African Journal of Pharmacy and Pharmacology

Volume 10 Number 42, 15 November, 2016
ISSN 1996-0816

Academic Journals

ABOUT AJPP

The **African Journal of Pharmacy and Pharmacology (AJPP)** is published weekly (one volume per year) by Academic Journals.

African Journal of Pharmacy and Pharmacology (AJPP) is an open access journal that provides rapid publication (weekly) of articles in all areas of Pharmaceutical Science such as Pharmaceutical Microbiology, Pharmaceutical Raw Material Science, Formulations, Molecular modeling, Health sector Reforms, Drug Delivery, Pharmacokinetics and Pharmacodynamics, Pharmacognosy, Social and Administrative Pharmacy, Pharmaceutics and Pharmaceutical Microbiology, Herbal Medicines research, Pharmaceutical Raw Materials development/utilization, Novel drug delivery systems, Polymer/Cosmetic Science, Food/Drug Interaction, Herbal drugs evaluation, Physical Pharmaceutics, Medication management, Cosmetic Science, pharmaceuticals, pharmacology, pharmaceutical research etc. The Journal welcomes the submission of manuscripts that meet the general criteria of significance and scientific excellence. Papers will be published shortly after acceptance. All articles published in AJPP are peer-reviewed.

Contact Us

Editorial Office: ajpp@academicjournals.org

Help Desk: helpdesk@academicjournals.org

Website: http://www.academicjournals.org/journal/AJPP

Submit manuscript online http://ms.academicjournals.me/

Editorial Board

African Journal of Pharmacy and Pharmacology

Table of Contents: Volume 10 Number 42 15 November, 2016

ARTICLES

academicJournals

Vol. 10(42), pp. 883-891, 15 November, 2016
DOI: 10.5897/AJPP2016.4662
Article Number: 4FE928961651
ISSN 1996-0816

http://www.academicjournals.org/AJPP

African Journal of Pharmacy and Pharmacology

Review

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

Igor Victor Ferreira dos Santos[1,2], Jonatas Lobato Duarte[1], Caio Pinho Fernandes[1,3], Hady Keita[1,2], Jesus Rafael Rodríguez Amado[1,2], Josué Arturo Velázquez-Moyado[4], Andrés Navarrete[4] and José Carlos Tavares Carvalho[1,2]*

[1]Programa de Pós-graduação em Ciências Farmacêuticas, Departamento de Ciências Biológicas e da Saúde, Universidade Federal do Amapá, Brazil.
[2]Laboratório de Pesquisa em Fármacos, Curso de Farmácia, Departamento de Ciências Biológicas e da Saúde, Universidade Federal do Amapá, Brazil.
[3]Laboratório de Nanobiotecnologia Fitofarmacêutica, Curso de Farmácia, Departamento de Ciências Biológicas e da Saúde, Universidade Federal do Amapá, Brazil.
[4]Facultad de Química, Departamento de Farmacia, Universidad Nacional Autónoma de México, México D.F., México.

Received 20 August 2016, Accepted 20 September, 2016

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 unprecise. 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

*Corresponding author. E-mail: farmacos@unifap.br.

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 extrauterine 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

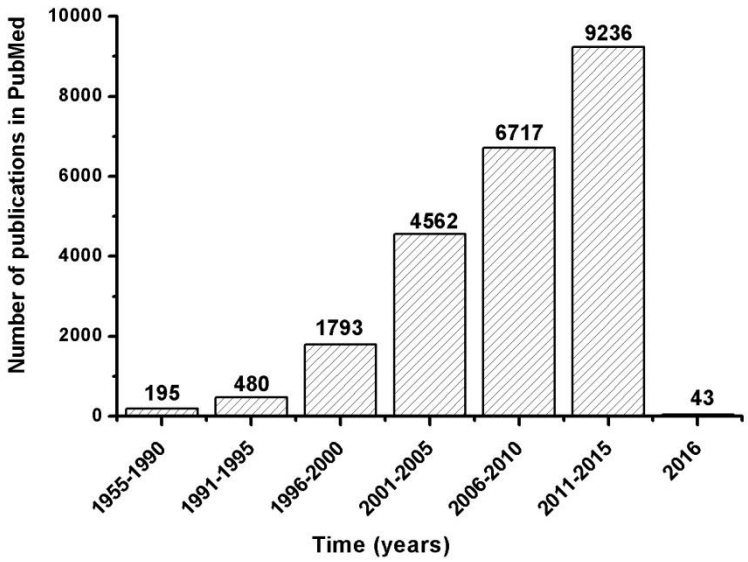

Figure 1. Number of citation in PUBMED for zebrafish as experimental model from 1955-2015. Only zebrafish keywords was used.

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

Activity assayed in zebrafish model	References
Angiogenic/anti-angiogenic	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
Antithrombotic activity	Song et al., 2012; Shi et al., 2015
anticonvulsant	Orellana-Paucar et al., 2012; Buenafe et al., 2013; Challal et al., 2014
Anti-inflammatory	Kao et al., 2010; Bohni et al., 2013; Wang et al., 2013
Antilipemic	Jin et al., 2011; Kim et al., 2012; Pardal et al., 2014; Littleton et al., 2014
Melanogenic	Kim et al., 2008; Ding et al., 2011; Park et al., 2013; Kim et al., 2014; Wang et al., 2014
Neurodegenerative diseases	Zhang 2012; Chong et al., 2013; Guo et al., 2014
Toxicity	Gertch et al., 2003; Kelleher et al., 2009; Wang et al., 2011; Fang et al., 2011; Zhang et al., 2014; Ding and Chen, 2012; Bernadi et al., 2013
Others studies	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

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 (Spitbergem 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; Biil et al., 2009; Doitsidou 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-SceI 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 multiwell 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 epimedii*, 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 (rhynchovisina, genistein, sophoroisoflavona A, licoisoflavona and 3-o-methylorobol). It was proven that genistein and sophoroisoflavone 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 (*Lourus 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 aquose 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 pigment 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 ethanol 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 miltiorrhizae*; 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 (Jevgenij et al., 2013). Furthermore, ZF has been very usefully 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

The authors thank CAPES (no. 3292/2013 AUXPE) and CNPq Proc. 402332/2013-0 for the financial support.

REFERENCES

Alex D, Lam IK, Lin Z, Lee SMY (2010). Indirubin shows anti-angiogenic activity in an in vivo zebrafish model and an *in vitro* HUVEC model. J. Ethnopharmacol. 131:242-247.

Amatruda JF, Shepard JL, Stern HM, Zon LI (2002). Zebrafish as a cancer model system. Cancer Cell. 1(3): 229-231.

Bailey J, Oliveri A, Levin ED (2013). Zebrafish model systems for developmental neurobehavioral toxicology. Birth Defects Res. Part C - Embryo Today Rev. 99(1):14-23.

Bernardi MM, Dias SG, Barbosa VE (2013). Neurotoxicity of neem commercial formulation (Azadirachta indica A. Juss) in adult zebrafish (Danio rerio). Environ. Toxicol. Pharmacol. 36: 1276-1282.

Bill BR, Petzold AM, Clark KJ, Schimmenti LA, Ekker SC (2009). A primer for morpholino use in zebrafish. Zebrafish 6: 69-77.

Bohni N, Cordero-Maldonado ML, Maes J, Siverio-Mota D, Marcourt L, Munck S, Kamuhabwa AR, Moshi MJ, Esguerra CV, de Witte PAM, Crawford AD, Wolfender JL (2013). Integration of Microfractionation, qNMR and Zebrafish Screening for the In Vivo Bioassay-Guided Isolation and Quantitative Bioactivity Analysis of Natural Products. PLoS One 8.

Broughton RE, Milam JE, Roe BA (2001). The complete sequence of the zebrafish (Danio rerio) mitochondrial genome and evolutionary patterns in vertebrate mitochondrial DNA. Genome Res. 11: 1958-1967.

Challal S, Buenafe OEM, Queiroz EF, Maljevic S, Marcourt L, Bock M, Kloeti W, Dayrit FM, Harvey AL, Lerche H, Esguerra CV, De Witte PAM, Wolfender JL, Crawford AD (2014). Zebrafish bioassay-guided microfractionation identifies anticonvulsant steroid glycosides from the Philippine medicinal plant Solanum torvum. ACS Chem. Neurosci. 5:993-1004.

Chong CM, Zhou ZY, Razmovski-Naumovski V, Cui GZ, Zhang LQ, Sa F, Hoi PM, Chan K, Lee SMY (2013). Danshensu protects against 6-hydroxydopamine-induced damage of PC12 cells in vitro and dopaminergic neurons in zebrafish. Neurosci. Lett. 543: 121-125.

Crawford AD, Bohni N, Maes J, Kamuhabwa A, Moshi M, Witte P, Wolfender J (2010). Zebrafish bioassay-guided microfractionation combined with CapNMR a comprehensive approach for the identification of anti-inflammatory and anti-angiogenic constituents of Rhynchosia viscosa. Planta Med. 2010: 76:544.

Crawford AD, Esguerra CV, De Witte PAM (2008). Fishing for drugs from nature: Zebrafish as a technology platform for natural product discovery, in: Planta Medica. pp. 624-632.

Crawford AD, Liekens S, Kamuhabwa AR, Maes J, Munck S, Busson R, Rozenski J, Esguerra C V, de Witte PAM (2011). Zebrafish bioassay-guided natural product discovery: Isolation of angiogenesis inhibitors from East African medicinal plants. PLoS One 6. http://dx.doi.org/10.1371/journal.pone.0014694

Cruz-Vega D, Verde-Star MJ, Salinas-Gonzalez NR, Rosales-Hernandez B, Estrada-Garcia I, Mendez-Aragon P, Carranza-Rosales P, Gonzalez-Garza M, Castro-Garza J (2009). Review of pharmacological effects of Glycyrrhiza radix and its bioactive compounds. Zhongguo Zhong Yao Za Zhi, 22: 557-559.

