In vitro CULTURE, CRYOPRESERVATION AND MEDICINAL PROPERTIES OF *Byrsonima intermedia*

MILENE ALVES DE FIGUEIREDO CARVALHO

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Tese apresentada à Universidade Federal de Lavras como parte das exigências do Programa de Pós-Graduação em Agronomia, área de concentração em Fisiologia Vegetal, para a obtenção do título de "Doutor".

Orientador

Prof. Renato Paiva, Ph.D.

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APROVADA em 26 de fevereiro de 2010

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LAVRAS

MINAS GERAIS - BRASIL

A Deus,

pela benção da vida e presença constante ao meu lado,

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À minha família,

Heider e Marlene,meus amados pais,

Hedeilson, Madeleine e Marislaine, meus irmãos queridos,

Vovó Nida, Jack minha cunhada,

Henrique, o afilhado mais lindo da dinda

e Flavinho meu amor, marido, companheiro e amigo,

pelo amor incondicional, apoio, paciência e incentivo

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BIOGRAFIA

Milene Alves de Figueiredo Carvalho, filha de Marlene Alves de Paula Figueiredo e Heider Francisco Barros de Figueiredo, nasceu em Varginha, MG, no dia 17 de julho de 1983. Cursou o ensino fundamental e médio no Instituto Presbiteriano Gammom, concluindo-o em 2000. Em 2001, foi aprovada no vestibular para o curso de Agronomia da Universidade Federal de Lavras (UFLA). De 2002 a 2005, foi bolsista de Iniciação Científica do CNPq no Laboratório de Cultura de Tecidos Vegetais da UFLA. Em agosto de 2005, graduou-se e iniciou o mestrado em Agronomia/Fisiologia Vegetal na UFLA, concluindo-o em fevereiro de 2007. Em março de 2007 iniciou o doutorado em Agronomia/Fisiologia Vegetal da UFLA, realizando parte de seu trabalho na Katholieke Universiteit Leuven, Bélgica, Leuven no período de agosto de 2008 a agosto de 2009 e, trinta e seis meses depois, encerra esta etapa profissional com a presente tese.

SUMMARY

GENERAL ABSTRACT

CARVALHO, Milene Alves de Figueiredo. ***In vitro* culture, cryopreservation and medicinal properties of *Byrsonima intermedia*.** UFLA, 2010. 203p. Thesis (Ph.D. in Agronomy/ Plant Physiology) - Federal University of Lavras, Lavras. [*]

The genus *Byrsonima*, which belongs to the Malpighiaceae family, is widely used in Brazil not only as food but also because of its interesting medicinal properties. The aim of the present study was to increase knowledge about medicinal properties of *Byrsonima intermedia*. For this, we studied *B. intermedia* cell suspension cultures, callus induction, cryopreservation; and we evaluated differences among extracts of *B. intermedia* leaves, bark, flowers, calli and cell suspensions cultures, using Thin Layer Chromatography technique and later on use representative lines to be tested in an anti-microbial assay and in zebrafish for their anti-inflammatory activity. First of all we established a protocol for callus induction and cell suspensions cultures. Next, we checked the effects of different techniques and cryoprotectants types and concentrations in the pre-culture and pre-treatment periods on cell growth and re-growth after cryopreservation. The anti-microbial study was executed on *Candida albicans*, *Saccharomyces cerevisiae* and *Staphylococcus epidermidis*. The crude methanolic extracts as well as purified compounds were tested in zebrafish. Phytochemical analysis was performed in order to characterize the active compounds present in the extracts. As results we observed that calli were successfully induced for *B. intermedia* species and different aspects were observed in these explants when cultured in different light conditions. Growth curves of *B. intermedia* cell suspensions were established. No regrowth of *B. intermedia* cells was observed within 4 weeks after cryopreservation. None of the crude *B. intermedia* extracts tested showed antimicrobial activity against the microorganisms used. Crude methanolic extracts from bark, leaves and flowers at 10µg mL^{-1} present anti-inflammatory effect on zebrafish larva with tail fin injury. The strong anti-inflammatory properties of *B. intermedia* are confirmed in a purified fraction from bark, leaf and flower methanolic extracts. The main bioactive compounds present in *B. intermedia* bark and leaf methanolic extracts are likely β-amyrin and epicatechin. Gallic acid is another secondary metabolite

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present in bark extracts. Red cell suspensions of *B. intermedia* contain the secondary metabolite epicatechin.

Key-words: calli; cell suspensions; growth curves; long-term preservation; TLC; anti-microbiology; zebrafish; GC-MS; HPLC.

RESUMO GERAL

CARVALHO, Milene Alves de Figueiredo. **Cultivo *in vitro,* criopreservação e propriedades medicinais de *Byrsonima intermedia*.** UFLA, 2010. 203p. Tese (Doutorado em Agronomia/Fisiologia Vegetal) – Universidade Federal de Lavras, Lavras. *

O gênero *Byrsonima*, pertencente à família Malpighiaceae, é amplamente utilizado no Brasil, não só como alimento, mas também por causa de suas propriedades medicinais. O objetivo do presente trabalho foi aumentar o conhecimento sobre as propriedades medicinais de Byrsonima intermedia. Para isso estudou-se suspensões celulares, indução de calos, criopreservação de *B. intermedia*; e avaliou-se diferenças entre folhas, cascas, flores, calos e suspensões celulares desta espécie, utilizando a técnica de Cromatografia em Camada Delgada, para posteriormente testar as linhagens representativas em ensaios anti-microbianos e em zebrafish para testar a atividade anti-inflamatória. Primeiramente estabeleceu-se um protocolo para indução de calos e culturas de suspensão celular. Em seguida, verificou-se o efeito de diferentes técnicas de criopreservação e crioprotetores (tipos e concentrações) em diferentes períodos de pré-cultivo e pré-tratamento no crescimento celular pós-criopreservação. O estudo anti-microbiano foi testado contra *Candida albicans*, *Saccharomyces cerevisiae* e *Staphylococcus epidermidis*. Os extratos metanólicos bruto e purificados foram testados em zebrafish. A análise fitoquímica foi realizada com o objetivo de caracterizar os compostos ativos presentes nos extratos. Como resultados observa-se que calos foram induzidos com sucesso para a espécie *B. intermedia* e diferentes aspectos foram observados nestes explantes quando cultivados em diferentes condições de luz. As curvas de crescimento das suspensões celulares de *B. intermedia* foram estabelecidas. Não observa-se crescimento pós-criopreservação durante o período de quatro semanas. Nenhum dos extratos brutos de *B. intermedia* testados apresentam atividade antimicrobiana contra os microrganismos utilizados. Extrato metanólico bruto das cascas, folhas e flores na concentração de 10μg mL^{-1} apresentam atividade anti-inflamatória nas larvas de zebrafish com lesão na nadadeira caudal. As propriedades anti-inflamatórias de *B. intermedia* são confirmadas em uma fração

* Comitê Orientador: Dr. Renato Paiva - UFLA (orientador), Dr. Bartholomeus J. Panis – KULeuven, Prof. Rony Swennen – KULeuven.

purificada a partir de cascas, folhas e flores dos extratos metanólicos. Os principais compostos bioativos presentes na casca e folhas dos extratos metanólicos de *B. intermedia* são possivelmente β-amirina e epicatequina. O ácido gálico é outro metabólito secundário presente em extratos de casca. Suspensões celulares de *B. intermedia* de coloração vermelha contêm epicatequina como metabólito secundário.

Palavras-chave: calos; suspensões celulares; curvas de crescimento; preservação a longo prazo; CCD; anti-microbiologia; zebrafish; CG-EM; CLAE.

CHAPTER 1

In vitro culture, cryopreservation and medicinal properties of *Byrsonima intermedia*

1 GENERAL INTRODUCTION

The genus *Byrsonima* composed of approximately 150 species belongs to the Malphigiaceae family and is widely spread in Central and South America. Their bark, leaves, flowers and fruits are used for medicinal purposes, such as anti-asthmatics and in skin infections. Among the species that emcompass the genus, there is limited phytochemical research, especially related to the species *Byrsonima intermedia*.

Although other species of the *Byrsonima* genus have wide ethnopharmacological application there are only a few citations of *B. intermedia* species related to the identification of chemical constituents and pharmacological application, justifying the present study.

The production of pharmaceutically active natural products is challenging due to the slow growing nature of plant species, low yield, and unpredictable variability in accumulation. In order to arrive to an efficient production system, plant cell culture technology could be an attractive alternative.

There are many plant cell culture technologies available as tools for both studying and producing medicinally valuable plant secondary metabolites.

2 THEORETICAL BACKGROUND

2.1 *Byrsonima* species

The genus *Byrsonima,* composed of approximately 150 species, belongs to the Malphigiaceae family and is widely spread in Central and South America (Aguiar et al., 2005). Their bark, leaves, flowers and fruits are used for medicinal purposes, such as anti-asthmatics and in skin infections (Caceres et al., 1993). Among the species that emcompass the genus, there is limited phytochemical research, especially related to the species *Byrsonima intermedia*.

Historical records confirmed the medicinal proprieties of several native Brazilian medicinal plant species, some which were also described in Pharmacopoeia, that have a long tradition of use (Brandão et al., 2008). These records demonstrate the importance of these medicinal plants in official medicine and just only a few have been studied in detail.

Species that have a promising phytotherapeutic potential should be regarded as a priority for pharmacological studies (Brandão et al., 2008). Most of the plant therapeutic proprieties mentioned by folk people are confirmed in scientific studies, demonstrating the importance of ethnopharmacology.

Scientific validation for the popular use of the medicinal plants is important to serve as a guide which may help in selection of plants with such potential activity.

Every day scientific studies that involve medicinal plants have being reported. These studies are very important for both economical and social issue. Plant medicinal treatments have been used for centuries by people. In this context, the pharmaceutical and cosmetics industries are very interested in these plants and they are looking for natural sources of crude materials and active compounds for their products (Lira et al., 2008).

Byrsonima species leaves are commonly known to contain several medicinal properties. Among these proprieties the treatment against fever, gastrointestinal and skin diseases are described. Michelin et al. (2008) studied the antimicrobial activity of *B. fagifolia*, *B. basiloba* and *B. intermedia* extracts obtained from the leaves by using the disc-diffusion method. The results showed that methanol (MeOH) extracts of leaves had an antimicrobial activity against all the microorganisms tested. The classes of compounds found in the MeOH leaves extracts of *B. intemedia* were catechins, tannins, gallic acid derivates and flavonoids. Sannomiya et al. (2007) observed signs of mutagenic activity in *B. intermedia* in the assays with *Salmonella* which was related to presence of the amentoflavone biflavonol. Quercetin, another compound found in the extract by the authors, has been the subject of numerous studies regarding its genetic toxicity and carcinogenicity. On the other hand, Lira et al. (2008) observed the chloroform and MeOH extracts of *B. basiloba* presented no mutagenicity in any of the strains of *Salmonella typhimurium* used. Phytochemical studies of the MeOH extract demonstrated the presence of (+)-catechin, quercetin-3-O-α-□-arabinopyranoside, quercetin, gallic acid, methyl gallate, and the amentoflavone.

Folk medicine related to *B. intermedia* reports that this plant is known to have antiulcer and healing activity. An aqueous portion obtained from this species leaves was effective on reducing the gastroprotector ulcerative lesion and also showed effective healing action in chronic gastric disease (Santos et al., 2009).

Confirming that some typical Brazilian plants may be used as a potential source for compounds with biological activity, *B. fagifolia* presented significant activity against gastric ulcers (Wagner & Souza Brito, 2006).

In methanolic extracts of *B. crassa* catechins and flavonoids were found which were considered the secondary metabolites responsible for the protection found against gastric ulcers. These metabolites can also be related to the

scavenging of the reactive oxygen species on the surface of gastric mucosa, thus protecting cells from gastric injury (Sannomiya et al., 2005). In this same species, the methanolic extract of leaves presented antioxidant activity which can also be related to its flavonoids content (Sannomiya et al., 2004).

The following compounds naphthoquinones, a derivative and flavanol, which were isolated from the wood of *B. microphylla*, did not show antioxidant activity (Aguiar et al., 2005).

Important behavioural changes were observed by Cifuentes et al. (2001) in the aqueous extracts of bark and leaves of *B. crassifolia* (L.) Kunth. Also Souza et al. (2008) affirmed that this species could be used for medicinal and food applications because of the large amounts of polyphenols found in it. The main source of polyphenols with antioxidant properties found by these authors was leaves compared to bark and fruits. In the phytochemical analysis of *B. crassifolia* extracts was found the presence of glycosides, saponins and flavonoids, with tannins presented in the bark extract (Martínez-Vázquez et al., 1999).

Alves & Franco (2003) found that the different flavor present in *B. crassifolia* fruit is normally attributed to the presence of butanoic acid, hexanoic acid and methyl butyrate which gives its cheese aroma.

Another species of the *Byrsonima* genus, *B. crassa*, was found to produce secondary metabolites with anti-*Helicobacter pylori* activity (Bonacorsi et al., 2009). Also the methanolic leaf extracts of *B. verbascifolia* were found to present an antiviral activity (Lopez et al., 2001).

The traditional use of *B. fagifolia* against gastrointestinal diseases was confirmed by Lima et al. (2008) who found the gastroprotective, healing and antidiarrheal activities in this species. According to these authors phenolic compounds were the substances which caused the mucus protection. The antidiarrheal activity was also presented in these plant extracts, however no

toxicity and mutagenicity were observed. Also bark of *B. crassifolia* is used in the treatment of diarrhoea and is rich in tannins (Leonti et al., 2002).

2.2 Phytochemistry

More than 30.000 natural products represent the secondary metabolites. They have been used as medicines, perfumes, dyes and food supplements in human life. They also have a complex chemical structure and have a great importance in our life (Yazaki, 2004).

Secondary metabolites from plants can be defined as bioactive compounds which present a low molecular weight and execute many roles in the interaction of plants with the environment (Estrada-Zúñiga et al., 2009).

Several diseases were often treated with valuable herbal products obtained from medicinal plants (Michelin et al., 2008). These plants are a tremendous source for the discovery of new products of medicinal value for drug development. New plants and interesting metabolites are continuously being revealed since the pharmaceutical potential and the research on bioactive compounds obtained from plants are still ongoing (Vanisree et al., 2004; Wilken et al., 2005).

Various ailments have been cured for centuries by using natural products from plants. The side effects of synthetic drugs used by the pharmaceutical industry to cure diseases can be even more dangerous than the diseases themselves. In this context the use of bioactive compounds derived from plants is increasing. These compounds contain natural products that can promote health and alleviate illness. So, nowadays the knowledgement and aplicability of the healing power of plants is necessary (Kaur et al., 2005).

The secondary metabolites have complex structures, low abundance and strong inhibitory effects on many enzymes, including their own biosynthetic enzymes. All these characteristics make the study of secondary metabolite

biosyntheses difficult, despite their potential of becoming a great resource for biochemical research (Yazaki, 2004). Moreover, secondary metabolites are currently being mainly obtained commercially by extraction from whole plants (Sajc et al., 2000) and desired products may unfortunately be converted to other metabolites or degraded (Kim et al., 2002).

To optimize the biosynthesis of key secondary metabolites a good knowledge of the kinetics and modelling for cell growth is needed to maximize the product production for pharmaceutical purposes (Kim et al., 2002; Ling et al., 2008).

2.3 Tissue culture

The plant tissue culture, the science of growing plant cells, tissues or organs isolated from a mother plant, on artificial media, is an important tool for clonal plant propagation free from pathogens, haploid production, genetic modification, embryo rescue, *in vitro* fertilization, germplasm conservation and to produce secondary metabolites (Razdan, 2003; George, 2008).

Typical *in vitro* plant growth can be organized (meristem, shoot, node, isolated root and embryos cultures) as well as unorganized (callus, cell suspension, protoplast and anther cultures). The unorganized plant callus (a coherent and amorphous tissue formed when plant cells multiply in a disorganized way) and/or cell suspension (cells that are freely dispersed in agitated liquid media) has different stages of development which can be distinguished if cell growth is measured (George, 2008).

2.3.1 Secondary metabolites in tissue culture

Primary and secondary metabolites are an almost unlimited source of phytochemicals in plants. The different functions, interactions with the environment, several stress factors, development of resistance against pathogen

attack (Sudha & Ravishankar, 2002) and an important source of active pharmaceuticals make the secondary metabolites interesting in various aspects (Bourgaud et al., 2001).

Several distinct chemicals derived from plants are important drugs currently used in one or more countries in the world. The majority of the drugs sold today are simple synthetic modifications or copies of natural substances. The slow growth behavior of some plant species, their low yield, and unexpected variability in their natural products accumulation make the production of pharmaceutically active natural products a real challenge. Plant cell culture technology has made it possible to alter the production of bioactive plant secondary metabolites in recent years (Vanisree et al., 2004; Zhong, 2008).

Since regulatory alterations allowed the introduction of herbs into the main supermarkets and pharmacies, the demand for herbal plants and extracts have been on rise. These plants and extracts can be used to improve human health and well being. However, the basic mechanisms on how the phytochemicals accumulation is regulated by the environment are still quite unknown. This gap can be fulfilled by using *in vitro* techniques to analyse the plant species response (Smith et al., 2002).

Plant cells are theoretically biosynthetically totipotent, since each cell in culture retains complete genetic information and is hence theoretically able to produce the range of similar or superior chemicals found in the parent plant. Several problems related to the industrial yield of the natural plant products by extraction from field grown plants can be overcome by the production of these phytochemicals by cell culture. In this context there are many advantages of using in vitro technique to produce plant secondary metabolites which contain interesting bioactive compounds for the pharmaceutical and cosmetic industry in comparison of their extraction in plants grown in fields. Among them the fact that *in vitro* cultures are independent of geographical and seasonal variations and

various environmental factors. It offers a defined and controllable production system, which ensures the continuous supply of products, uniform quality and yield. It is also possible to produce novel compounds that are not normally found in the mother plant. Moreover, the mass extraction of natural populations is avoided and then the risk of extinction is reduced (Bhojwani & Razdan, 1996; Sajc et al., 2000; Rao & Ravishankar, 2002; Matkowski, 2008).

There are three key aspects that must be considered when performing a plant cell culture; (i) first the plant part of interest must be removed from the intact plant, (ii) next the appropriate environment to promote optimal growth must be discovered and applied (this may vary depending on the cells of interest) and (iii) finally, these procedures must be executed in a sterile environment to prevent growth of unwanted microorganisms. These conditions have already been applied to callus and cell suspension culture of several different plant speceis (Torabi et al., 2008).

The initial step of developing *in vitro* production systems, on the whole plant or organ or the cell culture level, can facilitate making a deep analysis of phytochemical accumulation as a whole (Smith et al., 2002).

Cell cultures can supply means for *de novo* synthesis of natural products, also satisfactory bioconversion of low value compounds into high value products, and furthermore they can produce new physiologically active substances, which present medicinal interest, and that are not produced in intact plants (Bhojwani & Razdan, 1996; Vanisree et al., 2004). The main aim of producing biologically active compounds in plant cell culture systems is to produce the highest value of these products as possible. So, the bioreactors contribute widely with this production in large-scale (Sajc et al., 2000; Bourgaud et al., 2001; Kim et al., 2002; Sudha & Ravishankar, 2002).

Different *in vitro* systems have been widely studied objectifying to improve the production of secondary plant compounds. Biotic and abiotic

elicitation can induce several plant secondary compounds which are involved in the interaction of the plant with its environment. Assuming that the culture conditions necessary for growth of the plant cells are not inhibitory to the production of the metabolites under study, elicitation is one of the most effective approaches for increasing yields of plant secondary metabolites. Elicitation is then one of the most effective approaches for over-producing of desired plant secondary metabolites, which is an area of commercial importance mainly for high value low volume products (Sudha & Ravishankar, 2002; Vanisree et al., 2004).

The synthesis of many secondary metabolites, which normally possess a complex biosynthetic pathway, is determined by environmental factors and nutrient availability. The application of genetic engineering which can allow the control of the mechanisms and/or enzymes responsible for synthesis of specific secondary metabolites, can contribute for increasing the production of such metabolites (Kim et al., 2002).

Environmental factors as well as the artificial selection and/or the induction of variant clones can improve the biosynthetic activity of cell cultures. Culture systems allow the production of important medicinal compounds in morphologically specialized tissues or organs of native plants. These products can not only be induced by specific organized cultures such as hairy roots, but also by undifferentiated cell cultures such as callus and cell suspensions (Vanisree et al., 2004).

Calli and cell suspension cultures are sometimes not the best source of metabolites. For example, there are some plants (*Hypericum*, *Cymbopogon* and *Fabiana*) which the *in vitro* production of interesting secondary metabolites was always higher in shoot cultures compared to cell or callus cultures (Wilken et al., 2005).

Considerable attention has been given to medicinal plants with antioxidant properties. The production of identical material cultured independent from environmental factors supply the development of *in vitro* cell lines which present interesting antioxidant compounds (López-Laredo et al., 2009).

There are successful examples of tissue cultures for several medicinal plants where an increased content of the bioactive secondary metabolites has been achieved compared to that from wild plants. The stress factors can be overcome by plants through formation and accumulation of secondary metabolites. This formation and accumulation can be triggered by external stimuli which unchain a series of reactions in the plant cell. Several biotechnological advances have been developed in tissue culture that initiate or improve secondary metabolite production such as optimization of cultural conditions, nutrient stress, selection of high-producing strains, precursor feeding and biotic and abiotic elicitation inducting the biosynthetic enzymes of the secondary metabolite pathways, metabolic engineering, transformed root cultures, micropropagation, and bioreactor cultures, among others. The receptors capture the stimuli and activate the secondary messengers which, through the signal transduction pathways, send the signals. Thereafter the gene expression and biochemical changes take place. Polyamines, calcium, jasmonates, salicylates, nitric oxide and ethylene are the main factors found in the literature which influence signal transduction pathway(s) (Nezbedová et al., 1999; Bourgaud et al., 2001; Sudha & Ravishankar, 2002; Sarin, 2005). Hairy roots resulting from genetic transformation by *Agrobacterium rhizogenes* have interesting properties for secondary metabolite yield (Kim et al., 2002).

Light is also an important factor in the secondary metabolites production/induction, as described by López-Laredo et al. (2009) in the phenylpropanoid and flavonoid biosynthesis of *Tecoma stans* calli, which was regulated in response to light.

Much effort has been made in the last decades in plant biotechnologies that have led to some successful protocols for the production of valuable secondary compounds. Some examples of the use of tissue culture technique regarding this production are extensively found in the literature and are shown in table 1.

TABLE 1 Example of *in vitro* culture technology used to induce and/or improve some plant secondary metabolites production.

Species	**Plant part used**	**Compounds mainly found**	**Reference**
Aphelandra sp.	calli, cell suspensions	verbascoside	Nezbedová et al., 1999
Azadirachta indica	calli	azadirachtin-A	Babu et al., 2006
Centella asiatica	calli, cell suspensions	saponins	James et al., 2008
Echinacea angustifolia	cell suspensions	caffeic acid derivatives, echinacosides	Smith et al., 2002
Panax ginseng	hairy roots	saponins	Yoshimatsu et al., 1996
Piper methysticum	Calli, *in vitro* regenerated roots	kavapyrone	Smith et al., 2002
Tabernaemontana elegans	cell suspensions	serotonin	Lucumi et al., 2002
Tecoma stans	calli	phenolic compounds, flavonoids	López-Laredo et al., 2009
Valeriana glechomifolia	*in vitro* plants	valepotriate	Russowski et al., 2006
Valeriana glechomifolia	*in vitro* plants	valepotriates	Russowski et al., 2006

2.4 Zebrafish

2.4.1 Advantages of using zebrafish

2.4.1.1 General advantages

Zebrafish (*Danio rerio*) are small freshwater tropical fish that have recently emerged as an important vertebrate model for studying the development of embryos; human diseases like cancer, cardiovascular disorders and angiogenesis; toxicological assays; and identification of bioactive natural products with therapeutic potential (Zon & Peterson, 2005; Beckman, 2007; Drahl, 2007; Kari et al., 2007; Eimon & Rubinstein, 2009; He et al., 2009).

The main advantages which have enabled the zebrafish to become a widely used model organism are:

(i) their high fecundity, where hundreds of offspring per breeding pair are produced per week;

(ii) their high genetic, morphological, physiological and pharmacological similarity to mammals, presenting discrete organs and tissues such as a brain, sensory organs, heart, liver, pancreas, kidneys, intestines, bones, muscles, etc.;

(iii) their small size for a vertebrate, where adults reach 3 cm in length, but during the embryonic and larval stages of life, the zebrafish is only about 1–5 mm long and can then live for days in a single well of a standard 384-well plate, surviving on nutrients stored in their yolk sacs;

(iv) the ease and cost-effectiveness of breeding and maintaining zebrafish; and

(v) the optical transparency of their embryos and larvae, which facilitates the vizualization of every internal organ and structure in the intact, living organism, removing the need to sacrifice or dissect the animal and permitting multiple observations of dynamic processes. The transparency of zebrafish embryos becomes even more useful when combined with fluorescent markers (e.g. green fluorescent protein, GFP) that highlight the locations or activities of

specific populations of cells. These transgenic lines greatly facilitate detection of anatomical changes caused by small molecules once zebrafish can rise outside the uterus;

(vi) their embryos are permeable to small molecules and drugs, providing easy access for drug administration and vital dye staining. So, compounds can simply be added to the (non-sterile) water surrounding zebrafish and then hundreds of samples can be tested per day per researcher. Such assays are rapid - approximately 1-3 days from addition of compound to assay results;

(viii) embryonic zebrafish development is rapid. For example, embryos can execute evasive maneuvers upon touch within 24 h postfertilization (hpf), most major organs including the gut and the vasculature are in place by 2 days postfertilization, and embryogenesis is complete 5 days after fertilization (Serbedzija et al., 1999; Kidd & Weinstein, 2003; MacRae & Peterson, 2003; Zon & Peterson, 2005; Berger & Currie, 2007; Drahl, 2007; Kari et al., 2007; Lieschke & Currie, 2007; Baldessari & Mione, 2008; Crawford et al., 2008; Eimon & Rubinstein, 2009; Wheeler & Brändli, 2009).

All these characteristics are valuable and explain the increase of zebrafish-based research with an approximately 100-fold increase in the number of publications over the last two decades (Berger & Currie, 2007).

2.4.1.2 Natural product discovery

2.4.1.2.1 Models for discovery of small molecules

Natural products are (often small) molecules produced by living organisms. Among them, secondary metabolites have been emphasized as being an attractive but underused source of chemical diversity for drug discovery (Crawford et al., 2008).

Novel, bioactive small molecules for drug development were originally discovered randomly by phenotypic observations in whole organisms such as

rats and mice. However, in last decades, this organism-based process became more systematic. The use of *in vitro* tests, such as cell- or tissue-based screening assays using pathway-specific reporters may not always be representative of the *in vivo* response mainly because drug action involves metabolism and interplay among different tissues. Moreover, many diseases affect organs as a whole, and most organs cannot be reestablished *in vitro*. In this context, small model organisms present a practical option to conduct high-throughput screening in a whole organism at a very early stage of drug discovery and they are also required for biomedical research to understand human diseases at molecular and cellular level as well as for drug discovery and testing of new therapies. So, the combination between the advantages of organism-based small molecule discovery and the technologies and through-put of modern screenings becomes possible (MacRae & Peterson, 2003; Zon & Peterson, 2005; Drahl, 2007; Baldessari & Mione, 2008; Barros et al., 2008; Berghmans et al., 2008; Crawford et al., 2008; Eimon & Rubinstein, 2009; Wheeler & Brändli, 2009).

The use of model organisms for chemical screening has two main purposes. First is to promote research in a given area by obtaining small molecules that can be used as conditional research tools to investigate fundamental questions in development, physiology, and behavior. Secondly and more important application is to identify drug candidates that can be potentially used for therapeutic purposes (Wheeler & Brändli, 2009).

Caenorhabditis elegans, *Drosophila melanogaster*, *Arabidopsis thaliana*, and the zebrafish are all small model organisms which can grow in screening microplates. Among these organisms, emphasis is given to the zebrafish mainly because it is the most closely related to humans and is perhaps best established as a tool for small molecule discovery (MacRae & Peterson, 2003).

The presage of drug risk in humans is better seen in mammalian toxicity studies like in primate, mice, rat, or pig models by the fact that they clearly better reflect human physiology. However these traditional *in vivo* tests are very expensive, time-consuming and require large amounts of test compound, which make them less suitable for early stage toxicology screening. Then, such studies are usually conducted at the end of the drug discovery process (Crawford et al., 2008; Eimon & Rubinstein, 2009).

In this context, due to the limitations of both traditional mammalian models and *in vitro* approaches, researchers are showing increased interest in zebrafish-based assays to assess safety and toxicological end points (mortality, malformations and embryonic growth) and methodology (to determine the concentrations which cause lethality and developmental malformation) (Kari et al., 2007; Eimon & Rubinstein, 2009). However, several challenges still must be addressed to prove the use of zebrafish assays on early drug discovery worthy. Among them the determination of compound absorption and tissue distribution, demonstration of the predictive validity of specific zebrafish end points, and standardization and full automation of assays can be mentioned (Eimon & Rubinstein, 2009). The potential of *in vivo* zebrafish assays for the bioactivity-guided fractionation of complex natural extracts is only beginning to be explored (Crawford et al., 2008).

2.4.1.2.2 The use of zebrafish for drug discovery

The identification of new chemical products that change specific biological processes is possible through chemical genetics. Chemical genetics is the elucidation of biological function through the use of small molecules. Chemical screening applications using have increased in recent years. Indeed, zebrafish-based chemical genetics combined with modern chemistry enables the discovery of potent compounds which can be used not only as specific and

reversible tools to manipulate molecular pathways, but also as a starting point to discover new drugs that develop medical therapies to improve human health (Berger & Currie, 2007; Crawford et al., 2008).

The qualities that an animal model has to have to be considered as organism-based chemical screen include small size, low cost, high embryos producing, compatibility with simple culture conditions and highly permeable to small molecules (Wheeler & Brändli, 2009). In this context, the role of zebrafish system became more important in the field of drug development mainly after the completion of the zebrafish genome project and the establishment of a large infrastructure for genetic and physiological studies. This system can then provide a shorter and cheaper path to developing novel therapies for human disease since it contribute to target identification and validation, drug lead discovery and toxicology (Zon & Peterson, 2005). The reasons for the increasing use of zebrafish as a model for lead compound discovery and exploration are due to both government funded basic science and industry researches (Berger & Currie, 2007).

The action mechanism and ultimately the biological target, of natural products and other small molecules can be explored by using zebrafish system. However, it is important that these studies be combined with mammalian experiments in order to confirm the findings (Crawford et al., 2008). Therefore, zebrafish assays are not substitute of traditional *in vivo* models rather; they are best used in colaboration with these models to reduce the time and resources expended to identify candidate compounds (Zon & Peterson, 2005; Kari et al., 2007; Baldessari & Mione, 2008; Eimon & Rubinstein, 2009). Diseases that were previously untreated by target-based methods on drug discovery may also be handled by using zebrafish screens (MacRae & Peterson, 2003). With the technology advance, zebrafish assay can be capable of substituting the

mammalian models once their applications are in crescent complexity (Zon & Peterson, 2005).

Chemical compounds and/or bioactive extracts are usually stored as stock solutions in dimethyl sulfoxide (DMSO), which also serves as a vehicle to improve solubility of the compounds in the aqueous solutions used to culture embryos, either through their skin or in the gastrointestinal tract, and facilitates compound permeation into cells. Normally, concentration used in the medium which zebrafish is tolerant is 1% DMSO (Barrett et al., 2006; Barros et al., 2008; Berghmans et al., 2008; Wheeler & Brändli, 2009).

The fact that the zebrafish is transparent during embryogenesis does not means that all their tissues are easily identified and monitored by light microscopy. During their development pigmentation will difficulty phenotype detection. This problem can be overcome by supplementing the growth medium with the 1-phenyl-2-thiourea (PTU), a tyrosinase inhibitor which is routinely used to inhibit pigment production (Peterson et al., 2000; Peterson & Fishman, 2004) and is usually well tolerated by zebrafish embryos and larvae (Wheeler & Brändli, 2009).

The procedure of a small molecule screen with zebrafish is described by Zon & Peterson (2005), Murphey & Zon (2006) and Berger & Currie (2007). In this procedure, male and female zebrafish are paired in small mating tanks with a divider, which is removed simultaneously from all pairs to initiate mating. This enables the collection of synchronised embryos of the same developmental stage. Adult zebrafish are mated to produce 200–300 embryos per female After the eggs are cleaned from dead or unfertilised eggs they are cultivated in embryo medium and stored in an incubator until the developmental stage desired for screening is reached. The embryos are then distributed to 96- or 384-well assay plates and the small molecules, which can also be arranged in multititer plates at the desired concentration beforehand, are added to the water surrounding the

zebrafish. After a period of incubation, the embryos are analysed for their response to the small molecules usually by microscopy.

Nowadays, the pharmaceutical industry is responsible for the majority of small molecule discoveries. Maybe the main advantage of using organism-based screening is to increase the viability and resource to perform small molecule discovery in academic settings. Any laboratory which contains fish tanks, a chemical library, and a microscope can perform a zebrafish chemical screen. The comprehension and biological questions are limited only by the researcher's imagination and can lead to increased biological insight, to the discovery of new therapeutic compounds and to model genetic diseases (MacRae & Peterson, 2003; Baldessari & Mione, 2008).

2.4.2 Disadvantages of using zebrafish

As with any experimental system, there are clearly also limitations in working with zebrafish as a model organism. The first drawback to the model is that most of the advantages of zebrafish as a model for small molecule screens are limited to embryonic and larval stages and do not apply to adult fish. So, given that most screens are carried out at early life stages, it is important to consider the physiological differences between embryonic or larval fish and adult mammals. Another limitation to be noted is the necessity for a balance between the hydrophobicity and hydrophilicity of small molecules. Depending on their molecular weight and hydrophobicity, not all small molecules are readily absorbed by embryos and larvae, possibly leading to false negatives during screening. In a zebrafish-based screen, small molecules are usually simply added to the fish water. Extremely hydrophobic substances, which do not dissolve in water, are unlikely to affect the fish. On the other hand, poor absorption of extremely hydrophilic compounds by the fish can also lead to a

lack of an effect of the small molecule (Berger & Currie, 2007; Crawford et al., 2008).

There are also some country-specific animal rights legislation and institutional bioethics regulations that can limit the zebrafish use (Crawford et al., 2008) and there is also an absence of some mammalian tissues and organs (lung, prostate, skin, and mammary gland) in this model organism (Kari et al., 2007).

Despite these limitations, all characteristics considered above have made the zebrafish an excellent model of choice for whole organism-based small molecule screens (Berger & Currie, 2007).

2.4.3 Zebrafish as a model for inflammation

Phytotherapies have been widely used in folk medicine in many countries to treat several inflammatory diseases. However, there is a lack of scientific study to confirm the real efficacy of the medicinal properties of these plants (Maldini et al., 2009).

Nowadays there is a need of new treatments to cure inflammatory diseases. The current anti-inflammatory treatments are often inefficient and present side effects. Understanding how inflammation works would facilitate to find its cure and thus identify targets for the development of novel classes of drug (Renshaw et al., 2006b). In this context, inflammation can be defined as a complex biological response of vascular tissues to harmful stimuli, such as pathogens, damaged cells, or irritants. It is a defence mechanism aimed to remove the injurious stimuli and initiate the tissue healing process (Maldini et al., 2009).

The only way of studying inflammation as a whole is using *in vivo* models, mainly because of the diverse and dynamic cellular processes involved. In the inflammation process the endothelial cells are activated, first the

neutrophils and then the macrophages which go in the circulation and localize to the inflammatory focus. The adaptive immune system is also activated in the inflammatory response to foreign agents. These processes occur while guaranteeing the vascular integrity (Lieschke, 2006).

Zebrafish larvae are now well-established as an *in vivo* model for inflammation. Lieschke et al. (2001) first showed that leukocytes could be readily visualized in zebrafish larvae though whole-mount histochemical staining for myeloperoxidase activity within the first 48 hours after fertilization, and that transection of part of the larval tail stimulated the migration and accumulation of leukocytes within hours after injury.

