Assessment of the Antioxidant Activity of Two Plant Extracts Containing Isoflavonoids by Different In Vitro Methods

Sandra R. GEORGETTI 1, Rúbia CASAGRANDE 1,2, Waldiceu A. VERRI, JR. 3, Maria F. BORIN 1, Janice A. RAFAEL 1, José R. JABOR 1 and Maria J.V. FONSECA 1

1 Department of Pharmaceutical Science, Faculty of Pharmaceutical Sciences of Ribeirão Preto - USP, Av. do Café s/n, CEP 14040-903, Ribeirão Preto, SP, Brasil
2 Department of Food and Drugs Technology, Agricultural Sciences Center-UEL
3 Department of Pharmacology, Faculty of Medicine of Ribeirão Preto - USP

SUMMARY. The reactive oxygen species (ROS) may ultimately cause or participate in the induction of a wide range of diseases including cancer, atherosclerosis, rheumatoid arthritis and diabetes. Thus, many studies focus in the possible anticancerogenic activity of isoflavonoids present in soy and red clover. However, achieving therapeutic effect and a broader use depends on the extract standardization and quality. Therefore, in the present study two commercial extracts (Isoflavin Beta® - mixture of isoflavonoids and the red dry clover extract) were characterized on their constituents (total flavonoids, isoflavonoids, total polyphenols and total proteins), and in vitro antioxidant activities. Both extracts presented significant antioxidant activity in all tests. Moreover, the active doses depended on each test, probably because of extract composition. Concluding, chemical composition and in vitro antioxidant activity might help to standardize plant extracts.

RESUMEN. “Evaluación de la Actividad Antioxidante de Dos Extractos Vegetales que Contienen Isoflavonoides por Diferentes Métodos In Vitro”. Las especies reactivas del oxígeno (EROS) han sido relacionadas con diferentes enfermedades. Las EROS inducen daños y perjuicios a las membranas celulares vía la peroxidación lipídica (LPO), lo que contribuye a la oxidación del ADN y carcinogénesis. En ese sentido, varios estudios se centralizaron en la actividad anti-cancerosa de isoflavonoides presentes en la soja y en el trébol rojo. Sin embargo, para alcanzar su efecto terapéutico y permitir su amplia utilización, todo depende de la regularización y calidad de los extractos. En este estudio dos extractos comerciales, Isoflavin Beta® (mezcla de los isoflavonoides) y el extracto de trébol rojo, fueron caracterizados teniendo en cuenta sus constituyentes (flavonoides totales, isoflavonoides, polifenoles totales y proteínas totales). Además, sus actividades antioxidantes también fueron evaluadas por diferentes métodos. Los dos presentaron la actividad antioxidante significativa en todas las pruebas. Las concentraciones eficaces dependieron de cada prueba, lo que probablemente esté relacionado con sus constituyentes. Se concluye que la composición química y la evaluación in vitro de la actividad antioxidante deben contribuir para la estandarización de los extractos de las plantas.

INTRODUCTION

In the last 10 years, herbal medicines and natural products became a topic of increasing global interest on both world health and international trade 1. According to The World Health Organization (WHO), the primary assistance to health of nearly 65 to 80% of the population from developing countries depends mainly on medicinal plants, because of the low access to synthetic drugs 2. Furthermore, 20% of the population from developed countries uses 85% of the manufactured available drugs. Brazil show similar data, 20% of the population uses 63% of the manufactured drugs and the other 80% of the population has the natural products as the only therapeutic source 3.

Therefore, in the last decades there was a strong increase in the interest for plant-derived drugs 2. Corroborating, it is estimated a worldwide market of nearly 22 billion dollars for this type of drugs 4. In fact, many plant extracts are commercialized in Brazil including soybean and red clover extracts.

Although the popular observation about the

KEY WORDS: Antioxidant, Control quality, Free radical, Glycine max, Isoflavonoids.

PALABRAS CLAVE: Antioxidante, Control de calidad, Glycine max, Isoflavonoides, Radicales libres, Trífio - lium pratense.

* Autor a quem correspondência deve ser enviada. E-mail: magika@fcfrp.usp.br
plants efficacy gives an important contribution to reinforce their therapeutic effects stimulating their use \(^5\), the popular and traditional uses are not enough to validate them as safe and efficient treatments. Before the official approving for medicinal use of a plant-derived drug, it is necessary to guarantee the chemical and pharmacological integrity of the components comprised in the plant, aiming at the safety in use in additional to the therapeutic potential \(^6\).

In this context, the different components of an extract may proportionate potentiation or additive pharmacological and toxicological actions (e.g. may be beneficial or deleterious). Therefore, slight variations in the concentration of determined component may represent deleterious effects. However, at the same time, composition differences might help to standardize and identify plant extracts. Thus, key issues to use these products as therapeutic treatments are the quality control, safety and efficacy assurance. Therefore, the present study evaluated whether in vitro antioxidant activity analysis would help to standardize plant extracts and the chemical composition relation to the antioxidant activity.