Da-Song Y, Qiu-Xia HE, Yong-Ping Y, Ke-Chun L, Xiao-Li L (2014). Chemical constituents of Euphorbia tibetica and their biological activities. Chin. J. Nat. Med. 12:38-42.

Del Valle-Mojica LM, Ortíz J (2012). Anxiolytic properties of Valeriana officinalis in the zebrafish: A possible role for metabotropic glutamate

receptors. Planta Med. 78:1719-1724.
Ding HY, Chang TS, Shen HC, Tai SSK (2011). Murine tyrosinase Inhibitors from Cynanchum bungei and evaluation of in vitro and in vivo depigmenting activity. Exp. Dermatol. 20: 720-724.
Ding YJ, Chen YH (2012). Developmental nephrotoxicity of aristolochic acid in a zebrafish model. Toxicol. Appl. Pharmacol. 261: 59-65.
Doitsidou M, Reichman-Fried M, Stebler J, Köprunner M, Dörries J, Meyer D, Esguerra CV, Leung T, Raz E (2002). Guidance of primordial germ cell migration by the chemokine SDF-1. Cell 111:647-659.
Duan J, Yu Y, Shi H, Tian L, Guo C, Huang P, Zhou X, Peng S, Sun Z (2013). Toxic Effects of Silica Nanoparticles on Zebrafish Embryos and Larvae. PLoS One 8. http://dx.doi.org/10.1371/journal.pone.0074606
Esguerra CV, Nelles L, Vermeire L, Ibrahimi A, Crawford AD, Derua R, Janssens E, Waelkens E, Carmeliet P, Collen D, Huylebroeck D (2007). Ttrap is an essential modulator of Smad3-dependent Nodal signaling during zebrafish gastrulation and left-right axis determination. Development 134: 4381-4393.
Ferri-Lagneau KF, Moshal KS, Grimes M, Zahora B, Lv L, Sang S, Leung T (2012). Ginger stimulates hematopoiesis via Bmp pathway in Zebrafish. PLoS One 7. http://dx.doi.org/10.1371/journal.pone.0039327
Grabher C, Wittbrodt J (2008). Recent advances in meganuclease and transposon-mediated transgénesis of medaka and zebrafish. Methods Mol. Biol. 461: 521-539.
Gebruers E, Cordero-Maldonado ML, Gray AI, Clements C, Harvey AL, Edrada-Ebel R, De Witte PAM, Crawford AD, Esguerra CV (2013). A phenotypic screen in zebrafish identifies a novel small-molecule inducer of ectopic tail formation suggestive of alterations in non-canonical Wnt/PCP signaling. PLoS One 8: 1–14.
Gertsch J, Niomawë Gertsch-Roost K, Sticher O (2004). Phyllanthus piscatorum, ethnopharmacological studies on a women's medicinal plant of the Yanomamï Amerindians. J. Ethnopharmacol. http://dx.doi.org/10.1016/j.jep.2003.12.022
Gertsch J, Thöni Tobler R, Brun R, Sticher O, Heilmann J (2003). Antifungal, antiprotozoal, cytotoxic and piscicidal properties of justicidin B and a new arylnaphthalide lignan from Phyllanthus piscatorum. Planta Med. 69:420-424.
Guo B, Xu D, Duan H, Du J, Zhang Z, Lee SM, Wang Y (2014). Therapeutic Effects of Multifunctional Tetramethylpyrazine Nitrone on Models of Parkinson's Disease in Vitro and in Vivo. Biol. Pharm. Bull. 37: 274-285.
Hammond CL, Moro E (2012). Using transgenic reporters to visualize bone and cartilage signaling during development in vivo. Front. Endocrinol. (Lausanne). 3. http://dx.doi.org/10.3389/fendo.2012.00091
Han L, Yuan Y, Zhao L, He Q, Li Y, Chen X, Liu X, Liu K (2012). Tracking antiangiogenic components from Glycyrrhiza uralensis Fisch. based on zebrafish assays using high-speed countercurrent chromatography. J. Sep. Sci. 35: 1167–1172.
He MF, Liu L, Ge W, Shaw PC, Jiang R, Wu LW, But PPH (2009) a. Antiangiogenic activity of Tripterygium wilfordii and its terpenoids. J. Ethnopharmacol. 121:61-68.
He ZH, He MF, Ma SC, But PPH (2009) b. Anti-angiogenic effects of rhubarb and its anthraquinone derivatives. J. Ethnopharmacol. 121: 313-317.
He ZH, Zhou R, He MF, Lau CBS, Yue GGL, Ge W, But PPH (2011). Anti-angiogenic effect and mechanism of rhein from Rhizoma Rhei. Phytomedicine 18: 470-478.
Heasman J (2002). Morpholino oligos: making sense of antisense? Dev. Biol. 243:209-214.
Higashijima S, Okamoto H, Ueno N, Hotta Y, Eguchi G (1997). High-frequency generation of transgenic zebrafish which reliably express GFP in whole muscles or the whole body by using promoters of zebrafish origin. Dev. Biol. 192:289-299.
Hill AJ, Teraoka H, Heideman W, Peterson RE (2005). Zebrafish as a model vertebrate for investigating chemical toxicity. Toxicol. Sci. 86(1): 6-19.
Holbech H, Schröder KD, Nielsen ML, Brande-Lavridsen N, Holbech BF, Bjerregaard P (2013). Estrogenic effect of the phytoestrogen biochanin A in zebrafish, Danio rerio, and brown trout, Salmo trutta. Aquat. Toxicol. 144-145, 19-25.
Howe K, Clark MD, Torroja CF, Torrance J, Berthelot C, Muffato M, Collins JE, Humphray S, McLaren K, Matthews L, McLaren S, Sealy I, Caccamo M, Churcher C, Scott C, Barrett JC, Koch R, Rauch GJ, White S, Chow W, Kilian B, Quintais LT, Guerra-Assunção JA, Zhou Y, Gu Y, Yen J, Vogel JH, Eyre T, Redmond S, Banerjee R, Chi J, Fu B, Langley E, Maguire SF, Laird GK, Lloyd D, Kenyon E, Donaldson S, Sehra H, Almeida-King J, Loveland J, Trevanion S, Jones M, Quail M, Willey D, Hunt A, Burton J, Sims S, McLay K, Plumb B, Davis J, Clee C, Oliver K, Clark R, Riddle C, Elliot D, Threadgold G, Harden G, Ware D, Begum S, Mortimore B, Kerry G, Heath P, Phillimore B, Tracey A, Corby N, Dunn M, Johnson C, Wood J, Clark S, Pelan S, Griffiths G, Smith M, Glithero R, Howden P, Barker N, Lloyd C, Stevens C, Harley J, Holt K, Panagiotidis G, Lovell J, Beasley H, Henderson C, Gordon D, Auger K, Wright D, Collins J, Raisen C, Dyer L, Leung K, Robertson L, Ambridge K, Leongamornlert D, McGuire S, Gilderthorp R, Griffiths C, Manthravadi D, Nichol S, Barker G, Whitehead S, Kay M, Brown J, Murnane C, Gray E, Humphries M, Sycamore N, Barker D, Saunders D, Wallis J, Babbage A, Hammond S, Mashreghi-Mohammadi M, Barr L, Martin S, Wray P, Ellington A, Matthews N, Ellwood M, Woodmansey R, Clark G, Cooper J, Tromans A, Grafham D, Skuce C, Pandian R, Andrews R, Harrison E, Kimberley A, Garnett J, Fosker N, Hall R, Garner P, Kelly D, Bird C, Palmer S, Gehring I, Berger A, Dooley CM, Ersan-Ürün Z, Eser C, Geiger H, Geisler M, Karotki L, Kirn A, Konantz J, Konantz M, Oberländer M, Rudolph-Geiger S, Teucke M, Lanz C, Raddatz G, Osoegawa K, Zhu B, Rapp A, Widaa S, Langford C, Yang F, Schuster SC, Carter NP, Harrow J, Ning Z, Herrero J, Searle SM, Enright A, Geisler R, Plasterk RH, Lee C, Westerfield M, de Jong PJ, Zon LI, Postlethwait JH, Nüsslein-Volhard C, Hubbard TJ, Roest Crollius H, Rogers J, Stemple DL (2013). The zebrafish reference genome sequence and its relationship to the human genome. Nature 496: 498–503.
Huang SY, Feng CW, Hung HC, Chakraborty C, Chen CH, Chen WF, Jean YH, Wang HMD, Sung CS, Sun YM, Wu CY, Liu W, Hsiao C. Der, Wen ZH (2014). A novel zebrafish model to provide mechanistic insights into the inflammatory events in carrageenan-induced abdominal edema. PLoS One 9. http://dx.doi.org/10.1371/journal.pone.0104414
Jin S, Cho KH (2011) a. Water extracts of cinnamon and clove exhibits potent inhibition of protein glycation and anti-atherosclerotic activity in vitro and in vivo hypolipidemic activity in zebrafish. Food Chem. Toxicol. 49: 1521–1529.
Jin S, Hong JH, Jung SH, Ch KH (2011) b. Turmeric and laurel aqueous extracts exhibit in vitro anti-atherosclerotic activity and in vivo hypolipidemic effects in a zebrafish model. J. Med. Food 14: 247-256.
Jones RW, Huffman MN (1957). Fish embryos as bio-assay material in testing chemicals for effects on cell division and differentiation. Trans. Am. Microsc. Soc. (1957): 177-183.
Jones RW, Stout MG, Reich H, Huffman MN (1964). Cytotoxic activities of certain flavonoids against zebrafish embyos. Cancer Chemoter. Rep. 34(1):19-20.
Kang MC, Kim SY, Kim YT, Kim EA, Lee SH, Ko SC, Wijesinghe WAJP, Samarakoon KW, Kim YS, Cho JH, Jang HS, Jeon YJ (2014). In vitro and in vivo antioxidant activities of polysaccharide purified from aloe vera (Aloe barbadensis) gel. Carbohydr. Polym. 99: 365-371.
Kang Y, Mallikarjuna PR, Fabian DA, Gorajana A, Lim CL, Tan EL (2013). Bioactive molecules : current trends in discovery , synthesis , delivery and testing. Int. e-Journal Sci. Med. Educ. 7: 32-46.
Kao TT, Tu HC, Chang WN, Chen BH, Shi YY, Chang TC, Fu TF (2010). Grape seed extract inhibits the growth and pathogenicity of Staphylococcus aureus by interfering with dihydrofolate reductase activity and folate-mediated one-carbon metabolism. Int. J. Food Microbiol. 141: 17-27.
Kawakami K, Takeda H, Kawakami N, Kobayashi M, Matsuda N, Mishina M (2004). A transposon-mediated gene trap approach identifies developmentally regulated genes in zebrafish. Dev. Cell 7:133-144.
Kim DJ, Seok SH, Baek M-WW, Lee H-YHKY, Na YR, Park SH, Dutta NK, Kawakami K, Park JH (2009). Developmental toxicity and brain aromatase induction by high genistein concentrations in zebrafish embryos. Toxicol. Mech. Methods 19:251-256.

