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RESEARCH ARTICLE

Design of a bioassay for ecopharmacovigilance studies in *Phaseolus vulgaris*

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Abstract

Phaseolus vulgaris is a plant species recommended and used for Eco toxicity and phytotoxicity bioassays due to its sensitivity and the production of secondary metabolites that can act as exposure indicators. The objective of this research work was to design a bioassay for the cultivation of *P. vulgaris* in agar-agar to facilitate its exposure by homogeneous diffusion of chemical substances in the agar and the integrity of the plant root when harvested. The growth of *P. vulgaris* was evaluated at different concentrations of agar and at different days of culture and morphometric measurements of the plants and determination of total chlorophyll, total carotenoids, total polyphenols and antioxidant activity of the leaves of the cultivated plants were carried out. It was found that the best conditions due to growth and production of secondary metabolites were growing *P. vulgaris* in 0.5% agar for 7 days. This bioassay is available for use in Eco pharmacovigilance studies of drugs as environmental contaminants.

Keywords: Eco pharmacovigilance, Polyphenols, Antioxidant activity

Introduction

The common bean (*Phaseolus vulgaris*) is a plant species that is grown for human consumption. In Mexico it is the most important legume for human consumption since pre-Hispanic times and has great nutritional, social and economic importance (Aguilar et al., 2019; Chico et al., 2021). Because it is a very sensitive plant species, it is used as a bioindicator in ecotoxicity evaluation studies because it provides useful information about the toxic potential of pharmaceutical substances and therefore the US FDA (Food and Drugs Administration, United States) recommends Phaseolus. Vulgaris as a plant species for phytotoxicity testing (Chico et al., 2021; Jităreanu et al., 2011).

The assessment criteria frequently used in ecotoxicity tests are germination, growth and biomass of *P. vulgaris*; Furthermore, this plant species is rich in secondary metabolites such as polyphenols that, together with photosynthetic pigments (chlorophylls and carotenes), can be used as biomarkers of damage due to exposure to chemical substances such as allopathic drugs (Chico et al., 2021; Jităreanu et al., 2011; Groth et al., 2016; Khaled et al., 2016; González et al., 2001).

However, the main problem with common tests is that they are carried out on cotton or absorbent paper 2-4, this entails different difficulties, among which the first one stands out is that the roots of the germinated seedlings become

entangled in the material and tend to break and also break. Humidity must be controlled to prevent the seed from rotting or the proliferation of microorganisms, the second obstacle is that the absorbent material acts as a stationary chromatographic phase and the seeds are exposed to a less controlled concentration of the substance evaluated than if it were in a substrate that adequately allows the diffusion and homogenization of the evaluated substance.

As a result of the above, the interest arose in designing an Eco toxicity bioassay that could be used for future Eco pharmacovigilance and phytotoxicity studies that would be rapid, reliable and reproducible; For this reason, the kinetics of the growth of *P. vulgaris* on agar-agar as a substrate was evaluated to optimize the conditions of the bioassay so that it is useful in the analysis of the Eco toxicity of different substances, in particular to study the effect of drugs as environmental contaminants.

Materials and Methods

Certified bean seeds (*Phaseolus vulgaris*) were used that were selected according to their physical appearances that did not show signs of decomposition and that they were intact; The seeds were washed with running water to remove dust and then disinfected by immersion in a 5% aqueous solution of sodium hypochlorite for five minutes and finally washed with plenty of water to eliminate the rest of the hypochlorite. The seeds were placed in a sterile absorbent paper to dry them and subsequent planting (Aguilar et al., 2019).

Individual and group growth of *P. vulgaris* on agar

First, the growth of *P. vulgaris* was tested in different concentrations of agar-agar; for this, mixtures of four concentrations of agar-agar (0.5%, 1.0%, 1.5% and 2.0%) were prepared in sterile distilled water and each mixture was heated to boiling. for 10 minutes, the evaporated volume of water was replaced and distributed in germination trays (volume 250 mL of agar) and in 20-well vials (well volume of 20 mL of agar) to evaluate their growth as a group and individually. Each test was done in triplicate, in the group trays 12 seeds were placed equidistantly distributed and in the individual trays there were 12 wells with one seed in each well for a total of 36 seeds per test. Each container was placed in a transparent germination chamber containing sterile distilled water to provide humidity and prevent desiccation of the solid medium; The seeds were incubated in natural light and a temperature of 25°C ± 1°C for seven days, then the seedlings were harvested to determine their sizes and weight.