Renshaw et al. (2006b) also established a model of inflammation in the zebrafish tail. The authors affirm the transparency of the larvae makes these an ideal model for the study of *in vivo* inflammation. Indeed, Mathias et al. (2006) consider zebrafish embryos an ideal model system to analyze leukocyte migration and inflammatory processes *in vivo*.

The earliest signal recruiting leukocytes to the wound implicate reactive oxygen species (ROS), specifically hydrogen peroxide (H_2O_2), which are released by the inflammatory cells to kill invading microbes (Niethammer et al., 2009; Wheeler & Brändli, 2009).

Other groups have generated transgenic lines expressing enhanced green fluorescent protein (EGFP) under the control of the myeloperoxidase promoter, enabling the direct visualization of live leukocytes and therefore the observation of the inflammatory response in real time (Mathias et al.; 2006; Renshaw et al.; 2006a). These works show that inflammation is induced after transection of the tail of zebrafish larvae and that this inflammation subsequently resolves over a similar time course to mammalian systems. So, it can be concluded that the zebrafish use in combination with other possibilities to analyze the inflammatory response has the potential to be an effective system for the identification of anti-

inflammatory natural products and other small molecules (Renshaw et al.; 2006b; Crawford et al., 2008).

The leukocyte behavior has therefore been studied by several authors in the wounded tail and/or wounded fin in order to obtain information about inflammation and wound healing (Lieschke et al., 2001; Mathias et al., 2006; Renshaw et al., 2006a).

2.4.4 Angiogenesis in zebrafish

The cardiovascular system is one of the first organs composed during vertebrate development and it is essential to life by providing oxygen, nutrients and hormones to organs; controlling the circulation of metabolites; and maintaining tissue homeostasis. For these reasons, the process of vessel formation has being widely studied in the last decades (Baldessari & Mione, 2008). To better screen new compounds that affect blood vessel formation, the assay should ideally be carried out in the natural environment where blood vessels grow. This is possible by using a whole living organism such as zebrafish (Serbedzija et al., 1999; Cross et al., 2003; Baldessari & Mione, 2008).

Blood vessels are primordial to grow embryo organs and to fix wounded adult tissues (Velasquez, 2007). When wounding occurs blood vessels are damaged and neutrophils are the first white blood cells that migrate to the site of tissue damage (Martin & Feng, 2009).

The difference between vasculogenesis and angiogenesis is that in the former, the progenitor stem cells differentiate and give rise to a replacement vascular network, while in the latter, the resident endothelial cells of the wound's adjacent mature vascular network are activated within an existing vessel to proliferate, migrate, and remodel into new vessels (Serbedzija et al., 1999; Velasquez, 2007). So, once the basic pattern of major vessels and plexuses is formed by vasculogenesis, the following sprouting and growth of new vessels

from the pre-existing vessels is named angiogenesis (Baldessari & Mione, 2008). In zebrafish embryos after 28 hpf the main vital processes (for example, all the major vessels, the beat of heart, the circulation presence and angiogenesis) are complete (Kari et al., 2007).

An instability between angiogenesis and vasculogenesis cooperates to the development of several ailments, among them inflammatory and wound-healing illness (Velasquez, 2007). Zebrafish assays can be used not just to identify compounds that reduce angiogenesis but also that ones which stimulate it (Serbedzija et al., 1999).

The technique known as confocal microangiography, allowed Isogai et al. (2001) to examine and describe the vascular anatomy of the developing zebrafish. In this technique fluorescent microspheres of various sizes injected into the blood circulatory system allow the visualization of patent blood vessels in live embryos. This technique led to the first complete and detailed atlas of vascular anatomy of the developing zebrafish embryo and early larva (Isogai et al., 2001) and it is widely used to score vascular phenotypes, such as patterning- and lumen formation-defects. In transgenic lines a fluorescent protein expressed under a specific gene promoter, allows *in vivo* imaging of the organs or cell structures where the endogenous gene expression is normally driven by that promoter (Kidd & Weinstein, 2003; Baldessari & Mione, 2008; Crawford et al., 2008). For the study of angiogenesis, transgenic zebrafish lines have been developed which express EGFP under the control of vasculature-specific promoters such as *fli-1* (Lawson & Weinstein, 2002) and *flk* (Covassin et al., 2009).

2.5 Cryopreservation

2.5.1 Concept and advantages

Cryopreservation is the conservation of plant materials at ultra-low temperatures which guarantees that all metabolic processes be arrested. The most common refrigerant used in the cryopreservation is liquid nitrogen (LN) (González-Benito et al., 2004; Zhu et al., 2006). Cryopreservation is a key tool not only for the conservation of genetic resources but also for storage of specific plant tissues that present medicinal proprieties, requiring a minimum of space and maintenance (Joshi & Teng, 2000; Urbanová et al., 2006; Xue et al., 2008; Lambert et al., 2009; Lu et al., 2009; Popova et al., 2009). Cryotherapy can also allow pathogen eradication at a high frequency (Helliot et al., 2003; Wang & Valkonen, 2009).

In order for plant cells and tissues to be able to be successfully cryopreserved, it is fundamental to optimize each experimental procedure step (Urbanová et al., 2006) and to understand the basic principles of cryobiology (Day et al., 2008). This success can be characterized by high survival rate, maintenance of physiological competences, biosynthetic ability and genetic stability (Urbanová et al., 2006)

So, among the several advantages cryopreservation presents the low risk of contamination and human error, less expensive and prolonged storage time (Winkelmann et al., 2004), no requirement of subcultures, reduced threat of somaclonal variation and limited space requirement (González-Benito et al., 2004) can be mentioned.

Despite the fact that research on plant cryopreservation is considerably increasing over the last few years, its application to preserve plant biodiversity in germplasm collections is still limited (Panis & Lambardi, 2005; Zhu et al., 2006).

2.5.2 Basic principles

A wide diversity of different biological materials have been successfully cryopreserved (Winkelmann et al., 2004; Verleysen et al., 2005a,b; Bekheet et al., 2007; Xue et al., 2008) and all of them demand the knowledge of basic principles (water behavior, cryoinjury and cryoprotection) and fundamental processes (during sample pre-treatment, cooling, and warming). The difference in cryopreservation success can be related to the variation of plant samples, its water status and changes in original protocols which not means that it will be achieved success each time, mainly for highly desiccation-sensitive species (Block, 2003; Day et al., 2008).

To understand water behavior it is first important to know that it presents four states (liquid, glass, solid and vapor) which are widely influenced by temperature (and pressure). The cryoconservationists manipulate the liquid, glass and solid water states in order to avoid the lethal intracellular ice formation (Day et al., 2008).

The intracellular water crystallisation can occur during the cooling and/or the thawing steps. During the thawing, the crystallisation of a vitrified solution is termed devitrification (or sometimes cold-crystallization). The more vacuolized the plant cells are, the more water that needs to be withdrawn from the cells to prevent lethal ice crystallisation. The key to successful cryopreservation thus lies more in the induction of a tolerance towards dehydration instead of a tolerance to the freezing process itself (Helliot et al., 2003; Wang et al., 2004; Gonzalez-Arnao et al., 2008).

To better understand freezing processes and to improve the cryopreservation of cells in suspensions the development of specific protocols should be executed for each line species (Bernemann et al., 2007).

2.5.2.1 Cristallization – concept and how to avoid it

The crystallization, also known as ice crystal formation, provokes damage in the cellular integrity. This complex process can occur during freezing as well as during warming procedures and comprise a number of critical steps (nucleation, growth of crystals and/or recrystallization) (Gonzalez-Arnao et al., 2008).

There are two types of nucleation: homogeneous and heterogeneous. The homogeneous nucleation is a process which occurs spontaneously during cooling at a temperature lower than the melting point of the pure crystalline phase (ice). For pure water, this temperature is approximately –40ºC. This nucleation type is a thermodynamic process, simultaneous with ice formation, in which exothermic energy is released. Heterogeneous nucleation is the formation of ice nuclei (seeds) on a catalytic surface. The loss of post-thaw viability is normally caused by cell injuries which occur during the the rapid transition of extra- and intracellular supercooled water into ice. So, to avoid the intracellular ice formation, which causes irreversible damage to cell membranes thus destroying their semi-permeability, many procedures are available. One such procedure is cooling at a controlled rate which requires the control of extracellular ice formation when ice crystals are initiated (Helliot et al., 2003; Panis & Lambardi, 2005; Day et al., 2008; Gonzalez-Arnao et al., 2008).

There are many cryopreservation techniques which ensure high propagule recovery by minimizing the desiccation and freezing injury. Among them, slow cooling, air drying, vitrification, encapsulation–dehydration and encapsulation–vitrification (González-Benito et al., 2004; Xue et al., 2008).

In the controlled rate system, or slow cooling, water molecules move across the cell membrane to maintain the equilibrium. It is, however, important that this does not result in excessive, deleterious concentration of solutes. In this context, the addition of cryoprotectants is essential because they offer not only

physical protection against cells damage but also stabilization of proteins and membranes acting in this way as antioxidants and or molecular chaperones. This protection occurs as water is lost from the cell and obviates the potentially lethal reduction in cell volume. There are two main types of chemical cryoprotectants: (i) penetrating/colligative ones and (ii) non-penetrating/osmotic active ones and they are frequently used in combination. After this, and when cells are exposed to LN, the intracellular medium can be so concentrated that it becomes vitrified. Glasses are highly viscous solidified liquids which have unorganized structure and because of this they are far less damaging to cells compared to ice crystals. Their behavior in biological tissues is highly complex as water can devitrify and convert back to ice. In addition, if some water molecules remain available for nucleation, the ice crystals formed are so small they are presumed to be harmless. It is important to emphasize that a minimal amount of water remains essential for a cell to survive freezing (Chen et al., 1984; Ishikawa et al., 2006; Bekheet et al., 2007; Day et al., 2008).

Another cryoprotective strategy used in cryopreservation is known as ultra fast cooling technique. In this system, cells and tissues are allowed to be plunged into LN directly, but for this, a vitrified state is demanded. There are two types of vitrified systems: (i) system where formation of ice just occurs in the extracellular medium (that is also the case with slow cooling), and, (ii) system where both extra- and intracellular compartments are vitrified, therefore they are completely ice free (Day et al., 2008).

A preculture phase is also often used to increase tolerance of explants to dehydration and subsequent freezing in liquid nitrogen. Sucrose is frequently added to culture media for this purpose (Danso & Ford-Lloyd, 2004; Wang et al., 2004; Winkelmann et al., 2004; Verleysen et al., 2005a,b; Wang et al., 2005) due to its e□ects on the water content of the cells and its possible protecting e□ects on proteins and membranes (Panis et al., 2006).

Rewarming is another critical step to successfully cryopreserve cells and tissues since small harmless ice crystals formed during cooling are capable of growing during rewarming to a size that may cause damage. This mainly occurs if rewarming is conducted too slowly. Also, a previously formed glass can devitrify and form ice crystals which will fracture cell structures (Day et al., 2008).

Identification and consequently correction of all the critical points mentioned above during cryopreservation process would help to improve post-storage recovery of cryopreserved plant material (Urbanová et al., 2006).

2.5.3 Applications of cryopreservation

Recent advances in tissue culture technology have greatly increased the variety of organs and tissues that have been tested for storage in liquid nitrogen (Panis & Lambardi, 2005), among them cell suspensions, pollen, embryogenic cultures, somatic and zygotic embryos, shoot apices or meristems (González-Benito et al., 2004). For each species and tissue type, a cryogenic procedure needs to be developed or adapted to the natural freezing resistance of the species, explant size and type and water content (Danso & Ford-Lloyd, 2004; Wang et al., 2005; Panis et al., 2006).

The cryopreservation of non-organized tissues (cell suspensions and calli) normally aims at the maintenance of specific characteristics of tissues that can be lost during normal *in vitro* maintainance. For example, cryopreservation is important to preserve biosynthetic capacity of valuable compounds of pharmaceutical importance which are produced by cells of medicinal plants. The method that is often used for these tissues is slow-cooling system (Panis & Lambardi, 2005; Urbanová et al., 2006).

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CHAPTER 2

In vitro culture and cryopreservation of *Byrsonima intermedia*

1 ABSTRACT

The *Byrsonima* genus, especially *B. intermedia* species, is an interesting shrub from the Brazilian Cerrado which has been extensively used for both feed purposes and therapeutic activities. The aim of the present study was to gather information about *B. intermedia* cell callus induction, cell suspension cultures, their growth and cryopreservation. For this we established a protocol for callus induction at different environmental conditions (under light and dark conditions); cell suspensions culture protocols were established and the effects of explant choice which originated calli and inoculum densities were studied. Moreover, growth curves of cell suspension cultures were established and their different aspects observed (heterogeneous and homogeneous). We also checked the effects of applying different techniques and cryoprotectants types and concentrations during the pre-culture and pre-treatment phases on cell growth and re-growth after cryopreservation. We were able to successfully induce calli for *B. intermedia* species and when cultured at different light conditions, different characteristics were observed. Growth curves of *B. intermedia* cell suspensions with exponential and stationary phase were established. No regrowth of *B. intermedia* cells could be observed within 4 weeks after cryopreservation.

Key-words: calli; cell suspensions; growth curves; long-term preservation.

2 RESUMO

O gênero *Byrsonima*, especialmente a espécie *B. intermedia*, é um arbusto do Cerrado brasileiro que tem sido amplamente utilizado por suas características alimentares e terapêuticas. O objetivo do presente estudo foi obter informações sobre a indução de calos, suspensão celular e seu crescimento e criopreservação de células de *B. intermedia*. Estabeleceu-se então um protocolo para indução de calos em diferentes condições ambientais (claro e escuro); protocolos de cultivo de suspensões celulares foram estabelecidos e os efeitos da escolha do explante que originou calos e densidades do inóculo inicial foram estudados. Além disso, as curvas de crescimento de suspensões celulares foram estabelecidas e seus diferentes aspectos observados (heterogêneo e homogêneo). Verificou-se também os efeitos da aplicação de diferentes técnicas e tipos e concentrações de crioprotetores durante as fases de pré-cultivo e pré-tratamento sobre o crescimento celular após criopreservação. Calos são induzidos com sucesso para a espécie *B. intermedia* e quando estes são cultivados em diferentes condições de luz, diferentes características são observadas. Curvas de crescimento com fases exponencial e estacionária são estabelecidas para suspensões celulares de *B. intermedia*. Nenhum crescimento celular de *B. intermedia* pode ser observado dentro de 4 semanas após a criopreservação.

Palavras-chave: calos; suspensões celulares; curvas de crescimento; preservação a longo prazo.

3 INTRODUCTION

The two essential reasons which are responsible for the increasing interest of much research and pharmaceutical associations in plants from the Brazilian Cerrado are: (i) the extended use of land for both cattle raising and agricultural purposes which result in devastation of large areas and; (ii) the use of some plant species, which contain biologically active secondary metabolites, in folk medicine (Cunha et al., 2009).

The study of plants that have traditional medicinal use is worthy not only because they are potential source of chemotherapeutic drugs, but also as a safety-measure for the continued use of medicinal plants (Cardoso et al., 2006).

The genus *Byrsonima* which belongs to the Malpighiaceae family is known in Brazil to be employed as food to make juice, fruit-jelly and liquer as well as in folk medicine (Sannomiya et al., 2005).

The constant loss of information about native plants used in the folk medicine in Brazil is mainly caused by the rapid destruction of Brazil's botanically rich native ecosystems over the last years (Brandão et al., 2008).

Byrsonima intermedia, commonly known as murici-pequeno, is a shrub belonging to the family Malpighiaceae, and is a very important resource for feed purpose but also for its physiological and therapeutic effects. Sannomiya et al. (2007) evaluated the leaf extracts of *B. intermedia* in mutagenic assay with *Salmonella typhimurium* and mice. They observed that the methanolic extract presented signs of mutagenic activity for some *Salmonella strains*, however mutagenicity was not observed *in vivo*. The phytochemical analysis of methanol extract from *B. intermedia* leaves furnished (+)-catechin, quercetin-3-*O*-α-L-arabinopyranoside, quercetin-3-*O*-β-D- galactopyranoside, quercetin-3-*O*-(2"-*O*-galloyl)-β-galactopyranoside, quercetin-3-*O*-(2"-*O*-galloyl)-α-

arabinopyranoside, methyl gallate, gallic acid, amentoflavone and quercetin. Other data showed that aqueous portion of *B. intermedia* was effective healing action in chronic gastric disease (Santos et al., 2009). These studies lead to the possible use of the *B. intermedia* compounds as "new" medicament for the treatment or prevention of the metabolic syndrome.

Plant tissue (or sometimes called "*in vitro*") culture techniques allow the induction and maintenance of aseptic proliferating undifferentiated masses of cells known as callus or cell suspensions (Paz et al., 2006). *In vitro* cultures have been used as an alternative to agricultural processes for producing interesting medicinal compounds. In this context, callus and cell suspensions (the non-organized tissues) could be stored for the long term in order to maintain specific tissues features that could be lost during normal *in vitro* maintenance such as production of secondary metabolites that have pharmaceutical importance (Kim et al., 2002; Estrada-Zúñiga et al., 2009). Cell suspension and callus culture is a key tool not only to establish an efficient protocol to produce valuable secondary metabolites but also to study cell growth parameters (Khiet et al., 2006).

Many medicinal wild plants are threatened mainly because they are used as extracts or infusions by the folk population. One way to decrease this problem is by using plant tissue cultures which provide an alternative extraction procedure that may use cell, tissue and organ of plants in an aseptic, controllable, automatized way and result large-scale cultivation. In this context, cell suspension cultures represent one of the best systems for producing secondary metabolites because (i) they can guarantee homogeneous production since they offer a homogeneous system and also (ii) they grow quickly (Estrada-Zúñiga et al., 2009).

In this context, *B. intermedia* suspension cell lines can provide an important genetic resource used for secondary metabolite studies. However, the maintenance of *B. intermedia* cells by weekly subculture is laborious and entails

the risk of losing the line because of contamination, somaclonal variation, or technical error. Cryopreservation in liquid nitrogen (LN) provides a reliable method for long-term storage of *B. intermedia* cells and helps to secure against loss of the cell line. Moreover it will save labor and prevent further genetic changes during storage in these unique suspension cultures.

To date there have been no reports on suspension culture and cryopreservation of *Byrsonima intermedia* species.

In the study reported here we established a protocol for culture cell suspension that can eventually be used for the production interesting secondary metabolites and examined the effects of applying different cryoprotectants in the pre-culture and pre-treatment periods on cell growth and re-growth after cryopreservation. In addition, the influence of the length of the pre-culture period was investigated. The aim of the present work was then to gather information about *Byrsonima intermedia* cell suspension cultures, callus induction, growth curves and cryopreservation.

4 MATERIAL AND METHODS

4.1 *Byrsonima intermedia* callus induction

Mature fruits of *Byrsonima intermedia* (Malpighiaceae) were collected from native plants growing in Ijaci, state of Minas Gerais, MG, Brazil. The voucher of the plant sample was registered at the Herbarium Esal of the Biology Department of the Universidade Federal de Lavras (17.601), Brazil and their seeds and embryos excised and used as explants. For this, by using a hammer, the endocarps were opened and the seeds removed. Seeds were then immersed in 70% alcohol (v/v) for 30 seconds and then in a solution of 0.5% NaOCl active chlorine for 10 minutes. Under aseptic conditions the seeds were subsequently washed three times in distilled and autoclaved water and their teguments were removed to extract the embryos. Then, embryos were put on MS medium (Murashige & Skoog, 1962) with 4.52µM 2,4-D, 0.09M sucrose and 0.6% agar (Nogueira et al., 2007). They were cultured using two conditions: (i) at 25±2ºC under continuous 50 µE m^{-2} s^{-1} illumination provided by 36 W Osram cool-white fluorescent tubes (L) and (ii) at 25±2ºC under dark conditions (D) for 60 days. The calli thus obtained were then as a whole subcultured on the same culture medium and from each condition (L or D), they were maintained under the two different conditions for 45 days, giving rise to 4 treatments; LL, DL, LD and DD. After this period, calli from dark were put in light (giving rise to LDL; DDL) and calli from light were put in dark conditions (giving rise to LLD; DLD) (without subculturing) where they remained for another two months. After this, we evaluated the difference in calli appearance and colour.

4.2 Growth curves of *Byrsonima intermedia* cell suspension cultures

4.2.1 Cell suspension cultures

To obtain heterogeneous (Figure 1a) and yellow homogeneous (Figure 1b) cell suspension cultures, *in vitro* germinated plants of *Byrsonima intermedia* were used as explants. Leaf segments (≈ 0.25 cm^2) were transferred onto MS medium supplemented with 4.52µM 2,4-D, 0.09M sucrose and 0.6% agar. The explants were maintained in dark conditions at 25±2°C for 60 days. The friable heterogeneous and/or yellow calli (≈ 0.2 cm^3) thus obtained were then transferred into a 25 mL Erlenmeyer flask containing 10 mL maintenance medium (M medium) which consisted of MS liquid medium with 2.26µM 2,4-D, 4.65µM kinetin and 0.09M sucrose. They were transferred onto a rotary shaker (80 rpm) and incubated in the culture room at 25±2°C under darkness. The growth rate experiments (giving rise to growth curves) were initiated 15 days after their last subculture.

To obtain red homogeneous cell suspension cultures (see Figure 1c) the calli were cultured as described in item 4.1 (i) (under condition L). The friable red calli (≈ 0.2 cm^3) obtained were then transferred to 10mL M medium into a 25 mL Erlenmeyer flask and transferred to a rotary shaker (80 rpm) and incubated in the culture room under continuous 50 µE m^{-2} s^{-1} illumination provided by 36 W Osram cool-white fluorescent tubes 25±2°C.

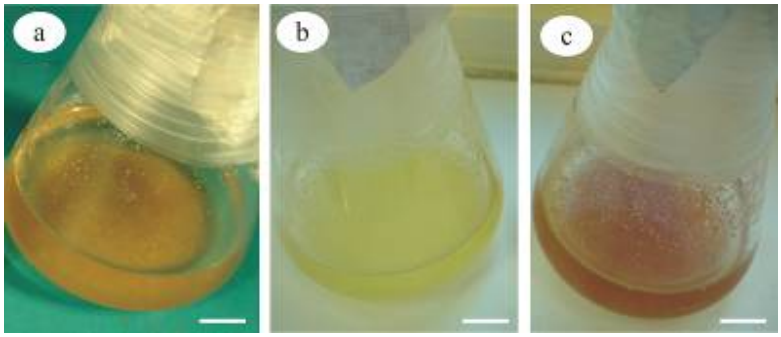

FIGURE 1 *Byrsonima intermedia* cell suspensions used in the growth curve experiments: (a) heterogeneous; (b) homogeneous yellow and (c) homogeneous red. Scale bars = 2 mm.

4.2.2 Growth curve

Using a heterogeneous suspension culture of *B. intermedia*, cells and clumps were inoculated in fresh M medium two weeks after the last subculture. The dilution was adjusted to 1/10 (=volume of the inoculum (old medium + cells)/ volume of fresh medium). Different inoculums were used (Settled cell volume (SCV) of 0.25, 0.5, 0.75, 1.0, 1.5 and 3.0 mL). Three 250 mL Erlenmeyer flasks were inoculated with 88 mL of the dilution for each SCV after which they were transferred to a rotary shaker (80 rpm) and incubated in the culture room at 25°C. Every 2 to 5 days, the SCV of each sample was measured by allowing the cells to settle for approximately one hour in a 10 mL sterile graduated tube.

For the homogeneous cell suspensions culture, 3mL SCV inoculums were used two weeks after the last subculture. The red ones were incubated in the culture room under light conditions while the yellow were incubated under dark conditions.

Each average (three repetitions) was transformed into a logarithm scale (with base 10).

4.3 Cryopreservation of *Byrsonima intermedia* cell suspension cultures

Cryopreservation has been carried out according to the techniques developed and used for routine cryopreservation of banana accessions held by Bioversity at the Laboratory of Tropical Crop Improvement, Katholieke Universiteit Leuven (KULeuven), Belgium (Panis, 2009). This laboratory hosts, under the authority of the International Network for the Improvement of Banana and Plantain (INIBAP), the largest collection of bananas in the world.

4.3.1 Plant material and maintenance of cell suspension

Cell suspensions derived from *Byrsonima intermedia* leaves were used, 7-10 days after their last subculture. At this stage cells were in their mid-exponential phase. For the first test (see below) different suspensions colors (yellow, white, red), culture conditions (dark and light) and culture medium (MS medium supplemented with 0.09M sucrose and 2.26µM 2,4-D x 4.65µM kinetin (M medium – maintenance medium) or 2.26µM 2,4-D or 4.52µM 2,4-D were used. In the second to fourth experiment (see experimental design), only yellow cell suspension cultures kept in M medium and under dark conditions were used. For the fifth experiment besides yellow cell suspensions we used red ones (obtained from calli originated from embryos cultured as described in item 4.1 (i)) cultured in the same medium but under continuous light at 50 µE m^{-2} s^{-1}. Cultures were kept at 25±2ºC at all times and around two weeks after the last subculture.

4.3.2 Experimental set-up

4.3.2.1 Slow-freezing (experiment 1)

Cells were allowed to settle in a graduated centrifuge tube and the old medium was removed. New liquid M-medium with 0.5 M sucrose was added until a final settled cell volume (SCV) of 30% (v/v) was obtained. An equal volume of sterile M + 0.5M sucrose medium containing 15% (v/v) dimethylsulfoxide (DMSO) was gradually transferred to the concentrated cell suspension over a period of one hour at room temperature. As such, the final cryoprotective solution contained 7.5% (v/v) DMSO and 0.5M sucrose.

For slow freezing, the cryotubes containing 2 mL of cryoprotected cell suspensions were placed in a Nalgene^{TM} cryo 1ºC Freezing Container ("Mr Frosty"). This simple freezing device consists of a plastic container holding 250 mL of isopropanol. This container was then transferred into a freezer (-80ºC) that allowed a cooling rate of about 1ºC min^{-1}. As soon as the temperature of -40ºC was obtained, cryotubes were plunged in liquid nitrogen (-196ºC) for further storage.

After at least 30 minutes storage, cryotubes were rapidly thawed in a beaker filled with sterile water at 40ºC for about 1.5 to 2 minutes until most of the ice had melted. Thawed cells were plated on semi-solid M or MS medium with 3% sucrose growth regulator free in 90 mm Petri dishes. Semi-solid M medium was used to try to re-establish the cell suspension. MS medium with 0.09M sucrose growth regulator free was employed to try to regenerate plants. During the first week following cryopreservation, Petri dishes with a sterile filter paper were always placed in the dark.

4.3.2.2 Slow-freezing and Encapsulation-vitrification techniques

Both techniques were executed for *B. intermedia* cell suspensions as well as for embryogenic cell suspensions cultures of *Musa* sp. (line 175 of the

cultivar Grande Naine). This experiment was executed in order to compare different plant species.

4.3.2.2.1 Slow-freezing with pre-culture and DMSO effect (experiment 2)

A pregrowth phase was included to increase the freezing tolerance of tissues cultures. The osmotically active compounds sucrose (0.4M) and sorbitol (0.4M) were tested for one and three days. The cell suspension culture was cultured at 25±2ºC in darkness.

The same procedure described above, in experiment 1, was executed. However in this experiment, we also tested the effect of a 7.5% (v/v) DMSO treatment (without freezing) on the cell suspensions.

4.3.2.2.2 Encapsulation-vitrification (experiment 3)

Alginate beads are formed by dropping small volumes of 3% (w/v) low viscosity alginic acid made up in liquid M medium with a 2mL plastic Pasteur pipette into 100mM calcium chloride solution, also prepared in liquid M medium. The amount of cells used was at a rate of 1:1 (v/v) (sedimented cells: alginate solution). Each 100 mL of this mixture contained approximately 140 beads, so the amount of cells in each bead was ≈ 0.04mL. A minimum of 5–20 similar-sized beads were used for each treatment. The beads were allowed to polymerise in the calcium chloride solution for 20 minutes and were then retrieved with a small sterile sieve and washed three times in liquid M medium. The filter sterilized loading solution (LS) contains 2M glycerol and 0.4M sucrose dissolved in MS medium (pH 5.8). The beads were left in the LS for varying time periods (1, 2, 3 and 4h). After 2 of hours loading, the solution was replaced by ice-cooled PVS2 solution. The PVS2 solution consists of 30% (w/v) glycerol, 15% (w/v) ethylene glycol (EG), 15% (v/v) DMSO and 0.4M sucrose. All these compounds were dissolved in MS medium, pH adjusted to 5.8

followed by filter sterilization. The beads were subjected to the PVS2 solution for 30 min, 1 and 2h at 0ºC. After the PVS2 treatment, the beads were plunged into liquid nitrogen in a sterile small sieve. The beads were kept in liquid nitrogen for at least 20 min. For re-warming, beads were placed in recovery solution (RS) in a big Petri dish at room temperature for 30 min. The RS consists of 1.2M sucrose dissolved in MS medium (pH 5.8).

Unloaded beads were placed onto semi-solid M medium containing 0.3M sucrose instead of 0.09M. After 24 h, the beads were transferred onto normal M medium.

Alternatively, recovery could be executed in liquid medium. For that, re-warmed beads were transferred to 100 mL Erlenmeyer flasks containing 15 mL of liquid M medium and placed on a rotary shaker at 80 rpm.

After one week of culture in dark conditions, Petri dishes and flasks were transferred to continuous 50 μE m^{-2} s^{-1} illumination provided by 36 W Osram cool-white fluorescent tubes.

4.3.2.3 Toxicity tests (experiment 4)

The different cryoprotective solutions that are tested were gradually transferred to the concentrated cell suspension over a period of 60 minutes at room temperature with exception of the treatments were different exposure times were tested. All treatments are presented in Table 1.

TABLE 1 Cryoprotective solutions used to test their toxicity on *Byrsonima intermedia* cell suspension cultures.

Treatments	Medium used	Control	Label
DMSO 7.5% (v/v) exposure time (15, 30, 45 and 60 min)	modified M medium	1 = normal M medium 2 = modified M medium	-
DMSO 5% (v/v)	normal M medium	A = normal M old medium B = normal M fresh medium C = modified M fresh medium	A
DMSO 5% (v/v)	modified M medium		B
DMSO 7.5% (v/v)	normal M medium		C
DMSO 7.5% (v/v)	modified M medium		D
Glycerol 5% (w/v)	normal M medium		E
Glycerol 5% (w/v)	modified M medium		F
Glycerol 10% (w/v)	normal M medium		G
Glycerol 10% (w/v)	modified M medium		H
Glycerol 15% (w/v)	normal M medium		I
Glycerol 15% (w/v)	modified M medium		J
Cryoprotective mixture 1	normal M medium		K
Cryoprotective mixture 1	modified M medium		L
Cryoprotective mixture 2	normal M medium		M
Cryoprotective mixture 2	modified M medium		N
PVS2 exposure time (15, 30, 45 and 60 min) at room temperature	-	normal M medium	-

Cryoprotective mixture 1 = 0.5M DMSO, 0.5M glycerol and 1M sucrose; cryoprotective mixture 2 = 0.25M DMSO, 0.25M glycerol and 0.5M sucrose; modified M medium = M medium that contains 0.5M sucrose; normal M medium = M medium that contains 0.09M sucrose; old medium = medium that has spend 7-10 days in the presence of a cell suspension.

Another treatment consisted of a pre-culture medium added for 1 and 3 days. Mannitol 6% (w/v), sucrose (0.1, 0.2 and 0.3M) and sorbitol (0.3M) were the pre-culture tested. The control consisted of M medium containing 0.09M sucrose, being Control 0 related to 0 day period culture and Control 1 for 1 and 3 days.

4.3.2.4 Slow-freezing – cryoprotectors and pre-culture (experiment 5)

Cryopreservation using slow-freezing was executed as described above, however using (i) pre-culture medium that consisted of sucrose (0.1, 0.2 and 0.3M) for 1 and 3 days followed by cryoprotective modified M medium with/without 5% (v/v) DMSO and; (ii) the following cryoprotectant solutions:

- normal M medium (0.09M sucrose) added with DMSO 5 (A) and 7.5% (C) (v/v), glycerol 5% (E) (w/v) and the cryoprotective mixture 2 (0.25M DMSO, 0.25M glycerol and 0.5M sucrose) (M) and;
- modified M medium (0.5M sucrose) added with DMSO 5 (B) and 7.5% (D) (v/v) and glycerol 5% (F) (w/v).

4.3.3 Re-growth

For all experiments, three to four weeks following cryopreservation/toxicity tests, re-growth was evaluated according to their callus formation capacity (%). The visual classification used is shown in Figure 2. In the first experiment, chemical cell viability of the thawed cells was determined by the fluorescein diacetate (FDA) test (Widholm, 1972) whereby surviving cells fluoresce very brightly under ultraviolet illumination.

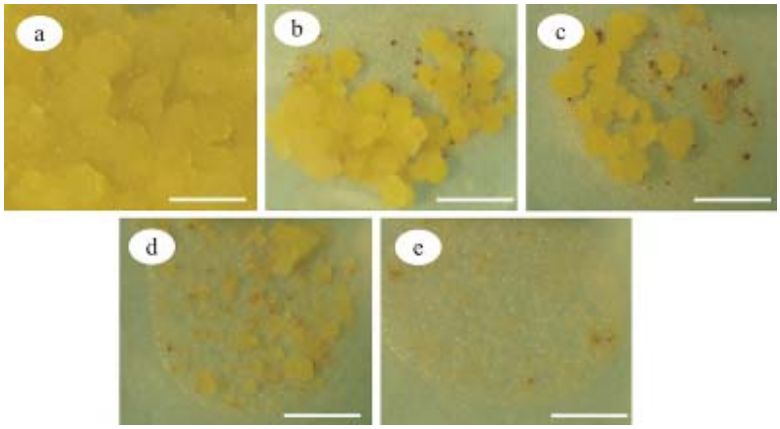

FIGURE 2 Classification used to evaluate re-growth after three to four weeks following cryopreservation/toxicity tests according to the calli formation (%): (a) 100; (b) 75; (c) 50; (d) 25; (e) 0. Scale bars = 5mm.

4.3.4 Statistics

All experiments were carried out with at least 5 repetitions/treatment and the values were given as means ± S.E.M. (Standard error of the mean). Data were analyzed using Sisvar® software and statistical significance was assessed by one-way ANOVA. P values less than 0.01 were considered significant.

5 RESULTS AND DISCUSSION

5.1 *Byrsonima intermedia* callus induction

When explants were initiated on 4.52µM 2,4-D, callus formation could be observed, 60 days after inoculation. Different types of calli arose from explants cultured under different conditions (Figure 3a, b). They presented a fast growth occupying almost all medium surface in the test tube; their structure is variable containing friable and hard parts; and the color was differed from the different conditions used, being calli cultured in dark conditions more homogeneous with yellow and white colors predominance while calli grown in light conditions presented an heterogeneous aspect with red, yellow, white and green color.

When the calli were exposed to different environmental conditions some differences in color could be observed (Figure 3c, j). Calli from dark conditions (D) (Figure 3a) subcultured after 60 days and maintained in dark conditions (DD) (Figure 3c) for 45 days continued to be yellow with friable and hard parts, while that ones that were transferred to light conditions (DL) (Figure 3d) became more heterogeneous, presenting many brown cells.

When calli from light conditions (L) (Figure 3b) were subcultured after 60 days and then transferred to the dark (LD) (Figure 3e), heterogeneity stopped. However calli from this condition (L) maintained in light (LL) (Figure3f) continued differentiation and generally became brown.

When DD and LD calli were transferred to the light (DDL; LDL) (Figure 3h,j), both calli type became brown. However, when DL/LL calli were put in the dark (DLD; LLD) (Figure 3g,i), calli differentiated, but not all cells turned completely brown.