Kim JH, Jeong SC, Hwang JS, Lee ES, Lee SM (2014). Modulation of melanin synthesis by rengyolone isolated from the root of eurya emarginata in melan-a cells. Phyther. Res. 28:940-945.
Kim JY, Hong JH, Jung HK, Jeong YS, Cho KH (2012). Grape skin and loquat leaf extracts and acai puree have potent anti-atherosclerotic and anti-diabetic activity in vitro and in vivo in hypercholesterolemic zebrafish. Int. J. Mol. Med. 30:606-614.
Kishi S, Bayliss PE, Uchiyama J, Koshimizu E, Qi J, Nanjappa P, Imamura S, Islam A, Neuberg D, Amsterdam A, Roberts TM (2008). The identification of zebrafish mutants showing alterations in senescence-associated biomarkers. PLoS Genet. 4. http://dx.doi.org/10.1371/journal.pgen.1000152
Koehn FE, Carter GT (2005). The evolving role of natural products in drug discovery. Nat. Rev. Drug Discov. 4, 0.
http://dx.doi.org/10.1038/nrd1657
Kovrižnych JA, Sotníková R, Zeljenková D, Rollerová E, Szabová E, Wimmerová S (2013). Acute toxicity of 31 different nanoparticles to zebrafish (Danio rerio) tested in adulthood and in early life stages - comparative study. Interdiscip. Toxicol. 6: 67–73.
Krill D, Madden J, Huncik K, Moeller PD (2010). Induced thyme product prevents VEGF-induced migration in human umbilical vein endothelial cells. Biochem. Biophys. Res. Commun. 403:275-281.
Lam HW, Lin HC, Lao SC, Gao JL, Hong SJ, Leong CW, Yue PYK, Kwan YW, Leung AYH, Wang YT, Lee SMY (2008). The angiogenic effects of Angelica sinensis extract on HUVEC in vitro and zebrafish in vivo. J. Cell. Biochem. 103:195-211.
Langheinrich U (2003). Zebrafish: A new model on the pharmaceutical catwalk. BioEssays. http://dx.doi.org/10.1002/bies.10326
Lee SH, Lee J, Jung MH, Lee YM (2013). Glyceollins, a novel class of soy phytoalexins, inhibit angiogenesis by blocking the VEGF and bFGF signaling pathways. Mol. Nutr. Food Res. 57:225-234.
Lele Z, Krone PH (1996). The zebrafish as a model system in developmental, toxicological and transgenic research. Biotechnol. Adv. 14:57-72.
Li JW-H, Vederas JC (2009). Drug discovery and natural products: end of an era or an endless frontier? Science 325:161-165.
Lin S, Lin S, Zhao Y, Nel AE (2013). Zebrafish: An in vivo model for nano EHS studies. Small. http://dx.doi.org/10.1002/smll.201202115
Littleton RM, Haworth KJ, Tang H, Setchell KDR, Nelson S, Hove JR (2013). Automated In Vivo Platform for the Discovery of Functional Food Treatments of Hypercholesterolemia. PLoS One 8. doi:10.1371/journal.pone.0052409
Liu CL, Cheng L, Kwok HF, Ko CH, Lau TW, Koon CM, Zhao M, Lau CP, Lau KM, Wong CW, Leung PC, Fung KP, Lau CBS (2011). Bioassay-guided isolation of norviburtinal from the root of Rehmannia glutinosa, exhibited angiogenesis effect in zebrafish embryo model. J. Ethnopharmacol. 137: 1323–1327.
Lu ZG, Li MH, Wang JS, Wei DD, Liu QW, Kong LY (2014). Developmental toxicity and neurotoxicity of two matrine-type alkaloids, matrine and sophocarpine, in zebrafish (Danio rerio) embryos/larvae. Reprod. Toxicol. 47:33-41.
McChesney JD, Venkataraman SK, Henri JT (2007). Plant natural products: Back to the future or into extinction? Phytochemistry 68: 2015-2022.
Miranda MM, Cuéllar A (2001). Pharmacognosia and natural product (In Spanish). Editorial Felix Varela. La Habana.
Muth-Köhne E, Sonnack L, Schlich K, Hischen F, Baumgartner W, Hund-Rinke K, Schäfers C, Fenske M (2013). The toxicity of silver nanoparticles to zebrafish embryos increases through sewage treatment processes. Ecotoxicology 22:1264-1277.
Orellana-Paucar AM, Serruys ASK, Afrikanova T, Maes J, De Borggraeve W, Alen J, León-Tamariz F, Wilches-Arizábala IM, Crawford, AD, de Witte PAM, Esguerra, CV (2012). Anticonvulsant activity of bisabolene sesquiterpenoids of Curcuma longa in zebrafish and mouse seizure models. Epilepsy Behav. 24:14-22.
Pardal D, Caro M, Tueros I, Barranco A, Navarro V (2014). Resveratrol and piceid metabolites and their fat-reduction effects in zebrafish larvae. Zebrafish 11: 32-40.
Park H, Song KH, Jung PM, Kim JE, Ro H, Kim MY, Ma JY (2013). Inhibitory effect of arctigenin from fructus arctii extract on melanin synthesis via repression of tyrosinase expression. Evidence-based Complement. Altern. Med. http://dx.doi.org/10.1155/2013/965312
Peterson RT, Macrae CA (2012). Systematic approaches to toxicology in the zebrafish. Annu. Rev. Pharmacol. Toxicol. 52:433-453.
Poon KL, Brand T (2013). The zebrafish model system in cardiovascular research: A tiny fish with mighty prospects. Glob. Cardiol. Sci. Pract. P 4.
Rao VS, Rao A, Karanth KS (2005). Anticonvulsant and neurotoxicity profile of Nardostachys jatamansi in rats. J. Ethnopharmacol. 102:351-356.
Rennekamp AJ, Peterson RT (2015). 15 years of zebrafish chemical screening. Curr. Opin. Chem. Biol. doi:10.1016/j.cbpa.2014.10.025
Scholz S, Fischer S, Gündel U, Küster E, Luckenbach T, Voelker D (2008). The zebrafish embryo model in environmental risk assessment—applications beyond acute toxicity testing. Environ. Sci. Pollut. Res. 15: 394-404.
Seth A, Stemple DL, Barroso I (2013). The emerging use of zebrafish to model metabolic disease. Dis. Model. Mech. 6: 1080-1088.
Shi YN, Shi YM, Yang L, Li XC, Zhao JH, Qu Y, Zhu HT, Wang D, Cheng RR, Yang CR, Xu M, Zhang YJ (2015). Lignans and aromatic glycosides from Piper wallichii and their antithrombotic activities. J. Ethnopharmacol. 162:87-96.
Song SJ, Li LZ, Gao PY, Yuan YQ, Wang RP, Liu KC, Peng Y (2012). Isolation of antithrombotic phenolic compounds from the leaves of crataegus pinnatifida. Planta Med. 78:1967-1971.
Spitsbergen JM, Kent ML (2007). The state of the art of the zebrafish model for toxicology and toxicologic pathology research--advantages and current limitations. Toxicol. Pathol. 31:62-87.
Stern HM, Zon LI (2003). Cancer genetics and drug discovery in the zebrafish. Nat. Rev. Cancer 3: 533–539.
Stewart, AM, Kalueff AV (2013). The behavioral effects of acute Δ 9 - tetrahydrocannabinol and heroin (diacetylmorphine) exposure in adult zebrafish. Brain Res. 1543:109-119.
Streisinger G, Walker C, Dower N, Knauber D, Singer F (1981). Production of clones of homozygous diploid zebrafish (Brachydanio rerio). Nature 291:293-296.
Thomas RJ (1975). The toxicologic and teratologic effects of ??9-tetrahydrocannabinol in the Zebrafish embryo. Toxicol. Appl. Pharmacol. 32:184-190.
Ulloa PE, Peña AA, Lizama CD, Araneda C, Iturra P, Neira R, Medrano JF (2013). Growth response and expression of muscle growth-related candidate genes in adult zebrafish fed plant and fishmeal protein-based diets. Zebrafish 10:99-109.
van der Greef J, McBurne RN (2005). Innovation: Rescuing drug discovery: in vivo systems pathology and systems pharmacology. Nat. Rev. Drug Discov. 4: 961-967.
Villefranc JA, Amigo J, Lawson ND (2007). Gateway compatible vectors for analysis of gene function in the zebrafish. Dev. Dyn. 236:3077-3087.
Wang S, Liu K, Wang X, He Q, Chen X (2011). Toxic effects of celastrol on embryonic development of zebrafish (Danio rerio). Drug Chem. Toxicol. 34: 61–65.
Wang TJ, An J, Chen XH, Deng QD, Yang L (2014). Assessment of Cuscuta chinensis seeds' effect on melanogenesis: Comparison of water and ethanol fractions in vitro and in vivo. J. Ethnopharmacol. 154: 240-248.
Wang YH, Wen CC, Yang ZS, Cheng CC, Tsai JN, K CC, Wu HJ, Chen YH (2009). Development of a whole-organism model to screen new compounds for sun protection. Mar. Biotechnol. 11: 419-429.
Wang YM, Xu M, Wang D, Yang CR, Zeng Y, Zhang YJ (2013). Anti-inflammatory compounds of "qin-Jiao", the roots of *Gentiana dahurica* (Gentianaceae). J. Ethnopharmacol. 147:341-348.
Wei Y, Li P, Wang C, Peng Y, Shu L, Jia X, Ma W, Wang B (2012). Metabolism of tanshinone IIA, cryptotanshinone and tanshinone i from radix salvia miltiorrhiza in zebrafish. Molecules 17: 8617-8632.
White RM, Sessa A, Burke C, Bowman T, LeBlanc J, Ceol C, Bourque C, Dovey M, Goessling W, Burns CE, Zon LI (2008). Transparent Adult Zebrafish as a Tool for In Vivo Transplantation Analysis. Cell Stem Cell 2:183-189.
Yang J, He S, Li S, Zhang R, Peng A, Chen L (2013). In vitro and in vivo antiangiogenic activity of caged polyprenylated xanthones isolated from garcinia hanburyi Hook. f. Molecules 18:15305-15313.
Yu SY, Lee IS, Jung SH, Lee YM, Lee YR, Kim JH, Sun H, Kim JS (2013). Caffeoylated phenylpropanoid glycosides from Brandisia

