The GBC group also showed statistical significance (P < 0.001) from the 10th day of treatment when compared to NTD group and at the end of the treatment, it showed a reduction of 36.04% (200.08 ± 14.9 mg/dL) in glucose level. INS group had reduced levels of blood glucose since the beginning of treatment (P < 0.001). In due course, glucose levels approached quite to that of the NDC group, with an average of 180.28 ± 17.870 mg/dL, a reduction equivalent to 67.52% (374.80 mg/dL).

It has been discussed in the literature that a drug is considered effective as anti-diabetic when it can lower plasma glucose levels by at least 15% (Martha et al., 2000). According to this criterion, it can be concluded that treatment with CBE in STZ-induced diabetic rats was effective because it was able to reduce blood glucose levels by 24.09% and moderate the display of clinical symptoms resulting from diabetes, such as: Weight loss, polydipsia, polyuria and glycosuria.

Histopathological findings of the pancreas (Figure 7) show that the dose of STZ caused endocrine cells destruction in the islets of Langerhans compared to the NTD group and to the NDC group, but there were remaining cells that characterize this model as type 2 diabetes. Similar results were obtained by Silva et al. (2011), who observed partial destruction of pancreatic β-cells, and the STZ cytotoxicity in β-cells was proportional to the administered dose. The cytotoxicity effects of STZ on the pancreatic β-cells are due to its similarity with
Figure 6. Effect of CBE500, GBC and INS on the blood glucose levels of diabetic rats. Where significance: *P < 0.05, **P < 0.01, ***P < 0.001, and (a) compared with NTD group, (b) compared to the group and GBC, (c) compared to all groups treated diabetics. Values express the mean ± S.D (n = 5/group).

Figure 7. Histological photomicrographs showing pancreatic islets of Langerhans, 40x magnification, stained by the method (H/E). NDC, non-diabetic; NTD, untreated diabetic; CBE500, treated with extract; INS, insulin treated; GBC, treated glibenclamide.

The glucose molecule, allowing their intracellular passage via transporters GLUT 2 (Schnedl et al., 1994). The cellular action involves the production of reactive oxygen species (ROS) that promote alkylation of DNA chain, causing irreversible damage to the metabolism of β-cells, resulting in the depletion of nicotinamide adenine dinucleotide (Asplunk et al., 1984).

The groups treated with CBE500 and INS showed a slight increase in the number of cells in the islets of Langerhans compared to the NTD group. GBC group also showed an increase, but was less than that of CBE500 and INS groups. The increase of cells observed in the INS group is due to the reduction of GLUT-2 expression in β cells, and therefore the limited damage caused by STZ (Thulesen et al., 1997). The higher number of β-pancreatic cells evidenced by CBE500 group is possibly because of its antioxidant potential, that possibly reduces reactive oxygen species (ROS) produced by STZ, restoring the β-pancreatic cells from oxidative damage (Sezik et al., 2005).

This possible mechanism was observed in several studies, which reported the hypoglycemic activity of plant extracts that have high content of polyphenolic compounds, such as: Cinnamomum parthenoxylon (Jia
et al., 2009), Lactuca indica (Hou et al., 2007), Dimocarpus longan (Li et al., 2015), Bauhinia monandra (Alade et al., 2012) and Sphaeranthus indicus (Ramachandran et al., 2011).

Despite these assumptions, the actual mechanism involved in the anti-diabetic activity of the CBE cannot be clearly described, thus, more detailed studies are needed to clarify and confirm the mechanism of CBE action.

Medicinal plants have different mechanisms for the control of carbohydrate metabolism, and these mechanisms reduce hyperglycemia by acting in the restoration of the functions of beta-pancreatic cells. Some of them involve: The increased stimulation of insulin release, the elevated glucose uptake and utilization, the reduction of the oxidative damage caused by reactive oxygen species (ROS) in the beta-pancreatic cells, the increase in the number and sensibility of insulin receptor sites, the reduction of gluconeogenesis and gastrointestinal absorption of glucose and/or the release of glucagon (Pepato et al., 2003; Rocha et al., 2006). Authors propose some mechanisms of action for polyphenolic compounds, which include: Protection of pancreatic beta-cells from oxidative damage, increased secretion of insulin, increased sensitivity of peripheral tissues in response to insulin and reduced gastrointestinal absorption of glucose.

Conclusion

Based on the results obtained in this study, the hydroethanolic extract of the bark of C. brasiliense species was able to improve clinical and laboratory parameters caused by diabetes in induced diabetic mice by streptozotocin, and this effect may possibly be related to the high content of polyphenols. Therefore, it can be concluded that the extract of this species has anti-diabetic effect and can probably develop similar effect in humans. These results confirm the information observed and described in popular medicine.

Conflict of Interests

The authors have not declared any conflict of interests.

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