**MATERIALS AND METHODS**

**Chemicals**

Isoflavin Beta\(^\circledast\) and dried red clover extract from France were donated by Galena (Campinas, SP, Brazil). Thiobarbituric acid (TBA) and 2,2-diphenyl-1-picrylhydrazyl (DPPH\(^\bullet\)) were obtained from Sigma Chemical Co. (St. Louis, MO, USA). 2-deoxy-D-ribose was obtained from Acros (New Jersey, USA). All other reagents used were of pharmaceutical grade.

**Chemical characteristics of Isoflavin Beta\(^\circledast\) and red clover**

**Protein concentration**

Samples of the extracts (0.1 g) were homogenized with 10 mL of deionized water for 10 min. The mixtures were centrifuged at 1600 \(g\) for 30 min and the supernatant fractions were used for protein quantification by the method of Lowry *et al.* \(^7\). The standard used was bovine serum albumin.

**Total polyphenol and flavonoid contents**

Samples of both extracts (0.1 g) were stirred with 80% ethanol for 15 min. The ethanol suspensions were centrifuged at 1660 \(g\) for 10 min and the supernatant fractions collected. Another extraction was done in the precipitates using 5 mL of 80% ethanol. Finally, the supernatant fractions were combined and the volume adjusted to 25 mL with deionized water obtaining the ethanol extracts (EE).

Total polyphenol contents in EE were determined by Folin-Ciocalteu colorimetric method using the gallic acid as standard \(^8\). EE (0.5 mL) was mixed with 0.5 mL of the Folin-Ciocalteu reagent and 0.5 mL of 10% \(Na_2CO_3\), and the absorbance was measured at 760 nm after 1 h incubation at room temperature.

Total flavonoids contents in the EE were measured as described by Chang *et al.* \(^9\). To 0.5 mL of EE, 0.5 mL of 2% \(AlCl_3\) ethanol solution was added. After 1 hour at room temperature, the absorbance was measured at 420 nm. Total flavonoids contents were calculated using quercetin (mg/g) as standard.

**High performance liquid chromatography (HPLC)**

Genistein and daidzein contents in Isoflavin Beta\(^\circledast\) and red clover were determined by reversed-phase HPLC analysis. Isoflavin Beta\(^\circledast\) and red clover samples were dissolved in 25 and 50 mL of methanol, respectively, diluted in the mobile phase 1:2, filtered with a 0.45 µm filter and 20 µL injected into the HPLC system. Genistein and daidzein separation was performed employing the SuperPac Sephasil C18 (5 µm) column, 250 x 4 mm- attached to a pre-column. The mobile phase consisted of 0.1% acetic acid in acetonitrile, 0.1% acetic acid (30:70), 1 mL/min. Eluted isoflavonoids were detected by their absorbance at 250 nm. Quantitative data for daidzein and genistein were obtained by comparing to their standards \(^10\).

**Antioxidant activities assays**

**Lipid peroxidation induced by Fe\(^{2+}\)/citrate system**

Lipid peroxidation was assayed by malondialdehyde (MDA) generation \(^11-12\) in the presence of different concentrations of Isoflavin Beta\(^\circledast\) and red clover. Ten µL of each sample in different concentrations (12.5 to 300 µg/mL in DMSO) were added to 1.0 mL of a medium 1 (125 mmol/L sucrose, 65 mmol/L KCl and 10 mmol/L Tris-HCl, pH 7.4). Mitochondria (lipid source) were added to yield a final concentration of 1 mg of protein, 50 µM (NH\(_4\))\(_2\)Fe(SO\(_4\)) and 2 mM sodium citrate were used as catalyst of the lipid peroxidation reaction. The reaction medium was incubated for 30 min at 37 °C. For MDA determination, 1 mL of 1% thiobarbituric acid (TBA), 0.1mL of 10M NaOH and 0.5 mL of 20% H\(_3\)PO\(_4\)
were added, followed by incubation at 85 °C for 20 min. The MDA-TBA complex was extracted using 2 mL of n-hexanol. The samples were then centrifuged at 1660g for 10 min. Absorbance was determined at 535 nm. Blank was prepared from the reaction mixture without mitochondria. Two controls were used for this test, a positive control (samples absence) and a negative control (iron absence). All measurements were made in triplicate.