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.
Yue GGL, Chan BCL, Kwok HF, Wong YL, Leung HW, Ji CJ, Fung KP, Leung PC, Tan NH, Lau CBS (2013). Anti-angiogenesis and immunomodulatory activities of an anti-tumor sesquiterpene bigelovin isolated from Inula helianthus-aquatica. Eur. J. Med. Chem. 59: 243–252.
Yunes RA (2001). Plantas medicinais sob a ótica da química medicinal moderna: métodos de estudo, fitoterápicos e fitofármacos, biotecnologia, patente. Argos.
Zhang Y, Hu G, Li S, Li ZH, Lam CO, Hong SJ, Kwan YW, Chan SW, Leung GPH, Lee SMY (2012)a. Pro-angiogenic activity of astragaloside IV in HUVECs in vitro and zebrafish *in vivo*. Mol. Med. Rep. 5:805-811.
Zhang J, Wang ZM, Liu KC, He QX, Qi YD, Zhang BG, Liu HT, Xiao PG (2014). Chemical constituents of Kadsura oblongifolia and evaluation of their toxicity. Yao xue xue bao 49:1296-1303.
Zhong Z, Hoi P, Wu G, Xu Z, Tan W (2012) b. Anti-angiogenic effect of furanodiene on HUVECs in vitro and on zebrafish *in vivo*. J. Ethnopharmacol. 141: 721-727.
Zhou X, Siu W, Fung C, Cheng L (2014). Phytomedicine Pro-angiogenic effects of Carthami Flos whole extract in human microvascular endothelial cells in vitro and in zebrafish in vivo. Eur. J. Integr. Med. 21: 1256-1263.
Zhu W, Fu A, Hu J, Wang T, Luo Y, Peng M, Ma Y, Wei Y, Chen L (2011). 5-Formylhonokiol exerts anti-angiogenesis activity via inactivating the ERK signaling pathway. Exp. Mol. Med. 43:146-52.
Zon LI, Peterson RT (2005). In vivo drug discovery in the zebrafish. Nat Rev Drug Discov 4:35-44.

academicJournals

Vol. 10(42), pp. 892-899, 15 November, 2016
DOI: 10.5897/AJPP2016.4683
Article Number: DBDB39861653
ISSN 1996-0816

http://www.academicjournals.org/AJPP

African Journal of Pharmacy and Pharmacology

Full Length Research Paper

Antinociceptive and antioxidant activity of *Calliandra umbellifera* Benth

Tainá Souza Silva*, Juliana de Medeiros Gomes, Diogo Vilar da Fonsêca, Camila Holanda de Albuquerque, Maria de Fátima Agra, Marcelo Sobral da Silva, Reinaldo Nóbrega de Almeida and Josean Fechine Tavares

Universidade Federal da Paraíba, Pós Graduação em Produtos Naturais e Sintéticos Bioativos Laboratório Multiusuário de Caracterização e Análise, João Pessoa, Paraíba, Brazil.

Received 13 October, 2016: Accepted 10 November, 2016

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 EC_{50} and higher flavonoid content as compared to the hydrobutanolic 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

*Corresponding author. taina.farmacia@gmail.com. Tel: +55 83 32167175. Fax: +55 83 32167804.

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, antidiarrhetic, antispasmodic, antibacterial and antioxidant (Orishadipe et al., 2010; Agunu et al., 2005; Aguwa 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 37% (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 Paraiba 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 35°C 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 hidrobuthanolic 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 LD_{50} 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 mice. 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-plantar (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 ($AlCl_3$) 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; Woisky 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 solution (25, 50, 100, 150 and 200 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 violet 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:

$$SA(\%) = \frac{(A\ negative\ control - A\ sample)}{A\ negative\ control\ x\ 100}$$

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 EC_{50} (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 LD_{50} was determined using non-linear regression and the EC_{50} was determined using linear regression.

RESULTS AND DISCUSSION

Acute toxicity of the MCE

The determination of the acute toxicity and the lethal dose 50 (LD_{50}) 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 (Duarte 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.

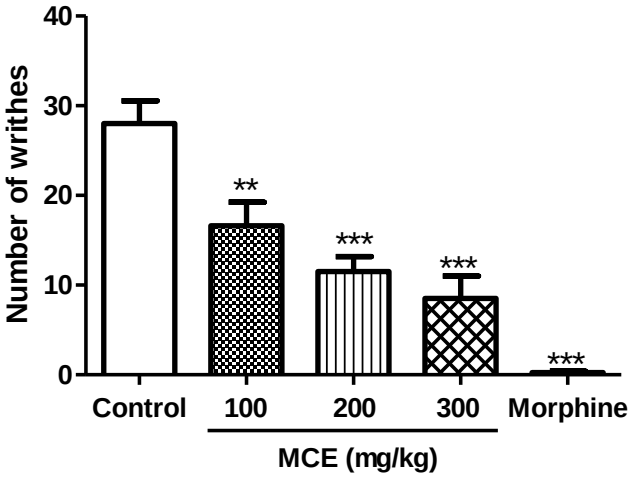

Figure 1. Effect of oral administration of methanolic extract of *C. umbellifera* (MCE) in the number of abdominal contortions induced by acetic acid in mice (n = 8 per group). **p<0.01, *** p< 0.001 versus the control group. The values are expressed as means ± S.E.M. (ANOVA - Dunett test).

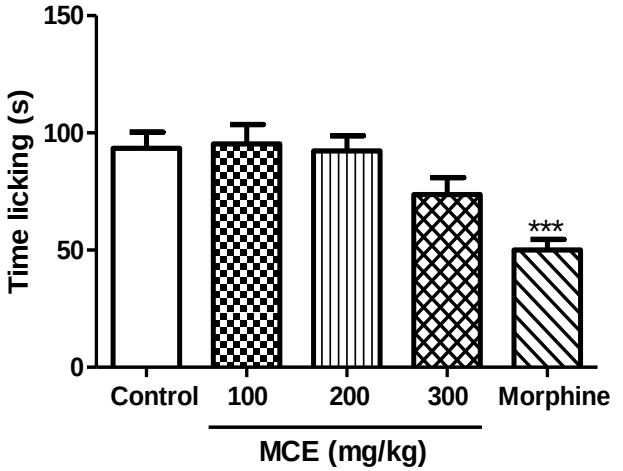

Figure 2. Effect of oral administration of methanolic extract of *C. umbellifera* (MCE) on the licking time in the first phase of the formalin test in mice (n = 8 per group). *** p <0.001 versus the control group. The values are expressed as means ± S.E.M. (ANOVA - Dunett test).

Effect of the MCE on the nociception induced by formalin

In order to better evaluate the activity of extract of *C. umbellifera*, the formalin test was used, which constitutes a valid and safe nociception model and sensitive to several classes of analgesic drugs. Formalin produces a distinct biphasic response where analgesic drugs may act differently in the first and second phases of the test (Morteza-Semnani et al., 2002).

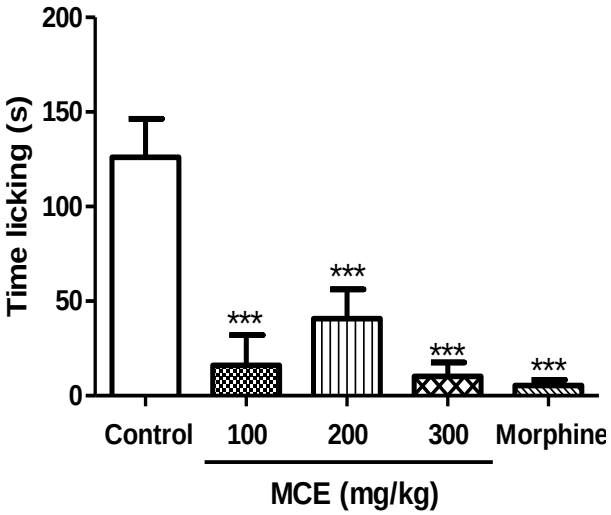

Figure 3. Effect of oral administration of methanolic extract of *C. umbellifera* (MCE) on the licking time in the second phase of the formalin test in mice (n = 8 per group). *** p < 0.001 versus the control group. The values are expressed as means ± S.E.M. (ANOVA - Dunett test).