Kinetics of *P. vulgaris* growth on agar

144 seeds (36 seeds per treatment) of *P. vulgaris* were grown in individual wells on 0.5% agar in a transparent germination chamber containing sterile distilled water to provide humidity and prevent desiccation of the solid medium; The seeds were incubated in natural light and a temperature of 25°C ± 1°C for 4 days, 7 days, 10 days, 15 days and 18 days and at the end of each period the seedlings that grew were harvested for morphometric analysis and the determination of their photosynthetic pigments, polyphenols. and DPPH inhibitory activity; To do this, the leaves were extracted by maceration with absolute methanol in a 1:5 (W/V) ratio, allowed to rest for 30 minutes and then the extract was filtered and centrifuged at 14,000 rpm for 10 minutes.

Determination of chlorophylls and carotenes

Chlorophyll quantification was performed spectrophotometrically at wavelengths of 649 nm and 665 nm and total chlorophyll was calculated using Sumanta equations 1 and 2 for methanol as a solvent. Likewise, the concentration of total carotenoids was quantified spectrophotometrically by measuring the absorbance of the extract at a wavelength of 470 nm and using equation 3 of Sumanta et al., each determination was performed in triplicate (González et al., 2001; Serpa Ibáñez et al., 2006; More et al., 2016; Sumanta et al., 2014).

$$C_a = 12.47A_{665} - 3.62A_{649} \qquad (1)$$

$$C_b = 25.06A_{649} - 6.5A_{665} \qquad (2)$$

$$C_c = \frac{1000A_{470} - 1.63C_a - 104.96C_b}{221} \qquad (3)$$

Quantification of total polyphenols

100 mL of *the P. vulgaris* leaf extract was added to 1.0 mL of distilled water and 100 mL of the Folin Ciocalteu Reagent was added. The mixture was allowed to react for 30 minutes and then 500 mL of a solution was added of 10% Na_2CO_3 in water and let it rest for 30 minutes at room temperature in the dark. Finally, the absorbance was measured at 760 nm; a five-point calibration curve was prepared using gallic acid as a standard. The polyphenol content was expressed as mg equivalents of Gallic Acid (GAE) per g of fresh leaf; each determination was performed in triplicate.

Determination of the inhibitory capacity of radical DPPH

2.0 mL of a 150 mM DPPH (2,2-diphenyl-1-picrylhydrazyl) solution in 80% methanol was used and 25 mL, 50 mL and 100 mL of the extract were added, incubated for 30 minutes in the dark and at room temperature. Subsequently, the absorbance was measured at 520 nm in a UV Visible spectrophotometer, gallic acid was used as a standard solution. Each sample was analyzed in triplicate and the results are expressed as mg EAG/g fresh leaf.

Statistical analysis

The data obtained were processed in the Microsoft Excel® program and the analyzes of variance and comparison of means (Tukey's test, $p<0.05$) were performed in the SPSS statistical program. For the calibration curves, a Pearson linear correlation analysis was performed and the respective straight-line equations were obtained for determining the concentrations of the analytes.

Results and Discussion

Fig. 1 shows the recorded weights of the *P. vulgaris* seedlings grown in different concentrations of agar-agar. It can be seen that the best growth was obtained in the 0.5% agar concentration and that there is not much variation between the individual and group cultivation; but a slight increase in the weight of the plants grown individually is observed. This allows selecting the 0.5% agar concentration and the individual culture to carry out the bioassays, with the advantage that less agar is consumed (economic savings) and the exposure to substances of each P seed can be individually evaluated. vulgaris to avoid variations and control the exposure concentration (Rochín et al., 2021; Oliveira et al., 2018; Mujica et al., 2012).