Scavenging effect on hydroxyl free radicals

The scavenging free radical ability was determined using a modification of the Halliwell et al. method. Briefly, 10 µL of Isoflavin Beta® and red clover extracts samples in different concentrations (0.005-1.0 µg/mL) were added to 1.0 mL of the reaction medium (20 mM KH₂PO₄-KOH buffer, pH 7.4). Hydroxyl radical was generated by incubation, for 30 min at 37 °C, of the reaction mixtures containing 50 µM FeCl₃, 52 µM EDTA, 1 mM H₂O₂, 2.8 mM deoxyribose and 200 mM ascorbate in 20 mM KH₂PO₄-KOH buffer, pH 7.4. Deoxyribose degradation caused by hydroxyl radical was estimated using the thiobarbituric acid method, as described before.

Scavenging activity of 2,2-diphenyl-1-picrylhydrazyl radicals (DPPH*)

DPPH* is a stable free radical that potentially reacts with the compounds able to donate H*. For radical scavenging activity of the Isoflavin Beta® and red clover extracts measurements, DPPH* ethanolic solutions 100 µM and 250 µM were used, respectively. The DPPH* reduction was determined from the change in absorbance measured at 517 nm as previously described Blois (1958). In order to evaluate the scavenging activity, 10 µL of each extract sample in different concentrations (Isoflavin Beta® from 30 to 500 µg/mL, and red clover from 10 to 125 µg/mL) were added in the reaction mixture containing 1 mL of 0.1 M acetate buffer (pH 5.5), 1mL of ethanol, and 0.5 mL of DPPH* ethanolic solution. The change in absorbance was measured after 10 min at room temperature. The positive control was prepared in the absence of the extracts, and it indicates the maximum odd electrons of DPPH*, which was considered 100% of free radicals in the solution to calculate the hydrogen-donating ability (%) of Isoflavin Beta® and red clover. The blank was prepared from the reaction mixture without DPPH* solution. All measurements were performed in triplicate.

Statistical analyses

The concentration of Isoflavin Beta® and red clover necessary to inhibit the oxidative process in 50% (IC₅₀) was determined by GraphPad Prism® software, version 3.02, using hyperbolic curve (one site binding and two site binding hyperbole).

RESULTS AND DISCUSSION

The plant extracts are mixture of substances with different chemical-structures and physicochemical properties as well as different pharmacological and toxicological actions. Thus, key issues to use these products as therapeutic treatments are quality control, safety and efficacy assurance. Therefore, the present study evaluated whether chemical composition and in vitro antioxidant activity analysis could help to standardize plant extracts.

Taking into account that extracts are constituted by different chemical compounds, and their activity is directly related to the constituents structure and concentration, two issues are important to be evaluated in that kind of antioxidant containing vegetal drugs: i) the concentration of phenolic compounds and ii) the antioxidant activity of the extracts using different assays in an attempt to acquire standardized extracts. First, the chemistry composition of Isoflavin Beta® and red clover were evaluated by measuring total flavonoid, polyphenol and protein contents, using chemical methods, and by determination of the isoflavones (daidzein and genistein) amount employing HPLC analysis (Table 1).

It was detected that Isoflavin Beta® extract has more isoflavonoids (daidzein and genistein), total polyphenols and total protein than red clover extract. On the other hand, red clover has more total flavonoids than Isoflavin Beta®. Others have also reported the presence of daidzein and genistein in these extracts.

Regarding the antioxidant activity, the Isoflavin Beta® and red clover effects were concentration-dependent in the lipid peroxidation (LPO) assay. The IC₅₀ values were 21.03 and 10.34 µg/mL, respectively (Fig. 1). The maximum inhibition of LPO was 80% and 90% for Isoflavin Beta® (150 µg/mL) and red clover (100 µg/mL), respectively. The IC₅₀ value in the LPO-inhibiting activity assay and total flavonoids content of red clover was 2 fold lower and 1.2 folds higher than Isoflavin Beta®, respectively.

The relevant chemical characteristics that contribute to the flavonoids antioxidant or ox-
dant scavenging activity are: the 3', 4' hydroxyl (catechol) groups in the B ring, the 2, 3 double bond in conjugation with a 4-oxo group in the C ring, and the presence of hydroxyl groups in positions 3 and 5. For instance, quercetin has all of those characteristics, and thus, presents strong antioxidant activity. Younes & Siergers found out in their experiments that, in LPO, naringin and naringenin, which lack the 3'-OH-group, were the less active inhibitors. The only structural feature of the flavonoids which affected their antioxidative action appeared to be the 3',4'-dihydroxy-groups. Both daidzein and genistein have 4'- hydroxyl group, which could explain the Isoflavin Beta antioxidant activity in LPO assay.

Furthermore, mitochondrial membranes were used as lipid source in the LPO assay. Probably, the difference observed for Isoflavin Beta and red clover extracts in the LPO assay may be due to the difference of interactions between the flavonoids of both extracts with the mitochondrial membrane. One of the possible consequences of this interaction is a direct modulation of membrane physical properties acting on the capacity of flavonoids in free radicals scavenging.

