PHYTOCHEMICAL SCREENING OF FIELD-GROWN PLANTS AND *in vitro* TISSUE CULTURE OF *Hovenia dulcis* THUNB

PERFIL FITOQUÍMICO DE *Hovenia dulcis* THUNB. CULTIVADA *in vivo* E *in vitro*

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ABSTRACT

Hovenia dulcis Thunberg is a tree which belongs to the family Rhamnaceae and is known in Brazil as "uva-do-japão". Many species of Rhamnaceae have been investigated for medicinal properties and important pharmacological activities were reported, such as those related to malaria treatment and the prevention of Chagas Disease. Among the several medicinal studies from the Rhamnaceae species, antioxidant, hepatoprotective, antimicrobial, antineoplasic and antigiardic activities were reported. The aim of this study was to carry out phytochemical screening of leaves from a wild growing plant and compare to material produced from biotechnological methods based on tissue culture. The HPLC analysis revealed that plants grown under field conditions presenting more diverse chromatographic profile and indicating the presence of a large quantity of compounds. Gas chromatography showed that these extracts presented higher amounts of volatile compounds. Moreover, the absence of peaks in samples from calli and *in vitro* propagated plants suggests that volatile compounds are not present in these samples. Due to the differences observed in the phytochemical profiles, according to their origin, the data reinforce the use of *in vitro* technologies for compounds production, being able to produce secondary metabolites not found in the field plant, or allowing *in vitro* manipulation of these compounds.

Index terms: Rhamnaceae, medicinal plant, callus culture, *in vitro* propagation

RESUMO

Hovenia dulcis Thunberg é originária da Ásia Oriental, sendo encontrada mais frequentemente na China e no Japão. No Brasil, é conhecida popularmente como uva-do-Japão. Diversas espécies da família Rhamnaceae vêm sendo estudadas quanto ao seu potencial medicinal, tendo sido relatadas importantes atividades farmacológicas, com destaque no tratamento da malária e na prevenção da Doença de Chagas, apresentando ainda atividades antioxidante, hepatoprotetora, antimicrobiana, antineoplásica, e antigiárdica. Extratos alcoólicos foram preparados a partir de calos compactos com dois meses de cultivo, de folhas de um exemplar arbóreo e da parte aérea de plantas propagadas *in vitro*, sendo submetidos a análises cromatográficas. A análise por CLAE revelou um perfil cromatográfico mais diversificado nos extratos de planta de campo, indicando a presença de maior quantidade de substâncias. Porém, foram constatados picos de substâncias produzidas nas condições *in vitro*, não observados nos extratos da planta de campo. A cromatografia gasosa demonstrou que os extratos de planta de campo possuem maior quantidade de substâncias voláteis, enquanto a ausência de picos nas amostras de calos e de plantas propagadas *in vitro* sugere que substâncias voláteis não estão presentes. Dessa forma, ficou constatado que os extratos apresentam perfis fitoquímicos diferentes em função da sua origem. Esses resultados consolidam a proposta do uso da cultura de células e tecidos vegetais como estratégia para a produção de metabólitos, onde podem ser obtidas substâncias não encontradas na planta de campo, oferecendo ainda a possibilidade de manipulação dessa produção *in vitro*.

Termos para indexação: Rhamnaceae, planta medicinal, cultura de calos, propagação *in vitro*.

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INTRODUCTION

Natural products have been obtained through plant tissue culture techniques, such as callus and cell suspension culture. These methods of producing compounds with medicinal interest are of great value, since they allow controlled cultivation, providing continuous and homogeneous synthesis of raw material, regardless of environmental and seasonal factors. These compounds are not essential to plant metabolism and are known as secondary metabolites (SANTOS, 2004), belonging to different chemical classes with medicinal interest.

Hovenia dulcis Thunberg, traditionally known as "uva-do-japão",is a tree native from East Asia, mainly China and Japan, and is cultivated in Brazil especially in subtropical mountainous areas, ideal for its adaptation (COSTA, 1934; JOHNSTON & FREITAS SOARES, 1972; BASTOS, 1990).