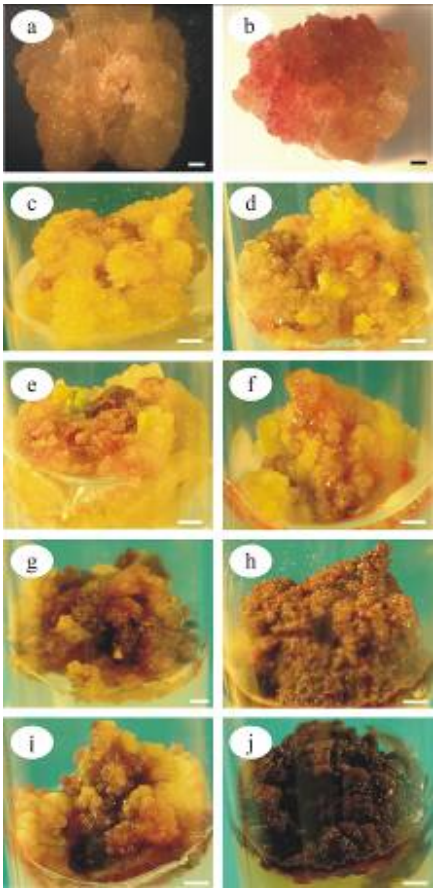

FIGURE 3 *Byrsonima intermedia* calli induction induced from embryos and cultured on M medium in different environmental conditions. (a) after 60 days in dark condition (D); (b) after 60 days in light condition (L); (c) D after 45 days subculture maintained in dark conditions (DD); (d) D after 45 days subculture maintained in light conditions (DL); (e) L after 45 days subculture maintained in dark conditions (LD); (f) L after 45 days subculture maintained in light conditions (LL); (g) DL after 2 months transferred to dark conditions (DLD); (h) DD after 2 months transferred to light conditions (DDL); (i) LL after 2 months transferred to dark conditions (LLD); (j) LD after 2 months transferred to light conditions (LDL). Scale bars = 2mm.

Ling et al. (2008) observed that 2,4-D was more suitable in inducing cell differentiation rather than for maintenance of cell growth of *Ficus deltoidea* cultures for the long-term. The 2,4-D treatments generally did not produce a healthy sigmoidal growth curve but it was rather linear and showed slow growth. In the case of *B. intermedia* cell cultures the addition of 2,4-D was effective in both induction and maintenance of calli. However, this growth regulator might also be involved in the differentiation observed in these calli.

Some calli can retains the ability to develop into any plant organ whether it is a root, shoot or leaf, and this using the appropriate growth hormone concentrations (Junaid et al., 2006; James et al., 2008). However, other undifferentiated tissues, like those obtained in the present study, lost their ability to regenerate. There is evidence in the literature (Nezbedová et al., 1999) that the accumulation of secondary metabolites is growth dependent and in some cases their presence can be positively related to the presence of 2,4-D in the medium. Thus, taking into account our main objective, establishing cell lines that produce interesting secondary metabolites, 2,4-D can be a good growth regulator not just for calli induction/maintenance, but also for secondary metabolites production.

Kinetin concentration variation can significantly influence callus induction in some species. This was the case of *Tecoma stans* calli induced from leaves (López-Laredo et al., 2009). They were initiated without kinetin but their induction was enhanced with increased concentration. However, to initiate *B. intermedia* calli kinetin addition was apparently not necessary.

Light plays a role in both growth and secondary metabolite production (Kim et al., 2002). Many of the enzymes in the terpenoid and other alkaloid pathways are regulated by light. According to Ling et al. (2008) light may inhibit or stimulate the production of specific secondary metabolites. For example, anthocyanins cannot be produced in some cells grown under dark conditions.

Zhou et al. (2008) studied influence of light on the anthocyanin level in transgenic tobacco calli. The average biomass of the red calli increased when they cultured under light conditions (16-h light/8-h dark) comparing with the completely dark culture condition. Also the anthocyanin biosynthesis changed depending on the enviromental cultures used. Anthocyanin levels in red calli reached approximately twofold under light condition compared to fully dark condition, showing that the biosynthesis of anthocyanins in red calli was enhanced by light. We observed similar reactions in *B. intermedia* calli under light conditions with respect to the production of red cell line (Figure 1b) that could indicate yield of anthocyanin pigmented calli.

Light stimulated the production of hydrolysable tannin in *Oenothera tetraptera* shoot tissue culture (Taniguchi et al., 2002). Light also influenced the content of phenolic compounds and flavonoids and antioxidant activity in *Tecoma stans* callus originated from leaves. These calli presented higher amount of the related compounds as well as antioxidant activity under a 16 hours photoperiod than in darkness, in despite of their growing slower (López-Laredo et al., 2009). These findings suggest a regulated response to light of the biosynthetic pathway of total concentration of phenolic compounds and flavonoids in *T. stans* callus.

Related to the cells growth light also could have a effect, such as the results obtained in the cell suspension culture of *Ficus deltoidea* which produced a higher growth rate under the light photoperiod than in darkness (Ling et al., 2008).

In contradiction to the studies of Zhou et al. (2008) and Ling et al. (2008), Almeida et al. (2001) studying the induction and development of callus of papaya observed that calli maintained in dark condition presented the best growth. Explants easily became brown and even necrotic in light conditions (Gow et al., 2009). Low growth, reduction of the regenerative ability and even

death can result from brown callus (He et al., 2009). The phenotypically brownish calli is probably due to the senesce of this cultured calli (Zhou et al., 2008). López-Laredo et al. (2009) controlled the oxidation by removing brown cells when subculturing the calli. All these findings could explain the high differentiation and callus browning found in *B. intermedia* cells.

He et al. (2009) investigated the effect of browning on *Jatropha curcas* callus and related this effect to enzymatic browning. These authors also observed that calli cells that were not brown had a tight arrangement, whereas browning callus cells were disorderly and loose. During subsequent callus subcultures the decrease of green and yellow pigments is followed by the gradual change into brown callus which first appear as small brown spots in the interior of callus and then gradually extended to the surface.

Buffa Filho et al. (2002) studied the influence of light during cell tissue culture on the accumulation of some secondary metabolites of *Maytenus ilicifolia* and observed no difference among the three treatments used (24 hours light, 16 hours light/8 hours dark photoperiod and 24 hours dark). Also plants of *Valeriana glechomifolia* presented a good *in vitro* growth and bioactive valepotriate production and the study of the light condition showed that plants grown under light and dark had similar weight increase and valepotriate yield (Russowski et al., 2006).

Kavapyrone distribution in roots (*in vivo*), callus, and roots (*in vitro*) from *Piper methysticum* were studied by Smith et al. (2002). The authors observed higher levels of methysticin and kavain in *in vivo P. methysticum* roots, while the relative levels of bioactive yangonin and desmethoxy-yangonin were comparable to callus tissues, showing that these tissues can provide a good alternative for the production of some secondary metabolites.

Estrada-Zúñiga et al. (2009) found that *Buddleja cordata in vitro* cultures (white callus, green callus and root) produced higher phenylpropanoid

concentrations compared to the leaves of wild plants. These authors concluded consequently that *in vitro* cultures of this species represent a potential source for producing phenylpropanoids instead of wild plants. Also Buffa Filho et al. (2002) observed the production of bioactive compounds of *Maytenus ilicifolia* was higher in the *in vitro* system when compared with the *in natura* plant.

All these studies prove the applicability of using tissue culture explants, mainly callus or cell suspensions, as source of secondary metabolites instead using wild *in vivo* plants.

5.2 Growth curves of *Byrsonima intermedia* cell suspension cultures

5.2.1 Heterogeneous cell suspensions

All six inoculum densities of *Byrsonima intemedia* heterogeneous cell suspensions followed a sigmoidal curve with respect to SCV, with distinct exponential and stationary phases (Figure 4). The 3 mL inoculum resulted in an optimal growth with growth which lasts until day 14. The 1.5 mL inoculum grows until day 19 and the remaining ones until the end of the measuring period.

The exponential growth curve can be better explained in practical terms by using a transformation. For this a logarithm scale (with base 10) was applied. When the points are following a line, growth is exponential (Figure 4b). The 3 mL inoculum resulted in an optimal growth with an exponential growth which lasts until day 7. So, the SCV reached the stationary phase after a culture period of 7 days for the 3 mL inoculum, while this was 14 days for the 1.5 and 1.0 mL inoculums while the 0.25; 0.5 and 0.75 mL inoculums increased exponentially until the last day.

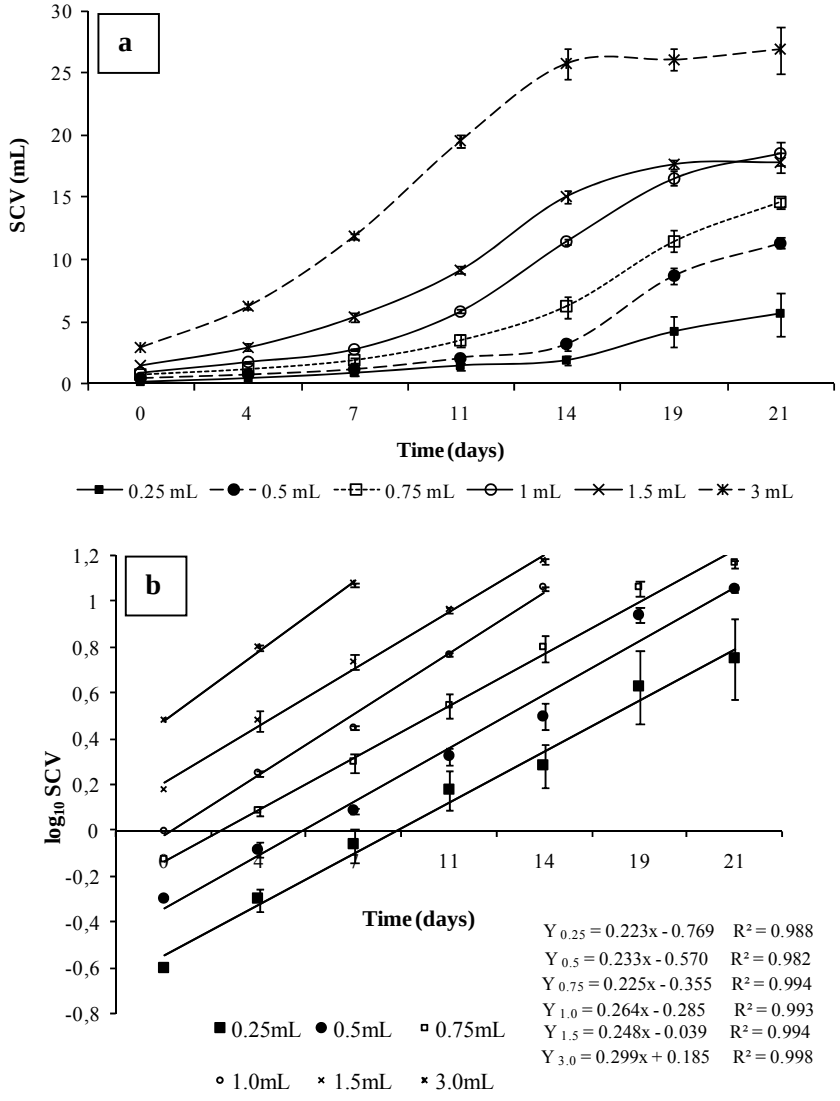

FIGURE 4 Growth curves of *Byrsonima intemedia* cell suspensions on several settled cell volume (SCV) using heterogeneous cell suspensions. (a) Growth curves and (b) growth curves represented by linear trendline by using a logarithm scale (with base 10). Error bar indicates standard error.

The growth rates during the exponential growth phase can be represented by fitting a line through the points. R^2 is for all lines close to 1 meaning that the line is well fitted. It can be concluded that (i) irrespective of the inoculums density growth rates during the exponential growth are similar and (ii) growth is from day 0 on going into the exponential phase. No lag phase is thus observed.

The different inoculum densities of *Byrsonima intermedia* heterogeneous cell suspensions also influenced their color. The largest amount of red cells was obtained using the smallest amount of inoculum (0.25 mL) (Figure 5a). All these cultures were taken from dark conditions and their red color was mainly found to be present in the cytoplasm content (microscopic observations). The cells presented different aspect with predominance of small ones which were generally dispersed into the medium. However some cells could be found in aggregates and/or in hard structures (clumps).

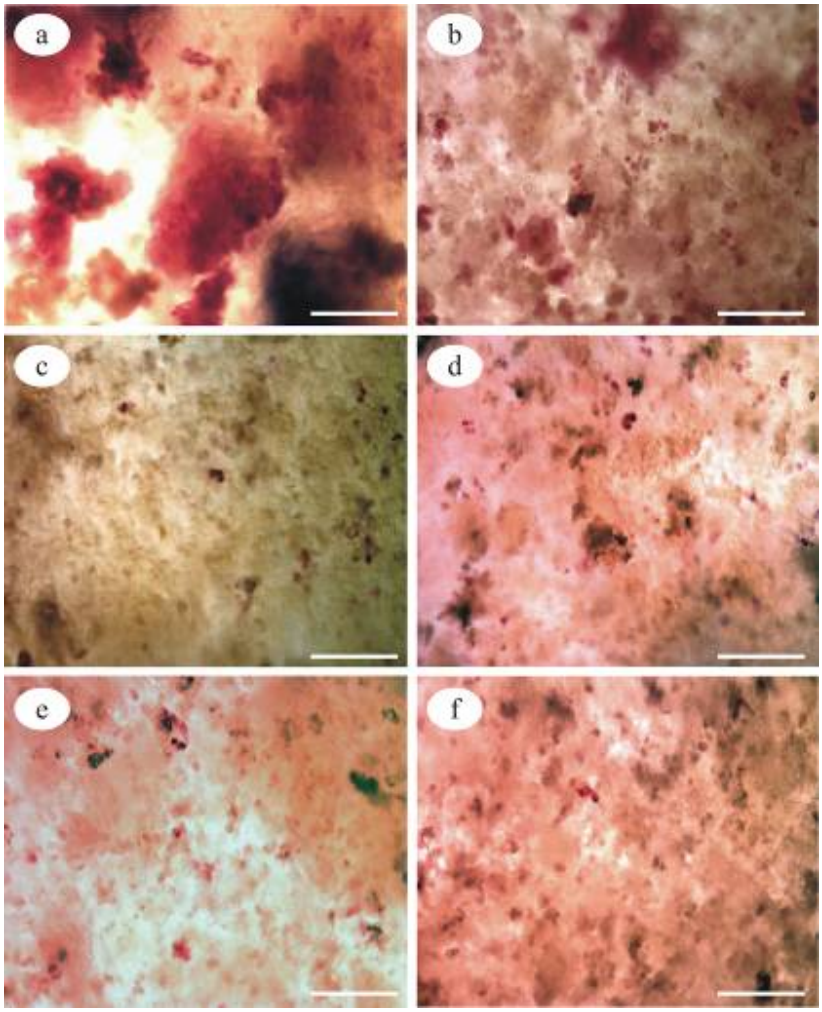

FIGURE 5 *Byrsonima intemedia* heterogeneous cell suspensions (21 days old), maintained under dark conditions, from growth curve experiment using several settled cell volume (SCV): (a) 0.25mL, (b) 0.5mL ,(c) 0.75mL, (d) 1.0mL, (e) 1.5mL and (f) 3.0mL. Scale bars = 200 μm.

The success in the establishment of a cell suspension culture depends, in a great extend, on the availability of "friable" callus tissue (i.e., a tissue that, when transferred to liquid medium, rapidly disintegrates into single cells and small clusters). Such a system is much more amenable for biochemical studies and process development compared to calli, because they generally grow in a faster rate and allow cells to be in direct contact with the medium nutrients (Godoy-Hernández & Vásquez-Flota, 2006). Callus cultures are still subjected to a decreasing "gradient" of nutrients, water and growth regulators with increasing distance from the culture medium.

Kinetin when used in combination with 2,4-D presented positive effects on cell growth as well verbascoside accumulation of *Aphelandra* sp (Nezbedová et al., 1999). These growth regulators when used together also influenced the callus induction of *Tecoma stans* which presented a high induction response (López-Laredo et al., 2009). In the present work, a combination of 2,4-D and kinetin was used to induce and maintain *B. intermedia* cell suspension showing positive effect on cell growth of this species.

For each explant and culture condition there is the best medium to induce friable calli (Almeida et al.; 2001). An alternative to produce worthy secondary metabolites is by using plant cell suspension cultures (Kim et al., 2002).

Settled cell volume (SCV), which it is obtained by allowing a cell suspension to sediment in graduated sterile tubes, is explained as the total volume of suspension occupied by the cell mass and it shows a reciprocal relation with dry weight data and thus, can be used for measuring cell growth. It allows the quick, non destructive estimation of culture growth, while maintaining sterile conditions and it is advantageous for accompanying growth in the same flasks along a culture cycle, because suspensions may be returned to prior culture conditions (Godoy-Hernández & Vásquez-Flota, 2006). In the

exponential growth phase, cells are adapted to the new environment and grow exponentially utilizing the nutrients provided. The sudden interruption in the increment of cell growth occurs in the stationary phase. This event might be due to the limitation of nutrients available in the culture medium or maybe because cells started to secrete toxins to the surrounding medium and further lead to cell death (George et al., 2008; Ling et al., 2008). Also pH changes might play an important role. For our *B. intermedia* cell culture, depending on the inoculums density used, a time period is needed to reach the stationary phase, although that for all of them the exponential phases always started at the same day (Day 1). For example, the 3 mL inoculum reached the stationary phase at approximately 14 days of culture while the 0.25mL inoculum did not reach this phase within the measured timeframe, showing an increase growth till the last day of the evaluation. This different behavior can be explained by the limitation of nutrients available in the 3mL inoculum comparing with the 0.25mL inoculum density and/or release of toxic compounds/change in pH. Finally prolonged culture periods will results in a changed (generally lower) concentration of plant growth regulators which will also influence growth.

When cultures are maintained in stationary phase for long periods, suspension cultures became opaque due to accumulation of polysaccharides (Smith et al., 2002) and as mentioned above cells begin to die mainly because of the exhaustion of components in the culture medium. Subculturing often becomes imperative to increase the volume of a culture when the density of cells, tissue or organs becomes excessive. This subculture is hence more conveniently carried out during the stationary phase when cell aggregation is least pronounced (George , 2008).

Ling et al. (2008) studied the growth profile of *Ficus deltoidea* cell suspension cultures. The authors observed a sigmoidal growth curve until the 12^{th} day. However, the complete growth curve of *F. deltoidea* cell suspensions

could not be observed as most of the cells were still in their mid-exponential phase at the end (on day 12). The growth rate was also highly influenced by the inoculum size used. Higher inoculum size (10.0 mL) produced the highest cell proliferation rate followed by the 5.0mL and 2.5mL inoculum size treatments, respectively. According to the authors, the suspensions require a critical minimum inoculum density for cell suspension cultures to grow. Similar findings were observed in the current work for some inoculum densities of *B. intermedia* cell suspension, where it was not possible to observe the stationary phase since cells were still growing. Like Ling et al. (2008) we also observed that the growth rate is highly influenced by the inoculum size used.

The growth curve of a cell suspension of *Centella asiatica* (L.) Urban was characterized by an initial lag phase until day 10 of incubation followed by a pronounced increase in the growth rate until the third week. In the fourth week, cells reached the stationary phase marked by the decline of growth in cell suspensions. As it was expected, the cells in suspension culture had a higher growth rate compared to the callus cells (Nath & Buragohain, 2005).

A typical sigmoidal growth curve was also observed in cell suspension cultures of three different species (*Psychotria carthagenensis*, *Azadirachta indica* and *Passiflora edulis* var. *flavicarpa*) (Lopes et al., 2000; Babu et al., 2006; Khiet et al., 2006).

An essential step to efficiently yield secondary metabolites on a large scale is to know in which growth phase a specific product is optimally formed. With this knowledge an effective production strategy can be developed. For instance, if it is known that the product under investigation is mainly produced during exponential growth, by using a biotechnological batch process which is based on feeding of a growth limiting nutrient substrate to a culture ("fed-batch") it could be possible to keep the culture actively growing, thereby maximizing production of the secondary metabolite. It is important to emphasize

that *in vitro* production of secondary metabolites can occur at almost any stage of culture growth (Kim et al., 2002; Ling et al., 2008).

Idiophase refers to the period in culture growth in which secondary metabolites are produced in preference to primary metabolites. This phase usually occurs when culture growth has slowed down or ceased, therefore in the stationary phase and/or the end of the exponential phase (James et al., 2008).

To exemplify how product formation is growth associated and species-dependent, a few examples are mentioned below. In *Rauwolfia sellowii* cell suspension cultures, alkaloids were produced preferably at the end of the exponential growth phase (Rech et al., 1998). Similar results were found for the verbascoside and linarin production of *Buddleja cordata* cell suspension which reached their highest metabolite production in the stationary growth phase (Estrada-Zúñiga et al.; 2009). For the transgenic tobacco calli the highest level of anthocyanin was found in the exponential phase, followed by a decreasing accumulation at the end of exponential and stationary phases (Zhou et al., 2008). Nezbedová et al. (1999) observed that the production of verbascoside in the callus lines of *Aphelandra* sp. increased together with the cell growth, reaching its maximum at 40 days. This maximum verbascoside production was achieved several days after the maximum cell growth was reached.

For neem cells, triterpenoids were found to be actively synthesized after the linear growth phase. Depending on the triterpenoid type different behavior was observed, but generally the highest accumulation was found at the end of the culture (in the stationary phase), when the growth rate of the cells was minimum. This proved that the accumulation of these compounds was enhanced by stress. It is interesting to mention that the presence of triterpenoids was not detected in the liquid medium (Babu et al., 2006).

Castro et al. (2008) also observed a higher activity and higher levels of total phenols in the initial explants of *Stryphnodendron adstringens* (Mart.)

Coville, with progressive reductions until the linear phase of calli growth. At the stationary phase there were significant increases in the activity of phenylalanine ammonia-lyase and total phenols.

In *Tabernaemontana divaricata* cells, specific alkaloids were present during the stationary phase of the growth cycle. After a period of 16 years, the enzymes responsible for the typical alkaloid biosynthesis of this species were still produced, and they were capable of a considerable production of alkaloids after feeding precursors. According to these results it appears that the production of secondary metabolites, though not utilized, can remain stable over prolonged periods of subculturing (Lucumi et al., 2001).

It is possible to produce specific bioactive compounds by making a choice of suitable cultivation conditions (Wilken et al., 2005). For example, callus and cell suspension cultures of *Rauwolfia sellowii* produced the same main alkaloids as the leaves of the mother plant. These undifferentiated cells were established in culture medium containing a combination of 2,4-D and kinetin growth regulators (Rech et al., 1998).

The ideal inoculum size depends on the species used and can have some effect on the synthesis of secondary metabolites. The difference in this synthesis can be related to the enhancement of the activity of the enzymes involving the metabolic pathway (Ling et al., 2008). So, the colour difference obtained in the different inoculum's size of *B. intermedia* could indicate some difference in the secondary metabolites synthesis.

5.2.2 Homogeneous cell suspensions

The 3 mL inoculums of homogeneous *Byrsonima intemedia* yellow and red cell suspensions (Figure 6) also followed a sigmoidal curve with respect to SCV, with distinct exponential and stationary phases, just like observed in the heterogeneous cell suspensions in previous sub-item (5.2.1) with the same

inoculum density. However exponential growth last until day 13 for homogeneous yellow and red cell suspensions while for the heterogeneous one it remained only until day 7 as observed in the previous sub-item.

At the end the evaluation period (on day 21) it was observed that yellow cell line remained yellow but with some brown cells into the medium. However, most of the red cells became brown in the red cell line confirming their higher differentiation compared to the yellow ones (data not shown).

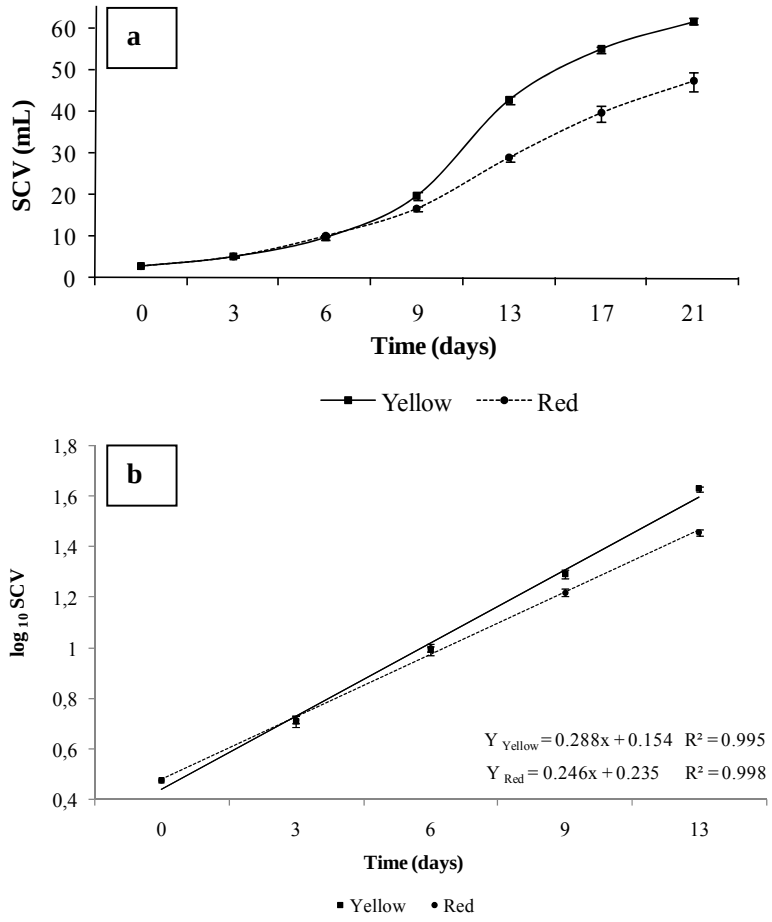

FIGURE 6 Growth curves of *Byrsonima intemedia* cell suspensions on 3 mL settled cell volume (SCV) inoculum using yellow and red homogeneous cell suspensions. (a) Exponential trendline of growth curves and (b) growth curves represented by linear trendline by using a logarithm scale (with base 10). Error bar indicates standard error.

Growth suspensions are different; (i) red ones grew more slowly than yellow ones and; (ii) homogeneous grew more slowly than heterogeneous cell suspensions.

Cell suspension cultures are commonly known for fast growing cells in liquid culture media. So, these cell suspensions are used for many purposes such as producing high amounts of cells for studying quanti or qualitatively the growth responses and metabolism of new chemicals, and also for analyses of cell cycle and plant regeneration system under standardized conditions (Torabi et al., 2008).

Species react differently towards the production of *in vitro* secondary metabolites and the growth curve understanding is fundamental to effectively reach desired responses. For example, *Lavandula officinalis* and *Hypericum perforatum* cells cultured in the same conditions but accumulating specific secondary metabolites reached the maximum production in different growth phases. For *L. officinalis* the highest content of rosmarinic acid was found in the exponential growth phase. However, for *H. perforatum* the highest content of hypericin was found in the stationary growth phase (Wilken et al., 2005). Also cell suspensions of *Centella asiatica* were found to produce the highest triterpenoids concentrations in the stationary phase (James et al., 2008). These findings just confirm that a complete study to optimize secondary metabolite production should be developed for each independently species.

A higher concentration of some secondary metabolites present in the callus as compared to that in the cell suspension cultures could be observed for some species, for instance alkaloids in *Rauwolfia sellowii* (Rech et al., 1998) and triterpenoids in *Azadirachta indica* (Babu et al., 2006). A possible explanation is that callus is more differentiated than cell suspensions, allowing better cell-to-cell contact and has consequently more chance to produce such metabolites. However, contradictory findings were reported by Nezbedová et al. (1999) who

found a higher verbascoside production in cell suspension cultures of *Aphelandra* sp. This behavior could be explained by the higher growth rate obtained in suspension culture when compared with callus cells.

5.3 Cryopreservation of *Byrsonima intermedia* cell suspension cultures

5.3.1 Slow-freezing (experiment 1)

5.3.1.1 First slow-freezing test

A first test was executed using the Slow-freezing protocol that has been developed for banana in the Laboratory of Tropical Crop Improvement, Leuven, Belgium. Different *B. intermedia* cell suspensions lines which were different in color (yellow, white, red), culture conditions (dark and light) and culture medium (normal or modified M medium) were tested and no re-growth after cryopreservation could be observed (Figure 7) irrespective of the cell line used. Most of the cell lines became completely white after cryopreservation (Figure 7 c,d), with the exception of cells that were red which became brownish (data not shown). All of them were apparently without any sign of life. Control cells (neither cryoprotected nor cryopreserved) but cultured in medium without growth regulator presence complete lost their regrowth ability (Figure 7b).

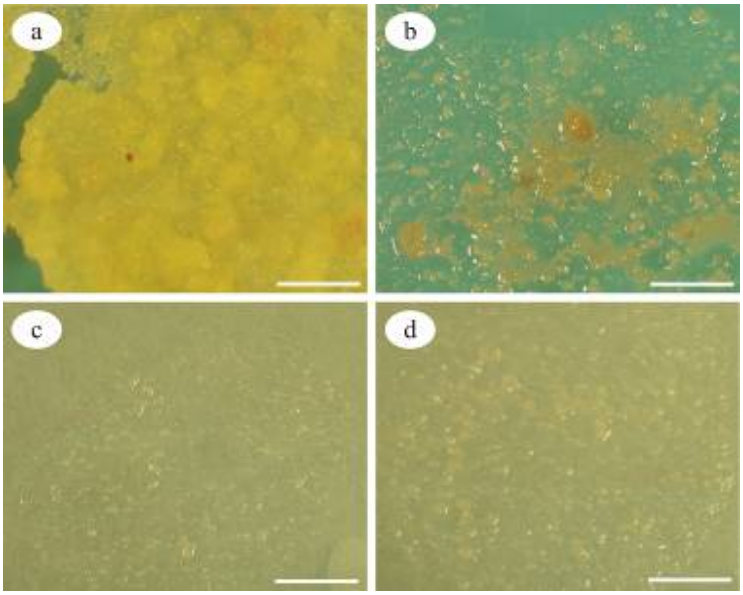

FIGURE 7 Slow-freezing first experiment 30 days after cryopreservation on line 1 (yellow cell line cultured in dark conditions) (a) control plus growth regulator; (b) control growth regulator free; (c) after cryo plus growth regulator; (d) after cryo growth regulator free. Scale bars = 5mm.

Plant cells growing in the undifferentiated state are known to be genetically unstable. Cell cultures producing secondary metabolites tend to loose their productivity during prolonged *in vitro* cultivation. To avoid long term frequent subculture events, long term preservation methods (like cryopreservation) are needed (Mannonen et al., 1990).

The two requirements that allow cell to vitrify upon exposure to ultra low temperatures are (i) application of rapid freezing rates and (ii) obtaining a concentrated cellular solution. Rapid freezing rates are commonly obtained by plunging samples into liquid nitrogen. The concentrated cellular solution is normally obtained through air drying, freeze dehydration and application of

penetrating or non-penetrating substances (cryoprotectants), among others. The classical slow-cooling procedure, also known as slow-freezing, is based on the application of slow cooling rates that can vary from 0.5 to 2°C/min, depending on the type and physiological state of the plant material. As plant cells seldom comprise ice-nucleating agents, crystallization during the slow cooling process is started in the extra-cellular solution, which does possess these agents. Since only a part of the water that contributes to the extra-cellular solution changes into ice, the remaining solution becomes concentrated and thus hypertonic to the cell. To reestablish the osmotic equilibrium, cellular water will leave the protoplast, resulting in cell dehydration avoiding intacellular ice formation (Menges & Murray, 2004; Panis & Lambardi, 2005). The result of slow freezing is thus not intracellular freezing but intracellular dehydration. Such slow-cooling rates are normally achieved by using computer-driven cooling devices, stirred methanol baths, and propanol containers held at -80°C. This slow-freezing process occurs in the presence of a cryoprotectant solution and for this the penetrating cryoprotector DMSO at a 5-15% concentration is generally used. During this process, when temperature reaches approximately -40°C, intracellular solution becomes so concentrated that allows its vitrification. So, the real freezing (vitrification) takes place when at this moment samples are plunged into liquid nitrogen. Nowadays this system is mainly used for cryopreservation of undifferentiated structures, like cell suspensions and calli (Panis & Lambardi, 2005).

With the aim of improvement of cell survival and re-growth after cryopreservation, cooling and thawing conditions must to be optimized for the different cell types to limit osmotic and thermal cell stresses (Bernemann et al., 2007). In our work we applied a slow-freezing protocol followed by rapid thawing which is found to be effective to cryopreserve embryogenic banana

cells at the Laboratory of Tropical Crop Improvement, Leuven, Belgium (Panis et al., 1990).

In the present work, slow-freezing was performed in cryotubes placed in a Nalgene™ cryo 1ºC Freezing Container that was transferred into a freezer (-80ºC) and at the moment that the temperature of -40ºC was reached, the cryotubes were plunged in LN. This system allows to cryopreserve a specific material using slow-freezing rate without the need to purchase expensive equipment. There are also some examples of using an effective inexpensive system to cryopreserve cell suspension cultures by using slow freezing method such as for *Arabidopsis* (Menges & Murray, 2004), tobacco (Kobayashi et al., 2005), *Tabernaemontana divaricata* (Suhartono et al., 2005) and *Taxus* (Škrlep et al., 2008).

Undifferentiated cell cultures are very sensitive towards environmental stresses. These tissues will be injured more severely by osmotic dehydration imposed by vitrification solutions compared to the moderate freezing-induced dehydration during cooling to around -30°C (Kobayashi et al., 2006).

It is believed that during the initial stages of freezing, a slower freezing rate of the extracellular solution could reduce the stress on the plasma membrane (Chen et al., 1984a; Menges & Murray, 2004).

A high vacuolation degree in cells often results in failure of cells to survive cryopreservation, mainly because of the high water content in these cells (Luo & Widholm, 1997; Mathur et al., 2003; Menges & Murray, 2004). The cells used in the current study show such high vacuolation, predominant in almost the complete intracellular space (data not shown). These characteristics could explain the negative results found for these *B. intermedia* cell suspension lines.

Studies of plant cell suspensions indicate the importance of the using the correct growth phase for successful cryopreservation (Chen et al., 1984b; Joshi

& Teng, 2000; Ishikawa et al., 2006). Cell suspensions are preferentially cryopreserved when they are in their exponential growth phase. For banana cells for example, the exponential cell growth usually occurs 7 to 10 days after last subculture (Panis, 2009).

Previous work has been based on the belief that cryopreservation of plant cell cultures should be executed with cells at the late linear or early exponential growth phase since that at these stages cells are small and non-vacuolated. The basis of these studies was done by using the classical slow prefreezing protocols. Survival was determined using fluorescein diacetate (FDA) staining or 2,3,5-triphenyltetrazolium chloride (TTC) assays (Ishikawa et al., 2006). A recent study on cryopreservation of ginseng cells confirmed these previous findings since cells were most tolerant to the freezing process when they were in their linear phase. However, these cells were most vulnerable when they were in their mid-exponential phase (Joshi & Teng, 2000).

The results found by Ishikawa et al. (2006) are in contradiction with the classical concept since the optimal growth phase may thus vary depending upon cell cultures, culture conditions or species from which the cells are derived. The traditional theory which generalizes a specific growth phase to be used in the cryopreservation procedure of cell suspension cultures should be then pondered for each cell culture.

In contrast to the requirement for freezing at a slow controlled cooling rate, generally high thawing rates (120ºC min^{-1}) are applied. Rapid thawing is ensured by rapid transfer of vials form LN into a 40ºC waterbath (Menges & Murray, 2004) avoiding the destabilization of the non-crystalline solid produced (Sakai & Engelmann. 2007; Gonzalez-Arnao et al., 2008).

Appropriate post-thawing culture conditions are essential to avoid loss of cellular viability and enhance organized cells growth (González-Benito et al., 2004; Menges & Murray, 2004).

A high survival rate and efficient growth of cells after cryopreservation is favoured when cells are transferred together as closely as possible in one jar, mainly because in this way they "feed" each other (Suhartono et al., 2005).