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 (Adeyemi 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

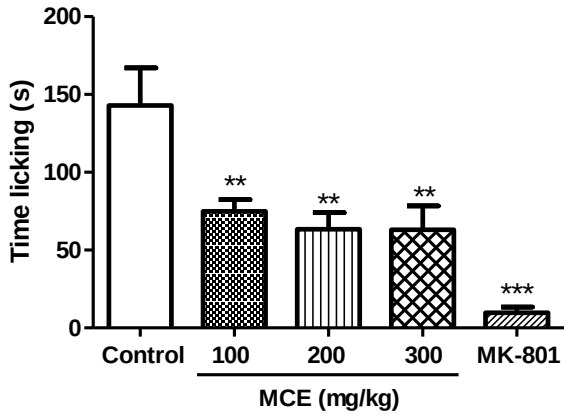

Figure 4. Effect of oral administration of methanolic extract of *C. umbellifera* (MCE) in the licking time in the glutamate test in mice (n = 8 per group) during the first 15 min. ** $p < 0.01$; ***$p<0.001$ versus the control group. The values are expressed as means ± S.E.M. (ANOVA - Dunett test).

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 r^2 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 r^2 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_{450} (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.

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

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.

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

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

Table 3. Means of EC_{50} ± SEM of sample analyzed by the DPPH method (n = 3).

Sample	**Mean of EC_{50} ± SEM (µg/mL)**			
	1st day	**2nd day**	**3rd day**	**P value**
MCE	17.15 ± 2.11	17.83 ± 1.02	17.95 ± 0.90	0.14
FHB	21.50 ± 0.19	21.78 ± 0.88	21.14 ± 1.01	0.63

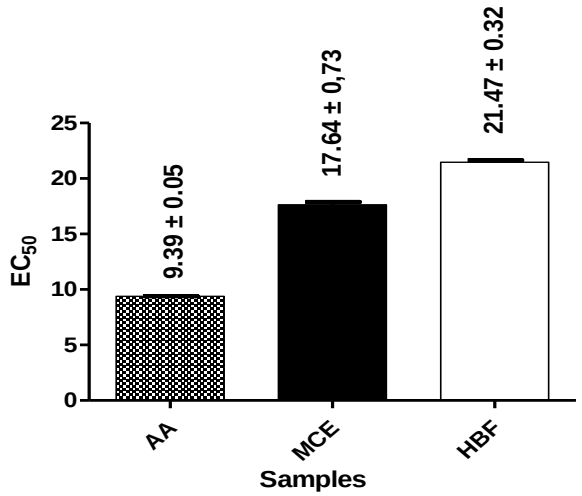

Figure 5. Means of EC_{50} ± SEM of sample for the DPPH radical sequestration test using AA as standard (n = 9).

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. umbellífera* showed the lowest EC_{50} (17.64 ± 0.43), presenting the optimum antioxidant activity as compared to hydro-butanolic fraction (EC_{50} 21.47 ± 0.32), though none of them have been effective as the standard (EC_{50} = 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 EC_{50} > 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 antinociceptive 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.

ACKNOWLEDGEMENTS

The authors express thanks to Federal University of Paraiba - UFPB and National Council for Scientific and Technological Development – CNPQ.

REFERENCES

Acuña UM, Jancovski N, Kennelly EJ (2009). Polyisoprenylated benzophenones from Clusiaceae: Potential drugs and lead. Curr. Top. Med. Chem. 9(16):1560-1580.

Adeyemi OO, Okpo SO, Okpaka O (2004). The analgesic effect of the methanolic extract of *Acanthus montanus*. J. Ethnopharmacol. 90(1):45-48.

Adesina GA (1976). Personal Communication. Chemistry Department, University of Ibadan, Ibadan Nigeria.

Agunu A, Abdurahman E, Shok M, Yusuf SA (2005). Analgesic activity of the roots and leaves extracts of *Calliandra portoricensis*. Fitoterapia 76(5):442-445.

Aguwa CN, Lawal AM (1988). Pharmacologic studies on the active principles of *Calliandra portoricensis* leaf extracts. J. Ethnopharmacol. 22(1):63-71.

Almeida MLB, Freitas WE, Morais PL, Sarmento JD, Alves RE (2016). Bioactive compounds and antioxidant potential fruit of *Ximenia americana* L. Food Chem. 192:1078-1082.

Asadbeigi M, Mohammadi T, Rafieian-Kopaei M, Saki K, Bahmani M, Delfan M (2014). Traditional effects of medicinal plants in the treatment of respiratory diseases and disorders: an ethnobotanical study in the Urmia. Asian Pac. J. Trop. Med. 7(Suppl. 1): S364-S368.

Barbosa AP, Da Silva BP, Parente JP (2008). Brevifoliasaponin with Adjuvant Activity from *Calliandra brevifolia*. Z. Naturforsch 63b:894-902.

Bastos GNT, Santos ARS, Ferreira VMM, Costa AMR, Bispo CI, Silveira AJA, Do Nascimento JLM (2006). Antinociceptive effect of the aqueous extract obtained from roots of *Physalis angulata* L. in mice. J. Ethnopharmacol. 103(2):241-245.

Beirith A, Santos ARS, Calixto JB (2002). Mechanisms underlying the nociception and paw edema caused by injection of glutamate into the mouse paw. Brain Res. 924(2):219-228.

Bleakman D, Alt A, Nisenbaum ES (2006). Glutamate receptors and pain. Semin Cell Dev. Biol. 17:592-604.

Boudet AM (2007). Evolution and current status of research in phenolic compounds. Phytochemistry 68(22-24):2722-2735.

Broizini PRB, Andrade-Wartha ERS, Oliveira AM, Silva AM, Novoa AJV, Torres RP, Azeredo HMC, Alves RE, Mancini-Filho J (2007). Avaliação da atividade antioxidante dos compostos fenólicos naturalmente presentes em subprodutos do pseudofruto de caju (Anacardium occidentale L.). Cienc. Tecnol. Aliment. 27(4):902-908.

Buzzi C, Franzoi FCL, Antonini G, Fracasso M, Cechinel Filho V, Yunes RA, Niero R (2009). Antinociceptive properties of caffeic acid derivatives in mice. Eur. J. Med. Chem. 44(11):4596-602.

Cavendish RL (2014). Atividade antinociceptiva do extrato hidroetanólico de própolis vermelha. Dissertação de Mestrado. Universidade Tiradentes – Aracaju.

Cervellati C, Romani A, Seripa D, Cremonini E, Bosi C, Magon S, Bergamini CM, Valacchi G, Pilotto A, Zuliani G (2014). Systemic oxidative stress and conversion to dementia of elderly patients with Mild Cognitive Impairment, BioMed. Res. Int. pp. 1-7.

Collier HOJ, Dinnen LC, Jonsnson CA, Schneider C (1968). The abdominal constriction response and its suppression by analgesic drugs in mouse. Br. J. Pharmacol. Chemother. 32(2):295-310.

Cunha WR, Silva MLA, Turatti ICC, Ferreira DS, Batarello HL (2003). Avaliação da atividade analgésica de Miconia ligustroides (Melastomataceae) utilizando o teste de contorção abdominal em camundongos. Rev. Bras. Farm. 84(2):47-49.

Degáspari CH, Waszczynskyj N (2004). Propriedades antioxidantes de compostos fenólicos. Visão Acadêmica 5(1):33-40.

Dimayuga RE, Espinoza JA, Garcia A, Delgado G, Molina-Salinas GM, Said-Fernandez S (2006). Two new cassane-type diterpenes from *Calliandra californica* with antituberculosis and cytotoxic activities. Planta Medica 72(8):757-761.

Duarte IDG, Nakamura M, Ferreira SH (1988). Participation of the sympathetic system in acetic acid-induced writhing in mice. Braz. J. Med. Biol. Res. 21:341-343.

Ferdinando MD, Brunettia C, Agatib G, Tattinic M (2014). Multiple functions of polyphenols in plants inhabiting unfavorable Mediterranean areas. Environ. Exp. Bot. 103:107-116.

Ferreira AA, Amaral FA, Duarte IDG, Oliveira PM, Alves RB, Silveira D, Azevedo AO, Raslan DS, Castro MAS (2006). Antinociceptive effect from *Ipomoea cairica* extract. J. Ethnopharmacol. 105:148-153.

Franzotti EM, Santos CVF, Rodrigues HMSL, Mourão RHV, Andrade MR, Antoniolli AR (2000). Anti-inflammatory, analgesic activity and acute toxicity of *Sida cordifolia* L. (Malva-branca). J. Ethnopharmacol. 72(1-2):273-277.

Garcez FR, Garcez WS, Hamerski L, Miguita CH (2009). Fenilpropanóides e outros constituintes bioativos de *Nectandra megapotamica***.** Quim. Nova 32(2):407-411.

Gilaberte Y, González S (2010). Novedades en photoprotection. Actas Dermo-Sifiliogr 1:659-672.

Gregoris E, Fabrisa S, Bertelle M, Grassato L, Stevanato R (2011). Propolis as potential cosmeceutical sunscreen agent for its combined photoprotective and antioxidant properties. Int. J. Pharm. 405:97-101.

Gulcin I, Sat IG, Beydemir S, Elmastas M, Kufrevioglu OI (2004). Comparison of antioxidant activity of clove (*Eugenia caryophylata Thunb*) buds and lavender (*Lavandula stoechas* L.). Food Chem. 87(3):393-400.

Hunskaar S, Hole K (1987). The formalin test in mice: dissociation between inflammatory and non-inflammatory pain. Pain 30: 103-104.

Koster R, Anderson M, DeBeer EJ (1959). Acetic acid for analgesic screening. Fed. Proc. 18:412-418.

Kumar S (2011). Free Radicals and Antioxidants: Human and Food System. Adv. Appl. Sci. Res. 2(1):129.