In Chinese folk medicine, pseudofruits are used as diuretic, febrifuge and against diarrhea (KENNEDY et al., 1988, HUSSAIN et al., 1990), while in Brazil it has been employed as antiasmatic (COSTA, 1934; CORRÊA, 1984; BASTOS, 1990). Recent studies report the antineoplasic potential by inhibition of growth of tumor cells cultures and tripanocid activity (CASTRO et al., 2002), besides antigiardic activity (GADELHA et al., 2005). The antioxidant activity was proposed in several extracts and related to different mechanisms. Antioxidant activity of pseudofruit extracts was associated with the control of diabetes (LEE et al., 2005). The hepatoprotective activity was reported in several studies and seems to be the most studied. Besides being used as a medicine against liver diseases in traditional Chinese and Korean medicine, extracts of *H. dulcis* act as detoxicant in alcohol poisoning and protect the liver against hepatotoxic substances (KIM, 2001; JI et al., 2001; HWANG et al., 2005).

The present work has been designed to evaluate the phytochemical profile of calli and *in vitro* propagated plants, establishing a comparison with the material collected under field conditions.

MATERIAL AND METHOD

Branches of *Hovenia dulcis* Thunb. were collected at Teresópolis city, Rio de Janeiro (RJ), Brazil. A voucher specimen (HRJ1426) is kept at the Herbarium of Rio de Janeiro State University (UERJ).

Plant material

The extracts were prepared from compact calli derived from two months old *in vitro* germinated seedlings, grown on MS medium with 13.43 mM NAA + 5.54 mM BAP; aerial parts of two months old *in vitro* propagated plants obtained from stem segments and cultivated on MS medium with 2.22 mM BAP + 2.32 mM of KIN (CASTRO, 2001) and expanded leaves of a tree grown under field condition located at Teresópolis city (RJ) (RIBEIRO, 2009). The material was dried at 45°C for 24 hours. The samples were immersed in ethyl alcohol PA (Merck) for two weeks at 25±2° C under agitation. After filtration on Whatman paper (n°.1), the extracts were concentrated in rotary evaporator under reduced pressure and 40° C, dried under vacuum until constant weight and kept in the dark, at 10°C (SIMÕES et al., 2006).

High pressure liquid chromatography (HPLC) analysis

The extracts were subjected to HPLC to evaluate and compare the chromatographic profiles. The samples were diluted in 77% aqueous acetonitrile to a concentration of 10 mg mL^{-1} and injected in a C18 column (BONDAPAK 27,324, particles 10 microns, 300 x 3.9 mm) with UV-VIS detector set at 210 nm (Castro, 2001). The elution flow was 0.7 mL/min for 15 minutes in linear gradient of 0-77%. The mobile phase consisted of 77% aqueous acetonitrile. 20 mL of each sample were injected in duplicate. The signs of absorption were identified according to the retention time (rt) of compounds.

Gas chromatography (GC) analysis

Gas chromatography was performed on a CP-SIL 31CB column (W-Cotinga, 25 m x 0.32 mm x 0.2 mm) with an injection temperature of 250 °C and detector at 300 °C. The temperature was progressively increased at a rate of 10 °C per minute. Nitrogen was used as the carrier gas. Samples were diluted in 77% acetonitrile to a concentration of 10 mg L^{-1} and 20 mL of each sample were injected in duplicate.

RESULTS AND DISCUSSION

High pressure liquid chromatography (HPLC) analysis

The extracts subjected to HPLC analysis are represented in Figure 1. At 210 nm wavelength it was possible to notice that the three types of extracts tested presented different profiles. The higher number of peaks showed that extracts obtained from plants grown under field conditions revealed that these plants presented a more diverse chromatographic profile indicating the presence of a larger amount of compounds. Furthermore, the different retention times observed in extracts of *in vitro* propagated plants compared to extracts of plants grown under field conditions, suggests that these two extracts presenting different compounds. Besides that, it was observed the presence of a high intensity peak with retention time around 3 minutes in all the three extracts, indicating the production of similar compounds occurring on the samples.

Callus cultures are very useful systems for the biosynthesis of natural products, but in some cases, the production of certain secondary metabolites depends on the differentiation of some tissues from callus, as observed in other species (YOSHIKAWA & FURUYA, 1985; ZHAO et al., 2001).