Rehydration and cryoprotectant removal after thawing is usual in most cryopreservation protocols (Verleysen et al., 2005a) and can be necessary for survival (Mathur et al., 2003). However, in banana cell suspensions it was observed that the removal of the 'potentially toxic' cryoprotectant solution directly after thawing and its replacement by cryoprotectant-free liquid medium, before transfer to a semi-solid medium, results in a complete loss of regrowth capacity and the cells becoming white. Direct transfer of cells to a liquid medium, which subjects the cells to similar post-thaw wash stresses, likewise results in growth failure. Regrowth can only be achieved when cells, still suspended in the cryoprotective solutions, are directly transferred to semi solid medium (Panis et al., 1990).

An adjustment is necessary that minimizes the time cells are exposed to toxic level of cryoprotectant (Menges & Murray, 2004). For this, we used a filter paper placed on solidified medium to absorb the toxic cryoprotectants from the cryopreserved cells.

Chen et al. (1984b) showed an efficient and simple method to improve cells viability of alkaloid-producing periwinkle cell lines after cryopreservation. The authors plated the thawed cells onto filter paper over agar medium without washing for 4 to 5 h to enable the cryoprotectants to spread into agar medium slowly. This method not only facilitated the post-thaw handling, but also avoided unnecessary washing damage.

During cryopreservation, cell undergo several stresses, such as osmotic stress, cold shock, chemical stress, ice crystal formation, membrane overstretch or bursting during thawing. Thus, the cryopreservation procedure kills a certain fraction of the cells and many other cells become damaged (Suhartono et al.,

2005). As a result, the cryopreserved cells or tissues may di□er physiologically (Urbanová et al., 2002; Urbanová et al., 2006; Zhu et al., 2006; Suhartono et al., 2005) and morphologically (Helliot et al., 2003; Yamazaki et al., 2009) from the normal (non cryopreserved) ones dependent on the degree of injury during freezing and thawing. Even during pre-culture treatment physiologic changes can occur, as is shown in *Ginkgo biloba* calli (Popova et al., 2009).

Kobayashi et al. (2006) confirm that tobacco cell regrowth depends on the number of cells that survive after cryopreservation.

Popova et al. (2009) studying the cryopreservation on *Ginkgo biloba* calli observed that they turned brown during and after cryopreservation procedure. However, three weeks after thawing, new white or yellowish tissues appeared on the surface of the brown calli indicating survival and regrowth. Similar results were observed on the recovery of cryopreserved *Panax ginseng* cells, which were initially brown-to-reddish, appeared that gradually became light-yellow again after various subcultures (Joshi & Teng, 2000).

Dark incubation for a limited time period following post-thawing prior to transfer to light conditions generally improves survival. This is probably attributed to damage repair of tissues that might take place during darkness (Yin & Hong, 2009). In the present study, cryopreserved tissues were cultured one week in dark conditions and subsequently transferred to the light.

5.3.1.2 Fluorescein diacetate (FDA) test

The chemical cell viability of the thawed cells was determined using the fluorescein diacetate (FDA) test whereby surviving cells fluorescence very brightly under ultraviolet illumination. The FDA assay measures the activity of the enzyme “esterase”. In Figure 8 it is shown that even after cryopreservation the cells showed a certain esterase activity, although not as bright as the control

cells. However, after the evaluation period (30 days) cells regrowth after cryopreservation could never be observed.

Thus, the FDA is not sufficiently effective to evaluate the recovery after cryopreservation. As already reported before, it can only give a rough indication. Therefore it was not used in the following experiments.

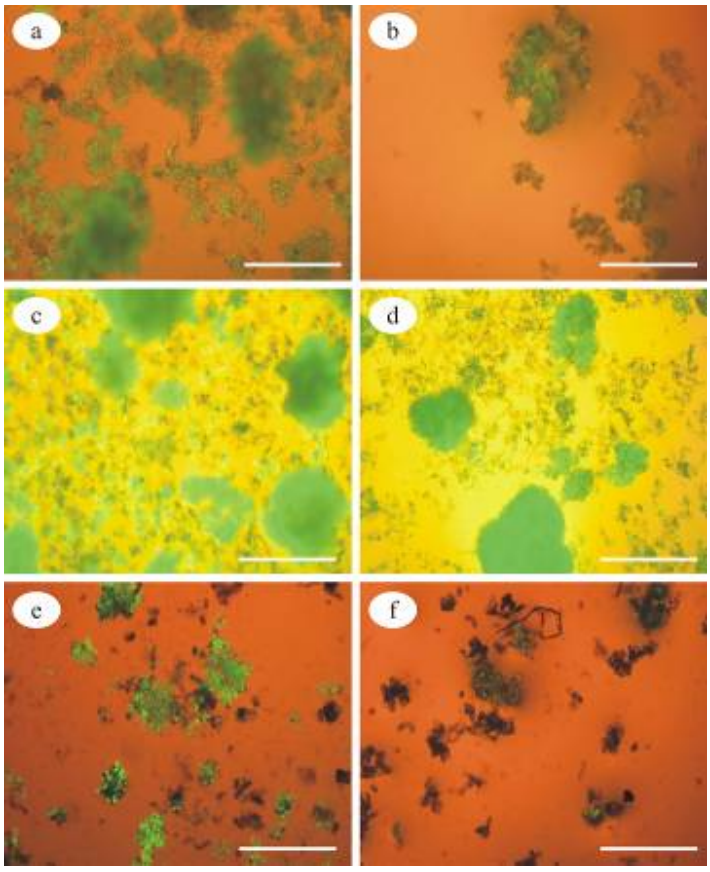

FIGURE 8 FDA (a,c,e) before and (b,d,f) after cryopreservation – (a,b) line 1 (yellow cell line – dark); (a,b) line 9 (yellow/white cell line – dark); (a,b) line 3 (red cell line – dark). Scale bars = 500µm.

Problems using a viability assay using fluorescein diacetate staining (FDA) are discussed by Pérez et al. (1997). They show that FDA staining performed immediately after thawing over-estimates cell survival. Also Panis et al. (1990) showed the contradiction between banana cells regrowth and FDA post-thaw viability estimation.

The viability of cryopreserved cells should be determined one day after cryopreservation when the cells start to resume their metabolism, because it is not correct to evaluate the viability of cells that are still recovering from plasmolysis immediately after re-warming (Kobayashi et al., 2005). This could also explain the "false" positive results obtained in the present work.

Regrowth is the most trustful and widely used method to evaluate cryopreservation survival (Ishikawa et al., 2006). Therefore this parameter was further used in our experiments.

5.3.2 Slow-freezing and Encapsulation-vitrification techniques

In order to develop a cryopreservation technique for *Byrsonima intermedia* cell suspensions different techniques were tested. These experiments were executed together with an embryogenic banana cell suspensions (line 175 of the cultivar Grande Naine) for prove of concept.

5.3.2.1 Slow-freezing with pre-culture and DMSO effect (experiment 2)

5.3.2.1.1 Slow-freezing with DMSO effect

Figure 9 (b,c) shows the effect of a one hour treatment of DMSO (7.5%) on *B. intermedia* yellow cell suspensions. Even, non-cryopreserved but DMSO treated cells did almost not survive to the DMSO treatment (Figure 9b). Different results were observed for the DMSO treated banana cells, which grew well after the DMSO addition (Figure 9e). It should be noted, however, that no

regrowth was observed nor in *B. intermedia* neither in banana, this probably because the M medium was supplemented with a normal concentrations of sucrose (0.088M) instead the 0.5M sucrose which is normally used.

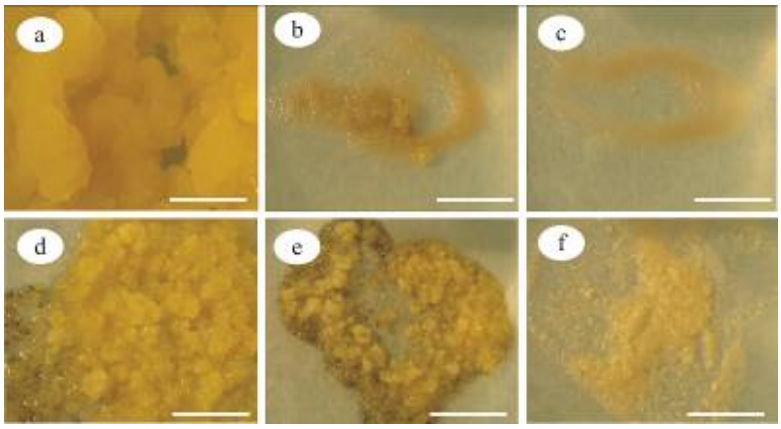

FIGURE 9 Control treatment of the slow-freezing technique after three to four weeks treatment. *Byrsonima intermedia* (a-c); banana (line 175 of the cultivar Grande Naine) (d-f); no DMSO before cryo (a,d); plus DMSO before cryo (b,e); plus DMSO after cryo (c,f). Scale bars = 5mm.

Mathur et al. (2003) reports that embryogenic cultures of *Pinus roxburghii* submitted to slow freezing and/or cryopreservation without any pre-treatment with cryoprotectants did not show any regrowth. This emphasizes the need of protecting the tissues with before they are exposed to the cryopreservation. These authors also showed that slow freezing step before plunging the culture in liquid nitrogen was essential for these cells.

Cryoprotection of embryogenic callus cultures of *Salustiana* sweet orange with 10% (v/v) DMSO, freezing by slow-cooling (0.1 to 0.5ºC min^{-1}) and fast thawing was suitable to recover viable growing cultures (Pérez et al., 1997).

It is observed that the concentration of DMSO has virtually no influence on the probability of intracellular ice formation when applied at low concentrations (<5%). However, higher concentrations of DMSO (up to 20%) tend to increase the probability of intracellular ice formation at most cooling rates, mainly because of the change it causes in the nucleation temperature (McGrath et al., 2007). Nucleation is a critical step that occurs in the crystallization process (Gonzalez-Arnao et al., 2008).

DMSO and glycerol are the the most commonly used penetrating cryoprotectants. Generally DMSO is prefered because of its extreme rapid penetration into the cells. However glycerol or amino acids (e.g., proline) are often applied when DMSO toxicity is a problem (Panis & Lambardi, 2005).

The combination of DMSO with other cryoprotectants may enhance cryoprotection for the following reasons; (a) addition of non-penetrating cryoprotectants such as sucrose and sorbitol, may reduce cellular water through osmosis and thus reduces the rate of initial ice crystallization; (b) the penetrating cryoprotectant DMSO enters the cells to reduce freeze-induced cellular dehydration and; (c) the combination of DMSO and sorbitol maximizes the effects due to the initial freezing stresses and subsequent dehydration (Chen et al., 1984a).

Winkelmann et al. (2004) reported that if cell suspensions were grown continuously in the standard medium containing 0.09 M sucrose, no re-growth was obtained for *Cyclamen persicum* cell suspensions after cryopreservation, even after pre-treatment with DMSO for one hour. A combination of sugar pre-culture (0.6M) for 2 to 4 days, followed by DMSO (10%) pre-treatment for 1

hour was essential for survival of this species cell suspensions submitted to cryopreservation.

The most frequently used method for cryopreservation of plant cell cultures is pre-culture in sucrose, mannitol or sorbitol followed by treatment with cryoprotectants and slow cooling of cells to subzero temperatures before storage in liquid nitrogen (Menges & Murray, 2004; Winkelmann et al., 2004; Škrlep et al., 2008). It is important to note the difference between pre-culture/pre-treatment and cryoprotection. Pre-culture (longer term) and pre-treatment (short term) are both responsible for protecting cells and prepare them against freezing injury at ultra-low temperatures (cryopreservation). They are procedures which involve a cellular metabolism. Cryoprotection does not envolve cells metabolism but has mainly a physical effect (osmotic and or colligative).

5.3.2.1.2 Slow-freezing with pre-culture

5.3.2.1.2.1 Sucrose pre-culture

The effect of a pre-culture with sucrose (0.4M) for 3 days is shown in Figure 10. In *B. intermedia* cells, sucrose pre-culture at 0.4M concentration proved to be toxic even before the DMSO treatment and subsequent cryopreservation takes place (Figure 10a). All cells turned completely white without any apparent sign of life. On the other hand, for banana cells, sucrose pre-culture was suitable in slow-freezing technique (Figure 10f).

The same behavior was observed for pre-culture cells with sucrose (0.4M) for 1 day (data not shown).

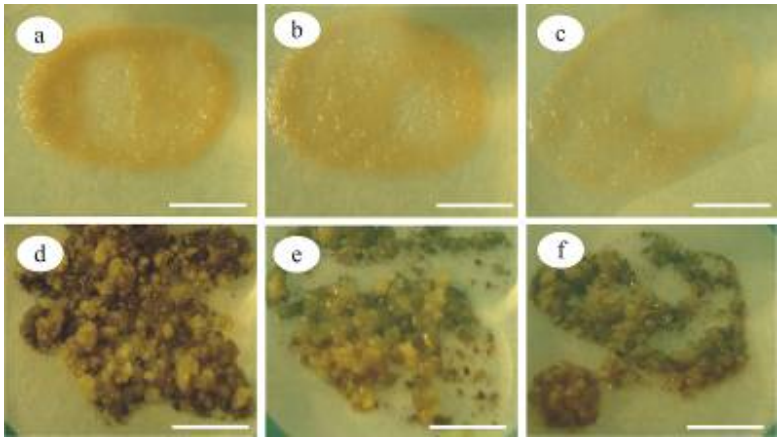

FIGURE 10 Slow-freezing with sucrose (0.4M) pre-culture (for 3 days) and after three to four weeks treatment. *Byrsonima intermedia* (a-c); banana (line 175 of the cultivar Grande Naine) (d-f); no DMSO before cryo (a,d); plus DMSO before cryo (b,e); plus DMSO after cryo (c,f). Scale bars = 5mm.

5.3.2.1.2.2 Sorbitol pre-culture

Since sucrose was not effective for *B. intermedia* cells, we tested sorbitol. The effect of a 3 days sorbitol pre-culture (at a concentration of 0.4M) is shown in Figure 11. As was observed with sucrose (0.4M), after a 3 days pre-culture treatment, the same reaction was observed. For *B. intermedia* cells, a sorbitol pre-culture was not effective at all, showing a toxic effect again even prior cryopreservation (Figure 11a,b). With respect to the banana cell lines, sorbitol showed very positive results protecting these cells against cryoinjury (Figure 11f).

The same behavior was observed for cells pre-culture with sorbitol (0.4M) for 1 day (data not shown).

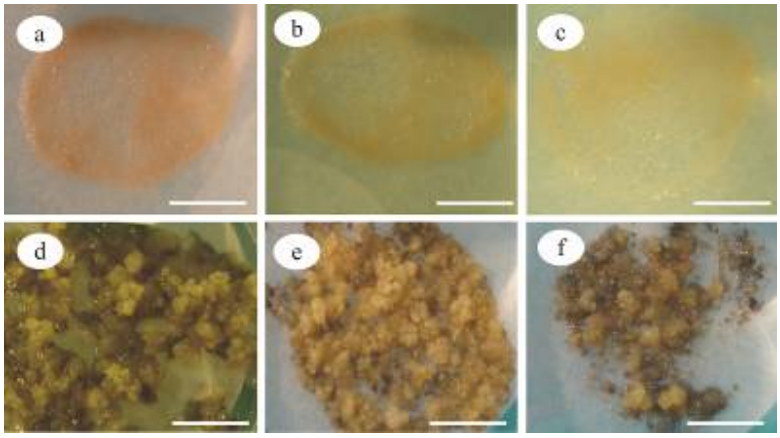

FIGURE 11 Slow-freezing with sorbitol (0.4M) pre-culture (for 3 days) and after three to four weeks treatment. *Byrsonima intermedia* (a-c); banana (line 175 of the cultivar Grande Naine) (d-f); no DMSO before cryo (a,d); plus DMSO before cryo (b,e); plus DMSO after cryo (c,f). Scale bars = 5mm.

The exact mechanism that explains the protective role of a sugar treatment before to cryopreservation is still obscure. The speculation of mode of action of sugars in cryoprotective medium is related its dehydration effect and more specifically the intracellular accumulation of solutes caused by osmotic caused stress that offers protection against further water stress and cryopreservation (Seijo, 2000; Bekheet et al., 2007; Popova et al., 2009). It has been proposed that in case of water limitation, OH groups of sugars can substitute for water and can interact with membrane phospholipids via hydrogen bonding (Suhartono et al., 2005), stabilizing and maintaining the integrity and function of cellular membranes.

Hatanaka & Sugawara (2008) observed the accumulation of a large amount of sucrose and *de novo* synthesis of several boiling-stable proteins after sucrose pre-culture. They also observed a very high level of desiccation

tolerance that was maintained in protoplasts isolated from sucrose pre-cultured *Marchantia polymorpha* cells.

The level of total soluble proteins, sugar contents and the viability of cryopreserved embryogenic cell suspensions of grapevine (*Vitis* spp.) pre-cultured with increased sucrose concentration increased and achieved an optimal value of viability (about 75%) when treated with 0.75 and 1.0M sucrose (Wang et al., 2004). Zhu et al (2006) found that a minimal amount of sugar in meristem cultures of banana is required to "theoretically" survive cryopreservation and this level is reached after a 2-week 0.4 M sucrose pre-culture (Panis et al., 2006). However, some plant species, like raspberry are extremely sensitive to high sucrose concentration when applied directly in the pre-cultured media (Wang et al., 2005). For *Robinia pseudoacacia* shoot-tips, the optimal protocol consisted in stepwise pre-culture up to high end-concentrations (0.3 + 0.5 + 0.7 M sucrose) (Verleysen et al., 2005b). Danso & Ford-Lloyd (2004) reported that a high sucrose concentration in the induction medium significantly reduced the viability and subsequent embryogenic competence of embryogenic calli, probably due to severe dehydration as the clumps were found to be dry and small in size following the high sucrose treatment. Different orchid species exhibited varying levels of tolerance to high sucrose concentrations (Yin & Hong, 2009).

Cells have to maintain their homeostasis and to survive dehydration when a high sucrose concentration is applied in order to osmoprotect them against injuries that can occur during the cryopreservation procedure. Carpentier et al. (2007) linked the tolerance of meristems of a banana to dehydration with proteins associated with both energy metabolism and stress adaptation.

Winkelmann et al. (2004) analysed the effects of sucrose, sorbitol and DMSO cryoprotectants on cell suspensions parameters of cyclamen. The authors observed that pre-culture which lasts 2-4 days in a medium containing 0.6 M

sucrose followed by pre-treatment in the same medium supplemented with 10% (v/v) DMSO showed the best regrowth response.

Different sugars (fructose, glucose, sorbitol, mannitol and sucrose) have been used in the pre-culture medium to osmoprotect cells against the injury caused in the cryopreservation procedure. However, for cryopreserved date palm tissue cultures the best survival was obtained by using sucrose in the pre-culture medium (Bekheet et al., 2007). Sucrose at high concentrations is known to have negative effects on morphogenesis (Danso & Ford-Lloyd, 2004). The required osmotolerance, however, is seldom reached by sucrose pre-culture solely (Yin & Hong, 2009; Wang et al., 2005; Wang et al., 2004).

Many ultra-structural changes were observed in banana meristems pre-cultured with sucrose. Among them, the fractionation of vacuoles into smaller ones, the differentiation of proplastids into amyloplasts containing starch, the swelling of organelles like the endoplasmic reticulum and modifications in the biomembranes were are described (Helliot et al., 2003).

The easy recovery of turgor after some hours of culture at normal water potential means that cell osmoregulation happened and then, they are able to deplasmolyze (Seijo, 2000).

5.3.2.2 Encapsulation-vitrification (experiment 3)

The results of applying the encapsulation-vitrification technique after cryopreservation of *B. intermedia* cells are shown in Figure 12c. No growth, only white dead cells can be observed. Even before cryopreservation took place the toxic effect of the cryoprotectors (two hours LS followed by 2 hours PVS2) is clear (see Figure 12b). It is important to emphasize that even after all the LS treatment times (1, 2, 3 and 4h) and PVS2 exposure times (30 min, 1 and 2h) (after LS of 2h) at 0ºC tried, the cells became white and without sign of live, not reaching re-growth after the evaluation period of 3 to 4 weeks (data not shown).

In case of banana cells, this technique showed some positive results, mainly after cryoprotection with 2 hours LS followed by 30 minutes and/or 1 hour PVS2 (data not shown). However, comparing this technique with slow-freezing (item 5.3.2.1) cells presented a slowlier regrowth and more blackening after cryopreservation (Figure 12f).

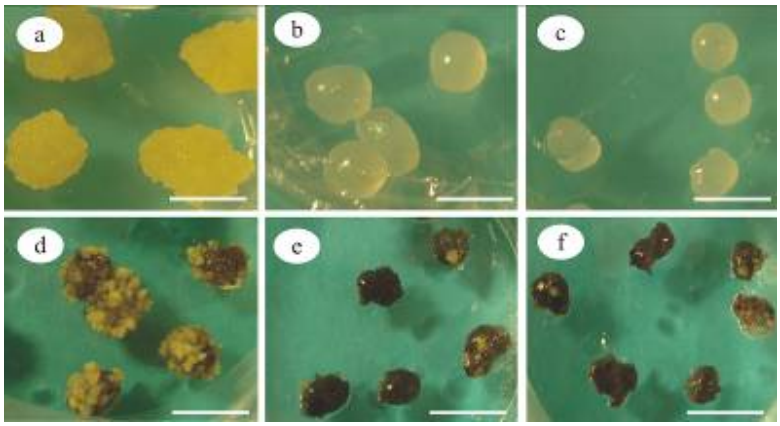

FIGURE 12 Encapsulation-vitrification technique. *Byrsonima intermedia* (a-c); banana (line 175 of the cultivar Grande Naine) (d-f); Control (a,d); LS (2 hours) followed by PVS2 (2 hours) before cryo (b,e); LS (2 hours) followed by PVS2 (2 hours) after cryo (c,f). Scale bars = 5mm.

Vitrification, the non-crystalline solidification of water, of solutions is achieved by reducing intra- and extracellular freezable water, preventing ice crystal formation at ultra-low temperatures without an extreme reduction in cellular water. This can be done either by exposure of plant tissues to highly concentrated cryoprotective mixtures (like in vitrification techniques) or by physical desiccation (for instance with slow-freezing technique or air drying),

and subsequent very rapid cooling, generally by direct immersion in liquid nitrogen (Helliot et al., 2003; González-Benito et al., 2004; Sakai & Engelmann, 2007; Gonzalez-Arnao et al., 2008).

In order for the vitrification technique to be successful, it is necessary that the cryoprotective (or vitrifying) solution is highly concentrated yet non-toxic and present an optimum exposure time (Bekheet et al., 2007).

A concentrated mixture of penetrating and non-penetrating cryoprotectors composes the vitrification solution. There are many factors that justify the success achieved by using the vitrification techniques such as their users -friendliness, high reproducibility and also because they are successfully applied to a wide range of tissues and plant species (Panis & Lambardi, 2005).

Different procedures have been used that result in a vitrified intracellular solution, such as pregrowth, air dehydration, freeze dehydration (through slow-freezing), pregrowth-dehydration, encapsulation-dehydration, vitrification, encapsulation-vitrification and droplet-vitrification (Gonzalez-Arnao et al., 2008).

When optimum conditions are used, vitrification techniques can produce higher levels of post-cryopreservation recovery growth and largely reduces the time required for dehydration of samples, compared to the encapsulation-dehydration technique. By contrast, despite the fact that the encapsulation-dehydration technique is more labor intensive, encapsulated samples are very easy to manipulate, thanks to the relatively large size of the alginate beads (Sakai & Engelmann, 2007). Thus, the technique known as encapsulation-vitrification combines the advantages of vitrification and encapsulation-dehydration to successfully cryopreserve samples (Panis & Lambardi, 2005; Wang et al., 2005; Gonzalez-Arnao et al., 2008; Xue et al., 2008).

The vitrification procedure normally consists of (i) pre-culture of samples on medium rich in sucrose, (ii) treatment with a cryoprotective solution

called the “loading solution” which has intermediate concentration (often composed of 2 M glycerol + 0.4 M sucrose) to prepare them for exposure to the vitrification solution; (iii) then the samples are dehydrated with a highly concentrated vitrification solution [e.g. the PVS2 – plant vitrification solution, which contains 30% (w/v) glycerol, 15% (w/v) ethylene glycol and 15% (w/v) DMSO and 0.4 M sucrose] at 0ºC which allows less toxicity; (iv) After they are frozen rapidly and (v) stored in liquid nitrogen; (vi) Then they are rapidly rewarmed; (vii) unloaded with basal culture medium supplemented with 1.2 M sucrose (recovery solution); and (viii) then transferred to standard culture conditions. In the encapsulation-vitrification technique, the plant tissue organs are encapsulated in alginate beads, treated with loading solution and dehydrated with a vitrification solution before rapid immersion in liquid nitrogen (Kobayashi et al., 2006; Sakai & Engelmann, 2007; Gonzalez-Arnao et al., 2008; Xue et al., 2008). Unlike the encapsulation/dehydration technique, this technique does not require physical desiccation (González-Benito et al., 2004).

The success of the encapsulation technique depends among others on the rate between encapsulated sample and alginate solution volume, and also on the physical changes and processes that can occur within the bead during pre-treatment and cryopreservation. Encapsulation within an alginate bead may bu□er the germplasm su□ciently to allow vitrification and prevent recrystallisation on rewarming (Block, 2003).

When cell suspensions of orange and carrot were pre-cultured in 0.4 M sucrose, the injurious effects of PVS2 were reduced to a large extent and very few clusters lost their viability. The most affected cells were the big ones containing large vacuoles (Seijo, 2000).

In most of the PVS2-treated (for 2 hours) cells of banana meristem tips, the nuclear content was less electron-dense compared to the nuclear content of untreated cells. When sucrose pre-culture was used, partial plasmolysis altered

the biomembranes. All these observations were done using the electron microscope (Helliot et al., 2003).

The duration of the PVS2 treatment has to be optimized for each species (which present variable water content and membrane permeability) since enough dehydration must take place to avoid the formation of lethal ice crystals during freezing. At the same time care has to be taken to prevent the potentially toxic solution (or more precisely 'dehydration tolerance' since PVS2 is considered as a dehydrating compound) from irreversibly damaging the tissue. The temperature at which dehydration takes place also plays an important role, for example, at 0ºC longer treatment periods are possible. Also the lengths of the loading treatment can influence the length of dehydration (Panis et al., 2005; Panis, 2009). The better reproducibility of results with the PVS2 exposure at 0°C (Sant et al., 2008) could be explained by the slower mobility of this solution to permeate cells at this temperature but which still allows enough water to be displaced (Volk & Walters, 2006), so extending the incubation time thus allowing work with large numbers of sample at the same time. Moreover, at this temperature the toxicity is reduced since the metabolism is slow.

Yin & Hong (2009) reported that pre-culture of protocorm-like bodies (PLBs) of orchid in 0.75 M sucrose for 5 days was found to be optimum for survival after cryopreservation using encapsulation-vitrification. Moreover, the highest frequency of survival of cryopreserved *Dendrobium candidum* PLBs was obtained when these were additionally treated for 80 min with a loading solution prior to dehydration. Beneficial e□ects of loading have been proved to be beneficial for most of plant tissues, for example as reported for *Robinia pseudoacacia* shoot tips (Verleysen et al., 2005b).

In the case of sucrose pre-cultured proliferating banana cultivars, it has been observed that optimal post-cryopreservation regeneration percentages are generally obtained after a 2 or 2.5 h PVS2 treatment. Survival after 3 h for most

cultivars is considerably lower, probably due to the toxicity of this highly concentrated solution (Panis, 2009).

The best survival of cryopreserved raspberry shoot tips is achieved by dehydration with PVS2 at 0°C for 5 h or at 24°C for 3 h, with similar survival rates following both treatments (Wang et al., 2005). Optimal viability of cryopreserved embryogenic cell suspensions of grapevine (*Vitis* spp.) was observed when they were pre-cultured with a final sucrose concentration of 0.75M and dehydrated with PVS2 at 0 °C for 270min (Wang et al., 2004). Verleysen et al. (2005b) observed for *Robinia pseudoacacia* when pre-culture with higher sucrose concentrations (0.6 or 0.9 M) was combined with a longer PVS2 incubation (at 0ºC) period (80 or 100 min), chances for survival (after cryopreservation) increased considerably. The frequency of survival of cryopreserved PLBs of orchid treated with PVS2 increased from 76.2% following dehydration at 25ºC for 120 min to 89.4% following dehydration at 0ºC for 150 min (Yin & Hong, 2009).

There are also reports that confirm the negative effect of PVS2 solution on the viability of plant material. For example, only very low percentages of the *Maesa lanceolata* and *Medicago truncatula* hairy roots survived incubation of 5 min of PVS2, without pretreatment with a loading solution. Roots that were first treated with loading solution became more resistant towards the PVS2 solution. However, none of the *Maesa* or *Medicago* hairy roots survived freezing in liquid nitrogen after vitrification (Lambert et al., 2009). With respect to ginseng cells PVS2 solution was also not advantageous since cells lost a half of their viability (Joshi & Teng, 2000).

Related to the *B. intermedia* cells, they were not dehydration tolerant in any the PVS2 solution exposure time tested (at 0ºC) after 2 hours in LS.

Although rapid cooling seems to be the most appropriate in case of cryopreservation techniques that involve encapsulation (González-Benito et al.,

2004; Wang et al., 2005; Xue et al., 2008), some studies showed that slow cooling could result in a higher survival rate (Scocchi et al., 2004; Kobayashi et al., 2005). A possible explanation is that not all freezable water was extracted from beads/apical meristem-tips during the desiccation. Therefore the remaining water could be extracted through freeze dehydration during the slow freezing process (Scocchi et al., 2004).

Tobacco suspension cells could be successfully cryopreserved using a vitrification method combined with the encapsulation technique. Cryopreserved cell cultures were thawed and could grow in such way that subculture into fresh medium was necessary within 14 days (Kobayashi et al., 2006). The encapsulation-vitrification method was, however, less effective for cryopreservation of tobacco suspension cells than a simplified slow prefreezing method also using an encapsulation technique (Kobayashi et al., 2005).

Slow and rapid freezing protocols using a mixture of cryoprotectants (sucrose:DMSO:glycerol 10:10:5% w/v for slow-freezing and sucrose 5% w/v, glycerol 30% w/v and 10mM $CaCl_2$ for rapid-freezing) resulted in higher cell survival of non-embryogenic bromegrass suspensions compared to the vitrification method which cells were pre-treated with the same cryoprotectant mixture slow-freezing was (Ishikawa et al., 2006).

The success of cryopreservation is species-dependent as was shown by Xue et al. (2008). When using the encapsulation–vitrification method, all the *Gentiana macrophylla* hairy roots died, whereas up to 6 and 73% of the *Astragalus membranaceus* and *Eruca sativa* hairy roots survived, respectively. Also Yin & Hong (2009) observed that responses of different orchid species towards cryopreservation by encapsulation-vitrification were different.

Some species are osmotolerant to PVS2, as have been reported for *Vitis* spp. (Wang et al., 2004) and *Robinia pseudoacacia* (Verleysen et al., 2005b).

This was, however, not the case for our *B. intermedia* cells, which showed no tolerance whatsoever even in the steps preceding cryopreservation.

For vitrification and encapsulation–vitrification, incubation in 1.2 M sucrose in liquid culture medium after freezing is most often applied (Sakai & Engelmann, 2007). For example, for azalea the detoxification of encapsulated tissues in concentrated sucrose solutions improved results after cryopreservation since regrowth rates were increased (Verleysen et al., 2005a).

In all the results obtained at the present work either using encapsulation-vitrification (at the present item) or slow-freezing (item 5.3.2.1) techniques, we observed no re-growth for *Byrsonima intermedia* species after cryopreservation. However compared with banana species we could see that the problem was not the protocol used but that *B. intermedia* species is too sensitive. Even in the control (before cryopreservation) pre-culture and pre-treatments caused cell death.

5.3.3 Toxicity tests (experiment 4)

These tests were performed because in previous experiments it was observed that none of the controls which contained cryoprotectants (in the pre-culture or in the pre-treatment) did survive even before cryopreservation.

5.3.3.1 DMSO exposure time

In previous experiment (item 5.3.2.1.1) pre-treatment with DMSO at 7.5% during 1 hour was toxic so different exposure time were tried.

According to Figure 13, the different controls used (control 1 in normal M medium which contains 0.09M sucrose and control 2 in modified M medium which contains 0.5M sucrose) did not differ in the callus formation, presenting values of 80% for both conditions. The 4 different DMSO exposure times tested did not show any difference in the toxic effect on *Byrsonima intermedia* cells

with a callus formation varying from 45.5% (60') to 50% (15' and 30'). However, independent of the DMSO exposure time used, they differed statistically from the controls, showing lower callus formation and though presenting some toxic effect.

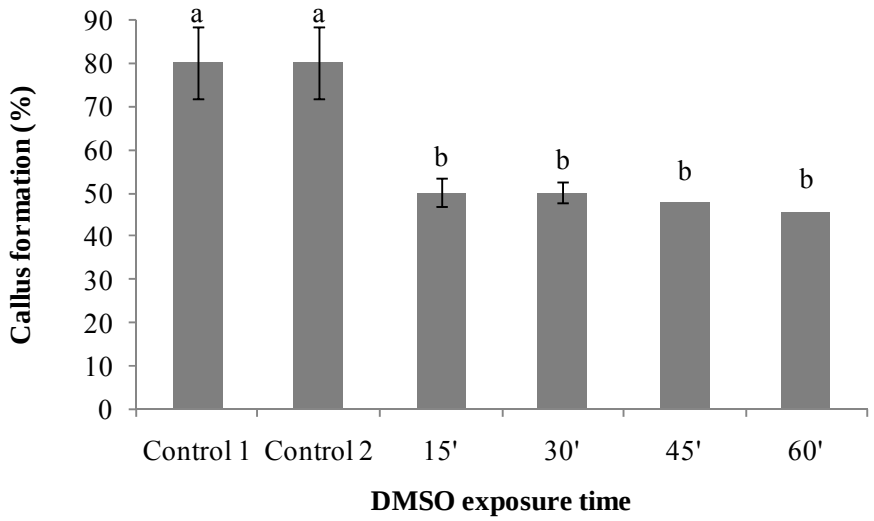

FIGURE 13 Effect of DMSO (7.5%) exposure time on callus formation from treated *Byrsonima intermedia* cell suspensions after 3-4 weeks treatment. Control 1 is the normal M medium which contains 0.09M sucrose and control 2 is the modified M medium which contains 0.5M sucrose. Each value represents the mean ± S.E.M. (n = 10) from a representative experiment and $P<0.01$ in one-way ANOVA followed by the Scott-Knott test.

The present experiment had different results (at DMSO 7.5% exposure time of 60 minutes) compared to previous ones (item 5.3.2.1.1) where cells treated with DMSO (7.5%) for 1 hour did almost not survive. This is explainable

because even that the same standard of cells were used (yellow cell suspensions cultured in the M medium under dark conditions and 7 to 10 days after last subculture), cells can change their characteristics in each subculture, so leading different behavior.

Mathur et al. (2003) observed that *Pinus roxburghii* embryogenic tissues do not tolerate DMSO at a concentration of 10%. However, 5 % DMSO combined with sorbitol pre-culture (for 24 hours) is suitable for the survival of these tissues. The authors also reported that sorbitol pre-culture was essential preceding DMSO pre-treatment, since no regrowth was observed in the cultures without it. Similar findings were found with periwinkle cells, where the combination of using sorbitol pre-culture followed by DMSO pre-treatment reduced the rate of ice crystallization in solutions (Chen et al., 1984a,b) and the amount of water frozen at sub-zero temperature (Chen et al., 1984a).

The mode of action of penetrating cryoprotectants like DMSO can be fully explained by their colligative properties by reducing lethal salt concentration and hence cells less suffer injury at a given temperature (Mcgann, 1999).

5.3.3.2 Cryoprotective solutions

Figure 14 shows the toxicity effect of different cryoprotective solutions on *B. intermedia* cell suspensions. M old (where the suspensions come from 7-10 days after their last subculture) medium (Control A), M fresh medium with 0.09M sucrose (Control B) and M fresh medium with 0.5M sucrose (Control C), used as control, were not different significantly, showing a high callus formation of 90%.

