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Enhancement of Growth Potential of *Duranta repens L.* by Ethanolic Extract Fractions of the *Cleome gynandra* L.

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Authors' contributions

This work was carried out in collaboration between all authors. Authors YAT and YPB designed and completed the laboratory studies, wrote the protocol, and wrote the first draft of the manuscript, managed the analyses and the literature searches with support from authors YVT and URC. Authors YGP, CSC and ENQ performed the statistical analysis, supported method development and helped authors YAT and YPB to write up the outputs. All authors read and approved the final manuscript.

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ABSTRACT

The study was conducted with the objective of investigating the ethanolic extract fractions of the stem of *Cleome gynandra* L. by analyzing its bioactivity in stakes of *Duranta repens*, L. The extracts were separated from a dry ethanolic extract (20% tincture m/V) with the solvents ethyl ether, chloroform, ethyl acetate and distilled water and evaluated at doses of 50, 100, 150 mg m L^{-1}, compared to a control to which only sterile distilled water was applied. The variables measured in *D. repens* at 45 days of cultivation were: number of shoots, length of shoots, and

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number of leaves in the apical buds, number of roots and length of the first root formed. In the different fractions evaluated, a stimulating effect was presented in the selected variables; therefore, the presence of bioactive metabolites of different polarity significatively influences the growth potential of C. gynandra. However, better results of plant growth were obtained in solvents of higher polarity, especially the acetate and aqueous fractions.

Keywords: Stimulating activity; ethanolic extract; Cleome gynandra L.; Duranta repens L.

1. INTRODUCTION

Cleome gynandra L. (Sin. *C. pentaphylla* L.; *Gynandropsis gynandra* Briq, *G. pentaphyla* D.C) in some countries such as India, Zimbabwe and Kenya is vulgar named spider flower, spider plant o cat whiskers [1,2]. This shrub is considered an invasive weed in many places in the United States of America and in the Pacific region. However, chemical analyzes performed on this species have shown that it has valuable nutrients such as amino acids, vitamins and minerals [3].

Duranta repens L. (*Duranta erecta* L., *Duranta arida* Britton & P. Wilson *var. domingensis* (Urb) Acev.- Rodr.) is a species of shrub belonging to the family Verbenaceae, which is distributed from Mexico to South America and the Antilles. It is widely cultivated as an ornamental plant in tropical and subtropical gardens around the world. In Cuba it is vulgarly known as "No me olvides". Due to the flowers and fruits are very showy, commonly is used as an aesthetic complement in ornamental gardens. In addition, the flowers attract butterflies and hummingbirds, favoring the pollination process. However, this plant is also considered an invasive species in Australia, China, Pakistan and several Pacific islands [4]. *D. repens* is distinguished from other similar species by the most numerous axillary inflorescences, by the length of the sepals and by the relatively small corolla tube of approximately 7-9 mm. However, the wide variation of growth, the presence of thorns, and shape of the leaves have led to the publication of approximately 20 synonyms [5].

Due to the rich chemical composition, *D. repens* have been used by larvicide effect by against *Culex quinquefasciatus* Say (Diptera: *Culicidae*), also, has been reported the use against mosquitoes, which can be transmitting agents of various diseases [6]. Also, from its leaves are obtained extracts with an important antifungal activity [7]. Also, the species possesses a significant antioxidant effect, and cytotoxic [8, 9] and antibacterial activities [10]. In addition, literature suggested that the aqueous extracts of *D. repens* help improve the uterine contractility in womens to facilitate chilbirth [11]. Moreover, some reports have been carried out about they uses in the mitigation of air pollution [12]. *D. repens* is multiplied by woody cuttings, for that reason is very useful apply hormones to accelerate the rooting process, especially in non-optimal periods of growth.