Several studies, in wich HPLC analysis was conducted, have reported that calli extracts presented a smaller number of compounds in comparison with *in vitro* propagated plants, especially in relation to plants grown under *in vivo* conditions (OLIVEIRA et al., 2001; ITAYA et al., 2005; ROOSTIKA et al., 2007). Callus cultures are supposed to produce less secondary metabolites mainly because of its undifferentiated and non-specialized cells. This way, the higher amount of compounds found on *in vivo* grown plants is somehow expected, once the extracts from these plants are composed by substances produced on several cell types with different functions and consequently with different metabolisms (PASQUA et al., 2003; GADZOVSKA et al., 2007). Besides that natural conditions play an important role in the synthesis of metabolites that would take effect in the protection against environmental stresses (EDREVA et al., 2008). Nevertheless, it is possible to manipulate the callus cultures for producing desired metabolites.

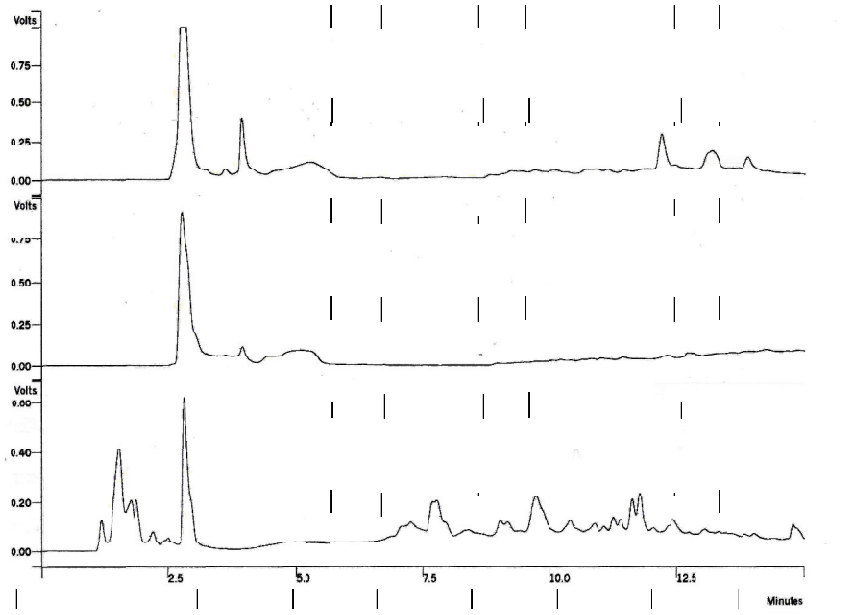

FIGURE 1 – Chromatograms of different extracts of *H. dulcis* submitted to HPLC analysis. A - *In vitro* propagated plants; B - Callus culture; C – Field grown plant.

Stafford (1991) suggests that an adjustment of growth regulators added to culture medium is, in most cases, the most important parameter to be considered, and therefore of highest priority. However, other factors such as nitrogen and phosphorus sources, temperature, presence or absence of light, addition of amino acids, metabolic engineering, genetic engineering and elicitors may be considered too (PANDA et al., 1992; CHOI et al., 1994; FETT-NETO et al., 1994; VEERPORTE et al., 1999; BOURGAUD et al., 2001). Temperature, for example, has proven to be an important factor affecting the biosynthetic activity of cells in several species (AKSU & EREN, 2005). The production of beta carotene was observed in callus cultures of *Capparis spinosa* L. maintained on media supplemented with PIC or 2,4-D, specially at 36±2 °C (ALBARELLO et al., 2007). The next step of this study is the identification of metabolites of interest in *H. dulcis* callus cultures. Thus, some of these parameters, specially temperature and light, will be evaluated in order to explore metabolite production.

Gas chromatography (GC) analysis

By the amount of peaks observed in the chromatograms (Figure 2), it was noticed that extracts of plant grown under field conditions presented higher amounts of volatile compounds. Moreover, the absence of peaks in samples from calli and *in vitro* propagated plants suggests that volatile compounds are not present in these samples. Gas chromatography has been widely used in the study of plant volatiles. Analysis by GC has shown that many volatile compounds produced by plants grown under field conditions may not be present in *in vitro* cultures (Hamada et al., 1991). Differences in phytochemical profiles obtained by GC have been reported for several species (GULLUCE et al., 2003; NIKOLAKAKI & CHRISTODOULAKIS, 2004; ZAYED et al., 2006; PACIFICO et al., 2008).