The ability of Isoflavin Beta and red clover to scavenge hydroxyl radicals was determined by the inhibition of thiobarbituric acid-reactive substances formation (MDA) originated from the degradation of deoxyribose by Fenton reaction (iron (III)-EDTA and H2O2 in the presence of ascorbate). The deoxyribose degradation decreased with increasing concentrations of Isoflavin Beta and red clover within the range of 0.005 - 1.0 μg/mL (Fig. 2). The level of inhibition reached a plateau of 90% at a concentration of 1.0 μg/mL of Isoflavin Beta and red clover. The IC50 of the extracts were 33.5 and 32.1 ng/mL for Isoflavin Beta and red clover, respectively, as calculated from the concentration-activity curves. Thus, although there were differences in chemical composition of Isoflavin Beta and red clover, the calculated IC50 values were not different. It is noteworthy that genistein inhibits peroxyl and hydroxyl radical-induced prooxidant effects and is an effective scavenger of hydrogen peroxide. Thus, the higher concentrations of the isoflavonoids daidzein and genistein in Isoflavin Beta than in the red clover might explain the scavenging effect on hydroxyl radicals similar to flavonoids and other polyphenols of red clover. Furthermore, structural differences might also contribute to explain the similar activities despite different composition. In fact, flavonoids that present hydroxyl substitution on C3 might have

<table>
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<th>Extracts</th>
<th>Total flavonoids (mg/g)</th>
<th>Isoflavonoids (mg/g)</th>
<th>Total polyphenol (mg/g)</th>
<th>Total protein (mg/g)</th>
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<tr>
<td>Isoflavin Beta®</td>
<td>6.8</td>
<td>196.0</td>
<td>87.0</td>
<td>75.0</td>
</tr>
<tr>
<td>Red clover</td>
<td>8.4</td>
<td>5.4</td>
<td>2.9</td>
<td>45.0</td>
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</tbody>
</table>

Table 1. Chemical characteristics of the Isoflavin Beta® and red clover.

Figure 1. Isoflavin Beta® (A) and red clover (B) concentration-dependently inhibited Fe²⁺-induced lipid peroxidation. One mg of mitochondrial protein was incubated for 30 min with the samples in the presence of 50 μM (NH4)2FeSO4, 2 mM citrate, 125 mM sucrose, 65 mM KCl and 10 mM Tris-HCl pH 7.4 at 37 °C (1 mL final volume). TBA-reactive substances were determined as described in material and methods.
higher antioxidant activity than isoflavonoids that with the B ring in iso position.

In addition, the high proteins content of Isoflavin Beta® (Table 1) could also intercept the hydroxyl radical and be regarded as antioxidants. It is the case of albumin, which may be degraded by hydroxyl radical attack and thus, preventing the hydroxyl radicals attack of other more important molecules. Therefore, the similar concentration of proteins and isoflavonoids, could also contribute to the Isoflavin Beta® ability to scavenge the hydroxyl radicals.

The hydrogen donating ability of Isoflavin Beta® and red clover were also evaluated by the use of the stable radical 2,2-diphenyl-1-picrylhydrazyl (DPPH*) as presented in Fig. 3. The IC₅₀ values suggested that red clover was more effective to scavenge DPPH* than Isoflavin Beta® (30.27 and 161.8 µg/mL, respectively). Sánchez-Moreno et al. 23 demonstrated efficient antioxidant activity of polyphenols (e.g. gallic acid, tannic acid and caffeic acid) present in red clover using the DPPH* assay. Moreover, Ko et al. 24 showed that monohydroxylated and dihydroxylated flavones possessed weak antioxidant activity against DPPH* radicals. Daidzein, present in high concentration in Isoflavin Beta® extract, is a dihydroxylated flavone, thus, besides the high concentration of isoflavonoids in that extract, the major composition by a flavone could explain the higher activity of red clover extract compared to Isoflavin Beta®, because it characteristically has a weak antioxidant activity against DPPH* radicals.

The present results demonstrated that de-
Despite chemical composition differences, the antioxidant activities may be very similar or different depending on the assay (type of antioxidant activity). Red clover has more total flavonoids than Isoflavin Beta®, but Isoflavin Beta® has more isoflavonoids and total polyphenols than red clover. The different methods used to evaluate the Isoflavin Beta® and red clover extracts indicate that red clover is an antioxidant/radical scavenger more efficient than Isoflavin Beta®, probably due to the OH substitution in some specific flavonoids rings. However, the protein content of Isoflavin Beta® could contribute to hydroxyl radical scavenging, that is the most deleterious of ROS.

Concluding, both extracts presented significant antioxidant activity in all tests with a direct relation to their constituents, although the active dose depended on each test. Moreover, the antioxidant activity is an efficient parameter to standardize and to evaluate quality of plant extracts.

Acknowledgments. This work was financially supported by grants from the Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP) and the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES).

REFERENCES