Different cryoprotective solutions show different toxic effects on *B. intermedia* cells. DMSO (5%) used in combination with 0.09M sucrose (= normal sucrose concentration in the culture medium) (A) was not toxic (80% of

callus formation) compared to the others cryoprotective solutions used in the present experiment. DMSO (5%) combined with 0.5M sucrose (B), DMSO (7.5%) with 0.09M (C) or 0.5M (D) sucrose and glycerol (5%) with 0.09M (E) or 0.5M (F) sucrose, resulted in similar toxic effects with 40, 35, 30, 30 and 25%, callus formation respectively. All other cryoprotective solutions tested were even more toxic to the *B. intermedia* cells. Glycerol at 15% with 0.09M (I) or 0.5M (J) sucrose showed some survival resulting in 10 and 5% callus formation, respectively.

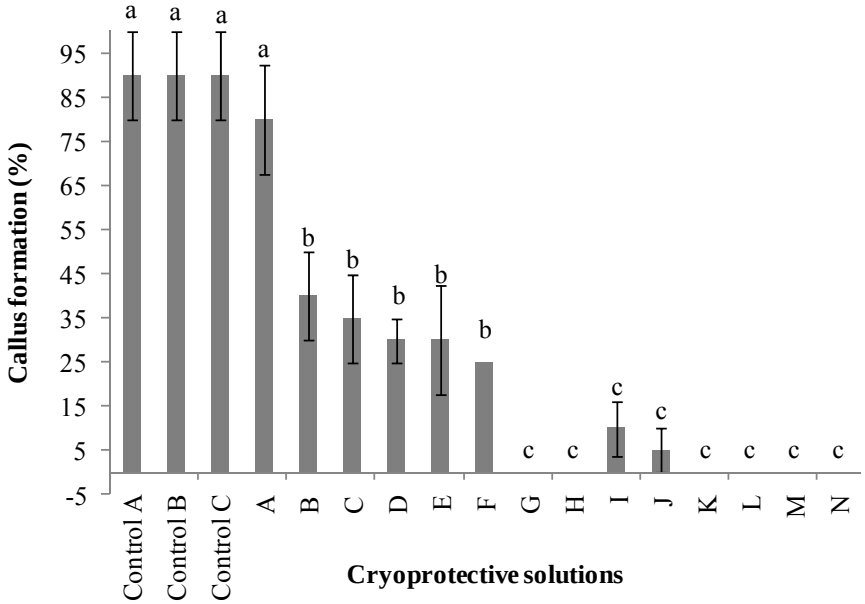

FIGURE 14 Toxic effect of different cryoprotective solutions on callus formation of *Byrsonima intermedia* cell suspensions 3-4 weeks after treatment. The cells were treated over a period of 60 minutes with the cryoprotective solutions. Each value represents the mean ± S.E.M. (n = 5) from a representative experiment and $P<0.01$ in one-way ANOVA followed by the Scott-Knott test.

Osmotically active compounds, also known as non-penetrating cryoprotectants, like sorbitol, mannitol and sucrose are added to reduce the intracellular water content before freezing, thus reducing the amount of water available for lethal ice formation (Panis, 2009).

Glycerol appears to be essential for surviving cryopreservation of tobacco BY-2 cells (Kobayashi et al., 2005). For the *Panax ginseng* cells a combination of 10% (v/v) glycerol and 4% (w/v) sucrose was chosen for presenting less cytotoxicity and high cell viability after thawing (Joshi & Teng, 2000).

Comparing single cells and cell clusters of *Cyclamen persicum*, Winkelmann et al. (2004) observed that cell clusters tolerated cryoprotectants pre-treatment better. These cells were mainly composed of small cells rich in cytoplasm with only a few small vacuoles or even missing them.

An evidence that osmotic compounds do not act just through dehydration is that different agents which present same osmolarity show both different preculturing and different cryoprotective effects during freezing (Chen et al., 1984b).

5.3.3.3 Pre-culture treatment

To increase the tolerance of *B. intermedia* cell suspensions towards the cryoprotectants, pre-culture treatments were checked for their toxicity effect.

Pre-culture medium enriched with 6% Mannitol, 0.3M Sucrose and 0.3M Sorbitol showed all toxic effects on the *B. intermedia* cells (Figure 15) when treated for 1 and 3 days resulting only in 15, 15 and 5% of the callus formation, respectively. The length of treatment used (1 and 3 days) did not statistically differ (data not shown). However, a pre-culture with 0.1 and 0.2M Sucrose affects the callus formation (65 and 85%, respectively) of this species, showing

similar results to the controls (85% for Control 1 and 100% for Control 0). The control consisted of M medium containing 0.09M sucrose, being Control 0 related to 0 day period culture and Control 1 for 1 and 3 days.

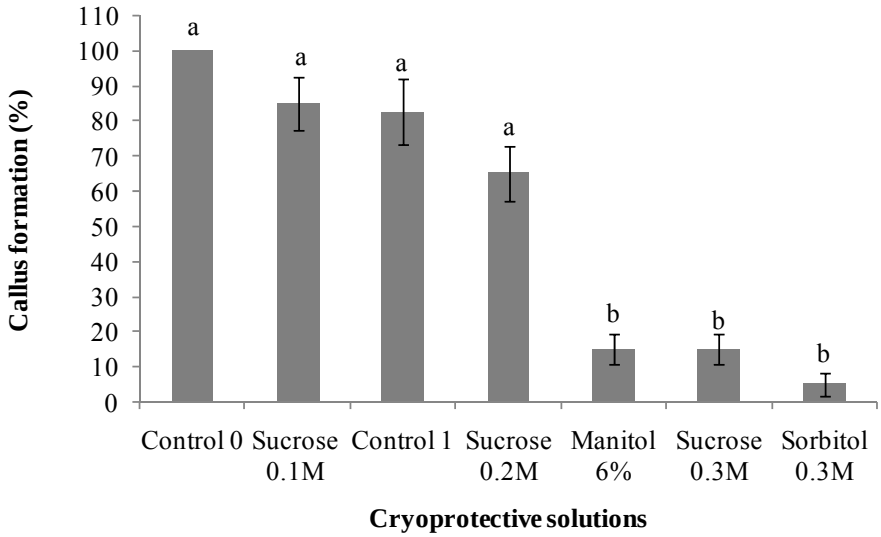

FIGURE 15 Toxic effect of pre-culture cryoprotective solutions on callus formation of *Byrsonima intermedia* cell suspensions suspensions 3-4 weeks after treatment. The cells were treated over a period of 1 and 3 days with the cryoprotective solutions. Each value represents the mean ± S.E.M. (n = 10) from a representative experiment and $P<0.01$ in one-way ANOVA followed by the Scott-Knott test.

An alternative to cryoprotect sensitive cells to long-term pre-culture is the use of cryoprotectant solutions in a short-term treatment (Kobayashi et al., 2005).

5.3.3.4 PVS2 exposure time

In order to check the toxic effect of PVS2 solution used with the aim to protect *B. intermedia* cell suspensions to cryopreservation, the present experiment was executed.

After the PVS2 exposure, no calli formation was observed for any of the exposure time tested (15, 30, 45 and 60 minutes at room temperature) (data not shown), showing the high toxicity of this cryoprotectant to the *B. intermedia* cell suspensions. The cells were completely white with no growth observed.

The toxicity tests were conducted in order to choose the best treatment to be used in cryopreservation trials of *B. intermedia* cell suspensions.

5.3.4 Slow-freezing – cryoprotectors and pre-culture (experiment 5)

In this part, the most promising cryoprotectant solutions from the toxicity experiment were applied for the cryopreservation of yellow and red *B. intermedia* cell suspensions.

5.3.4.1 Sucrose pre-culture followed by the presence and/or absence of 5% (v/v) DMSO

Figures 16 and 17 show the toxicity effect of sucrose pre-culture followed or not by DMSO pre-treatment on yellow and red *B. intermedia* cell suspensions 3-4 weeks before and after cryopreservation. It could be observed that for all the treatments used yellow cell line submitted to cryopreservation became white with no regrowth, whereas cryopreserved red cell line were brown and also no sign of life was observed. Also the effect of pre-culture followed or not by DMSO treatment was observed without cryopreservation, showing the similar response as reported in the previous item (5.3.3) on the toxicity tests.

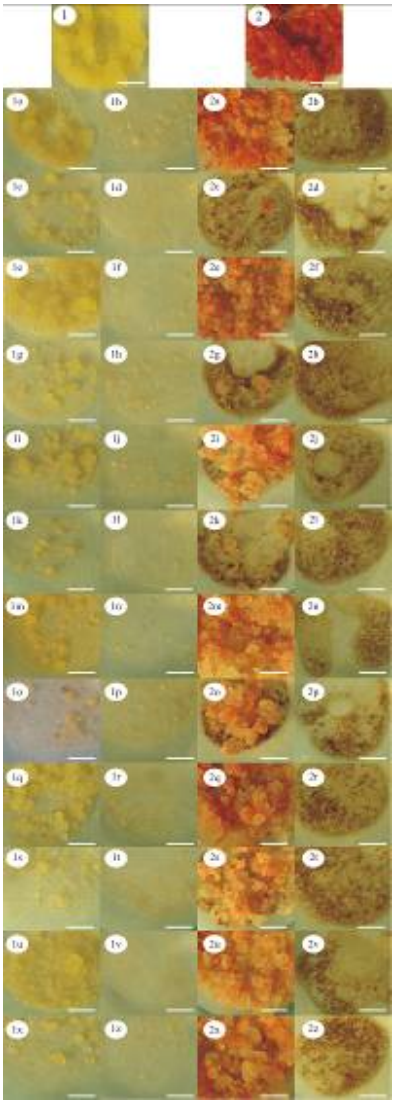

FIGURE 16 Toxicity effect of sucrose pre-culture and DMSO pre-treatment on *B. intermedia* yellow (1) and red cell lines (2). 1 day sucrose treatment (a-l); 3 days sucrose treatment (m-x); 0.1M sucrose (a-d, m-p); 0.2M sucrose (e-h, q-t); 0.3M sucrose (i-l, u-z); no 5% (v/v) DMSO (a,b,e,f,I,j,m,n,q,r,u,v); plus 5% (v/v) DMSO (c,d,g,h,k,l,o,p,s,t,x,z); before cryo (a,c,e,g,I,k,m,o,q,s,u,x) and after cryo (b,d,f,h,j,l,n,p,r,t,v,z), 3-4 weeks after treatments. Scale bars = 1.5cm.

Different cell suspensions showed different responses towards pre-culture toxicity using sucrose concentrations exposed to different culture time period (1 and 3 days) followed by the presence or absence of DMSO 5% (v/v) (for one hour) (Figure 17). Generally, red cell line showed a better response compared to the yellow ones, mainly after 3 days of pre-culture. Table 2 illustrates the difference of callus formation (%) after 3 days sucrose pre-culture (long-term) at different concentrations and followed or not by DMSO 5% (v/v) treatment (short-term).

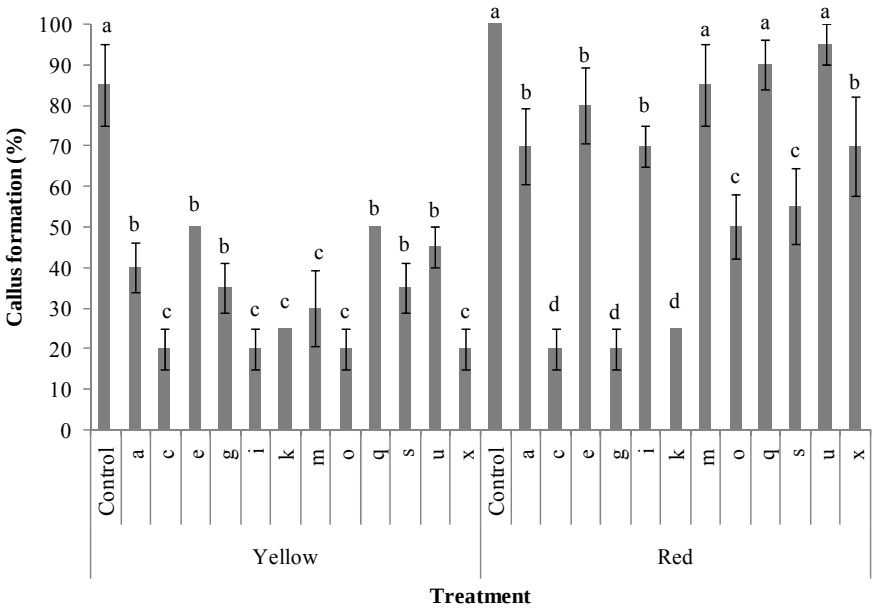

FIGURE 17 Toxicity effect of sucrose pre-culture and DMSO pre-treatment in the yellow and red cell lines of *B. intermedia* before cryopreservation and after 3-4 weeks treatment. 1 day sucrose (a,c,e,g,i,k); 3 days sucrose (m,o,q,s,u,x); 0.1M sucrose (a,c,m,o); 0.2M sucrose (e,g,q,s); 0.3M sucrose (i,k,u,x); no DMSO (a,e,i,m,q,u); plus DMSO (c,g,k,o,s,x). Each value represents the mean ± S.E.M. (n = 5) from a representative experiment and $P<0.01$ in one-way ANOVA followed by the Scott-Knott test.

TABLE 2 Callus formation (%) after 3 days sucrose pre-culture at different concentrations and followed or not by DMSO 5% (v/v) treatment in the yellow and red cell lines of *B. intermedia* before cryopreservation and 3-4 weeks after treatment.

Cell line (color)	**Sucrose concentration (M) pre-culture**	**DMSO 5% (v/v) pre-treatment**	**Callus formation (%)**	**Label**
Yellow	0.1	absent	30	M
Red	0.1	absent	85	M
Yellow	0.2	absent	50	Q
Red	0.2	absent	90	Q
Yellow	0.3	Absent	45	U
Red	0.3	Absent	95	U
Yellow	0.1	Present	20	O
Red	0.1	Present	50	O
Yellow	0.2	Present	35	S
Red	0.2	Present	55	S
Yellow	0.3	Present	20	X
Red	0.3	Present	70	X

5.3.4.2 Cryoprotectant solutions

When testing different cryoprotective solutions (normal M medium (0.09M) added with DMSO 5 and 7.5% (v/v), glycerol 5% (w/v) and the cryoprotective mixture 2 (0.25M DMSO, 0.25M glycerol and 0.5M sucrose) (M) and; modified M medium (0.5M sucrose) added with DMSO 5 and 7.5% (v/v) and glycerol 5% (w/v)) to cryopreserve red and yellow *B. intermedia* cell suspensions, similar results were observed as those described in the last sub-item (5.3.4.1), where yellow cell line became white and red cell line became brown and both cell lines showed no re-grew after cryopreservation (data not shown). The toxic effect of cryoprotective solutions before cryopreservation observed in the present experiment can be seen on item 5.3.3 since they showed similar responses.

In the *B. intermedia* cells, survival rates of control and cryopreservation experiments were completely different, indicating that the cells were not enough protected to prevent lethal ice crystal formation.

The cryopreservation research must advance in order to enough simplify and standardize the processes and though becoming the technology available to both public institutions and private companies (Panis & Lambardi, 2005).

The success of cryopreservation can vary because each material has its characteristics. For example, undifferentiated cell suspensions, which consist of large vacuolated cells (Luo & Widholm, 1997; Seijo, 2000; Mathur et al., 2003; Menges & Murray, 2004), is sensitive to rigorous cryoinjury compared with embryogenic cultures and apical organs, which contain small cytoplasmic-rich meristematic cells (Danso & Ford-Lloyd, 2004; Menges & Murray, 2004; Winkelmann et al., 2004). Moreover, suspension cells are sensitive to environmental stresses (like dehydration, high osmotic pressure, and low temperatures) (Kobayashi et al., 2005).

Several pre-culture assays were tried to improve survival after cryopreservation of azalea shoot tips. Sucrose and glucose were the only carbohydrates tested that assured survival after cryopreservation (Verleysen et al., 2005a). Pre-culture was also essential to cryopreserve *Eruca sativa* and *Astragalus membranaceus* hairy roots (HRs), but completely inefficient for those of *Gentiana macrophylla* HRs (Xue et al., 2008).

A pre-treatment of cyclamen embryogenic cell suspensions with cryoprotectants was essential for achieving good regrowth after cryopreservation (Winkelmann et al., 2004).

Most living plant cells have high quantities of water and are therefore extremely sensitive to temperatures below 0ºC (González-Benito et al., 2004). This was also the case of *B. intermedia* cell suspensions tested in the present work.

Without any selection pressure, plant cells are known to loose their productivity of secondary metabolites in time. Mannonen et al. (1990) proved that *Panax ginseng* and *Catharanthus roseus* cultures lost their productivity when maintained with continuous weekly subcultivations. Another example that lost its ability to produce secondary metabolites after several subcultures is the cell suspension culture of *Tabernaemontana elegans* that produces alkaloids. This loss is explained due to a change in the level of the regulation of the pathway, more than to the loss of the capacity to express an individual biosynthetic gene of the pathway (Lucumi et al., 2002).

There are many reports in the literature in which cryopreservation did not change the ability of tissues/cells to produce their main secondary metabolites. For example, hypericin content in plants regenerated from cryopreserved *Hypericum perforatum* meristems (Urbanová et al., 2006); the main alkaloid precursor tryptamine of *Tabernaemontana divaricata* suspension cell cultures (Suhartono et al., 2005); anthocyanins and other flavonoids in *Vaccinium pahalae* cells (Shibli et al., 1999); alkaloid in *Catharanthus roseus* cell suspension lines (Chen et al., 1984b); ginsenoside of *Panax ginseng* hairy roots (Yoshimatsu et al., 1996) and cell cultures (Mannonen et al., 1990)

Also some work related cryopreservation procedures that increase the production of specific secondary metabolites in some species were reported; one example is *Chrysanthemum cinerariaefolium* which after cryopreservation was observed to have a higher pyrethrin biosynthesis ability compared to the non-cryopreserved cells (Hitmi et al., 1997). Also the residual productivity of ajmalicine and catharanthine *Catharanthus roseus* cells was higher after cryopreservation (Mannonen et al., 1990).

The interest in cryopreservation has been increased and various successful reports have been published on the use of this technique to store cell cultures. Even the biosynthesis of secondary metabolites has been retained

during cryostorage of several plant species. Despite the successful reports cited above, it is, however, important to keep in mind that considerable experimentation is always needed to optimize all the steps during the cryopreservation procedure. No routine method is until this moment available for different species and cultures (Mannonen et al., 1990).

The unsolved problem of cryopreservation of *Byrsonima intermedia* may be related, in part, to their sensitivity to chilling (it is a tropical plant) and cryoprotective agents. *B. intermedia* is extremely sensitive towards chilling and toxicity and protective effects against chilling of cryoprotectants also affected this species, showing that cryopreservation procedures are species-dependent. Luo & Widholm (1997) observed that three out of the six different suspension culture lines submitted to cryopreservation did not survive any treatment, confirming this procedure is species and cell line dependent. Also results obtained by Škrlep et al. (2008) showed that recovery was line dependent.

It is likely that plasma membranes of *B. intermedia* cells were irreparably damaged, just like in *Polytrichum* cells (Yamazaki et al., 2009).

6 CONCLUSIONS

Calli were successfully induced for *Byrsonima intermedia* species and different aspects were observed in these explants when cultured in different light conditions.

Growth curves of *B. intermedia* cell suspensions were established.

No regrowth of *B. intermedia* cells was observed within 4 weeks after cryopreservation.

7 FUTURE WORK

As tissue culture explants, mainly callus or cell suspensions, showed in many reports to be suitable for producing secondary metabolites in several species, further work is needed to understand which compounds are produced and when exactly during the growth phase of *B. intermedia* cell suspensions they are formed. According to the literature the production of secondary metabolites *in vitro* can occur at almost any stage of culture growth. Also the ideal inoculum size used to initiate cell suspension cultures should be investigated in this species in order to observe its effect on the synthesis of interesting secondary metabolites.

The research of cryopreservation in *B. intermedia* species still requires further optimization with the aim of preserving potential cell suspensions (embryogenic and/or medicinal proprieties) for the long term as well as for biotechnological applications such as genetic engineering. A simple and efficient protocol for cryopreservation of highly productive cell lines of *B. intermedia*

should therefore be developed that allows high viability rates and regrowth without loss of secondary metabolite biosynthetic capacity. For this suitable pretreatment conditions need to be developed that allows these *B. intermedia* cells to have better tolerance of osmotic and freeze-induced dehydration stresses.

To better understand what the types of damage cryopreservation caused in *B. intermedia* cells, a complete study observing the intra and extracellular changes should be developed using morphologic and ultra-structural analyses such as scanning/transmission electron microscopy and light microscopy.

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CHAPTER 3

Phytochemical, anti-microbial and anti-inflammatory assays of different extracts of *Byrsonima intermedia*

1 ABSTRACT

Byrsonima intermedia, popularly known in Brazil as "murici-pequeno", is a shrub from the Brazilian Cerrado. This species has been extensively used for medicinal purposes by the folk population. The aim of the present study was to evaluate chemical differences among extracts of *B. intermedia* leaves, bark, flowers, calli and cell suspensions cultures, using Thin Layer Chromatography (TLC) technique and later on test representative lines in an anti-microbial assay and in zebrafish (*Danio rerio*) for their anti-inflammatory activity. The anti-microbial study was executed on *Candida albicans*, *Saccharomyces cerevisiae* and *Staphylococcus epidermidis*. The crude methanolic extracts as well purified compounds were tested in zebrafish. Phytochemical analysis was performed in order to characterise the active compounds present in the extracts. None of the crude *B. intermedia* extracts tested showed antimicrobial activity against the microorganisms used. Crude methanolic extracts from bark, leaves and flowers at 10μg mL^{-1} present anti-inflammatory effect on zebrafish larva using the tail fin injury test. The strong anti-inflammatory properties of *B. intermedia* are confirmed in a purified fraction from bark, leaves and flowers methanolic extracts. The main bioactive compounds present in *B. intermedia* bark and leaves methanolic extracts are β-amyrin and epicatechin. Gallic acid is another secondary metabolite present in bark extracts. Red cell suspensions of *B. intermedia* contain the secondary metabolite epicatechin.

Key-words: Thin Layer Chromatography (TLC); anti-microbiology; zebrafish; Gas Chromatography/Mass Spectrometer (GC-MS); High performance liquid chromatography (HPLC).

2 RESUMO

Byrsonima intermedia, popularmente conhecido no Brasil como "murici-pequeno", é um arbusto do Cerrado brasileiro. Esta espécie tem sido amplamente utilizada para fins medicinais pela população local. Objetivou-se avaliar as diferenças químicas entre os extratos de folhas, cascas, flores, calos e suspensões celulares de *B. intermedia*, utilizando-se a técnica de Cromatografia em Camada Delgada (CCD) e, posteriormente, testar as linhagens representativas em análise anti-microbiana e em zebrafish (*Danio rerio*) para a sua atividade anti-inflamatória. Para o teste anti-microbiano os microorganismos utilizados foram *Candida albicans*, *Saccharomyces cerevisiae* e *Staphylococcus epidermidis*. Os extratos metanólicos (bruto e purificado) foram testados em zebrafish. A análise fitoquímica foi realizada a fim de caracterizar os compostos ativos presentes nos extratos. Nenhum dos extratos brutos de *B. intermedia* testados apresentaram atividade antimicrobiana contra os microrganismos utilizados. Extrato metanólico bruto das cascas, folhas e flores na concentração de 10μg mL^{-1} apresenta efeito anti-inflamatório na larva de zebrafish utilizando o teste de lesão da nadadeira caudal. As propriedades anti-inflamatórias de *B. intermedia* são confirmadas em uma fração purificada dos extratos metanólicos de cascas, folhas e flores. Os principais compostos bioativos presentes nos extratos metanólicos de cascas e folhas de *B. intermedia* são β-amirina e epicatequina. Ácido gálico é outro metabólito secundário presente nos extratos de casca. Suspensões celulares de *B. intermedia* de coloração vermelha contêm o metabolito secundário epicatequina.

Palavras-chave: Cromatografia em Camada Delgada (CCD); anti-microbiologia; zebrafish; Cromatografia Gasosa/Espectrometria de massa (CG-EM); Cromatografia Líquida de Alta Eficiência (CLAE).

3 INTRODUCTION

The American flora has a wide diversity and possess one of the world's wealthiest sources of material with pharmacological proprieties. In Brazil, nowadays medicinal plants have been extensively used as home-made medicines mainly because the industrialized remedies present a high cost (Brandão et al., 2008). The genus *Byrsonima* which belongs to the Malphigiaceae family, contains around 150 species and is widely distributed in Central and South America (Aguiar et al., 2005). Data obtained in the NAPRALERT (Natural Products Alert Database) indicated these genus species are used for medicinal purposes, as anti-asthmatics, anti-thermic and for skin infections (Caceres et al., 1993). For the species that compose the genus, few phytochemical studies are executed, especially related to the species *Byrsonima intermedia*.

A common worldwide practice of plants use is its therapeutic activity and special dietary. Nowadays, drugs originated from plants are researched for the possibility of presenting biological activities (Cardoso et al., 2006).

In Brazil, many plant extracts are used in popular medicine to cure several kinds of illness, among them *B. intermedia* which is a medicinal plant with extensive use. All *B. intermedia* parts are used by the local population in the treatment of several ailments. Its leaves tea is used against diarrhea, intestinal infections and as a protector of intestinal mucosa. Its roots tea is utilized to treat chronic wounds, ulcers, mouth and throat diseases, through the application in the affected area with cotton soaked in tea. This same tea is also indicated for vaginal flowing in seat bath (Lorenzi & Matos, 2008).

Despite the popular use of *B. intermedia* as medicinal plants, there are no data available about the anti-inflammatory effect of their leaf, bark, flower as well as their cell suspensions extracts. There are just few data about the

antimicrobial effect of their extracts (Sannomiya et al., 2007; Michelin et al., 2008) and healing action in chronic gastric disease (Santos et al., 2009).

Traditional uses for *Byrsonima* species have been confirmed by phytochemical and pharmacological studies (Navarro et al., 1996; Martínez-Vázquez et al., 1999; Cifuentes et al., 2001; Leonti et al., 2002; Alves & Franco, 2003; Sannomiya et al., 2004; Aguiar et al., 2005; Sannomiya et al., 2005; Cardoso et al., 2006; Wagner & Brito, 2006; Sannomiya et al., 2007; Lima et al., 2008; Lira et al., 2008; Michelin et al., 2008; Souza et al., 2008; Bonacorsi et al., 2009; Cunha et al., 2009; Maldini et al., 2009; Santos et al., 2009).

The zebrafish (*Danio rerio*) has been one of the first developmental and genetic tool to study not only organogenesis but also diseases identifying small molecules that suppress their state. There are some attributes of using zebrafish as a model organism which make them easy to analyse development and bioactive natural products, various of which are likely to be attractive lead compounds for the development of new, effective drugs. Among several advantages of zebrafish the main are the small size of their embryos and larvae with which most tests can be executed, the high degree of genetic and physiological similarity between zebrafish and mammals, the high fecundity of adult zebrafish, the optical transparency of embryos and larvae, the speed at which these develop *ex utero*, the fact that phenotypes can be so rapidly assessed in high throughput systems and its quickness and and less costly (Zon & Peterson, 2005; Crawford et al., 2008). The zebrafish has been widely and increasingly used not only in industry research but also in government funded basic science (Berger & Currie, 2007).

All these advances prompted us to examine the anti-inflammatory and anti-microbial activities of *B. intermedia*, a species that presents a potential medicinal value. The aim of the present study was to evaluate differences among extracts of *B. intermedia* leaves, bark, flowers, calli and cell suspensions

cultures, using TLC (Thin Layer Chromatography). This will later on be used to test different lines in an anti-microbial and anti-inflammatory (using zebrafish) activities assays and phytochemical analyzes.

4 MATERIAL AND METHODS

Experimental design

The anti-microbial study was executed on the fungus *Candida albicans* (SC5314), the yeast *Saccharomyces cerevisiae* (BY4741) and the bacteria *Staphylococcus epidermidis* (LMG10474). For this, fresh material previously dried and homogeneized was extracted with methanol and water solvent. Using Thin Layer Chromatography (TLC), we could analyze and choose representative cell lines that were afterwards tested in an anti-microbial assay and in zebrafish for their anti-inflammatory effect. The crude methanolic extracts were tested in zebrafish. Using the TLC we could analyze and purify different components that were also tested in zebrafish separately. Phytochemical analysis was performed in order to characterise the active compounds present in the extracts.

4.1 Samples

Bark; leaves; flowers; red, green, brown callus from embryos; red and yellow cell suspensions from calli originated from *in vitro* leaves and embryos cultured in different conditions (dark/light) and in different medium were used. *Byrsonima intermedia* (Malpighiaceae) was collected from native plants growing in the Ijaci, state of Minas Gerais, MG, Brazil. The voucher of the plant sample was registered (by prof. Dr. Eduardo van der Berg) at the Herbarium Esal of the Biology Departament of the Universidade Federal de Lavras number 17.601, Brazil.

4.2 Extraction procedure

Fresh bark, leaves and flowers were dried for 3 days in an oven at 70ºC, while calli and cell suspensions were lyophylized till dry. The dried samples

were then homogenized before the extraction procedure. For this, bark, leaves and flowers were pulverized to a fine powder with a ball mixer (Retsch mixer mill MM200) and calli and cell suspensions using pistil and graal. Different extraction procedures were applied: (i) a methanol (MeOH) extraction and (ii) a water (H_2O) extraction. For the methanol extraction, 150 mg of dried samples were extracted successively two times with 1.5 mL of methanol using an ultrasonic bath (sonicator) for 2 hours and 10 minutes, respectively. For the water extraction, the same procedure was followed, however while in the sonicator, the samples were heated at 100°C. Then the sample was centrifuged for 5 minutes at 15000 rpm and the supernatant was collected in a pre-weighed test tube. The total supernatant fraction was completely dried in vacuum overnight. MeOH supernatants were evaporated in a rotovapor. Thereafter, the plant extracts were dissolved in DMSO at the final concentration of 5% (50 mg mL^{-1}) for use in the zebrafish and anti-microbial tests.

4.3 Thin layer chromatography (TLC)

Thin layer chromatography was performed on 20 x 20 cm silica-gel 60 F254 TLC plates, fluorescent indicator 254 nm, coated with 0.20 mm layer (Fluka). After preliminary saturation of the TLC chamber with solvent for at least 15 minutes, the samples (≅ 1 mg) (Table 1) were spotted on the TLC plates. For the development of the TLC, $CHCl_3$:MeOH (50:50) was used as the mobile phase. Afterwards, the plate was dried using cold air and subsequently visualized under UV light (254 nm and 366 nm). Visualization of the total organic matter in the extract bands was done by spraying plates with sulfuric acid (50% in methanol) and then heating them at 110°C for few minutes to better visualize the bands under UV detection.

TABLE 1 Plant extracts samples of *Byrsonima intermedia* analyzed on Thin Layer Chromatography (TLC).

Samples	Color	Aspect	Originated from	Culture medium	Culture conditions	Label
Cell suspension	Yellow	Homogeneous	Callus from *in vitro* leaves	A	Dark	1
Cell suspension	White	Homogeneous	Callus from *in vitro* leaves	A	Dark	2
Cell suspension	Red	Homogeneous	Callus from *in vitro* leaves	A	Dark	3.1
Cell suspension	Red	Homogeneous	Callus from embryos	A	Dark	3.2
Cell suspension	Red	Heterogeneous	Callus from *in vitro* leaves	A	Dark	4
Cell suspension	Red	Heterogeneous	Callus from *in vitro* leaves	A	Light	5
Cell suspension	White	Homogeneous	Callus from *in vitro* leaves	A	Light	6
Cell suspension	White	Homogeneous	Callus from *in vitro* leaves	B	Dark	7
Cell suspension	Red	Heterogeneous	Callus from *in vitro* leaves	B	Light	8
Cell suspension	White	Homogeneous	Callus from *in vitro* leaves	C	Dark	9
Callus	Red	Homogeneous	Embryos	D	Light	10.1
Callus	Green	Homogeneous	Embryos	D	Light	10.2
Callus	Brown	Homogeneous	Embryos	D	Light	10.3
Callus	Yellow	Homogeneous	Embryos	D	Dark	11.1
Callus	Brown	Homogeneous	Embryos	D	Dark	11.2
Bark	-	-	-	-	-	B
Leaves	-	-	-	-	-	L
Flowers	-	-	-	-	-	F

"…continue…"

"TABLE 1, Cont."

A - MS (Murashige & Skoog, 1962) liquid medium with 2.26µM 2,4-D, 4.65µM kinetin and 0.09M sucrose; B - MS liquid medium with 2.26µM 2,4-D and 0.09M sucrose; C - MS liquid medium with 4.52µM 2,4-D and 0.09M sucrose; D - MS medium with 4.52µM 2,4-D, 0.09M sucrose and 0.6% agar; Light - at 25±2ºC under continuous 50 µE m^{-2} s^{-1} illumination provided by 36 W Osram cool-white fluorescent tubes; Dark - at 25±2ºC under darkness.

4.4 Anti-microbial activity assay

The anti-microbial activity of plant extracts (using methanol and water) against different microorganisms was assayed using microspectrophotometry of planktonic cultures. The plant extracts used were bark (A) (B from table 1 on 4.3 item), leaves (B) (L from table 1 on 4.3 item), flowers (C) (F from table 1 on 4.3 item), red callus from embryos cultured in light conditions (D) (10.1 from table 1 on 4.3 item), yellow cell suspension from callus initiated from *in vitro* leaves cultured in dark conditions (E) (1 from table 1 on 4.3 item), red cell suspension from callus initiated from *in vitro* leaves cultured in dark conditions (F) (4 from table 1 on 4.3 item), red cell suspension from callus initiated from *in vitro* leaves cultured in light conditions (G) (8 from table 1 on 4.3 item) and green callus from embryos cultured in light conditions (H) (10.2 from table 1 on 4.3 item). The microorganisms used were the fungus *Candida albicans* (SC5314), the yeast *Saccharomyces cerevisiae* (BY4741) and the bacteria *Staphylococcus epidermidis* (LMG10474). *C. albicans* and *S. cerevisiae* were grown overnight at 30°C in YPD (1% Bacto Yeast Extract, 2% Bacto Peptone, 2% glucose) and *S. epidermidis* at 37°C in TSB (Tryptic Soy Broth (Bacto)). The precultures were further diluted in yeast minimal medium (YMM - 0.8 g L^{-1} complete supplement mixture [CSM, BIO 101, La Jolla, Calif.], 6.5 g L^{-1} yeast nitrogen base without amino acids [Difco], 20 g L^{-1} glucose) (*C. albicans* and *S. cerevisiae*) or TSB (*S. epidermidis*) till a cell density of 10^6 cells mL^{-1}. Five microliter of extract (25 mg mL^{-1}) was mixed with 95 µL of cell suspension in a

96-well plate and incubated for 24 hours at 37°C without shaking. As a measure of microbial growth, the absorbance at 595 nm (optical density – O.D.) was determined using a Versamax microplate reader (Molecular Devices). All determinations were made in triplicate and repeated three times. DMSO 2.5% was used as the negative control. As such, a dilution series (1250; 625; 313; 156; 78; 39; 20; 10; 5; 2.5; 1.3 µg mL^{-1}) of three water plant extracts (E, G, B) was tested. A dilution series [(1.0; 0.5; 0.25; 0.063; 0.031; 0.016; 0.008; 0.004; 0.002; 0.001 µg mL^{-1}) and (100; 50; 25; 12.5; 6.25; 3.125; 1.6; 0.8; 0.4; 0.2 µg mL^{-1})] of the antifungal agent miconazole (Sigma - Aldrich NV) was used as a positive control for *C. albicans* and *S. cerevisiae*, respectively. Miconazole is a widely used, commercially available antifungal drug. The minimal inhibitory concentration (MIC) of the extracts could be determined. The MIC is defined as the lowest dose of the extract that is responsible for complete growth inhibition of the microorganism, analogous to the MIC for pure compounds.