Litchfield JT, Wilcoxon FA (1949). A simplified method of evaluation dose-effect experiments. J. Pharmacol. Exp. Ther. 96:99-113.

Lewis G, Rico L (2005). Legumes of the World. In: Lewis G, Schride B, Mackinder B, Lock M. (Eds.). Royal Bot. Gardens, Kew, UK, P 577.

Lushchak VI (2014). Free radicals, reactive oxygen species, oxidative stress and its classification. Chem. Biol. Interact. 224:164-175.

Marques GS, Monteiro RPM, Leão WF, Lyra MAM, Peixoto MS, Rolim-Neto PJ, Xavier HS, Soares LAL (2012). Avaliação de procedimentos para quantificação espectrofotométrica de flavonoides totais em folhas de B*auhinia forficata* LINK**.** Quim. Nova 35(3):517-522.

Martínez-Flórez S, González-Gallego J, Culebras JM, Tuñón MJ (2002). Flavonoids: properties and anti-oxidizing action. Natr Hosp. 17(6):271-278.

Mattagajasingh I, Acharya L, Mukherjee AK, Panda PC, Das P (2006). Genetic relationships among nine cultivated taxa of *Calliandra* Benth. (Leguminosae: Mimosoidaee) using random amplified polymorphic DNA (RAPD) markers. Sci. Hortic. 110:98-103.

Mercurio DG, Wagemaker TAL, Alves VM, Benevenuto CG, Gaspar LR, Maia PMBG (2015). *In vivo* photoprotective effects of cosmetic formulations containing UV filters, vitamins, Ginkgo biloba and red algae extracts, J. Photoch. Photobio. B. 153:121-126.

Morteza-Semnani K, Saeedi M, Hamidian M, Vafamehr H, Dehpour AR (2002). Anti-inflammatory, analgesic activity and acute toxicity of *Glaucium grandiflorum* extract**.** J. Ethnopharmacol. 80:181-186.

Murillo BM, Sánchez A, Quevedo R, Pabón ML, Carulla JEF (2008). Rev. Colomb. Quim. 37(3):287-295.

Nichols JA, Katiyar SK (2010). Skin photoprotection by natural polyphenols: anti-inflammatory, antioxidant and DNA repair mechanisms. Arch. Dermatol. Res. 302:71-83.

Orishadipe A, Okogun J, Mishelia E (2010). Gas chromatography–mass spectrometry analysis of the hexane extract of *Calliandra portoricensis* and its antimicrobial activity. Afr. J. Pure. Appl. Chem. 4(7):131-134.

Petry RD, Souza KCB, Bassani VL, Petrovick PR, González-Ortega G (1998). Doseamento do teor de flavonoides totais em extratos hidroalcoólicos de *Passiflora alata* Dryander (maracujá). Rev. Bras. Farmacogn. 79:7-10.

Pietta PG (2000). Flavonoids as Antioxidants. J. Nat. Prod. 63:1035-1042.

Phang CW, Malek SNA, Ibrahim H, Wahab NA (2011). Antioxidant properties of crude and fractionated extracts of *Alpinia mutica* rhizomes and their total phenolic content. Afr. J. Pharm. Pharmacol. 5(7):842-852.

Rezaire A, Robinson JC, Bereau D, Verbaere A, Sommerer N, Khan MK, Durand P, Prost E, Fils-Lycaon B (2014). Amazonian palm *Oenocarpus bataua* ("patawa"): chemical and biological antioxidant activity--phytochemical composition. Food Chem. 149:62-70.

Rice-Evans CA, Miller NJ, Paganga G (1997). Antioxidant properties of phenolic compounds. Trends Plant Sci. 2:152-159.

Sá PGS, Guimarães AL, Oliveira AP, Filho JAS, Fontana AP, Damasceno PKF, Branco CRC, Branco A, Almeida JRGS (2012). Fenóis totais, flavonoides totais e atividade antioxidante de Selaginella convoluta (Arn.) Spring (Selaginellaceae). Rev. Cienc. Farm. Basica. Apl. 33(4):561-566.

Schmidt PC, Ortega GG (1983). Passionsblumenkraut. Bestimmung des Gesamtflavonoidgehaltes von Passiflorae herba. Deutsche Apotheker Zeitung, 133:17-26.

Sies H (2015). Oxidative stress: a concept in redox biology and medicine. Redox Biol. 4:180-183.

Souza RFV, Giovani WF (2005). Synthesis, spectral and electrochemical properties of Al (III) and Zn (II) complexes with flavonoids. Spectrochim Acta A 61:1985-1990.

Tjolsen A, Berge OG, Hunskaar S, Rosland JH, Hole K (1992). The formalin test: an evaluation of the method. Pain 51:5-17.

Tegeli V, Karpe P, Katve V (2014). Importance of free radical and antioxidant on human health. IJPCBS 4(4):1038-1050.

Woisky RG, Marcucci MC, Salatino A (1998). Uso de cloreto de alumínio na quantificação de flavonoides em amostras de própolis. Mensagem Doce 46:3-8.

Wojtunik-Kulesza KA, Oniszczuk A, Oniszczuk T, Waksmundzka-Hajnos M (2016). The influence of common free radicals and antioxidants on development of Alzheimer's Disease. Biomed Pharmacother. 78:39-49.

academicJournals

Vol. 10(42), pp. 900-908, 15 November, 2016
DOI: 10.5897/AJPP2016.4663
Article Number: 97840D161655
ISSN 1996-0816

http://www.academicjournals.org/AJPP

African Journal of Pharmacy and Pharmacology

Full Length Research Paper

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

Helison de Oliveira Carvalho[1], Grace Suzan Lopes Lacerda[1], Adriana Maciel Ferreira[1], Larissa Daniele Machado Góes[1], Beatriz Martins de Sá[1], Mona Saout[3], Didier Bereau[3], Jean-charles Robinson[3], Rafael Lima Resque[1], Caio Pinho Fernandes[1] and José Carlos Tavares Carvalho[1,2]*

[1]Laboratory of Research in Pharmaceuticals, Pharmacy Course, Department of Biological Sciences and Health, Federal University of Amapá, Rod. JK, KM 02, 68902-280, Macapá, Amapá, Brazil.
[2]Postgraduate Program in Pharmaceutical Sciences, Department of Biological Sciences and Health, Federal University of Amapá, Macapá, Amapá, Brazil.
[3]Université de la Guyane Francesa, UMR QUALITROP, Campus Universitaire de Troubiran, P. O. Box 792, 97337 Cayenne Cedex, French Guiana, France.

Received 23 August 2016; Accepted 20 September, 2016

***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 determinated 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 glucosemeasurements, it was shown that the groups treated with 500 mg/kg CBE and 3 mg/kg of Glibenclamide 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 (Millepore®) 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 × 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.

*Corresponding author. Email: farmacos@unifap.br.

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 (NTD), group treated (VO) with 3 mg/kg of glibenclamide (GBC), group treated (VO) with 250 mg/kg of CBE (CBE250), 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 0,01M (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 (Figure 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

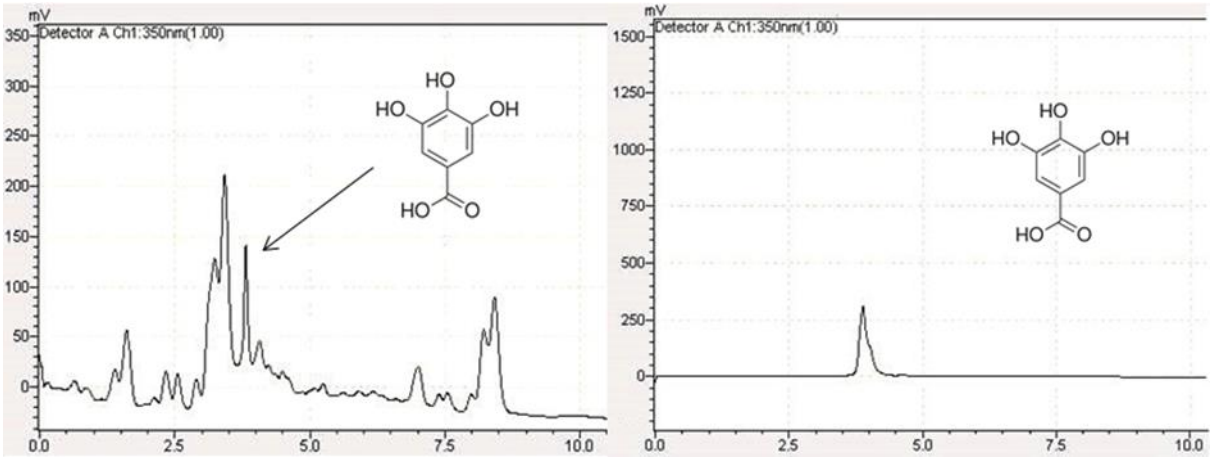

Figure 1. Chromatographic profile of the Cb extract and standard substance (gallic acid), obtained at 350 nm with a reversed phase C18 column (Shim-pack VP-ODS (150 × 4.6 mm; 5 µm), with retention time peak at 3.827 min.

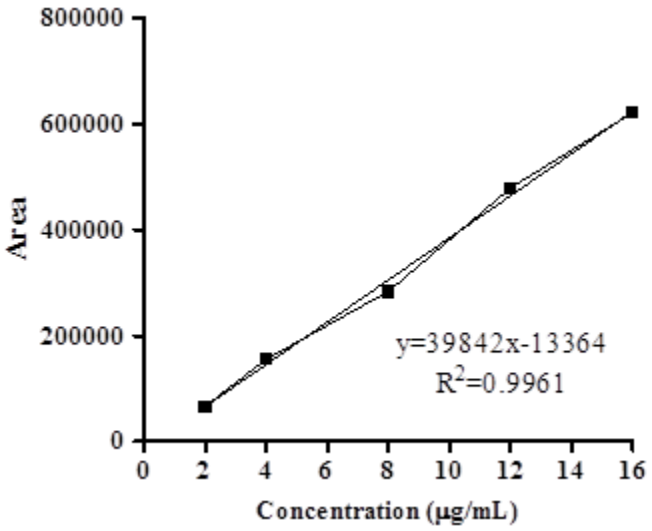

Figure 2. Standard curve of gallic acid by HPLC, concentrations from 2 to 16 µg/mL. Linear equation: y = 39842x-13364, correlation coefficient, R^2 = 0.9961.