The low amount of volatile substances found in *in vitro* cultures of *H. dulcis* does not imply that this kind of compounds cannot be produced in these conditions. For example, the analysis of wheat calli identified over 200 volatile compounds (LOZOVAYA et al., 2006).

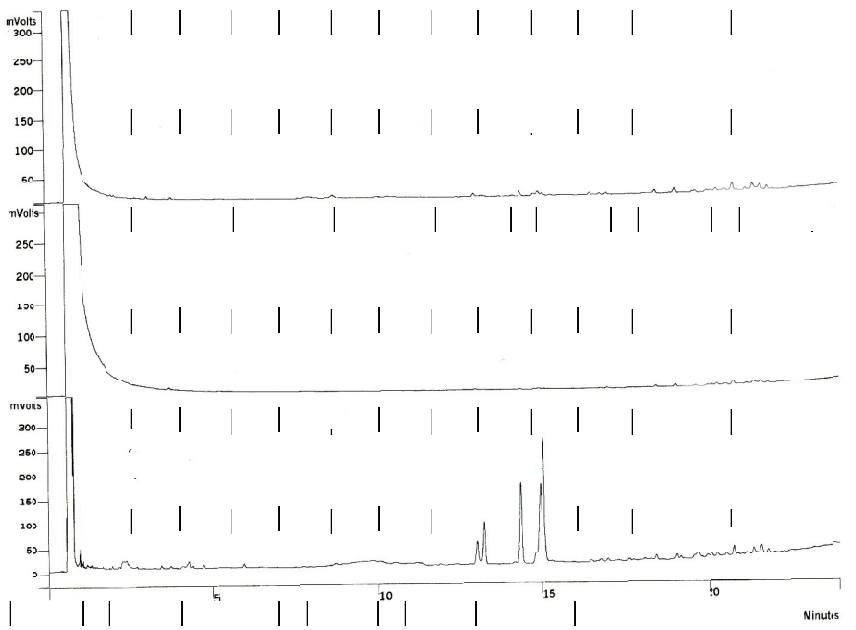

FIGURE 2 – Chromatograms of different extracts of *H. dulcis* submitted to GC. A - *In vitro* propagated plants ; B - Callus; C - Field grown plants.

Through the manipulation of culture medium, it is possible to stimulate or inhibit the synthesis of substances already present in *in vivo* plants, besides inducing the production of new substances (FIGUEIREDO et al., 1999; SIMÕES et al., 2009). Schmeda-Hirschmann et al. (2004) compared different types of *in vitro* cultures of *Fabiana imbricata* Ruiz & Pavón with plants collected in field during different months throughout the year analyzing the contents of oleanolic acid, rutin, chlorogenic acid and scopoletin. The authors found a high variation of these metabolites in plants under *in vivo* conditions, and in some cases showed that the callus cultures were more efficient in the production of these metabolites. Simões et al. (2009) observed similarities on the chromatographic profiles from calli and stems of field-grown plant extracts of *Cleome rosea* **Vahl ex DC.** and the presence of the anthocyanin peonidin was observed from callus culture, but not from field-grown plants. The use of elicitation methods may also be applied on the production of these compounds (DE ALWIS et al., 2009). This way some modifications may be applied on *H. dulcis* callus culture medium allowing the synthesis of volatile compounds such as terpenes.

Preliminary studies of *H. dulcis* by HPLC analysis showed that different compounds were detected mainly in extracts of leave and stem from *in vitro* plants. Tissue culture techniques were efficient to increase the production of some compounds in extracts from *in vitro* plants, when compared with extracts from seedlings and adult plant (CASTRO et al., 2001) In the present study, considering that different compounds were detected depending on culture conditions (*in vivo* and *in vitro*), new procedures should be adopted regarding the modification of culture medium, in order to increase *in vitro* production of desirable metabolites from *H. dulcis*.

CONCLUSIONS

It was possible to conclude that the synthesis of metabolites might vary according to the origin of plant material and culture conditions. These data reinforce the use of *in vitro* technologies for compounds production, being able to produce compounds not found in the field plant, or allowing *in vitro* manipulation of these compounds.

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