With these measurements it is possible to make dose-response curves of the water plant extracts and compare them with miconazole (*S. cerevisiae* and *C. albicans*).

An anti-microbial activity assay was executed for all the microorganisms by determining the minimal fungicidal/bactericidal dose (MFD/MBD) of two plant water extracts (E, G). Overnight grown cultures of the microorganisms mentioned above were diluted in PBS buffer till a cell density of 10^6 cells mL^{-1}. Five microliter of water extract (25 mg mL^{-1}) was mixed with 95 µL of cell suspension in a 96-well plate and incubated for 24 hours at 37°C without shaking. Amphotericin B (AMB) (125 µg ml^{-1}) (Sigma – Aldrich NV), an antifungal drug, was used as the positive control for *C. albicans* and *S. cerevisiae* while DMSO 2.5% was used as the negative control for all the microorganisms tested. The 96-well plates were incubated at 37 °C for 16 h. The determinations were done in triplicate for each extract. One hundred µL of a 10-

fold dilution series (10^{-2}, 10^{-3} and 10^{-4}) of each condition was plated on YPD or TSB agar medium and incubated for 1 day at 37°C, after which colony forming units (CFUs) could be determined. The minimal fungicidal/bactericidal dose (MFD/MBD) of the extract is defined as the minimal concentration of the extract necessary to kill more than 99.9% of the microorganisms as compared to the DMSO-control.

4.5 Zebrafish pigmentation

4.5.1 Zebrafish care

During the course of the experiments, the zebrafish line used was the fli (transgenic, with enhanced green fluorescent protein (EGFP) under the control of the fli-1 promotor). The zebrafish were kept in tanks of 150 L, each containing 200 to 300 individuals. The temperature in the tanks was kept constant at 28ºC. The zebrafish were fed brine shrimps (Silver Star). The brine shrimp eggs (3g) were incubated for 2 days in 2 L water containing 50 g salt (Reef Crystals, Aquarium systems), air was added at the bottom of the bowl, under a bright light. This amount of brine shrimps was needed to feed three tanks of zebrafish. Complementary to the brine shimps the zebrafish were also fed flakes (Vipan main feed, Sera). The osmolarity and hardness of the water were maintained with magnesium sulfate (60 mg L^{-1}), calcium sulfate (60 mg L^{-1}), sodium carbonate (95 mg L^{-1}) and potassium chloride (4 mg L^{-1}). All the embryos were reared in 0.3x Danieau's solution (a buffered embryo water consisted of 17,4 mM NaCl, 0.21 mM KCl, 0.12 mM $MgSO_4$, 0.18 mM $Ca(NO_3)_2$ and 1.5 mM Hepes, pH 7.6) and were dechorionated at 10-12 hours post-fertilization (hpf) prior to exposure to extracts or fractions. Each treatment group contained 10 embryos. Embryos were allowed to develop at 28 ± 1ºC. Collection of the zebrafish eggs was done using traps which were put in the tanks in the evening. A plant substitute was placed on the trap to provide an

extra stimulus for the fish to spawn. This took place in the morning. The traps consist of a dish with a porous lid which was covered with marbles to simulate pebbles. The eggs fell through the pebbles and the porous lid into the dish, safe from the adut zebrafish and waiting to be collected. Once the eggs were collected, the fertilized eggs were separated from the unfertilized ones.

4.5.2 Extracts used

Two types of extracts were used: (1) crude methanolic extracts of *Byrsonima intermedia* leaves, bark, flowers at different concentrations (0.1, 0.3, 1, 3, and 10µg mL^{-1}); and (2) fractions that were purified from crude methanolic extracts of *B. intermedia* leaves, bark, flowers, callus and cell suspensions cultures (Figure 1). "Yellow cell suspensions" are actually all white/yellow homogeneous cell suspension originated from callus obtained from *in vitro* leaves (1, 2, 7 and 9 from Table 1 on item 4.3) and cultured under dark conditions (at 25±2ºC under darkness); while "red cell suspensions" are all those originated from callus 10.1 (Table 1, item 4.3) and cultured under light conditions (at 25±2ºC under continuous 50 µE m^{-2} s^{-1} illumination provided by 36 W Osram cool-white fluorescent tubes). The raw material was chromatographed on silica gel eluted with $CHCl_3$:MeOH (50:50). Aliquots of 400 µL of the crude methanolic extracts (at the final concentration of 5% - 50mg mL^{-1}) were transferred in lanes on silica-gel plates for the development of the analytical thin layer chromatography (TLC). The plates were dried by cold air and visualized under UV light (254 nm and 366 nm). Visualization of the total organic matter in the extract bands was done by spraying a part of the TLC plates with sulfuric acid (50% in methanol) and then heating at 110°C for few minutes to better visualize the bands under UV detection. Only part of the TLC plates was sprayed and heated in order to scrape off the bands from the other part. The bands found (Table 2) were then scraped and after eluted from the

silica using $CHCl_3$:MeOH (50:50). Briefly, after evaporation, the dried residue was dissolved in DMSO (20 µL) and stored at -20ºC. Aliquots of 5 and 10µL of the bands dissolved in DMSO were used in the zebrafish assay.

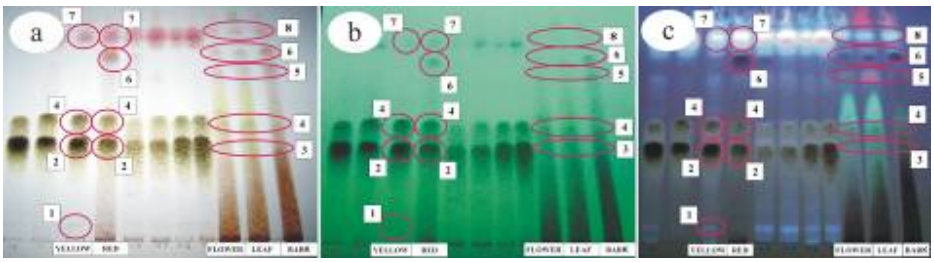


FIGURE 1 Bands tested in zebrafish assay on TLC plates of *Byrsonima intermedia* methanolic extracts under (a) normal light, (b) UV366nm detection and (c) UV254nm illumination after detection with sulfuric acid ($CHCl_3$:MeOH – 50:50 system).

TABLE 2 Bands tested in zebrafish assay from different *Byrsonima intermedia* methanolic extracts after detection with sulfuric acid ($CHCl_3$:MeOH – 50:50 system).

Bands number	**Samples**				
	Yellow cell suspensions	**Red cell suspensions**	**Flowers**	**Leaves**	**Bark**
1	X	-	-	-	-
2	X	X	-	-	-
3	-	-	X	X	X
4	X	X	X	X	X
5	-	-	X	X	X
6	-	X	X	X	X
7	X	X	-	-	-
8	-	-	X	X	X

4.5.3 Preliminary tests on vascular system

This test was performed only from isolated bands from crude methanolic extracts (the second extract type described in the item above and visualized in figure 1 and table 2).

The *fli-1*:EGFP transgenic line (Lawson & Weinstein, 2002) was obtained from the Zebrafish International Resource Center at the University of Oregon (Eugene, Oregon, USA). Zebrafish husbandry, embryo collection, and embryo and larva maintenance were performed as described (Westerfield, 1994; Volhard & Dahm, 2002). Zebrafish assays were standardly performed in 24-well microtiter plates using 10 embryos per well in 1ml of 0.3x Danieau's medium. Embryos were exposed to extracts and compounds at 16 hours post fertilization (hpf) – approximately 8 hours prior to the initiation of intersegmental vessel (ISV) outgrowth – and scored for relative vascular outgrowth at 40hpf. Extracts and compounds were solubilized in dimethyl sulfoxide (DMSO, Agros Organics, Geel, Belgium), and were added to the medium up to a maximum DMSO concentration of 1%. The extent of outgrowth of ISVs was determined using a scoring method that takes into account both the approximate number of outgrowing vessels (100, 75, 50, 25, or 0%) and the average degree to which these vessels have extended into the trunk from the the dorsal aorta/posterior cardinal vein (DA/PCV) (100, 75, 50, 25, or 0%). These two values are multiplied to give the relative vascular outgrowth (RVO) score.

4.5.4 Lipopolysaccharide (LPS)-induced leukocyte migration assay

The LPS-induced leukocyte migration assay was carried out in the tails of 4 days post fertilization (dpf) zebrafish larvae pre-treated with 1X PTU (1-phenyl 2-thiourea). PTU blocks all tyrosinase-dependent steps in the melanin pathway inhibiting melanogenesis, remaining the zebrafish embryos transparent as long as its treatment. However it has to be used with caution since it can be

toxic at high concentrations. PTU treatment not remove already formed pigment and therefore must be initiated before the initial pigmentation (Karlsson et al., 2001). First, a one hour pre-incubation step at 28° C in 24-well culture plates was performed. Ten larvae per well were incubated in 1ml of testing mix, prepared with specific concentrations of each drug/extract and the amount of 0.3X Danieau's necessary to complete 1ml. The drugs/extracts were first dissolved in DMSO (Agros Organics, Geel, Belgium) (1 to max. 2%). Immediately after pre-incubation, complete tail transection in each larva was performed. Zebrafish larvae were first anesthetized by immersion in 0.3X Danieau's containing 70µg mL^{-1} tricaine (ethyl 3-aminobenzoate, Sigma-Aldrich Chemical Co., St. Louis, MO) and then tail transection near the tip was done with a sterile scalpel. Larvae were then placed in fresh 0.3X Danieau's without anesthetic for a few minutes.

For the last step, a new testing mix was prepared including 10µg mL^{-1} lipopolysaccharide (LPS, Sigma-Aldrich Chemical Co., St. Louis, MO) to stimulate leukocyte migration to the injured zone. LPS induces local and systemic inflammation as well as activation of macrophages even at concentrations as little as 10pg mL^{-1} (Abbas & Lichtman, 2005; Watzke et al., 2007). The final incubation step of the injured larvae in contact with the pro-inflammatory agent and with the specific concentration of each drug at 28°C lasted seven hours. Within this period, sporadic evaluations of the larvae were done to examine their viability during the assay.

As reference, the non-steroidal anti-inflammatory drug (NSAID) indomethacin (10.74 and 35.78 µg mL^{-1}) was used. DMSO at 1% was used as a negative control.

4.5.5 Myeloperoxidase staining

To evaluate the inflammatory response and its resolution in zebrafish larvae, a histochemical approach was used (Siverio-Mota, D.; Cordero-Maldonado, M. L.; Esguerra, C. V.; de Witte P. A. M.; Crawford, A. D. unpublished data). It is based on the enzymatic action of a lysosomal protein called myeloperoxidase (MPO) most abundantly present in neutrophil granulocytes. Particularly neutrophils and eosinophils are clearly peroxidase-positive. The great majority of monocytes also react peroxidase-positively although the response is weaker than the one of the neutrophilic and eosinophilic granulocytes (black-brown). Basophils and all cells of the lymphatic and erytropoietic series are peroxidase-negative.

To perform the histochemical staining, larvae that completed the incubation time were fixed in 4% formaldehyde solution for five minutes and washed twice with PBS for one to two minutes. Fixed larvae were incubated at room temperature in 1ml of freshly prepared staining solution Leucognost® Pox (Merck), made by dissolving the content of reagent 1 (4-chloro-1-naphthol) in 15ml of ethanol. To this solution was added: 45ml of distilled water, 10 drops of reagent 2 (Tris{hydroxymethyl aminomethane}-HCl buffer) and 2 drops of reagent 3 (3% hydrogen peroxide solution). Observation of stained black-brown cells (leukocytes) in the larvae was possible within approximately ten minutes of incubation. Evaluation of the leukocyte migration specifically around the injured region in each larva was done under light microscopy using a migration scale established to quantify the amount of migrating leukocytes (Table 3). Figure 2 schematically explains the values assigned to each larva in the leukocyte migration assay. The average of the value achieved for each of the ten larvae tested for one sample is expressed as relative leukocyte migration (RLM) according the formula:

$$\text{RLM (\%)} \quad \frac{\text{Average \{leukocyte migration after treatment\}}}{\text{Average \{leukocyte migration without treatment\}}} \text{ x } 100$$

TABLE 3 Leukocyte migration scale.

Migrating leukocytes	**Assigned value**	**RLM (if control = 1)**
0 to 5	0	0
6 to 10	1	0.25
11 to 15	2	0.50
16 to 20	3	0.75
> 20	4	1

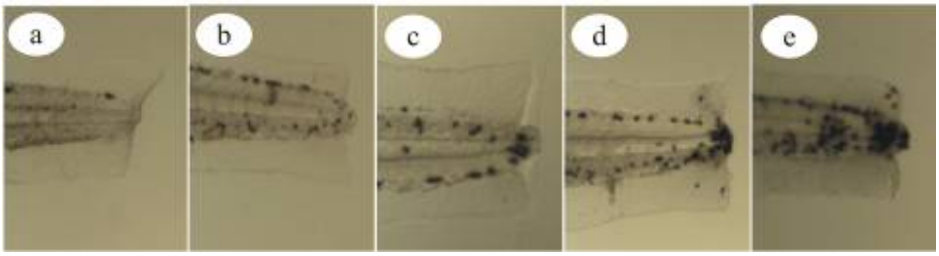


FIGURE 2 Example of the Leukocyte Migration Scale in tails of 4dpf zebrafish larvae. Dark-tip spots represent the migrating leukocytes to the injured zone. Relative leukocyte migration (RLM) (a) 0; (b) 0.25; (c) 0.5; (d) 0.75 and (e) 1.

4.5.6 Statistics

All experiments were repeated once and the values were given as means ± S.E.M. (Standard error of the mean). Data were analyzed using Sisvar® software and statistical significance was assessed by one-way ANOVA. P values less than 0.01 were considered significant.

4.6 Phytochemical analysis

4.6.1 Tannins and Carotenoids

4.6.1.1 Samples

Three types of cells suspensions were initiated from different calli. They varied according colour and culture conditions: (i): yellow cell suspensions (YCS) derived from yellow friable calli initiated on leaf segments (≈ 0.25 cm^2) of *B. intermedia* seed germinated *in vitro*; (ii) red cell suspensions (RCS) derived from red friable calli initiated on embryos of *B. intermedia*; and (iii) pink cells suspensions (PCS) derived from a mixture of yellow and red calli initiated on embryos of *B. intermedia*. All the cell suspensions were initiated and maintained as described in the item 4 of the previous chapter:"*In vitro* culture and cryopreservation of *Byrsonima intermedia*".

4.6.1.2 Tannin content

The tannin content was estimated using a colorimetric method based on the Folin-Denis reagent. The Folin-Denis reagent is specific for compounds containing the oxy phenyl group. This way, tannins can be determined spectrophotometrically (λ = 760 nm) using the Folin-Dennis colorimetric method, according to AOAC (1990) and Deshpande et al. (1986). The concentration was then calculated through a standard curve, prepared with a standard solution of tanic acid.

4.6.1.3 Carotenoid content

Total carotenoids were determined as described by Higby (1962) in extractor solution of isopropyl alcohol:hexane (3:1). The content was transferred to a separatory funnel (125 mL) involved in aluminum and the volume completed with distillated water. After 30 minutes of rest the material was washed. This procedure was repeated for at least four times. The content was filtered with cotton sprayed with the anhydrous sodium sulphate (99%) into a volumetric flask (50 mL) wrapped with aluminum. The next step was to add 5 mL of acetone (99.5%) and then to complete the volume with hexane (98.5%). The readings were made at 450 nm and the results calculated through the following formula:

$$\text{Carotenoids (mg } 100\text{mL}^{-1}) = (A_{450} \times 100) / (250 \times L \times W)$$

A_{450} = absorbance;
L = cuvette lenght (cm);
W = ratio between original sample mass (g) and the dilution final volume (mL).

4.6.1.4 Statistics

All experiments were carried out in triplicate and the values were given as means ± S.E.M. (Standard error of the mean). Data were analyzed using Sisvar® software and statistical significance was assessed by one-way ANOVA. P values less than 0.01 were considered significant.

4.6.2 Gas chromatography (GC)

Yellow cell suspensions (YCS), bark and leaves were used to identify their compounds. The extraction procedure used is described under item 4.2 of the present chapter.

The identification of the constituents was performed by GC-MS (GC coupled to a mass spectrometer - MS) in the Division of Organic and Pharmaceutical Chemistry of the Multidisciplinary Center of Chemical , Biological and Agricultural Research of the University of Campinas (CPQBA/UNICAMP), Campinas – Brazil. For this, a Hewlett-Packard Model (HP) 6890 gas chromatograph coupled to a HP-5975 mass selective detector and HP-5MS capillary column (30m x 0.25mm x 0.25μm) with a flow of carrier gas helium of 1 mL min^{-1} and heating with set temperature (60°C with a gradient of 3°C min^{-1} till 240°C) was used. The volume of sample injection (concentration of 10mg ml^{-1} in ethyl acetate (Synth)) was of 1.0μL and 30:1 split ratio. The injector and detector temperatures were maintained at 220°C and 250°C, respectively. The analytes relative percentage was obtained by integrating the ion total chromatogram (ITC). The retention indices (RI) were determined by injecting a standard mixture of hydrocarbons (C9-C20) (Sigma, USA). The identification of constituents was performed by RI comparison, literature data (Adams, 2001) and fragmentation of the mass spectra deposited in the library NIST-05.

4.6.3 High performance liquid chromatography (HPLC) and Thin layer chromatography (TLC)

4.6.3.1 Thin layer chromatography (TLC) analysis

Chromatographic aluminum plates (Merck - article 5554) of 10cm high were used. We applied 5 μL of the quercetin (Q), epicatechin (E) and gallic acid (GA) standard solutions to 2.5 and 5.0 μg μL^{-1} of the extracted samples (leaves (L), bark (B), yellow cell suspensions (YCS) and red cell suspensions (RCS)) at the concentration of 40μg μL^{-1}. Chloroform: methanol (1:1 v/v) was used as eluent.

For compounds detection, after plates elution, UV irradiation was used at 254 and 366 nm, followed by spraying the whole plates with two developers: (1) anisaldehyde solution (acetic acid: sulfuric acid: anisaldehyde, 50.0:1.0:0.5 v/v) and heating in an oven at 100ºC for 5 minutes; and (2) NP solution, followed by 400 polyethylene glycol solution (50g L^{-1} in methanol) and evaluated under UV light (Natural products-polyethylene glycol reagent) (Wagner & Bladt, 1996).

4.6.3.2 High performance liquid chromatography (HPLC) analysis

The samples analyses were performed by HPLC-DAD in the Waters Alliance chromatographic system equipped with 2695 pump, diode array detector 2996 (scan from 200 to 400 nm and reading at 276nm), column oven at 35°C and Empower® software.

The separation of compounds was performed on an Atlantis dC-18 (Waters) column, 100 x 2.1mm, 3µm, using as eluents: A = 0.1% formic acid (aq) (v/v) and B = methanol. The gradient elution was performed according to the table 4.

TABLE 4 Gradient elution of the 0.1% formic acid (aq) (v/v) (A) and methanol (B) eluents for standardizing the HPLC conditions.

Time (min)	A(%)	B(%)
0	98	2
5	98	2
40	0	100
45	0	100
60	98	2
80	98	2

The stock solutions of standards were prepared in methanol at a concentration of about 400 µg mL^{-1}. The following step was to dilute them in 0.1% formic acid (aq):methanol (90:10) in a proportion of 1:10 and then to filter through a 0.45µm membrane before the injection.

The following standards concentrations were applied: gallic acid (monohydrate, Sigma): 47.4 µg mL^{-1}; (-) epicatechin (Sigma): 40.2 µg mL^{-1}; and quercetin (dehydrate, Sigma): 40.6 µg mL^{-1}.

The stock solutions of samples were prepared by dissolving the dry methanol extract to a final concentration of approximately 40 mg mL^{-1}. For leaves and bark the stock solution was diluted 1:50 with 0.1% formic acid (aq): methanol (90:10); for red cell suspensions the stock solution was diluted 1:10 with formic acid 0.1% (aq): methanol (20:80); and for yellow cell suspensions the stock solution was diluted 1:10 with formic acid 0.1% (aq): methanol (80:20).

5 RESULTS AND DISCUSSION

5.1 Thin layer chromatography (TLC)

Using TLC, different patterns among the bark, leaves, flowers and cell lines could be observed. Analysing the mobile phase used ($CHCl_3$:MeOH (50:50)), it was possible to distinguish the difference among samples based on their bands.

Figures 3 to 5 show the different banding pattern observed on TLC plates using *Byrsonima intermedia* methanolic extracts under normal light (Figure 3), UV254nm illumination (Figure 4) and UV366nm detection (Figure 5) after being eluted with $CHCl_3$:MeOH (50:50) and detection with sulfuric acid/heating. In these figures the difference among some samples is clear and three main groups can be distinguished: (1) 7, 8, 9, 10.3, 11.1, 11.2; (2) 10.1, 10.2 and; (3) flower (F), leaf (L) and bark (B) (whose identification can be found on table 1 in item 4.3). Samples 1, 2 and 6 belong to the group 1 and; samples 3.1, 3.2, 4 and 5 to the group 2 (data not shown). The main differences observed among the cell lines groups are related to their color. Group 1 has yellow/white colour predominance; while group 2 has red colour as predominant.

After evaluating all the samples patterns, representative cell lines were tested together with leaves, bark and flowers extracts in an anti-microbial assay and in zebrafish to test their anti-inflammatory effect.

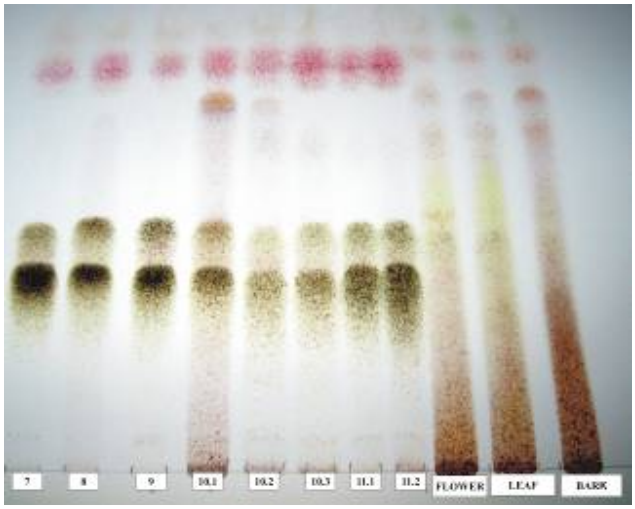


FIGURE 3 TLC plates of *Byrsonima intermedia* methanolic extracts under normal light after detection with sulfuric acid/heating ($CHCl_3$:MeOH - 50:50 system).

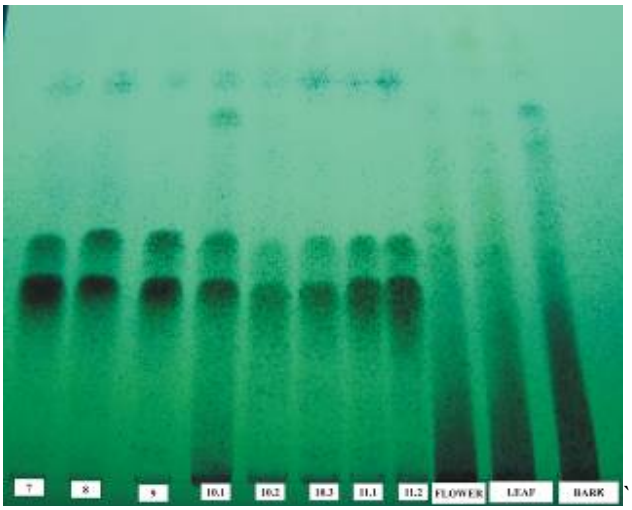


FIGURE 4 TLC plates of *Byrsonima intermedia* methanolic extracts under UV254nm detection after detection with sulfuric acid/heating ($CHCl_3$:MeOH - 50:50 system).

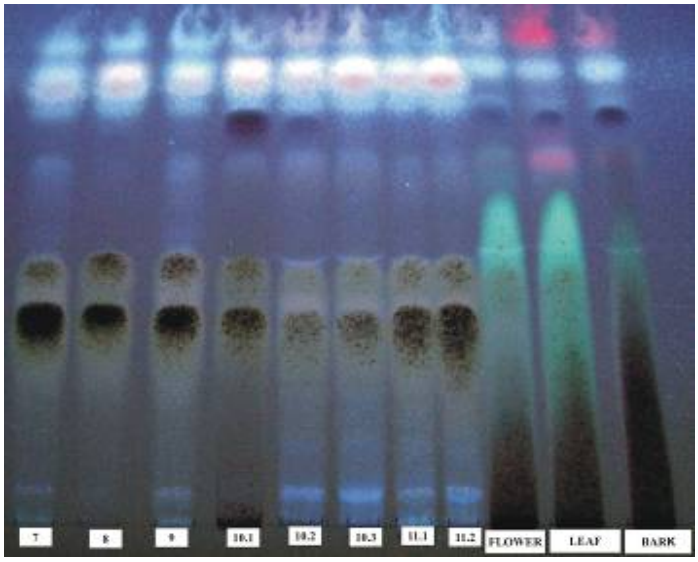


FIGURE 5 TLC plates of *Byrsonima intermedia* methanolic extracts under UV366nm illumination after detection with sulfuric acid/heating ($CHCl_3$:MeOH - 50:50 system).

TLC is an inexpensive methodology which does not require any sophisticated instrumentation for compounds detection in a rough screen. The major drawback of TLC is its low sensitivity and low specificity, thus negative results of TLC are not always negative using other methods. TLC relies on a reproducible migration pattern of compounds on a thin layer adsorbent (for instance, silica gel coated with glass or aluminum plates). Characterization of a particular medicine can be achieved by color reaction produced by spraying the plate with coloring reagents. To accomplish this, the sample is spotted along with known compounds on a TLC plate, which is put in a solvent chamber. The solvent runs up the plate, it is dried and then the plates are sprayed with various reagents to identify the components. The color spots of various known

compounds are then compared with unknown samples. The spots on TLC plate can be visualized under normal, ultraviolet or florescent light (Margoob et al., 2004).

López-Laredo et al. (2009) observed that for *Tecoma stans* species the explant type which originated from calli and the conditions (periodic light and/or darkness) in which they were cultured did not influence accumulation of phenolic compounds and flavonoids content.

Analyses of methanolic extract from *B. fagifolia* leaves using TLC silica-gel plates sprayed with β-carotene and 1,1-diphenyl-2-picrylhydrazyl (DPPH) reagents revealed several spots in the plate which could thus evidence the presence of several substances with potential antioxidant properties (Lima et al., 2008).

5.2 Anti-microbial activity assay

5.2.1 Anti-microbial test

Figure 6 shows the effect of 8 methanolic and water *B. intermedia* extracts (bark (A), leaves (B), flowers (C), red callus from embryos cultured in light conditions (D), yellow cell suspension from callus initiated from *in vitro* leaves cultured in dark conditions (E), red cell suspension from callus initiated from *in vitro* leaves cultured in dark conditions (F), red cell suspension from callus initiated from *in vitro* leaves cultured in light conditions (G) and green callus from embryos cultured in light conditions (H)) against *Candida albicans*, *Saccharomyces cerevisiae* and *Staphylococcus epidermidis*. The microorganisms grew better in presence of the plant extracts when compared to the solvent control. This is could be eplained because of the inactivity of the plant extracts (at 1.25 mg mL^{-1}) against the microorganisms used but also because the extracts still contain some compounds that contribute to microorganisms' growth. Another possibility is the influence of the color of the plant extracts, which

could absorb light and interfere with the O.D. measurement, resulting in a higher value. To observe this effect on the *B. intermedia* extracts tested, we performed an O.D. measurement and made a comparison with the controls (data not shown). This was done measuring a filled cuvette with sterile (clear) growth medium or plant extracts in the photometer. In some extracts we could observe a higher O.D. compared to the negative control, showing that the presumed "growth stimuli" of the microorganisms is actually due to the light absorption caused by the extract color.

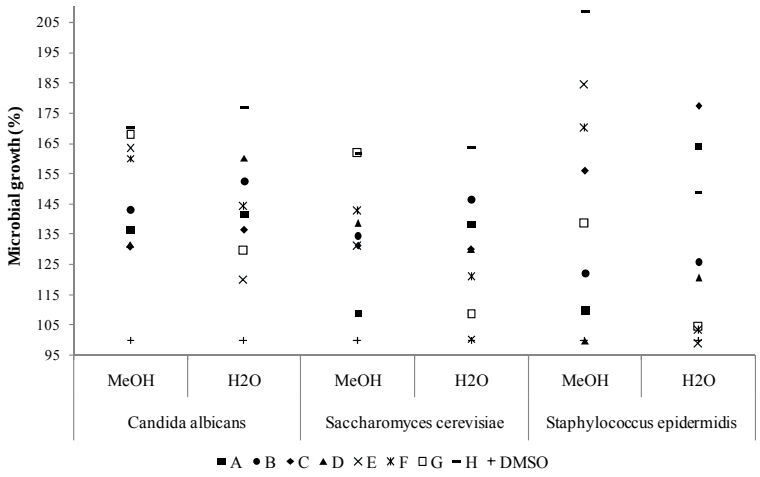


FIGURE 6 Relative antimicrobial growth of *Candida albicans*, *Saccharomyces cerevisiae* and *Staphylococcus epidermidis* in presence of methanolic and water *B. intermedia* extracts. Percentages are relative to the negative control 2.5% DMSO. Bark (A), leaves (B), flowers (C), red callus from embryos cultured in light conditions (D), yellow cell suspension from callus initiated from *in vitro* leaves cultured in dark conditions (E), red cell suspension from callus initiated from *in vitro* leaves cultured in dark conditions (F), red cell suspension from callus initiated from *in vitro* leaves cultured in light conditions (G) and green callus from embryos cultured in light conditions (H) were used as plant extracts.

Optical Density (O.D.) can be used as a measure of microbial growth. A light beam will be scattered by the cells, depending on the cell density that to our eye appears as turbidity. Actually, the scattering intensity or turbidity or O.D. is, within limits, proportional to the cell density. The microbial culture O.D., which is measured in a spectrophotometer, is primarily not an absorbance, as in the case of stained liquids. Cells of many microorganisms are almost colorless and real light absorption is marginal. It is therefore not correct (even though unfortunately common) to designate the O.D. of a culture as an absorption; a very appropriate term would be turbidity. Only strongly pigmented cells cause a significant absorption of light in addition to scattering (Widdel, 2007)

Because we only want to measure the O.D. caused by the cells, any light absorption caused by the medium (brownish yeast extract, etc.) can interfere in the measurement (Widdel, 2007). In our work we observed such influence in some plant extracts when compared to the controls.

The minimal inhibitory concentrations (MICs) against *Staphylococcus aureus*, *Escherichia coli*, *Pseudomonas aeruginosa* and *Candida albicans* were established for methanolic leaf extracts from *B. crassifolia* and the results showed just a good response against *S. aureus* (Navarro et al., 1996).

The methanolic *B. crassifolia* extract of roots showed activity against *Klebsiella pneumoniae*, *Staphylococcus aureus*, *Escherichia coli* and *Salmonella typhi*, whereas the methanolic extract of stems as well both hexanic extracts were ineffective (Martínez-Vázquez et al., 1999).

The strongest antibacterial activity of *B. fagifolia* and its acetate fraction was found against the respective standard strains of *Escherichia coli*, *Staphylococcus aureus* and *Helicobacter pylori*. The results showed that the MIC of acetate fraction against *Helicobacter pylori* or *Staphylococcus aureus*

was 0.25 mg mL^{-1}. The ethyl acetate fraction has weak anti-microbial action (Lima et al., 2008).

5.2.2 Dose-respons curves

Figure 7 shows the dose-respons curve of *Byrsonima intermedia* water plant extracts against *Candida albicans* (Figure 7a), *Saccharomyces cerevisiae* (Figure 7b) and *Staphylococcus epidermidis* (Figure 7c). Also the dose-respons curve of the antifunfal drug miconazole against *C. albicans* (Figure 7a) and *S. cerevisiae* (Figure 7b) was followed as a positive control. The dose-respons curves of *B. intermedia* water plant extracts against all the microorganisms used, showed that incubation with the highest concentration of *B. intermedia* extracts (1.25 mg mL^{-1}) resulted in a growth percentage comparable to the lowest concentration of plant extract (0.0013 mg mL^{-1}) and to the negative control (DMSO 2.5%) for yellow cell suspension from callus initiated from *in vitro* leaves cultured in dark conditions (E); red cell suspension from callus initiated from *in vitro* leaves cultured in light conditions (G) and leaves (B). However, it appears that extract B showed some effect against *S. cerevisiae*. However, comparing this result with the miconazole dose-respons curve against *S. cerevisiae*, it could be concluded that plant extract B was less effective in the growth inhibition of this microorganism. This result also confirmed the lack of activity of plant extract B against *S. cerevisiae* in comparison with the antifungal drug miconazole as a reference.

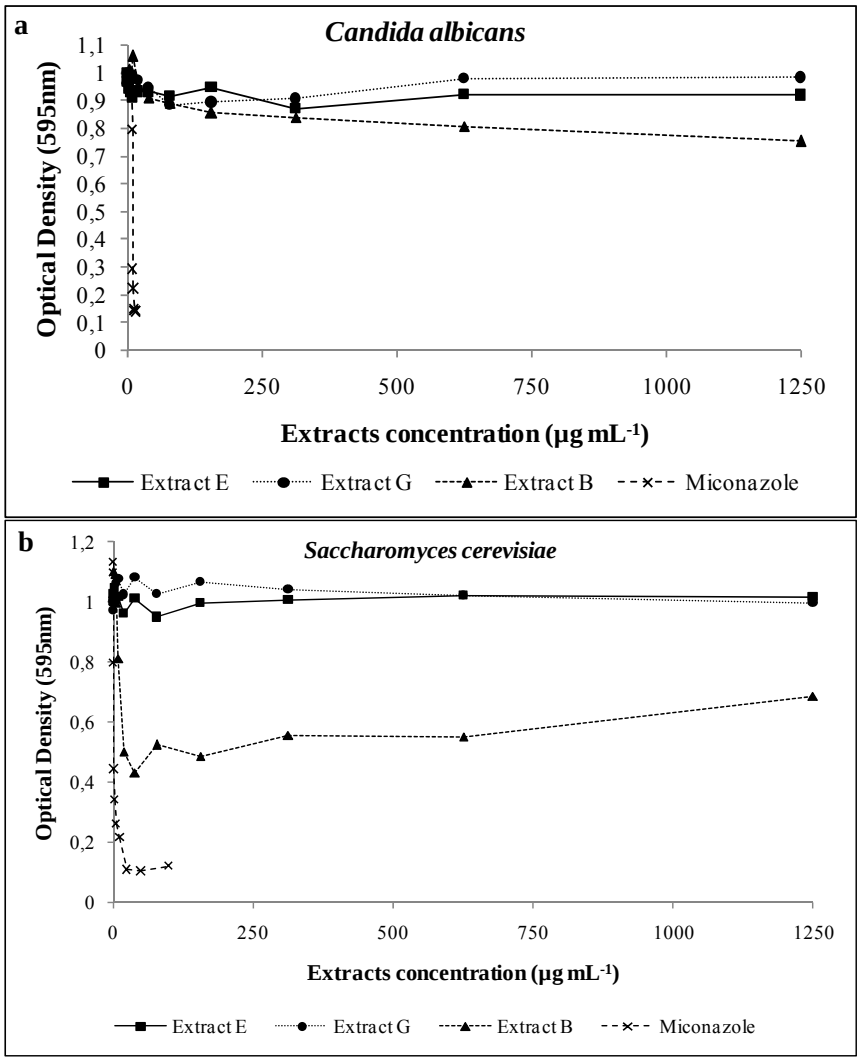
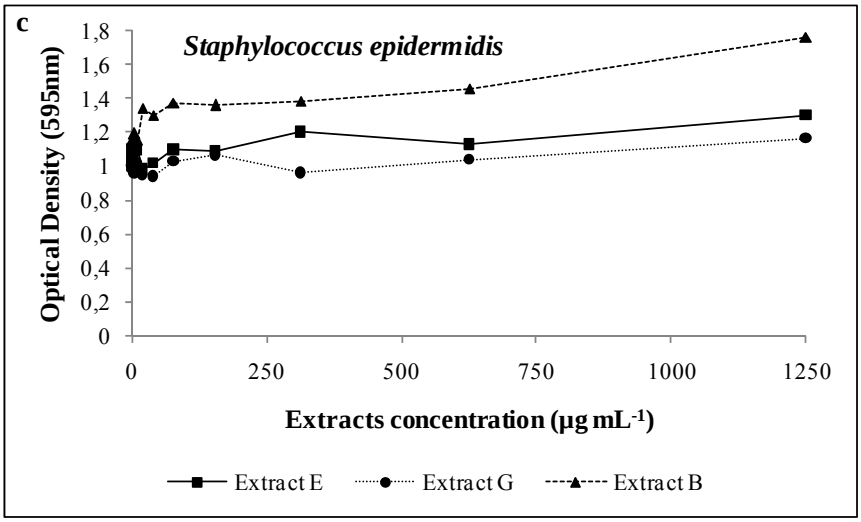


FIGURE 7 Dose-respons curves of water *Byrsonima intermedia* extracts (yellow cell suspension from callus initiated from *in vitro* leaves cultured in dark conditions (E); red cell suspension from callus initiated from *in vitro* leaves cultured in light conditions (G) and leaves (B)) against (a) *Candida albicans*, (b) *Saccharomyces cerevisiae*, (c) *Staphylococcus epidermidis*; (a,b) and antifungal miconazole as a positive control against (a) *C. albicans* and (b) *S. cerevisiae*. DMSO 2.5% was used as a negative control. "…continue…"

"FIGURE 7, Cont."