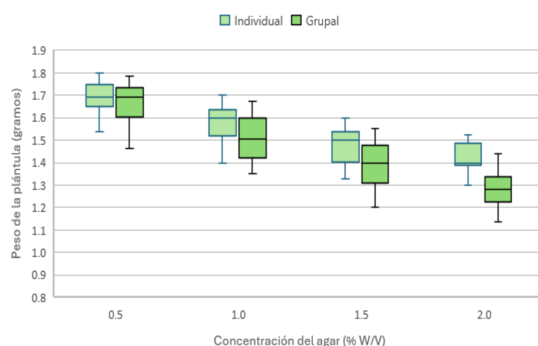

Figure 1: Weights of *Phaseolus vulgaris* seedlings grown individually and in groups in different concentrations of agar (Source: own elaboration)

The *Phaseolus vulgaris* seeds, used in all trials, correspond to the expected parameters reported in the literature. This was guaranteed to avoid discordance in the results, thus the dimensions, weights and germination percentage had values similar to those recorded by other authors; Furthermore, only agar-agar and distilled water were used to avoid salt stress that has a negative impact on germination (Morales et al., 2017; Can Chulim et al., 2014). The advantages of using this test initially are because the use of absorbent paper does not control humidity and does not guarantee a good distribution of the substances to be evaluated. Under these conditions, it is necessary to continually monitor the amount of water to avoid desiccation or waterlogging. Furthermore, if cotton or another similar substrate is used, the roots become entangled in the material and tend to break when harvested; this does not happen with agar (Fig. 2). The use of land, although it resembles the natural ecosystem, can complicate the first observations due to the interaction of its abiotic and biotic components with the substances to be evaluated. In this way, the agar bioassay simplifies the system and allows

the evaluation of the interaction between the plant and the chemical substance and the agar, being a hydrogel, control the availability of water without harming the seed (García et al., 2015; Anzalone et al., 2011; García et al., 2008).

Figure 2: Photograph of a *Phaseolus vulgaris* plant harvested after growth in agar (Source: own photographic collection)

Fig. 3 shows the weights of the *P. vulgaris* seedlings harvested on different days. The weights obtained increased according to the cultivation time until a stationary phase was achieved between day 15 and day 18. Under these conditions the plants collided with the roof of the chamber or were submerged in water, which caused signs of damage and microbial infections. This same trend is observed in fig. 4, which reports the results of the root and stem size of the seedlings, where a maximum size was reached between 10 days and 15 days of cultivation.

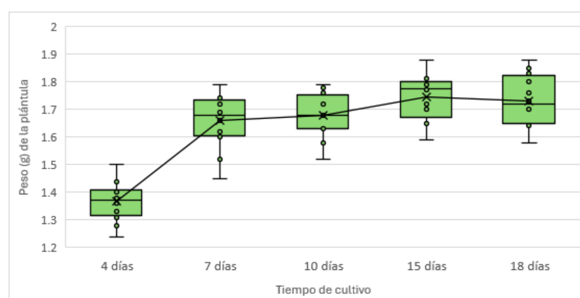

Figure 3: Weight of *Phaseolus vulgaris* seedlings harvested on different days of cultivation (Source: own elaboration)

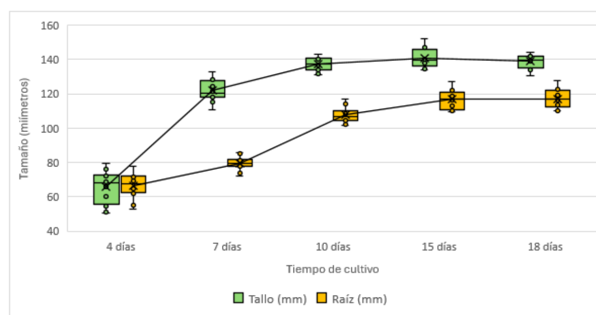

Figure 4: Size of the stems and roots of *Phaseolus vulgaris* seedlings harvested on different days of cultivation (Source: own elaboration)

The values of the morphometric characterization are similar to those reported in studies in which growth on filter paper was evaluated. In this case, practically only gelled water was provided to the seeds and the growth was very good because during the germination of the bean seed Several processes occur that depend solely on the reserves contained in the endosperm and cotyledon (Can Chulim et al., 2014; Domínguez et al., 2014; Maqueira et al., 2021; Isaza et al., 2013; Rodríguez et al., 2017; Mena et al., 2015). The growth of the stem and root coincides with the increase in size and is dependent on the days of cultivation, a stationary phase is observed around day 10, however from this day onwards due to the humidity conditions in which they are grown, it begins. to appear contamination by fungi and signs of deterioration of the plant parts, particularly the leaves (Domínguez et al., 2014; Mena et al., 2015; Recek et al., 2021), for this reason the cultivation is recommended for 7 days because the fullness of its growth is already reached without showing signs of infections or deterioration, by being in physical contact with the incubation chamber.