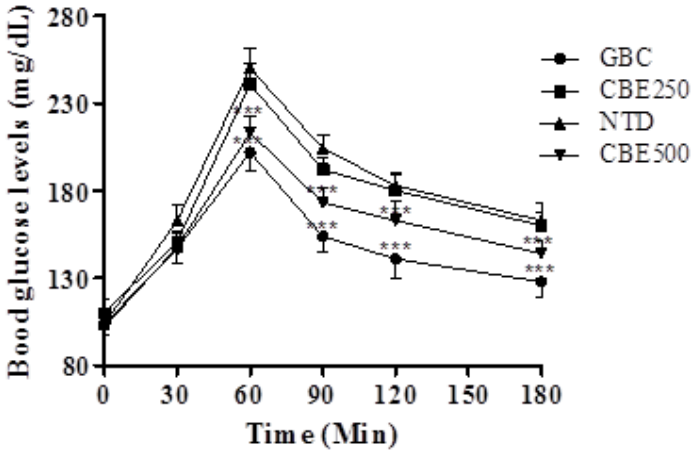

Figure 3. Acute effect of CBE250, CBE500 and GBC on normal rats's blood glucose levels. Where significance: *** P < 0.001 compared to non-treated group (NTD). Values express the mean ± S.D (n = 5/group).

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. parthenoxylon* extract were offered and the highest dose showed the greatest hypoglycemic activity in the OGTT

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

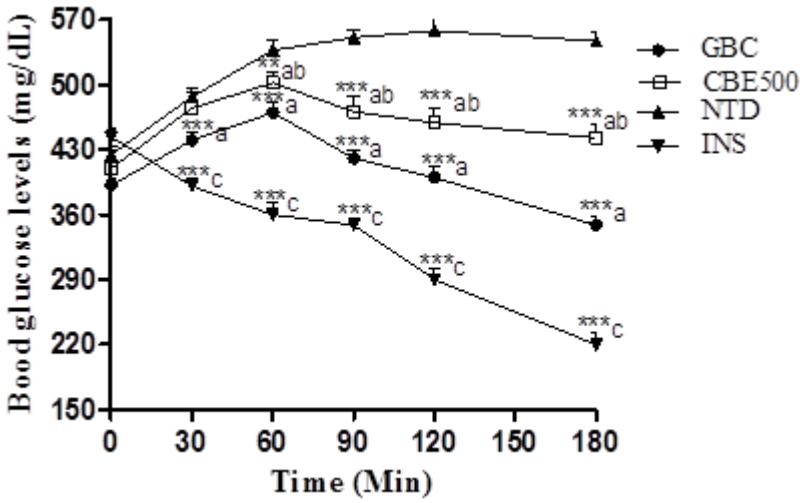

Figure 4. Acute effect CBE500, GBC and INS on diabetic rats' blood glucose levels. Significance: **P < 0.01, ***P < 0.001 (a) compared with the untreated group (NTD), (b) compared to the treated group and GBC (c) compared to all groups. Values express the mean ± S.D (n = 5/group).

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

Parameter	Groups				
	NDC	NTD	GBC	INS	CBE500
Weight (g)	292±9.2	241±7.1	259±4.5***	279±6.9***	257±6.9***
Feed (g)	33±4.4	44±4.7	34±5.4*	30±3.8**	37±6.3
Water (mL)	43±4.9	149±11.4	91±13.4***	47±7.5***	108±9.5***
Urine (mL)	10±3.5	118±6.8	72±5.5***	20±5.1***	58±7.2***

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).

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, characteristical 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

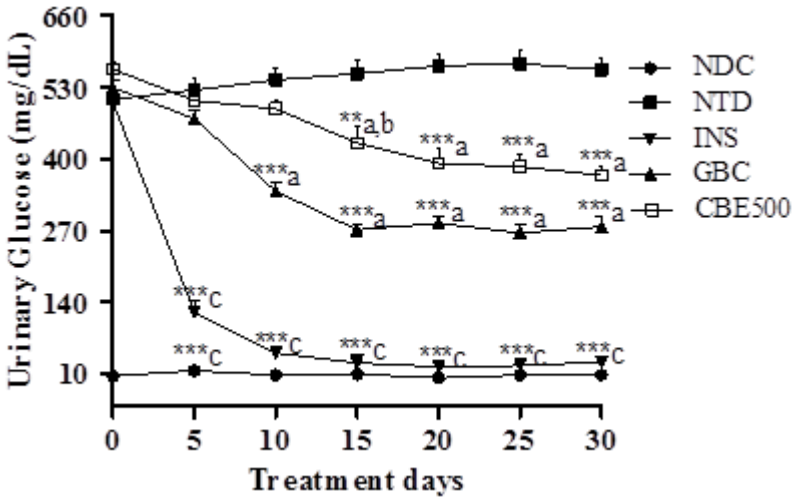

Figure 5. Effect of CBE500, GBC and INS on the urine glucose levels of diabetic rats. Where significance: ** P < 0.01 and *** P < 0.001 (a) compared with NTD group, (b) compared to the group and GBC, (c) compared to all groups treated diabetics. Values express the mean ± S.D (n = 5/group).

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

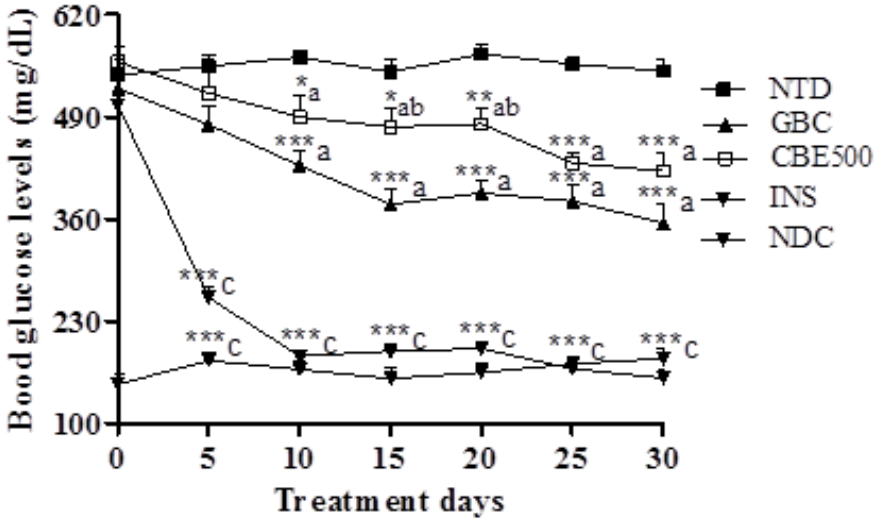

Figure 6. Effect of CBE500, GBC and INS on the blood glucose levels of diabetic rats. Where significance: *P < 0.05, **P < 0.01, ***P < 0.001, and (a) compared with NTD group, (b) compared to the group and GBC, (c) compared to all groups treated diabetics. Values express the mean ± S.D (n = 5/group).

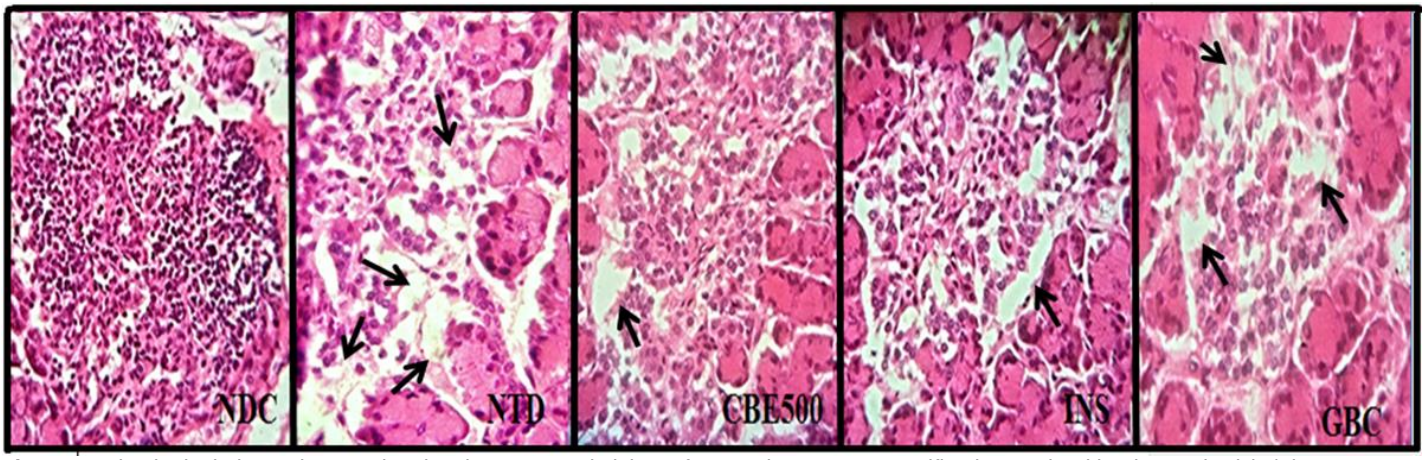

Figure 7. Histological photomicrographs showing pancreatic islets of Langerhans, 40x magnification, stained by the method (H/E). NDC, non-diabetic); NTD, untreated diabetic; CBE500, treated with extract; INS, insulin treated; GBC, treated glibenclamide.