The application of plant growth regulators promotes the formation a well-developed root system, obtaining seedlings with a better quality in a shorter period of time [13]. Since ancient times, is known that some plant species can influence the growth of others; that process is known as allelopathy. The phenomenon of allelopathy covers the biochemical interactions, both beneficial and harmful, between different plant species. In addition, the process characterizes the indirect chemical effects of a plant that influence the germination, growth and/or the development of another plant. The effect of allelopathy can be considered as a component of biological control, where plants are used to reduce or stimulate the vigor and development of other plants [14]. For that reason, allelopathy can be used to improve crop yields; also, is very important that the plants used as allelopathic sources are easily acquired and non-toxic to the man or environment. In preliminary studies carried out by this research team, was observed that some extracts from different organs of *C. gynandra* had a significantly positive allelopathic potential in the growth of *D. repens*, obtaining the best results with ethanolic extract of stems. However, the families of the chemical compounds present in this extract, which may be related to the stimulating activity of this plant, are currently unknown. The objective of this work is to perform a bioguided fractionation of the ethanolic extract of the stem of *C. gynandra*, and a study of its stimulating activity in stakes of *D. repens*.

2. MATERIALS AND METHODS

2.1 Selection of Plant Material

The work was carried out in the period between october/2016 to april/2017. The sample of *C.*

gynandra was collected from an area adjacent to the University of Granma, Cuba. A specimen was herborized, which was preserved until the subsequent experiments. The material separated from the stem (leaves, roots, flower and fruit), was wrapped in blotting paper and dried in heated chamber at 35ºC until obtaining a constant mass.

2.2 Obtaining the Extracts

The 20% extracts were obtained from small fragments of stems (average diameter of 2-5 mm), using as a menses a 70% hydroethanolic solution (v / v). A sample of 60 g of the crude drug was used to obtain 300 mL. An ultrasound-assisted extraction method was used as extraction method (Ultrasonic Cleaner SB -3200 DTD, China) at a constant-temperature of 40ºC and frequency of 40 KHz for two hours.

2.3 Fractionation of Plant Extract

A volume of 60 mL of 20% extract, from the stems of *C. gynandra*, were consecutively partitioned in ethyl ether (pa.PanReac, Spain), chloroform (pa.Panreac.Quality HPLC/$_{CG}$, Spain), ethyl acetate (p.a.Riedel-de-Haënm, Switzerland) and distilled water, using a separating funnel (POBEL, Spain) of 250 mL of volume. The samples were stirred for three minutes and allowed to stand until the separation of the phases occurred; each sample was performed in triplicate. Then, the fractions were concentrated to dryness using a rotoevaporator (IKA, RV05 Basic, Germany), which was connected to a bath with thermostat (IKA, HB4, Werke, Germany), with recirculation of water for condensation (MLW, Germany) and vacuum pump (VEM KMR 53 K4 FTH, Germany).

2.4 Experimental Procedure

For the tests, woody sticks of *D. repens* of 15 cm long in active growth were taken. Twelve treatments were evaluated, which were randomly selected, where 4 fractions of ethanolic extract and three doses were combined. Each combination was repeated 10 times. A control sample consisting of evaluating the same conditions in sterile distilled water was also selected. The stakes *D. repens* was embedded for 24 hours in extracts or water; then, these were placed in polyethylene bags of 0.5 kg capacity. Each stake was planted in a substrate consisting of screened and sterilized river sand. The number of sprouting buds, length of shoots and number of leaves were recorded every 15 days; meanwhile, at the end of the experiments, 45 days after the stakes were planted, the number of roots and length of the first root formed were determined. The samples were carried out in a greenhouse, with controlled temperature conditions (28±1ºC), maintaining a constant photoperiod of 12 hours of light and 12 hours of darkness.

2.5 Phytochemical Screening

Phytochemical screening was conducted to determine the presence of natural products in the fractions of selected plants using standard methods of as following [15].

