Review

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

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Research on natural products is facing significant difficulty. The analysis "high-throughput screening" has limited effectiveness in their evaluation. This report on *in vitro* screening of pharmacological activity of the drugs candidate molecule by evaluating a single target. The presence of many substances in a natural extract makes this process 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...
for new drugs discovery. Until now, bioactive components isolated from plants, fungi and bacteria gave rise to a wide range of therapeutic compounds. Also, a lot of these compounds are useful tools in pharmacology, biochemistry and cell biology and molecular (Crawford et al., 2008).

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

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

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

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

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

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

ADVANTAGE OF THE ZEBRAFISH AS EXPERIMENTAL MODEL

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

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

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

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

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

| 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; Yang 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; Bohn 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; Bill 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 alymphaphilide, a kind of lignans extracted from Phylanthus 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 epimedi*ii, 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* (Mie.). 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 thrombosis 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 pentylentetrazol. 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 pentylentetrazol. 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. Pentylentetrazol 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 (rhynchosvisina, genestin, sophorosiflavan A, licoisoflavona and 3-o-methylorobol). It was proven that genistin and sophorosiflavone possess anti-inflammatory and anti-angiogenic effects.

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

**Antilipemic activity in zebrafish model**

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

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

Cinnamon and clove 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 µM) 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 (Scholtz 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; Scholtz 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 (Jevgenj et al., 2013). Furthermore, ZF has been very useful in evaluating plant extracts and isolated compounds. Thus, the toxic effect of celestrol, one terpenoid isolated from Tripterygium wilfordii Hook F., was assessed. The results showed that celestrol, 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 ketogenic 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.

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