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 indicates* (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.

ACKNOWLEDGMENTS

The author extend their thanks to the Research Support Foundation of the State of Amapá for its financial support through project PPSUS EFP-00007609, the Brazilian Federal Agency for Support and Evaluation of Graduate Education (Coordenação de Aperfeiçoamento de Pessoal de Nível Superior – CAPES) for the grant bestowed, and Guyamazon project (FAPEAP – AIRD) and CNPq Process number 407768/2013-0.

REFERENCES

Abe F, Nagafuji S, Okabe H, Akahane H, Estrada-Muñiz E, Huerta-Reyes M, Reyes-Chilpa R (2004). Trypanocidal constituents in plants. 3. leaves of Garcinia intermedia and heartwood of Calophyllum brasiliense. Biol. Pharm. Bull. 27:141-143.

Alade GO, Adebajo AC, Omobuwajo OR, Proksch P, Verspohl EJ (2012). Quercetin, a minor constituent of the antihyperglycemic fraction of Bauhinia monandra leaf. J. Diabetes. 4:439-441.

Asplunk K, Grankvist K, Marklund S, Taljedal IR (1984). Partial protection against streptozotocin-induced hyperglycaemia by superoxide dismutase linked to polyethylene glycol. Acta Endo. 107:390-394.

Bhuiyan MJ, Do HV, Mun S, Jun HJ, Lee JH, Kim YR, Lee SJ (2011). Hypocholesterolemic and hypoglycemic effects of enzymatically modifield carbohydrates from rice in high-fat-fed C57BL/6J mice. Mol. Nutr. Food Res. 55:214-226.

Cambraia RPB (2004). Aspectos psicobiológicos do comportamento alimentar. Rev. Nutr. 17:217-225.

Carvalho HO, Medeiros BJL, Sá BM, Araújo JTC, Kawakami MYM, Favacho HAS, Carvalho JCT (2013). Study of dissolution profiles and desintegration of capsules containing the dried hydroethanolic extract of Calophyllum brasiliense. Rev. Bras. Farmacogn. 23:194-199.

Carvalho HO, Souza BSF, Santos IVF, Resque RL, Keita H, Fernandes CP, Carvalho JCT (2016). Hypoglycemic effect of formulation containing hydroethanolic extract of Calophyllum brasiliense in diabetic rats induced by streptozotocin. Rev. Bras. Farmacogn.: http://dx.doi.org/10.1016/j.bjp.2016.04.004.

Cottiglia F, Dhanapal B, Sticher O, Heilmann J (2004). New chromanone acids with antibacterial activity from Calophyllum brasiliense. J. Nat. Prod. 67:537-541.

Halpern MCMA (2002). Aspectos fisiológicos do balanço energético. Arq. Bras. Endocrinol. Metab. 46:230-248.

Hou CC, Lin SL, Cheng JT, Hsu FL (2003). Antidiabetic dimeric guianolides and a lignan glycoside from Lactuca indica. J. Nat. Prod. 66:625-629.

Huerta-Reyes M, Basualdo M, Abe F, Jimenez-Estrada M, Soler C, Reyes-Chilpa R (2004). HIV-1 inhibitory compounds from Calophyllum brasiliense leaves. Biol. Pharm. Bull. 27:1471-1475.

Ito C, Itoigawa M, Mishina Y, Cechinel-Filho V, Enjo F, Tokuda H, Nishino H, Furukawa H (2003). Chemical constituents of Calophyllum brasiliense, Structure of three new coumarins and cancer chemopreventive activity of 4-substituted coumarins. J. Nat. Prod. 66:368-371.

Jacobson AM (1996). The psychological care of patients with insulin-dependent diabetes mellitus. N. Engl. J. Med. 334: 1249-1253.

Jia Q, Liu X, Wu X, Wang R, Hu X, Li Y, Huang G (2009). Hypoglycemic activity of a polifenolic oligomer-rich extract of Cinnamomum parthenoxylon bark in normal and streptozotocin-induced diabetic rats. Phytomedicine 16:744-750.

King A, Young G (1999). Characteristics and occurrence of phenolic phytochemicals. J. Amer. Diet. Ass. 99: 213-218.

Lee SJ, Umano K, Shibamoto T, Lee KG (2005). Identification of volatile components in basil (*Ocimum basilicum* L.) and thyme leaves (*Thymus vulgaris* L.) and their antioxidant properties. Food Chem. 91:131-137.

Lerco MM, Spadella CT, Machado JLM, Schellini AS, Padovani CR (2003). Caracterização de um modelo experimental de diabetes mellitus, induzido por aloxana em ratos: estudo clínico e laboratorial. Acta Cir. Bras.18: 132-142.

Li L, Xu J, Mu Y, Han L, Liu R, Cai Y, Huang X (2015). Chemical characterization and antihyperglycaemic effects of polyphenol enriched longan (*Dimocarpus longan* Lour.) pericarp extracts. J. Funct. Foods. 13: 314-322.

Martha RCD, Poubel J, Ferreira LCL, Lima RS, Borras MRLP, Costa RC, Roland I (2000). Atividade hipoglicêmica de Averrhoa carambola

L. usada em Manaus como antidiabético. News Lab. 38:142-148.
Mather A, Pollock C (2011). Glucose handling by the kidney. Kidney Int. 79:1 -6.
Molina PDML, Angeleli AYO, Macedo AR, Burini RC (1989). Efeito da insulina sobre o catabolismo das proteínas musculares, estudo in vitro da composição nitrogenada e da atividade das proteases ácidas lisossômicas. Alim. Nutr. Araraquara 1:127-140.
Naczk M, Shahidi F (2004). Extraction and analysis of phenolics in food. J. Chromatogr. A, 1054: 95-111.
Noel PH, Pugh JA, Larme AC, Marsh G (1997). The use of traditional plant medicines for non-insulin-dependent Diabetes mellitus in south Texas. Phytother. Res. 11:512-517.
Panda S, Kar A (2007). Apigenin (4',5,7-trihydroxyflavone) regulates hyperglycemia, thyroid dysfunction and lipid peroxidation in alloxan induced diabetic mice. J. Pharm. Pharmacol. 59:1543-1548.
Pepato MT, Baviera AM, Vendramini RC, Perez MPMS, Kettelhut IC, Brunetti IL (2003). Cissus sicyoides (princess vine) in the long-term treatment of streptozotocina diabetic rats. Biotechnol. Appl. Biochem. 37:15-20.
Pereira MOS, Gottlieb OR, Magalhães MT (1966). Constituintes xantônicos do Calophyllum brasiliense. Acad Bras Cienc. 38:425-427.
Queiroz JCF, Alonso-Vale MIC, Curi R, Lima FB (2009). Controle da adipogênese por ácidos graxos. Arq. Bras. Endocrinol. Metab. 54:582-594.
Ramachandran S, Asokkumar K, UmaMaheswari M, Ravi TK, Sivashanmugam AT, Saravanan S, Rajasekaran A, Dharman J (2011). Investigation of Antidiabetic, Antihyperlipidemic, and *in vivo* Antioxidant Properties of *Sphaeranthus indicus* Linn. in Type 1 Diabetic Rats: An Identification of Possible Biomarkers. Evid. Based Complement. Alternat. Med. 26 Sep 2010.
Rocha FD, Teixeira VL, Pereira RC, Kaplan MAC (2006). Diabetes mellitus e estresse oxidativo: produtos naturais como alvo de novos modelos terapêuticos. Rev. Bras. de Farm. 87:49-54.
Sacks DB, Bruns DE, Goldstein DE, Maclaren NK, Mcdonald JM, Parrott M (2002). Guidelines and recommendations for laboratory analysis in the diagnosis and management of Diabetes mellitus. Clin. Chem. 48:436-472.
Sartori NT, Canepelle D, Sousa-Júnior PT, Martins DTO (1999). Gastroprotective effect from Calophyllum brasiliense Camb.bark on experimental gastric lesions in rats and mice. J. Ethnopharmacol. 67:149-156.
Sezik E, Aslan M, Yesilada E, Ito S (2005). Hypoglycemic activity of Gentiana olivieri and isolation of the active constituent through bioassay-directed fractionation techniques. Life Sci. 76:1223-1238.
Schnedl WJ, Ferber S, Johnson JH, Newgard CB (1994). STZ transport and cytotoxicity: specific enhancement in GLUT 2 expressing cells. Diabetes. 42:1326-1333.
Silva KL, Santos ARS, Matos PEO, Yunes RA, Delle-Monache F, Cechinel-Filho V (2001). Chemical composition and analgesic activity of Calophyllum brasiliense. Therapie 56:431-434.
Silva M, Lima WG, Silva ME, Pedrosa ML (2011). Efeito da estreptozotocina sobre os perfis glicêmico e lipídico e o estresse oxidativo em hamsters. Arq. Bras. Endocrinol Metab. 55:46-53.
Silva LE, Quadros DA, Neto AJM (2015). Estudo etnobotânico e etnofarmacológico de plantas medicinais utilizadas na região de Matinhos-PR. Ciência e Natura. 37:266-276.
Swain T, Hillis WE (1959). The phenolics constituents of prumus domestica, the quantitative analysis of phenolic constituents. J. Sci. Food Agric. 10:63-68.
Thulesen, J, Orskov C, Holst JJ, Poulsen SS (1997). Short term insulin prevents the diabetogenic action of streptozotocin in rats. Endocrinology 38:62-68.

African Journal of Pharmacy and Pharmacology

Related Journals Published by Academic Journals

- *Journal of Medicinal Plant Research*
- *African Journal of Pharmacy and Pharmacology*
- *Journal of Dentistry and Oral Hygiene*
- *International Journal of Nursing and Midwifery*
- *Journal of Parasitology and Vector Biology*
- *Journal of Pharmacognosy and Phytotherapy*
- *Journal of Toxicology and Environmental Health Sciences*

academicJournals