Phenols (Ferric chloride test):

To 1.0 mL of extract 2.0 mL of distilled water were added followed by few drops of 10% ferric chloride (FeC13). Appearance of blue or green colour indicates presence of phenols.

Tannins (Ferric chloride test):

A sample of 0.5 mL of the extract was boiled with 10 mL of distilled water in a test tube, then a few drops of 5% ferric chloride solution was added and the reaction mixture was observed for blue, greenish black colour change.

Coumarins:

To 1.0 mL of extract, 1.0 mL of 10% NaOH was added formation of yellow colour presents a positive result.

Quinones:

To 1.0 mL of extract, 1.0 mL of concentrated sulphuric acid (H2SO4) was added formation of red colour shows a positive result.

Dragendroff's Test (Alkaloids lest):

Filtrates were treated with Dragendroff's reagent (solution of Potassium Bismuth Iodide). Formation of red precipitate indicates the presence of alkaloids.

Test for saponins (Frothing test):

A sample of 0.5 mL of the extract was added to 5.0 mL of distilled water in a test tube. The

solution was shaken vigorously and observed for the stable persistent froth.

Resins test:

A volume of 2.0 mL of the ethanolic extract was added to 10 mL of distilled water. The appearance of a precipitate indicates a positive test.

Nihydrin test (free amino acids):

A volume of 1.0 ml of the ethanolic extract was taken and mixed with 2.0 ml of a 2% nihydrin solution. The mixture was heated for 10 min in a water bath. It was considered positive when a violet color developed.

Shinoda test (flavonoids):

A volume of 1.0 mL of the ethanolic extract was taken and diluted in 1.0 mL of 37% fuming hydrochloric acid (HCl) and a small piece of metallic magnesium tape. After the reaction, 5 min was waited, and 1.0 mL of amyl alcohol ($C_5H_{11}OH$) was added. Then, the phases were mixed and were waited until their separation. The test was considered positive when the amyl alcohol is colored to yellow, orange, brown or red, intense in all cases.

Anthocyanidin test:

A volume of 2.0 ml of the ethanolic extract was heated for 10 minutes; later, a volume of 1.0 ml of fuming hydrochloric acid was added. It was allowed to cool, and subsequently 1.0 mL of distilled water and 2.0 mL of amyl alcohol were added. The sample was stirred and waited until phase separation. The appearance of a red to brown color in the amyl phase is indicative of a positive test.

Kedde test (cardiotonic glycosides):

A volume of 1.0 mL of the ethanolic extract was taken and mixed with 1.0 mL of Kedde´s reagent, and the sample allowed standing for 10 min. A positive test develops a purplish, persistent color for 1-2 hours.

2.6 Experimental Design

The results were processed from the statistical package STATISTICA 12. A two-factorial design was used as experimental design, in which the effects of the i^{th}-levels of several quantitative factors and their interactions on a response of interest were studied. The variables representing the factors to be studied were: fraction extract (τ) and doses (β); while, the other parameter of the model characterize the effect of the interactions between both factors.

$$y_{ijkl} = \mu + \tau_i + \beta_i + (\tau\beta)_{ij} + \epsilon_{ijkl} \begin{cases} i = 1,2,3,4 \\ j = 1,2,3 \end{cases}$$

The levels of the factors were defined as follows: 4 fractions of ethanolic extract (ethyl ether, chloroform, ethyl acetate and distilled water) and 3 levels of doses (50, 100, 150 mg L^{-1}). Analysis of Variance (ANOVA) was used to determine the differences between average values, where the F-test value was calculated for a determinate probability value ($p<0.05$), in order to determine the significant differences between the means of the main effects and interactions.