Cardoso et al. (2006) studied the mutagenic activity of the methanol extract of *B. crassa* and amentoflavone and found mutagenic effect in the *Salmonella*/microsome assay and no mutagenic effect in the mouse micronucleus test. According to the authors, a possible justification for the difference in response between the tests is that the *in vivo* tests would reflect *in vivo* metabolism of the constituents. Thus, mutagenicity data obtained *in vitro* and *in vivo* in animal studies do not necessarily prove mutagenic risks to humans. Mutagenic action of methanolic extract from the leaves of *Byrsonima fagifolia* was also studied by Lima et al. (2008). The authors observed no *in vitro* mutagenic action of the extract and fractions against any of the strains used (*Escherichia coli*, *Staphylococcus aureus* and *Helicobacter pylori*).

The ethanol extracts of *B. crassifolia* leaves showed some trypanocidal activity (Berger et al., 1998). Also some antibacterial and antifungal activity was found in this species (Cáceres et al., 1998). However, a recent study showed that

aerial parts of *B. crassifolia* crude extracts were inactive against the trypomastigote forms of *Trypanosoma cruzi*, shown by lack of *in vitro* trypanocidal activity (Cunha et al., 2009). Martínez-Vázquez et al. (1999) observed that the ethyl acetate *B. crassifolia* extracts from both roots and stem were more effective agents against some microbial species than methanolic extracts were, showing a dose-dependent activity.

5.2.1 Minimal fungicidal/bactericidal dose (MFD/MBD)

In the MFD/MBD assay all three microorganisms grew well in the presence of plant water extracts (Figures 8-10). Amphotericin B (AMB), as a positive control, was very effective in controlling the growth of *Candida albicans* and *Saccharomyces cerevisiae* due to the relatively high concentrations used (Figures 8,9). AMB is clinically characterized by its high toxicity and its large number of side effects. The results confirmed the absence of antimicrobial activity of the *Byrsonima intermedia* extracts tested against the microorganisms *Candida albicans*, *Saccharomyces cerevisiae* and *Staphylococcus epidermidis*.

Figure 8 shows the inactivity of the water plant extracts against *C. albicans*.

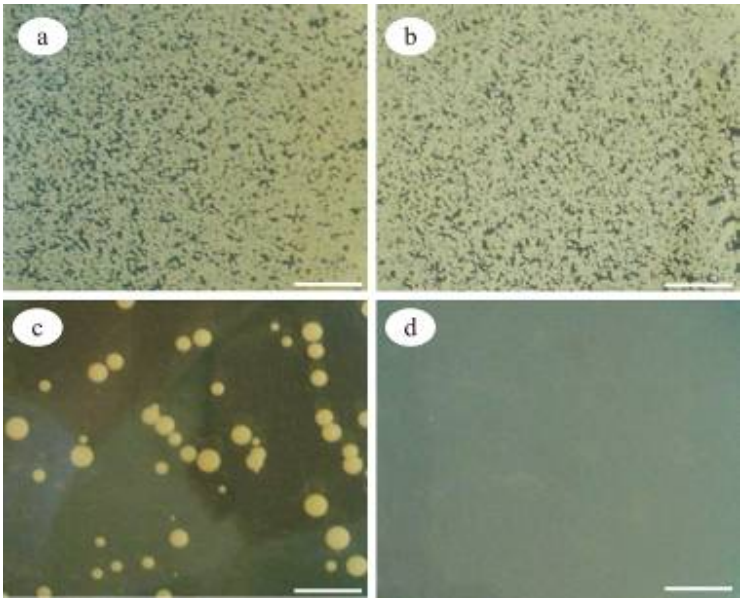


FIGURE 8 Anti-microbial activity of *Byrsonima intermedia* plant water extracts (1.25 mg mL^{-1}) against *Candida albicans* (10^{-2}). (a) yellow cell suspension from callus initiated from *in vitro* leaves cultured in dark conditions; (b) red cell suspension from callus initiated from *in vitro* leaves cultured in light conditions; (c) negative control (DMSO 2.5%); (d) positive control (Amphotericin B 125 µg mL^{-1}). Scale bars = 5 mm.

The same behavior of the microorganisms could be observed in figure 9, i. e., there was no anti-microbial effect of the plant water extracts against the yeast *S. cerevisiae*. On the contrary, the plant extracts seem to stimulate the growth of the yeast.

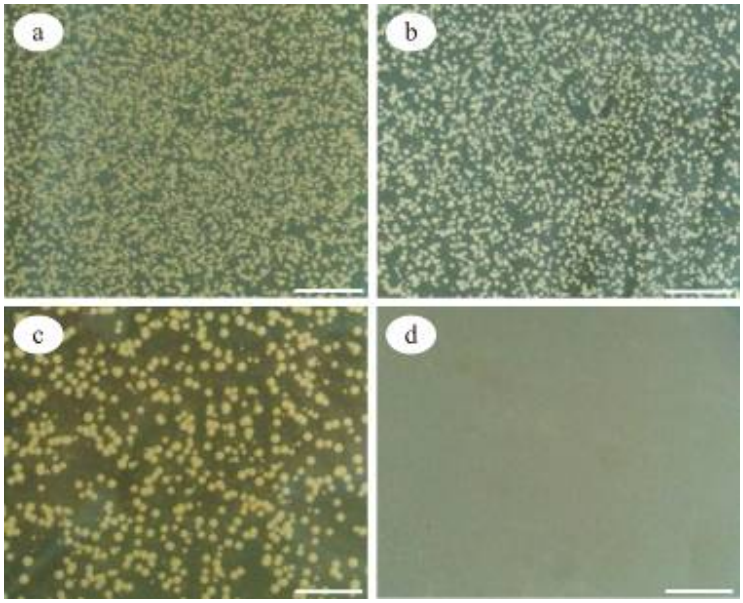


FIGURE 9 Antimicrobiological activity of *Byrsonima intermedia* plant water extracts (1.25 mg mL^{-1}) against *Saccharomyces cerevisiae* (10^{-2}). (a) yellow cell suspension from callus initiated from *in vitro* leaves cultured in dark conditions; (b) red cell suspension from callus initiated from *in vitro* leaves cultured in light conditions; (c) negative control (DMSO 2.5%); (d) positive control (Amphotericin B 125 µg mL^{-1}). Scale bars = 5 mm.

Figure 10a,b demonstrates the superior growth of *S. epidermidis* bacteria compared to the negative control (DMSO 2.5%) (Figure 10c). Therefore, like the yeasts, *S. epidermidis* showed a super-population growth when cultured with the plant water extracts of *B. intermedia*.

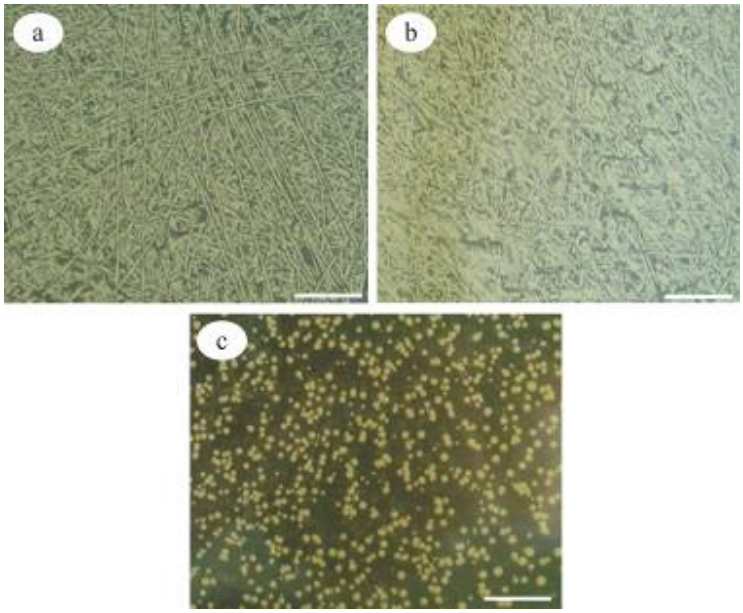


FIGURE 10 Antimicrobiological activity of *Byrsonima intermedia* plant water extracts (1.25 mg mL^{-1}) against *Staphylococcus epidermidis* (10^{-2}). (a) yellow cell suspension from callus initiated from *in vitro* leaves cultured in dark conditions; (b) red cell suspension from callus initiated from *in vitro* leaves cultured in light conditions; (c) negative control (DMSO 2.5%). Scale bars = 5 mm.

It is a common characteristic of complex extracts that they still contain other components, such as amino acids and often sugars that allow the microorganisms to grow in the buffer during the incubation period. This explains the difference in CFUs (colony forming units) between the DMSO-control (that does not contain extract) and extract samples. This also means that there is no fraction present in the plant extract with strong antimicrobial activity.

5.3 Zebrafish inflammation

5.3.1 Effect of crude extracts of bark, leaves and flowers

Crude methanolic extracts of bark, leaves and flowers at 10µg mL^{-1} presented a relative leukocyte migration (RLM) of 20, 32.5 and 32.5%, respectively (Figure 11). These values were not significantly different to indomethacin (10.74 and 35.78 µg mL^{-1}), a non-steroidal anti-inflammatory drug (NSAID) used as reference, which presented a reduced leukocyte migration (25 and 5% RLM, respectively) compared with the 1% DMSO negative control (70% RLM). These results indicate the potential of the methanolic *B. intermedia* extracts which are probably not yet 100% pure. The most active methanolic plant extracts, at 10µg mL^{-1}, were, as active as that of the NSAID indomethacin. This effect is maybe not be related to a single active component in the extracts, but actually to several anti-inflammatory compounds which are acting synergistically.

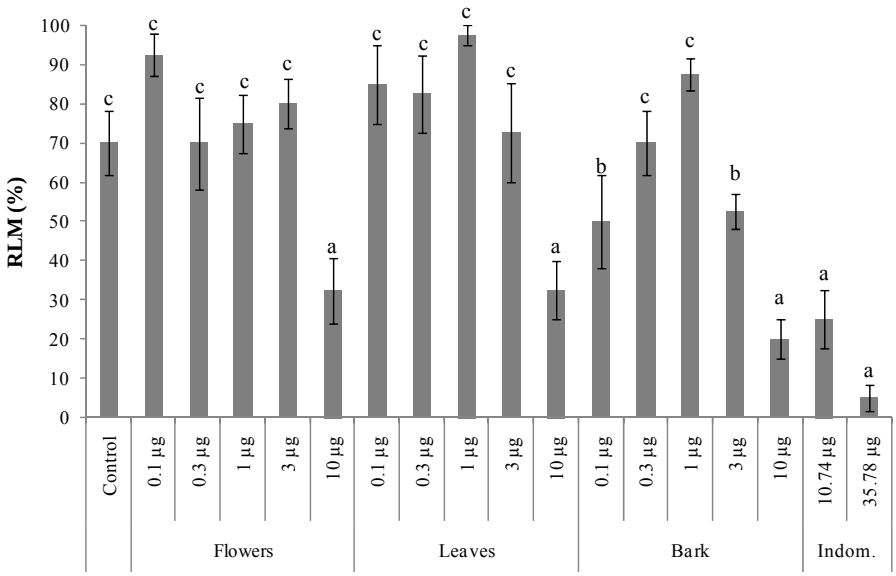


FIGURE 11 Anti-inflammatory activity of the crude methanolic extracts of *Byrsonima intermedia*. Each value represents the mean ± S.E.M. (n = 10) from a representative experiment and $P<0.01$ in one-way ANOVA followed by the Scott-Knott test.

Figure 12 shows the leukocyte migration with tail fin injury in crude methanolic extracts from bark (Figure 12f), leaves (Figure 12e) and flowers (Figure 12d) at 10µg mL^{-1}, showing the anti-inflammatory effect of these extracts compared with the commercial anti-inflammatory indomethacin (Figure 12b,c) as a positive control and DMSO (Figure 12a) as a negative control. Of the extracts tested, the bark extract displays the strongest effect.

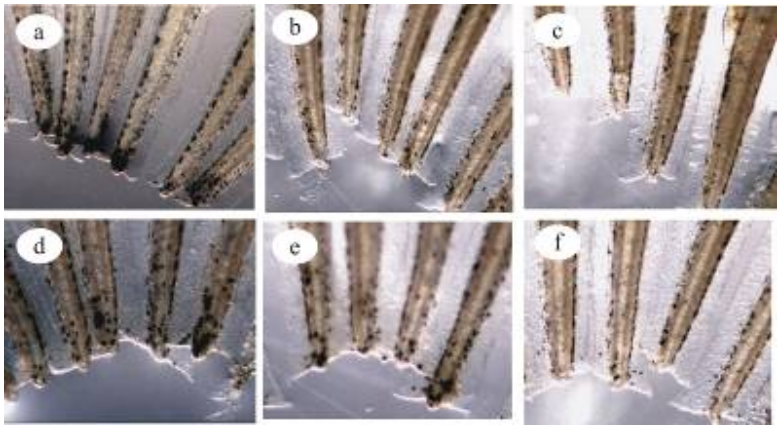


FIGURE 12 Leukocyte migration by whole-mount myeloperoxidase staining in larva at 4 dpf with tail fin injury. Larva with tail fin injury (a) not treated (negative control); treated with Indomethacin commercial anti-inflammatory (positive control) at (b) 10.74 µg mL^{-1} and (c) 35.78 µg mL^{-1} and treated with the crude methanolic extract (10µg mL^{-1}) of (d) flower, (e) leaf and (f) bark, exhibiting reduced leukocyte migration to site of injury.

Inflammation is the first response of the body to infection, irritation or other injuries and is considered as a non-specific immune response aiming to neutralize the aggressor agents, and to repair damaged tissues, assuring this way the survival of the organism (Gomes et al., 2008). Inflammation is featured by increased permeability of endothelial tissue and influxes of blood leukocytes into the interstitium resulting in edema. Several different biological mediators influence each step of the inflammation cascade, and typically, anti-inflammatory agents exhibit therapeutic properties by blocking the actions or syntheses of these mediators (Manthey et al., 2001).

Lipophilic extracts (solvents of increasing polarity grade - petroleum ether, chloroform, methanol) from *B. crassifolia* bark are considered as potential sources of anti-inflammatory proprieties, since the most active chloroform

extracts were comparable to that of the NSAID indomethacin. These results also indicated that apolar solvents would be useful to obtain anti-inflammatory preparations rather than the traditional aqueous ethnomedicinal medicines. (Maldini et al., 2009).

The fact that methanolic extracts of *B. crassifolia* bark generally presented higher values of total phenolics, flavanoids and flavonols than do the leaves and the fruits, is considered to be linked with the possibility of bark to be normally richer in tannins and procyanidins (Souza et al., 2008).

The confirmation and validation of the popular use of *B. fagifolia* Brazilian medicinal plant as an antiulcer, healing and antidiarrheal agent came from the integrative research based on ethnopharmacological studies, followed by the chemical, toxicological and pharmacological investigation of this species (Lima et al., 2008).

The plant species must be selected to be used in therapeutic applications. Different species from the same genus could react completely different in biological assays. This was the case, for example, of the ethanol *Hypericum perforatum* extracts which presented anti-inflammatory activity; while ethanol extract of *Hypericum scabrum* showed neither remarkable wound healing nor anti-inflammatory activity (Süntar et al., 2009).

5.3.2 Fractions effect of crude extracts of bark, leaves, flowers and cell suspensions

5.3.2.1 Preliminary tests on vascular system

The different fractions isolated and purified from crude methanolic extracts of bark, leaves, flowers and red and yellow cell suspensions (Figure 1 and Table 2 from item 4.5.2) were previously performed using the zebrafish vascular system test in order to rapidly investigate the effect of the different purified fractions on zebrafish anti-inflammatory effect. Fraction number 8

(Figure 1 and Table 2 from item 4.5.2) (from bark, leaves and flowers) presented a reduced lumen formation during the formation of new blood vessels (Figure 13b,d). All the other bands tested (Figure 1 and Table 2 from item 4.5.2) did not show any significant effect in zebrafish angiogenic model (data not shown).

Treatment with DMSO had no effect on the vessel formation and served as a vehicle control (Figure 13a,c).

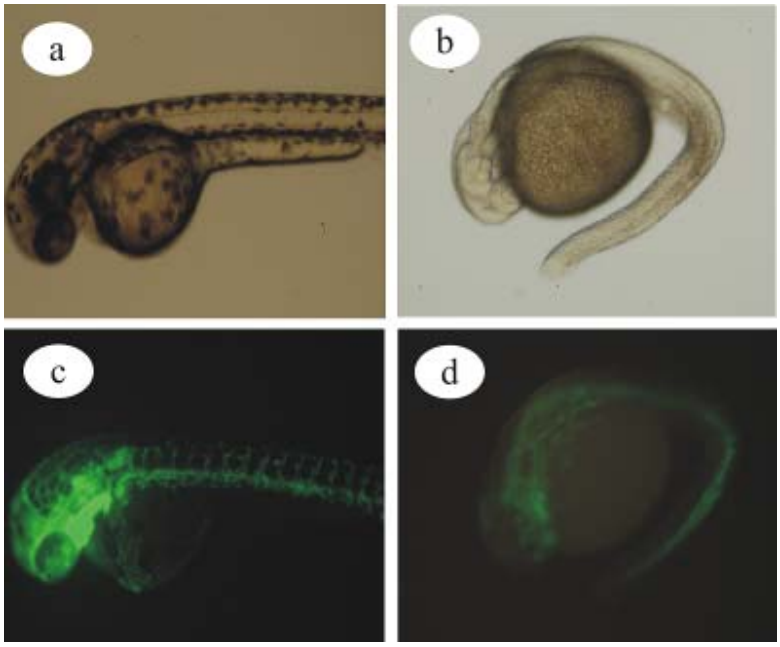


FIGURE 13 Transgenic zebrafish embryos (*fli-1*:EGFP line), 2 days post-fertilization (dpf), cardiovascular system. (a,c) negative control; (b,d) fraction number 8 (from bark, leaves and flowers) showing a reduced lumen formation during the formation of new blood vessels.

Vascular formation in zebrafish embryos have been linked with anti-inflammatory effects for several natural product extracts and compounds (Crawford, A.D.; Siverio-Mota, D.; Cordero-Maldonado, M. L.; Esguerra, C. V.; de Witte, P. A. M. unpublished data); Giving a positive effect with fraction 8, this bark fraction was further tested in zebrafish using the tail injury/LPS-induced leukocyte migration assay.

To better comprehend the cited bioactivity of various Brazilian herbal preparations, which consist of crude plant extracts, it is important to purify and to elucidate the structure of such active compounds. However, because of the complexity of mixtures in the most plant extracts, isolation is still a critical step. The separation of the compounds present in a crude extract is often performed by repeated processes based on adsorption column chromatography. Moreover, the isolated compounds should be submitted to in vivo pharmacological tests, which require fast separation and amounts of sample varying from few milligrams to grams of pure compound (Sannomiya et al., 2004).

5.3.2.2 LPS-induced leukocyte migration assay

Figure 14 shows that the migration of leukocytes in the fraction number 8 used from *B. intermedia* extracts was minimal (22.5 and 15% RLM for 5 and 10μL, respectively. An indication of the maximum plant extracts concentration (mg mL^{-1}) that can be applied can be given. Aliquots of 400 μL of the crude methanolic extracts (at the final concentration of 50mg mL^{-1}) were transferred to a silica-gel plate; therefore 20 mg of crude extract was loaded on a single TLC plate. As each band was extracted and resolubilized in 30 μL DMSO and we assume 50% recovery for each active constituent (depending on the chemical nature of each active constituent), 30 μL contains compounds in this fraction equivalent to what was originally in 10 mg of crude extract. Five microliters in a total of 1 mL of Danieau's medium actually contains the equivalent of ca. 1.67

mg mL^{-1} and 10 µL is equivalent of ca. 3.33 mg mL^{-1}, indicating the anti-inflammatory effect of these fractions. The band studied in the present work reduced the leukocyte migration as effectively as the NSAID indomethacin (20 and 6.25% RLM for 10.74 and 35.78 µg mL^{-1}, respectively) which confirms the strong anti-inflammatory properties of *B. intermedia* extracts.

DMSO negative control presented a RLM of 90%, confirming the findings described above.

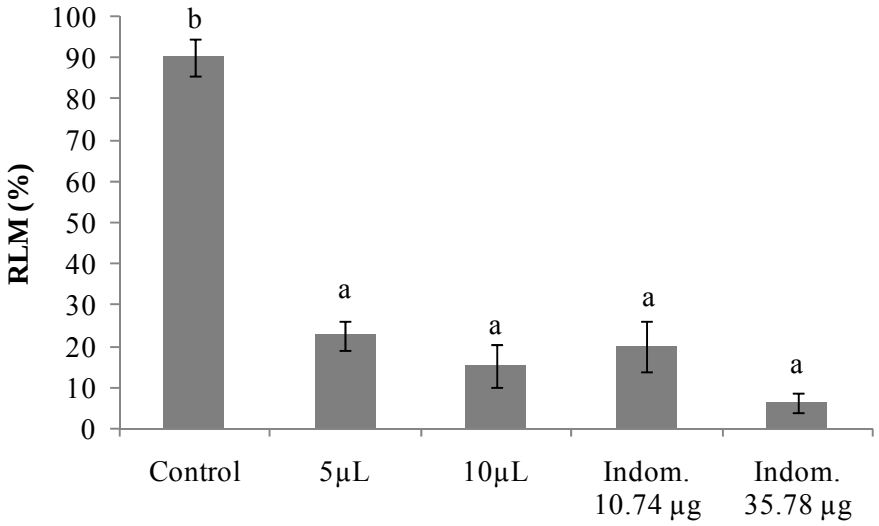


FIGURE 14 Anti-inflammatory activity of the fraction 8 from bark methanolic extract of *Byrsonima intermedia*. Each value represents the mean ± S.E.M. (n = 10) from a representative experiment and $P<0.01$ in one-way ANOVA followed by the Scott-Knott test.

The leukocyte migration with tail fin injury in fraction 8 methanolic bark extract is shown in Figure 15d (5µL) and 15e (10µL). The NSAID indomethacin

(Figure 15b,c) was comparable to the plant extracts and visual different of DMSO negative control (Figure 15a).

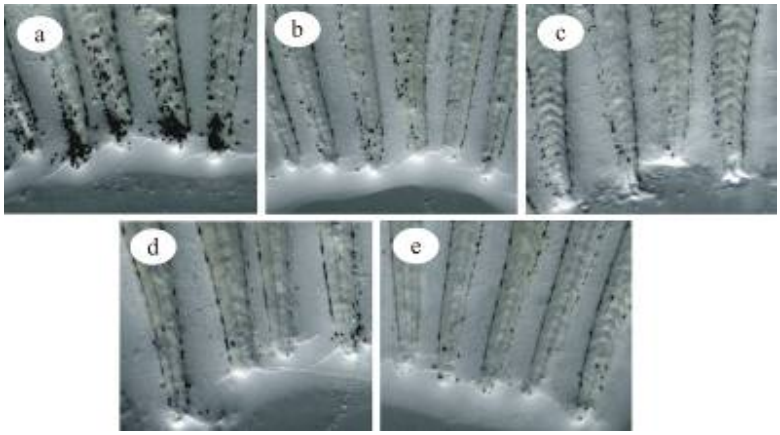


FIGURE 15 Leukocyte migration by whole-mount myeloperoxidase staining in larva at 4 dpf with tail fin injury. Larva with tail fin injury (a) not treated (negative control); treated with Indomethacin commercial anti-inflammatory (positive control) at (b) 10.74 µg mL^{-1} and (c) 35.78 µg mL^{-1}; and treated with the fraction 8 from bark at (d) 5µL and (e) 10µL, exhibiting reduced leukocyte migration to site of injury.

The interest in the utilization of botanical and herbal remedies complementing the treatment of inflammatory diseases is constantly increasing, mainly because of the adverse effects associated with the use of nonsteroidal anti-inflammatory drugs. A possibility to discover new anti-inflammatory compounds is to search for their presence in natural sources (Mazura et al., 2007).

A healing effect was already found in the ethyl acetate partition of *B. intermedia* leaf extracts by using an *in vivo* approach with mice (Santos et al., 2007). In *B. fagifolia* extracts the healing activity found could be related to the presence of components with antioxidant properties, mainly due the ability of such compounds on reducing the effect of reactive species that are harmful to the gastric mucosa (Lima et al., 2008).

The aqueous portion of *B. intermedia* leaf extracts presented potent gastroprotective effects, corroborating with popular indication. The gastroprotective action can be related to the activation of the compounds sulfhydryl, nitric oxide and glutathione that exert influence the protection together of the barrier mucous front to severe harmful agents (Santos et al., 2008). Expression of COX2 (cyclooxygenase-2) and SOD (superoxide dismutase) and intense angiogenesis are involved in cellular healing of *B. intermedia*, indicating a cicatrizing effect of this medicinal species in addition to activation of nitric oxide and sulfhydryl compounds that exert great influence on the protection against these severe harmful agents (Santos et al., 2009). Also the anti-ulcerogenic effect of the *B. intermedia* methanolic leaf extract was proven (Santos et al., 2006).

The pharmacological activity of other species of the *Byrsonima* genus and the possibility that these species show some flavonoids and terpenoids among its likely interesting metabolites, raises the possibility that *B. intermedia* could be studied in phytochemical and therapeutic terms.

5.4 Phytochemical analysis

5.4.1 Tannins and carotenoids

In table 5, the tannins and carotenoids contents of different cell suspensions culture of *Byrsonima intermedia* are shown. There is a higher amount of tannins present in red cell suspensions (RCS) with 8.96 $mg.100g^{-1}$

compared to pink cell suspensions (PCS) and yellow cell suspensions (YCS) where the obtained tannins values were 2.96 and 0.71 mg $100g^{-1}$, respectively.

It can also be observed in Table 5 that the difference among suspension colors was not caused by their carotenoids content. No statistical differences could be observed between the contents of carotenoids in the different cell suspension cultures. However, pink cell suspensions had a little more of this group of metabolites (0.043 mg $100g^{-1}$) compared to yellow cell suspensions (0.037 mg $100g^{-1}$) and red cell suspensions (0.027 mg $100g^{-1}$) which, on the contrary to the tannins, was found to contain the least amount of all.

TABLE 5 Tannins and carotenoids content in different cell suspensions culture of *Byrsonima intermedia*.

Cell suspension samples	**Tannins content (mg 100 g^{-1})**	**Carotenoids content (mg 100 g^{-1})**
Red cell suspensions (RCS)	8.96^{a*}	0.027^{a}
Pink cell suspensions (PCS)	2.96^{b}	0.043^{a}
Yellow cell suspensions (YCS)	0.71^{c}	0.037^{a}

*Different letters in the same column indicate significant statistical differences ($P<0.01$) in one-way ANOVA followed by the Scott-Knott test.

Plant tissue cultures represent a potential source for producing valuable secondary metabolites (Kim et al., 2002; Estrada-Zúñiga et al.; 2009).

Polyphenolic compounds are widely distributed in higher plants and are an integral part of the human diet. Tannins, substances present in vegetable extracts that are able to convert animal skin into leather, are classified into three major groups on the basis of their structural characteristics: the hydrolysable (gallotannins like gallic acid), the complex or partially hydrolysable, and the condensed or non-hydrolyzable (i.e., catechin and epicatechin) tannins. Condensed tannins do not have a polyol nucleus and are not readily hydrolyzed.

However, upon heating in acidic alcohols condensed tannins produce red anthocyanidin pigments and are therefore also termed proanthocyanidins (Cos et al., 2003).

Production of phenylpropanoids was increased in cell suspension culture originated from white callus of *Buddleja cordata* (Estrada-Zúñiga et al.; 2009).

The anthocyanin levels in the transgenic tobacco white calli at Day 0 and Day 5 were approximately four to fivefold lower than those in the red calli. However, it was noted that the difference of the absorbance values between white and red calli decreased at Day 15, 20, and 25. This probably resulted from both surface cells producing anthocyanins and calli browning when obvious senescence started in many cells (Zhou et al., 2008).

The carotenoid amounts found in the present work were very low. Carotenoids can, therefore, not be related to the color difference observed among the cell suspension cultures (yellow, red and pink). The tannins difference observed between the cell suspensions, however, can be related to the color difference. The red cell suspensions presented the highest tannin amount comparing with the others. Further work should be developed in order to identify the compounds responsible for the color difference.

5.4.2 GC-MS

Phytochemical analysis by GC-MS/GC-FID (see Annex A) for *Byrsonima intemedia* bark revealed the presence of 20 compounds, of which 12 could not be indentified; in the leaves 28 compounds were detected, of which 18 were not identified; and in the yellow cell suspensions, only seven compounds were detected and among these two were not identified (Table 6). For yellow cell suspensions, no good mass spectra could be obtained and only the presence of saturated and unsaturated fatty acids derivatives was proven. GC-MS was not executed for the red cell suspensions, mainly because of the lack of results

obtained in the yellow cell suspensions and also because of the preliminary TLC study which showed the presence of compounds with high polarity. The GC-MS technique is used to analyse compounds with intermediate and low polarity which volatilize when injected in the equipment. High polarity compounds are not volatilized under these conditions; actually they would be retained, so not justifying the use of GC-MS for identification. To analyse such compounds it would be necessary to proceed their chemical isolation and afterwards determine their structures using other spectrometry methods (such as nuclear magnetic resonance (NMR), among others).

TABLE 6 Identification of the compounds present in the methylated bark, leaves and yellow cell suspensions of *Byrsonima intemedia*.

RT (min)	**Identification**	**Relative %[a] (methylated sample)**		
		Bark	**Leaf**	**Yellow[b]**
6.48	MW = 182	nd[c]	11.61	nd
6.88	MW = 182	nd	4.10	nd
7.36	MW = 182	nd	4.00	nd
7.71	MW = 182	nd	1.77	nd
7.88	MW = 182	nd	1.30	nd
9.30	MW = 182	8.61	nd	nd
9.32	MW = 182	nd	6.08	nd
9.89	4-methylbenzenesulfonic acid ethyl ester	nd	nd	10.30
10.85	MW = 180.	20.60	nd	nd
10.88	MW = 182	nd	7.66	nd
11.34	MW = 182	0.65	nd	nd
12.35	MW = 178	2.14	nd	nd
12.58	MW = 212 (benzoic acid derivate)	nd	2.17	nd
12.59	MW = 222	1.81	nd	nd

"...continue..."

“TABLE 6, Cont.”

13.23	MW = 226	nd	1.55	nd
13.32	MW = 226	1.38	nd	nd
13.54	MW = 198 (benzoic acid derivate)	5.90	nd	nd
13.56	MW = 150	nd	nd	5.60
13.57	MW = 212 (benzoic acid derivate)	nd	3.95	nd
13.61	MW = 198	1.45	nd	nd
13.79	MW = 198 (benzoic acid derivate)	nd	1.13	nd
14.12	MW = 220	1.66	nd	nd
14.14	MW = 212 (benzoic acid derivate)	nd	5.13	nd
14.21	MW = 212	4.09	nd	nd
14.35	MW = 212 (benzoic acid derivate)	nd	2.26	nd
14.87	MW = 220 (benzoic acid derivate)	nd	2.46	nd
15.10	MW = 220 (benzoic acid derivate)	nd	2.61	nd
15.60	MW = 220 (benzoic acid derivate)	nd	3.29	nd
17.30	hexadecanoic acid methyl ester	5.02	1.62	22.13
17.96	hexadecanoic acid	nd	1.87	nd
20.42- 20.44	9,12-octadecadienoic acid methyl ester	2.01	0.60	10.45
20.54	12-octadecenoic acid methyl ester	5.04	1.58	28.48
21.01-21.02	octadecanoic acid methyl ester	2.51	0.92	14.95
21.18	MW = 282	nd	nd	8.09
21.21	MW = 282	nd	1.03	nd
27.63	docosanoic acid methyl ester	1.44	nd	nd
30.58	tetracosanoic acid methyl ester	1.38	nd	nd
31.95	squalene	nd	1.13	nd

“...continue...”

"TABLE 6, Cont."

32.89	nonacosane	nd	0.50	nd
36.26	E vitamin	0.72	0.91	nd
39.78	g-sitosterol	nd	0.60	nd
40.16	MW = 426	1.70	nd	nd
40.46-40.48	beta-amyrin	25.35	1.51	nd
41.55	MW = 426	6.78	nd	nd
41.60	MW = 426	nd	10.92	nd

Notes: **a)** fraction in percentage of the integral total area for the chromatogram.
b) cell suspension culture.
c) nd: not detected.
d) RT: retention time.
e) MW: molecular weight.

According to the GC-MS, the possible explanation for the potent anti-inflammatory activity found in the bark and leaves could be due to the presence of beta-amyrin. There are still some bark and leaves compounds without identification. In the yellow cell suspensions, beta-amyrin was not detected, but the five identified compounds that were to most present are 12-octadecadienoic acid methyl ester, hexadecanoic acid methyl, octadecanoic acid methyl ester, 4-methylbenzenesulfonic acid ethyl ester and 9,12-octadienoic acid methyl ester.

Fatty acids are essential molecules in living organisms and commonly analysed by gas chromatography (GC). They are normally separated as their methyl ester derivatives (fatty acid methyl esters, FAMEs) by polar stationary phases which allow separation according to carbon number and according to unsaturation degree (number of double bonds) (Bicalho et al., 2008).

The most critical step for GC determination of fatty acids is sample preparation, which normally needs prior derivatization of the fatty acids for appropriate chromatographic separation and high sensitive determination (Sánchez-Ávila et al., 2009).

Essential fatty acids are not only integral structural components of cell membranes, but also known as hormonal regulators of a variety of biological processes. Evidence suggests that the essential fatty acids may be safe and also very effective as anti-inflammatory and immunomodulatory agents (Wander et al., 1997; Kliewer & Willson, 1998). Fatty acids have unique roles as precursor molecules of chemical mediators of inflammation and regulators of the immune function such as the leukotrienes and the prostaglandins. These compounds are synthesized and released by almost all tissues in the body, and participate in many biological functions, including the inflammatory and immune processes (Hartmann & Endres, 1997).

Ledón et al. (2003) provided evidence that FAM, a mixture of fatty acids isolated and purified from sugar cane (*Saccharum officinarum* L.) whose main components are 9-octadecanoic; hexadecanoic; 9,12-octadecanoic and 9,12,15-octadecanoic acids, exerts anti-inflammatory and anti-nociceptive effects. These results confirmed the previously observed anti-inflammatory activity of several well known fatty acids (Hartmann & Endres, 1997).