Similarly, the results of the quantification of photosynthetic pigments, total polyphenols and antioxidant activity demonstrate that there is a plateau after ten days of culture (Fig. 5). These values correspond to those reported by different authors for *P. vulgaris.* The chlorophyll content of a plant is directly related to the amount of nutrients available through absorption, but by not supplying nutrients in the substrate it will depend on the nutritional quality of the seed. The amount of chlorophyll is an important indicator because the amount depends on it. of nutrients, such as carbohydrates and proteins, that the plant synthesizes for its development (More et al., 2016; Rochín et al., 2021; Oliveira et al., 2018; Maqueira et al., 2021; Dada et al., 2023). In this same sense, carotenoids are antioxidant pigments that protect against photochemical damage and free radicals that are found in the lipid matrix and are associated with proteins in the thylakoid membranes, which is why they are a good indicator of oxidative damage produced by harmful substances (Zengin., 2013; Parry et al., 1990; Guerrero et al., 2019).

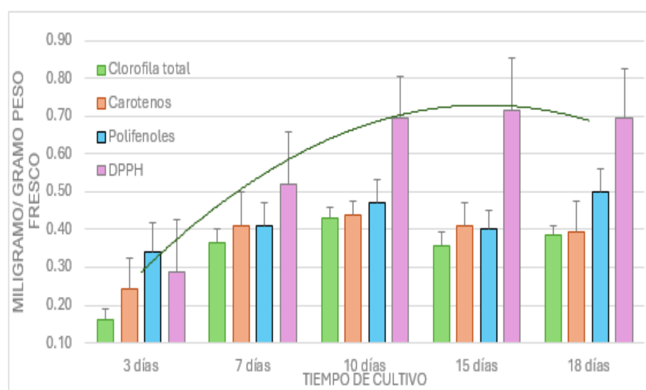

Figure 5: Photosynthetic pigments, polyphenols and antioxidant activity of extracts from *Phaseolus vulgaris* leaves grown on different days. Total chlorophyll values are divided by ten to better compare the dimensions of the other metabolites. (Source: self-made)

Another group of metabolites of biological and pharmacological interest that are produced by *Phaseolus vulgaris* are the polyphenol compounds that include a diversity of substances such as flavonoids, tannins and anthocyanins and that are related to the antioxidant activity of the plant, the content of the polyphenols is closely linked to the growing conditions of the plant and the different types of stress to which it can be subjected and thus the concentration of polyphenols produced by *P. vulgaris* can be monitored in an Eco pharmacovigilance test to deduce the damage caused by certain toxins (Arellano et al., 2017; Ahmed et al., 2023; Mendoza et al., 2016). The polyphenol content in the *P. vulgaris* extract largely determines the antioxidant activity because polyphenolic compounds are the most important antioxidants produced by this species; However, the antioxidant activity provides a higher amount of mg EAG compared to the concentration of polyphenols because other compounds such as ascorbic acid, vitamin E and carotenoids also contribute to the antioxidant activity (Arellano et al., 2017; Oh et al., 2023; Fonseca et al., 2021; Pereira et al., 2014). The DPPH radical inhibition assay is used to measure the capacity of a plant extract to neutralize free radicals that are chemical species toxic to biological systems and that are involved in damage to cellular systems (Oh et al., 2023; Fonseca et al., 2021; Pereira et al., 2014; García et al., 2021; Bento et al., 2021; Mendoza et al., 2020).

Conclusions

A *Phaseolus vulgaris* germination and growth bioassay on agar-agar was developed that can be used for ecotoxicity and Eco pharmacovigilance studies. The most appropriate conditions to carry out are a concentration of agar-agar at 0.5% in water and a culture for 7 days in humid chamber. This bioassay easily allows morphometric characterization and quantification of pigments and secondary metabolites of harvested plant extracts.

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