3. RESULTS AND DISCUSSION

The effect of the doses of the ethanolic fractions on the number of shoots, leaves and roots in the stakes of *D. repens* is shown in Fig. 1a. In the first 15 days, sprouting of 3 - 5 buds (of the total of 6 possible) was observed in the stakes treated with ethanolic extracts, which had a greater number compared with the control samples, which only developed values between 1 - 3 sprout per stakes. Likewise, after 15 days of experimentation, the shoots had two more leaves, on average, then the stakes of the control sample; this difference was greater at 45 days of the experiment, where all ethanolic treatments exceeded in four leaves per sprout to the control. The number of leaves in the stakes is an important factor for the production of *D. repens* seedlings, since this indicates the degree of adaptation of the plants. The leaves function as reservoirs of water, nutrients and hormones; in addition, it is responsible for the conduction of the sap in vegetables, which is also reflected in the development of the roots. Also, the amount of carbohydrates from photosynthesis directly influences the response of rooting [16]. The amount of biomass of the aerial part of the plant is directly related to the quality and quantity of the leaves formed. This characteristic is very important, since leaves are one of the main sources of metabolized energy and nutrients for adaptation in the post-plantation [17].

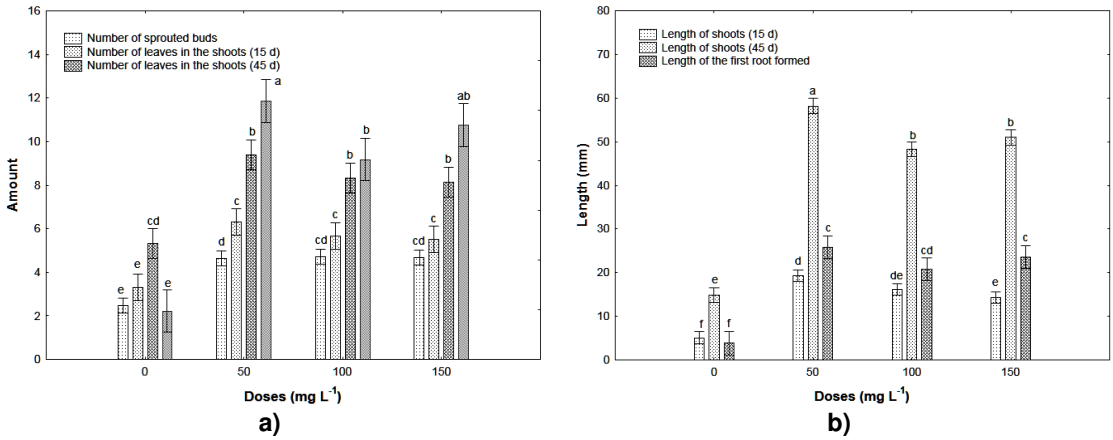

Fig. 1. Effect of the doses applied on the growth of *D. repens*: a) number of shoots and roots; b) length of shoots and roots.

Similarly, stakes treated with ethanolic fractions developed between 9-12 roots; meanwhile, the control only presented 2 roots as average. The rooting is a fundamental stage for the species with vegetative multiplication. Phytostimulation through the application of plant extracts can be used to promote this type of reproduction [18]. The length of shoots and roots were also significantly affected by the doses applied (Fig. 1b), where all ethanolic treatments also exceeded the control experiment. It should be noted that the length of the first root formed did not decrease with the increase in the doses applied; however, the length of the shoots decreased.

On the other hand, the analysis of variance (ANOVA) was conducted to study the significance of both factors (ethanolic extract and doses) over the variable responses for the experimental data obtained. The experimental results obtained at various treatment combinations using ANOVA are summarized in Table 1. The effect of the doses applied was significant for all the responses evaluated for a 95% probability level; also, significant differences were detected between the solvents used for length of shoots (15 and 45 d), as well as number of roots and length of the first root formed. The significant effects of both factors on the growth of *D. repens* are evident, fundamentally in the rooting of the plant, which directly influences the sprouting of the buds.