He et al. (2009) studied modifications in fatty acids during callus cultures of *Jatropha curcas*, an important woody oil plant which is widely used as source material to produce biodiesel. Fatty acid contents between non-browning and browning callus differed. Concentrations of saturated and unsaturated fatty acids in browning callus were about double compared to that in non-browned callus. Similar fatty acids distribution patterns were observed between non-browning and browning callus. The ideal conditions of the *in vitro* secondary metabolites production are just obtained after laboured procedures of culture medium optimization and making a profound study of the *in vitro* environment atmospheric conditions to increase the cells and interesting metabolites productivity (Buffa Filho et al., 2001).

James et al. (2008) compared two morphologically different phenotypes of *Centella asiatica* related to their triterpenoid saponins levels. The authors aimed to evaluate the potential of this species for biotechnological manipulation of triterpenoid synthesis. The metabolites investigated were produced in cultured undifferentiated cells (cell suspensions and calli) and leaves. Leaves contained the highest triterpenoid levels compared to undifferentiated cells, which had lower, but still quantifiable, levels of these targeted secondary metabolites.

Accumulation of the triterpenoids azadirachtin-A, nimbin and salannin was only detected at the end of the third subculture (120th day) of callus tissues of neem. The lack of morphological differentiation in callus cells may prevent the formation of secondary metabolites and disrupt regular metabolic pathways. But this morphological differentiation is not mandatory. For example, for the three triterpenoids studied, neem calli synthesized and accumulated them efficiently (Babu et al., 2006).

Sterols and triterpenes are widely distributed isoprenoids found in various organisms and constitute one of the most important classes of natural products. They exhibit a wide variety of functions in biological systems (Kushiro et al., 1998).

Triterpenes, such as β-amyrin, isolated from several species of medicinal plants are, in general, responsible at least in part for their biological activities (Aragão et al., 2006).

Generally, steroids are widely distributed in the plant kingdom. Most of these compounds have been related to determined activities (Lopes et al., 2000). Beta-amyrin, a precursor of oleanolic acid, is a popular type of triterpenes found in plants (Kushiro et al., 1998). The amyrins (α,β), pentacyclic triterpenes, have been proved to exhibit strong anti-inflammatory (Miranda et al., 2000; Mazura et al., 2007; Backhouse et al., 2008; Pinto et al., 2008a), anxiolytic and antidepressant (Aragão et al., 2006), antifungal (Johann et al., 2007),

gastroprotective (Oliveira et al., 2004) and antinoceptive/analgesic (Miranda et al., 2000; Otuki et al., 2005; Backhouse et al., 2008; Pinto et al., 2008b) activities.

The ethyl acetate extract of the air-dried leaves of *Diospyros blancoi* gave rise to a mixture of α-amyrin palmitate, α-amyrin palmitoleate, β-amyrin palmitate and β-amyrin palmitoleate in a 13:4:3:1 ratio, exhibited antimicrobial activity against *Escherichia coli*, *Pseudomonas aeruginosa*, *Candida albicans*, *Staphylococcus aureus* and *Trichophyton mentagrophytes*, and were found inactive against *Bacillus subtilis* and *Aspergillus niger*. This sample also exhibited significant analgaesic and anti-inflammatory activities (Ragasa et al., 2009).

The pharmacological properties reported in literature for steroids and fatty acids could also explain the anti-inflammatory effect found in our study.

For *Lavandula officinalis*, the content of rosmarinic acid was higher in *in vitro* grown plant material, especially in callus and cell cultures, than in the field grown plants (Wilken et al., 2005). Similar results were observed in the asiaticoside production in the suspension-cultured cells of *Centella asiatica*, which was found to be present in higher amount than in callus and leaf (Nath & Buragohain, 2005). These findings justify the present work where cell suspension cultures were evaluated for their biological effect and phytochemical composition.

5.4.3 High performance liquid chromatography (HPLC) and Thin layer chromatography (TLC)

5.4.3.1 Thin layer chromatography (TLC)

Samples of leaves (L), bark (B), yellow (YCS) and red (RCS) cell suspensions extracts and of the gallic acid (GA), epicatechin (E) and quercetin (Q) standards were separated by TLC and visualised using two developers:

anisaldehyde (an universal developer which works to many compounds classes, mainly to terpenes) and the NP solution (specific for phenolic compounds, i.e., flavonoids) (see figures 16 and 17, respectively).

The band of the epicatechin standard was well evident in the methanolic extracts of leaves and bark and this only for the anisaldehyde solution developer (Figure 16). In the red cell suspensions the epicatechin presence it appeared be in a lower amount. The gallic acid and quercetin standards presented a characteristic band, however, it was not so evident in the extracts used. In the cell suspensions extracts (YCS and RCS), the presence of more polar compounds (greenish) was observed only when anisaldehyde solution (Figure 16) was used. These gray-green compounds observed in the TLC plates probably belong to the saponins class (Wagner & Bladt, 1996).

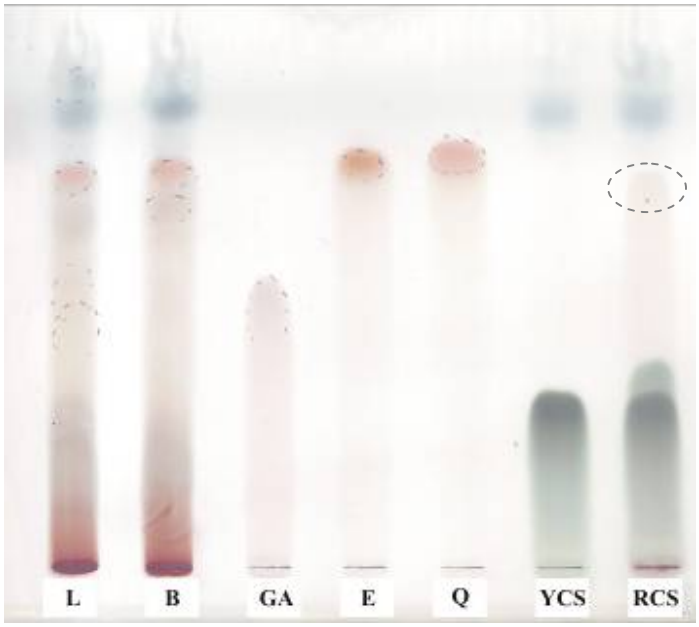


FIGURE 16 Chromatographic plates of leaves (L), bark (B), yellow (YCS) and red (RCS) cell suspensions extracts and of the gallic acid (GA), epicatechin (E) and quercetin (Q) standards. Mobile phase: Chloroform:Methanol (1:1 v/v). Developer: anisaldehyde solution.

The plate visualized with NP/polyethylene glycol showed only the epicatechin presence in leaves and bark (Figure 17). In the red cell suspensions this compound seemed to be present but in a lower amount. The gallic acid and quercetin standards were not present in the plant extracts by using this developer.

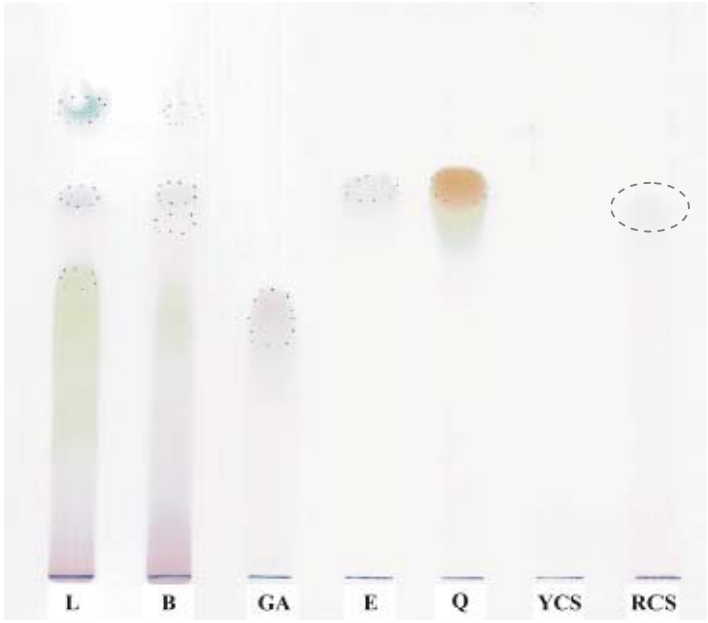


FIGURE 17 Chromatographic plates of leaves (L), bark (B), yellow (YCS) and red (RCS) cell suspensions extracts and of the gallic acid (GA), epicatechin (E) and quercetin (Q) standards. Mobile phase: Chloroform:Methanol (1:1 v/v). Developer: NP/polyethylene glycol solution.

Previously it was supposed that the anthocyanins should be present in the RCS thus causing the difference between the cell suspensions color (yellow and red). However, by using this TLC method we could not prove the presense of these secondary metabolites in the samples. The anthocyanins should normally be observed in normal light without developers, while in the present work the compounds that could be detected were only visualized under UV irradiation (254 and 366nm) and this after the developers use (data not shown).

Polyphenols in general and proanthocyanidins (condensed tannins like catechin and epicatechin) in particular play an important role as antioxidants.

This activity may play other important role in health care, e.g. by acting as cancer chemopreventive and anti-inflammatory agents and by reducing risk of cardiovascular mortality (Cos et al., 2003; Scalbert et al., 2005). The health effects of polyphenols depend on the amount consumed and on their bioavailability (Manach et al., 2004). A study conducted by Manach et al. (2005) revealed that gallic acid and isoflavones are polyphenols that are well absorbed, while proanthocyanidins, the galloylated tea catechins, and the anthocyanins are the least absorbed polyphenols.

Catechins, naturally occurring polyphenolic compounds, exert vascular protective effects through multiple mechanisms, including antioxidative, anti-hypertensive, anti-inflammatory, anti-proliferative, anti-thrombogenic, and lipid lowering effects (Lambert et al., 2007; Babu & Liu, 2008). The precaution of vascular inflammation performs a crucial role in the progress of atherosclerotic lesions. The anti-inflammatory activities of catechins may be due to their suppression of leukocyte adhesion to endothelium and subsequent transmigration through inhibition of transcriptional factor NF-kB-mediated production of cytokines and adhesion molecules both in endothelial cells and inflammatory cells (Babu & Liu, 2008). Catechin epicatechin gallate has been shown to present significantly improvement in the quality of wound healing and in scar formation (Kapoor et al., 2004).

Literature on flavonoids compounds is extensive, since these substances are well known for their widespread distribution in fruits and vegetables, as well as for their activity as active substances in medicinal plants (Raventós et al., 2005). The flavones (quercetin) and catechins are flavonoids which most protect the human body against reactive oxygen species (Pietryga et al., 2009). These antioxidant properties can explain the effects of polyphenols such as anti-inflammatory, anti-tumor and anti-atherogenic (Manthey et al., 2001; Rice-

Evans, 2001; Sagin & Sozmen, 2004; Selmi et al., 2006; Silva et al., 2009; Mejia et al., 2009).

Zhou et al. (2008) confirm that in tobacco both red and white cells produce similar patterns of other flavonoids. The compound rutin (quercetin-3-rutinoside) was not detected in bright-yellowish wild-type calli by HPLC-MS analysis. However, red and white callus produced similar levels of rutin. This result indicates that the branch of the flavonol pathway is activated in the two PAP1 transgenic calli. This observation is consistent with their previous metabolic analysis for PAP1 transgenic tobacco plants, in which quercetin was dramatically increased by the PAP1 transgene. The *Arabidopsis* PAP1 gene (At1g56650) encodes the MYB75 transcription factor, which has been demonstrated to regulate essentially the biosynthesis of anthocyanins.

5.4.3.2 High performance liquid chromatography (HPLC)

In order to confirm the presence of epicatechin in the samples, they were injected into the liquid chromatograph as well as the analytical standard. The HPLC-DAD analysis provides both the chromatogram as well as the spectrum of the measured peaks. In Table 7 the peaks of the compounds identified in the samples are shown (see Annex B). Thus, there is strong evidence that epicatechin is present in the leaves, bark as well as in red cell suspensions samples, because of the similarity of the obtained spectra and the retention time between sample and standard.

Quercetin and gallic acid standards were also injected into the HPLC, in order to verify their presence in the *B. intermedia* extracts used. The results indicated the presence of gallic acid in the bark. However, in leaves and in cell suspensions gallic acid and quercetin were not detected (Table 7).

TABLE 7 Identification of gallic acid, epicatechin and quercetin standards in the leaves, bark and yellow and red cell suspensions extracts of *Byrsonima intermedia*.

Standard	RT (min)	Leaves	Bark	Yellow cell suspensions	Red cell suspensions
Gallic acid	3.28	-	+	-	-
Epicatechin	16.37	+	+	-	+
Quercetin	25.15	-	-	-	-

(+) means presence and (-) means absence of the measured standards in the samples.

Proanthocyanidins show interesting anti-inflammatory properties (Cos et al., 2003). Epicatechin in the *in vitro* system works as an antioxidant. Some antioxidant properties of epicatechin have been proven by their capacity to remove free radicals, oxydase activity inhibition and anti-inflammatory effect (Pietryga et al., 2009).

Flavonoids (epicatechin and gallic acid) have been considered to have anti-inflammatory properties (Gomes et al., 2008). The anti-inflammatory activity of (-)-epicatechin 3-(3-*O*-methylgallate) and (+)-catechin 3-(3-*O*-methylgallate) was stronger than those of indomethacin, the normally used anti-inflammatory agents (Iijima et al., 2009).

Anti-inflammatory activity of the flavonoids and naphthoquinones active fractions might have a contribution in the wound healing effect of the *Hypericum perforatum* (Süntar et al., 2009).

Methanolic extract of *Centaurea iberica* shows notable wound healing and anti-inflammatory activity (Koca et al., 2009). This extract was then subjected to TLC analysis and when the plates were examined for the colored spots, phenolic components were shown as prominent ones. Eventually, anti-inflammatory activity of *Centaurea* species may be attributed to these

phytochemicals, their individual or synergistic effects might speed up their anti-inflammatory process.

The production of high amounts of interesting metabolites could be reached by stressing the cultures, by controlling their biosynthesis pathway in genetic engineering, by using the root transformation technique that represents an appropriate system for producing phenylpropanoid and likely terpenoid metabolites. Also, applying these methodologies in bioreactors could give rise to the commercial production of worthy metabolites (Estrada-Zúñiga et al.; 2009).

In vitro cultures and plant material of diverse plants were analyzed for their secondary metabolites contents. According to the literature, secondary metabolites production in calli and cell suspensions is possible (Nezbedová et al., 1999; Lopes et al., 2000; Nath & Buragohain, 2005); however, other studies could not reveal the presence of high amount of these specific secondary metabolites in these *in vitro* undifferentiated cells.

Epicatechin, gallic acid, fatty acids and β-amyrin were the main bioactive constituents found in *B. intermedia* in our present study. Eventually, anti-inflammatory activity of this species may be attributed to these phytochemicals, their individual or synergistic effects might increase the anti-inflammatory process. However, the responsible phytochemical(s) for this effect was not yet investigated in detail and the amount of each compound was not yet determined because of the sample complexity.

The findings in this work provide scientific evidence for the ethnomedicinal properties of *B. intermedia* in Brazil. Together with other recent reports which also provide additional pharmacological basis in the traditional use of *B. intermedia*, our work suggests that this species can be used against inflammatory diseases because of its potential to be inflammation inhibitors.

Results obtained in the present work are significant towards studies on enhanced synthesis and accumulation of secondary metabolites in *in vitro*

culture. The present system is advantageous because it depicts the ability of cells raised from *B. intermedia* to synthesize epicatechin. To the best of our knowledge, this is the first report of epicatechin produced in cell suspension cultures of *B.intermedia*.

6 CONCLUSIONS

The findings in the present work offer additional pharmacological information on the therapeutic efficacy of *Byrsonima intermedia*.

- None of the crude *B. intermedia* extracts tested showed antimicrobial activity against the microorganisms used. This might be because there is no anti-microbial activity present or that the concentration of active compounds in the crude extract is too low and further fractionation is needed to reveal its activity.
- Crude methanolic extracts from bark, leaves and flowers at 10µg mL^{-1} present anti-inflammatory effects on zebrafish larvae with tail fin injury.
- The strong anti-inflammatory properties of *B. intermedia* is confirmed in a purified fraction from bark, leaves and flowers methanolic extracts.
- The main bioactive compounds found in the present work in *B. intermedia* bark and leaves methanolic extracts are β-amyrin and epicatechin. Gallic acid is another secondary metabolite present in bark extracts.
- Red cell suspensions of *B. intermedia* contain the secondary metabolite epicatechin.
- Our results suggest that β-amyrin, epicatechin and/or gallic acid could be responsible for the anti-inflammatory effects of *B. intermedia* used in popular medicine.

7 FUTURE WORK

Phytochemical studies should now be executed, where the methanolic extract will be subjected to further fractionation and purification for the identification/isolation of the compound(s) responsible for the activity found in the present work. For this, techniques like nuclear magnetic resonance (NMR) of isolated chemicals could be use.

The sample complexity together with the low concentration of analytes in the samples lead to the need for a more refined analytical study to quantify these compounds. Further work should be developed with the aim of optimizing the compounds separation.

Also a screening in other type of tissues, calli and cell suspensions for the specific presence of the active compounds found in the present work should be done.

To increase the production of the interesting secondary metabolites obtained *in vitro* in the present work, elicitation techniques such as optimization of cultural conditions, nutrient stress, selection of high-producing strains, precursor feeding and biotic and abiotic elicitation inducting the biosynthetic enzymes of the secondary metabolite pathways, metabolic engineering, transformed root cultures, micropropagation, and bioreactor cultures, can be used.

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ANNEXES

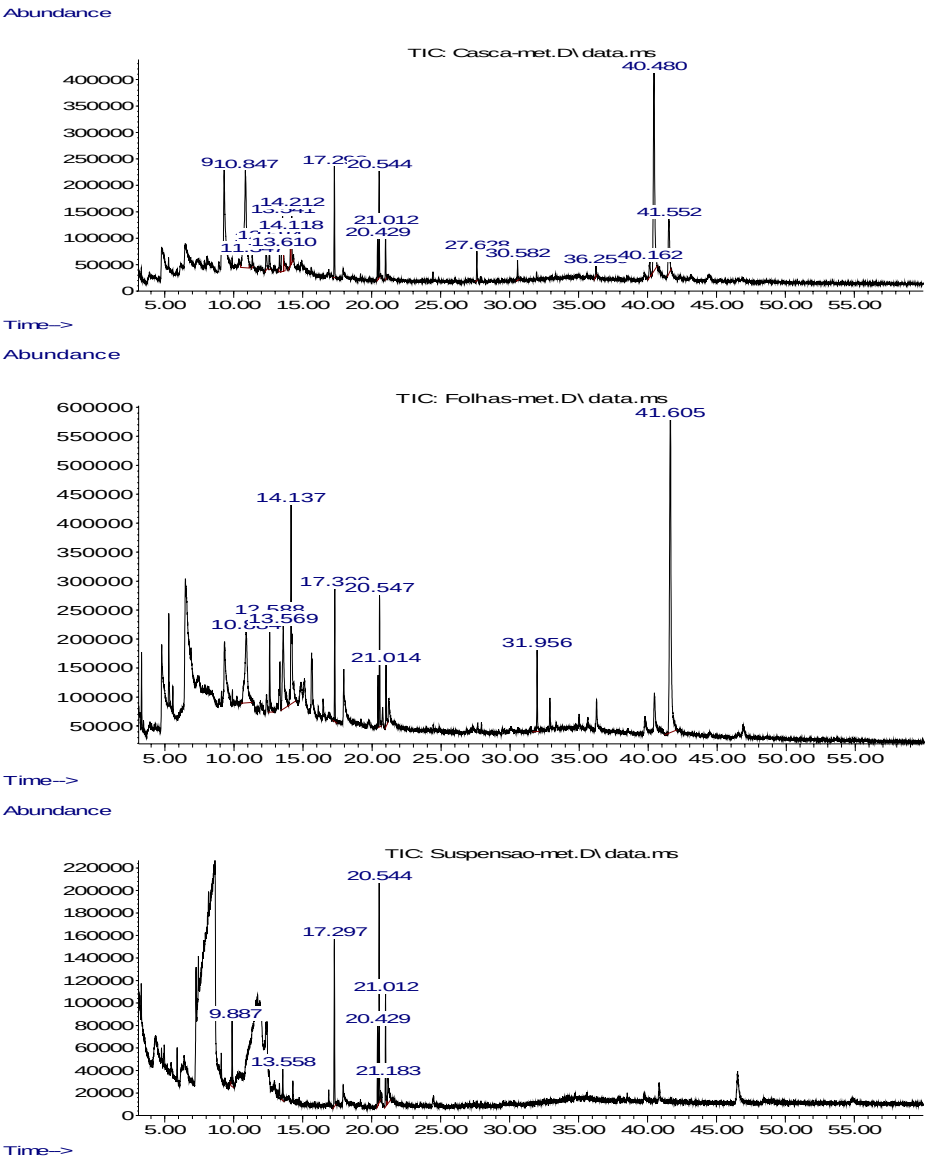

FIGURE 1A Chromatogram of the methylated bark sample (Casca-met), methylated leaves sample (Folhas-met) and methylated yellow cell suspensions sample (Suspensao-met).

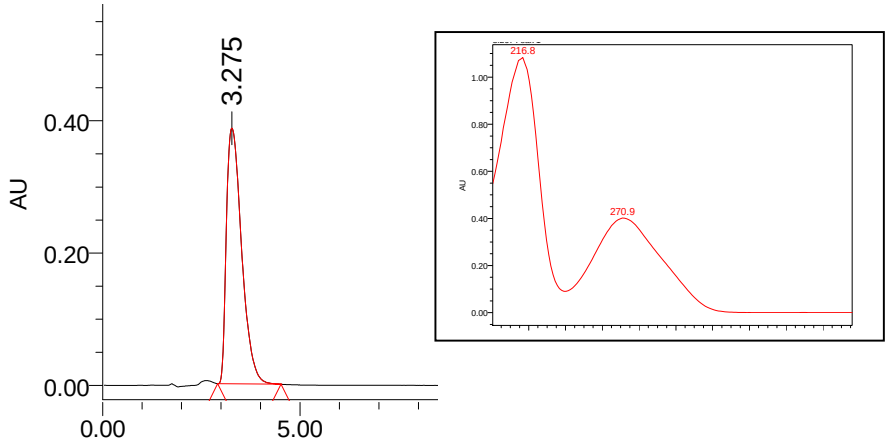

FIGURE 1B Chromatogram obtained by HPLC at 276 nm of the gallic acid standard and its corresponding UV-Vis spectrum with bands at 217 nm and 271 nm.

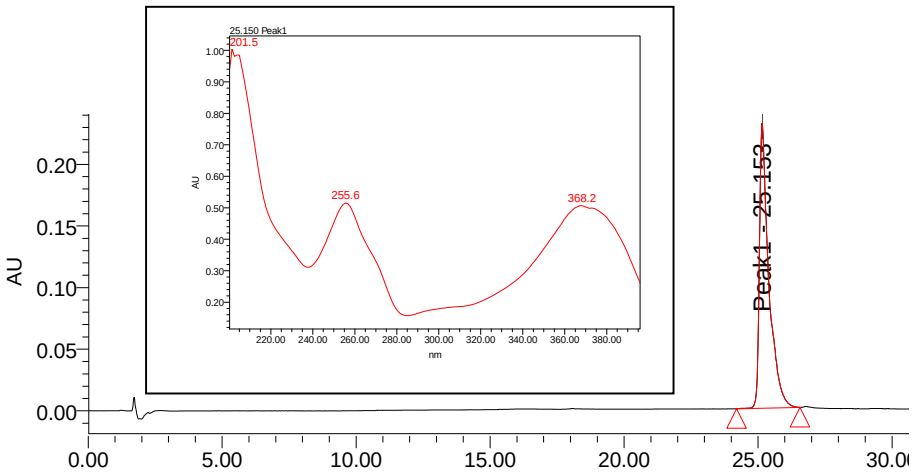

FIGURE 2B Chromatogram obtained by HPLC at 276 nm of the quercetin standard and its corresponding UV-Vis spectrum with bands at 201 nm, 255 nm and 368 nm.

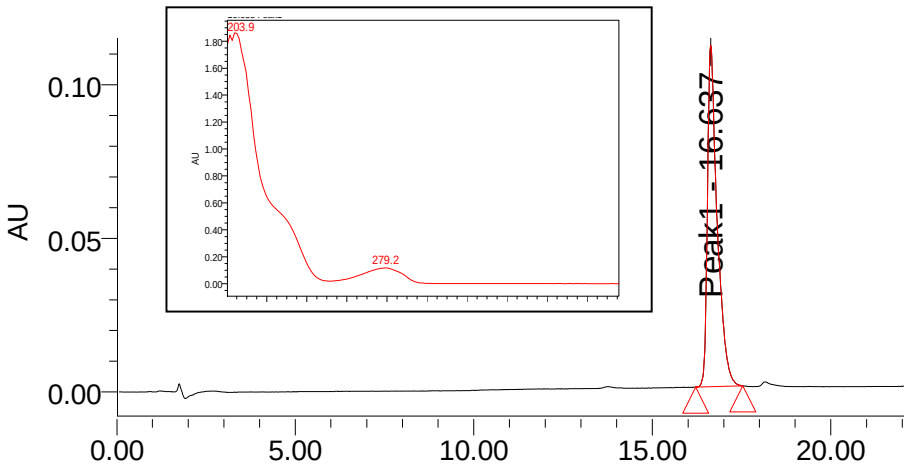

FIGURE 3B Chromatogram obtained by HPLC at 276 nm of the epicatechin standard and its corresponding UV-Vis spectrum with bands at 204 nm and 279 nm.

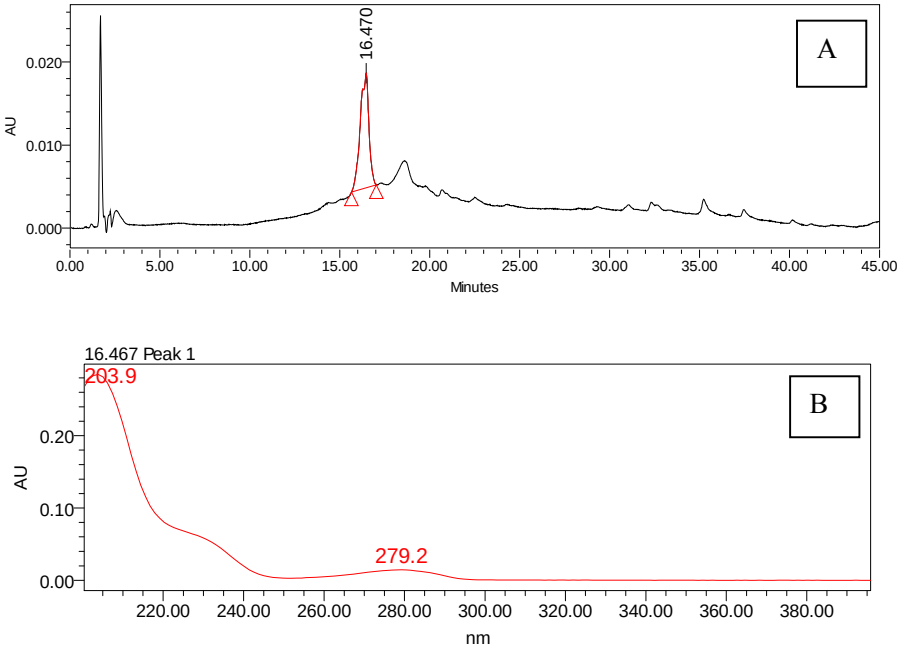

FIGURE 4B Chromatogram obtained by HPLC at 276 nm of the red cell suspensions (A) and the UV-Vis spectrum of the 16.5 min peak (B).

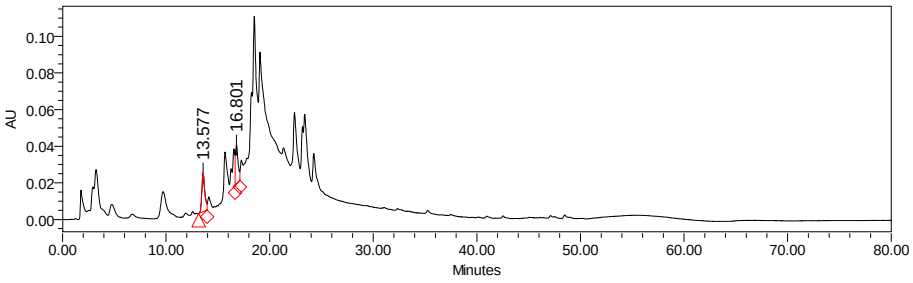

FIGURE 5B Chromatogram obtained by HPLC at 276 nm of the leaves sample.

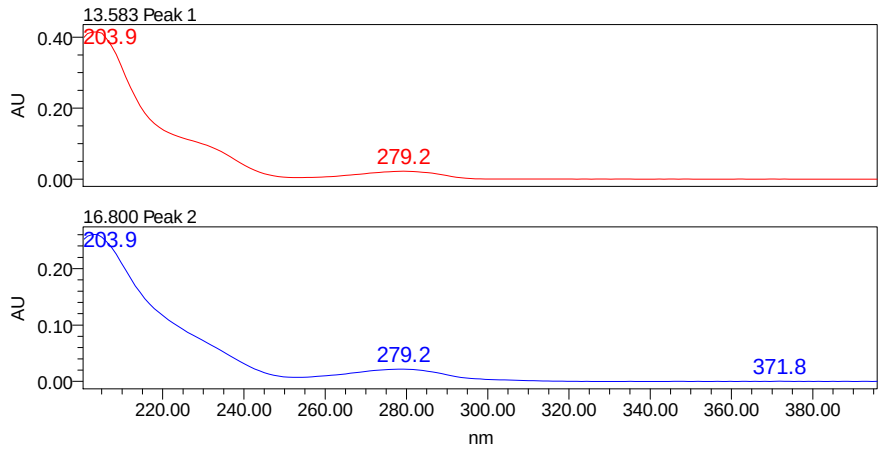

FIGURE 6B UV-Vis spectrum of the 13.6min and 16.8 min peaks present in the leaves sample chromatogram.

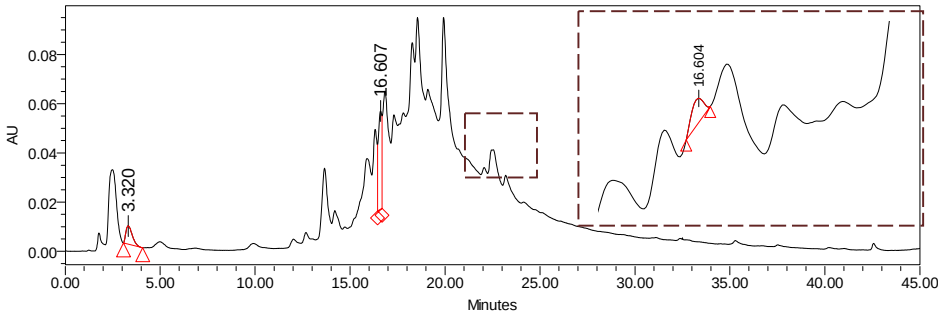

FIGURE 7B Chromatogram obtained by HPLC at 276 nm of the bark sample with expanded window showing the 16.6 min peak.

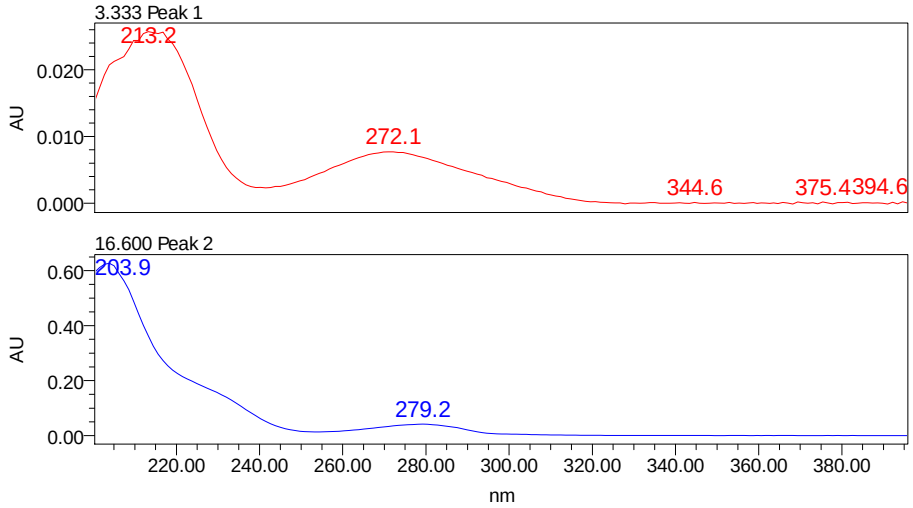

FIGURE 8B UV-Vis spectrum of the 3.3min and 16.6 min peaks present in the bark sample chromatogram.

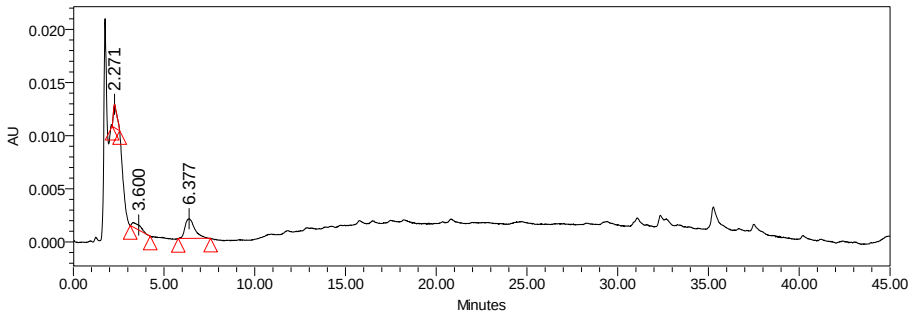

FIGURE 9B Chromatogram obtained by HPLC at 276 nm of the yellow cell suspensions sample.

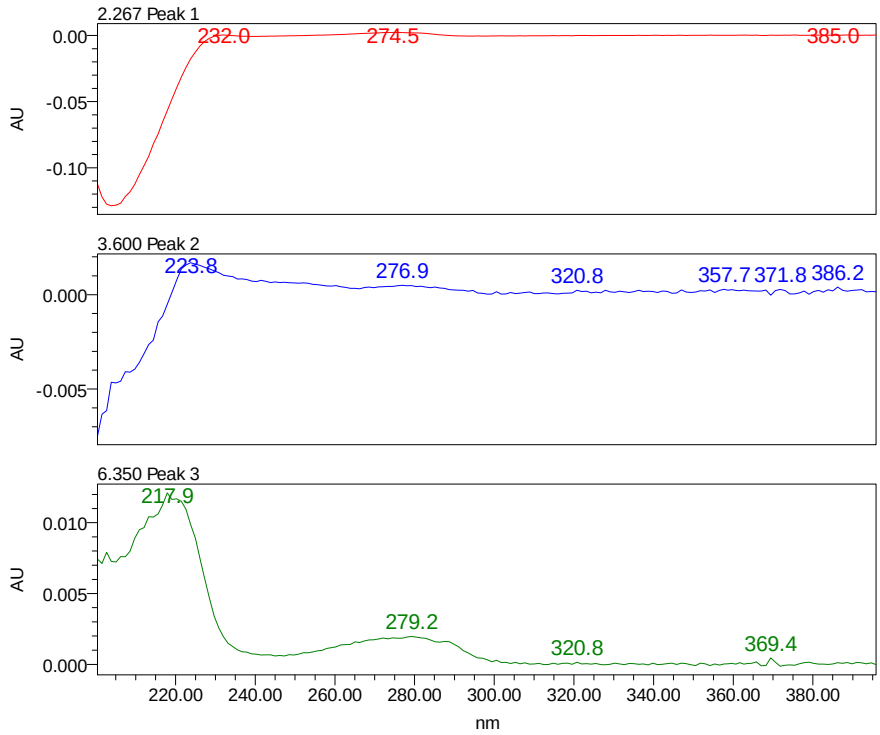

FIGURE 10B UV-Vis spectrum of the 2.3; 3.6 and 6.35 min peaks present in the yellow cell suspension sample chromatogram.