Fig. 2 shows the interactions between the evaluated factors for the response variables length of shoots, first root formed and number of roots. In general, among the solvents evaluated, water had the best results for the variable length of the shoots, where the increase in the doses applied did not affect the responses (Fig. 2a). The control sample (Dose 0) showed the shortest length of shoots, statistically different from the rest of the treatments evaluated. Regarding the interaction between the factors analyzed, different trends were detected. In case of ethyl ether fraction, by tripling the initial dose, a significant decrease in the stimulating activity was observed; however, for the ethyl acetate fraction the response was different. In the fraction obtained from chloroform, and for a concentration of 50 mg L^{-1}, the highest values of length of shoot were obtained. However, these values decreased with the increase of the applied doses: the values obtained at the dose of 100 mg L^{-1} were significantly lower than achieved with the 150 mg L^{-1} dose; for that reason, these results suggesting a non-linear dose-effect interaction.

The lowest number of roots was obtained at a dose of 150 mg L^{-1} of the ethyl ether fraction; these results were similar to that achieved by the control sample (Fig. 2b). The largest rooting was obtained with the most polar fractions (ethyl acetate, at a dose 150 mg L^{-1}, and water, at doses of 50 and 150 mg L^{-1}). Meanwhile, no significant differences were detected among the 3 concentrations evaluated for the chloroform fraction. On the other hand, in the aqueous and ethyl acetate fractions, the intermediate dose (100 mg L^{-1}) obtained the lowest results, indicating a non-linear dose-effect relationship.

Table 1. Analysis of variance (ANOVA)

Effects	Sum of squares	Mean squares	F-value	p-value
Number of sprouted buds				
Solvent	0,61	0,20	0,17	0,9137
Doses	144,21	48,07	40,60	0,0000*
Interaction	11,40	1,26	1,07	0,3882
Error	170,50	1,18		
Total	326,74			
Length of shoots 15 d (mm)				
Solvent	36,61	1488,48	83,28	0,0000*
Doses	4465,44	141,53	7,91	0,0000*
Interaction	1273,75	17,87		
Error	2573,60			
Total	8349,40			
Number of leaves in the shoots 15 d				
Solvent	11,89	3,96	1,04	0,3766
Doses	206,36	68,78	18,05	0,0000*
Interaction	64,21	7,13	1,87	0,0604
Error	548,57	3,81		
Total	831,04			
Length of shoots 45 d (mm)				
Solvent	3499,80	1166,60	39,61	0,00*
Doses	44605,10	14868,40	504,83	0,00*
Interaction	7401,40	822,40	27,92	0,00*
Error	4241,10	29,50		
Total	59747,40			
Number of leaves on the shoots 45 d				
Solvent	12,65	4,21	0,87	0,4573
Doses	362,66	120,89	24,98	0,0000*
Interaction	51,01	5,66	1,17	0,3176
Error	696,85	4,83		
Total	1123,19			
Number of roots				
Solvent	171,25	57,08	5,79	0,0009*
Doses	2244,65	748,22	75,89	0,0000*
Interaction	870,50	96,72	9,81	0,0000*
Error	1419,60	9,86		
Total	4706,00			
Length of the first roots formed				
Solvent	1096,15	365,38	5,21	0,0019*
Doses	11982,15	3994,05	56,96	0,0000*
Interaction	5118,90	568,77	8,11	0,0000*
Error	10096,80	70,12		
Total	28294,00			

**p-value<0.05 was considered as significant.*

To fractionate extracts with the use of solvents of different polarity, initiate the dissolution with apolar solvents or of low polarity is recommended, fundamentally with hexane or ethyl ether. Then, with the objective of dragging substances of intermediate to low polarity, chloroform can be used to increase the polarity of the solvent. Finally, the solvents ethyl acetate, methanol, acetic acid or water can be used, since these separate the more polar compounds. Terpenes are mainly concentrated in low polarity solvents (hexane or ethyl ether); however, phenolic compounds, saponins and free amino acids are found mainly in the most polar fractions (ethyl acetate and water).

The doses of 150 mg L^{-1} of the chloroform fraction and 100 mg L^{-1} of the aqueous fraction had the lowest length of the first root formed (Fig. 2c). Statistical similarity of the values obtained

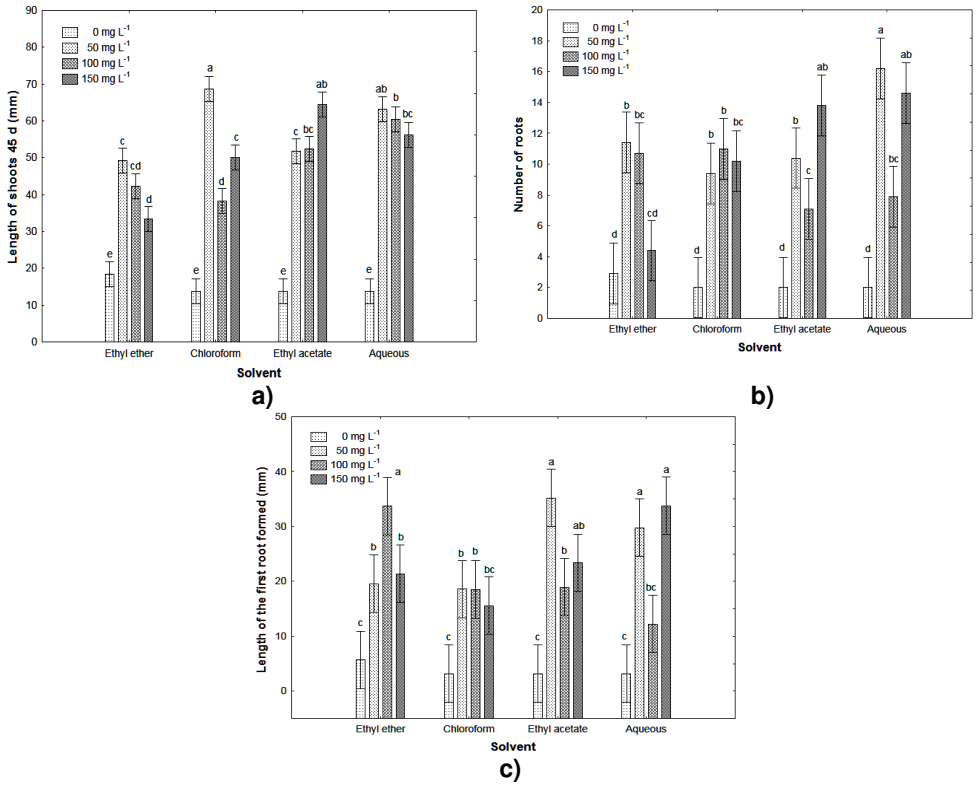

Fig. 2. Significant interactions between the solvents used and the doses applied: a) length of the shoots (45 d); b) number of roots; and c) length of the first root formed

between the different doses and fractions evaluated was significant. However, significantly higher data were obtained at concentrations of 50 mg L^{-1} and 150 mg L^{-1} of aqueous and ethyl acetate fractions, respectively, and 100 mg L^{-1} for the ethyl ether fraction. These results indicate that the length of the root was affected by metabolites of different nature. In addition, the dose or concentration applied plays an important role, but its dose-effect relationship is not linear. A high percentage of rooting directly influences the root volume and sprouting of the vegetative buds [19].

A greater volume of roots will absorb a greater amount of water and nutrients, causing the elongation of the shoots as it happened with the stakes of *D. repens*. The radicular system of plants is sensitive to the action of allelopathic substances, since their elongation depends on cell divisions, which can be inhibited or stimulated by the presence of secondary metabolites [20]. Also, the length of the roots is another important variable, since the individuals or plants with a greater root development can increase the survival rate of the seedlings when they are transferred to the field [21].

The technique of arranging data according to priority or importance in a problem-solving framework is called Pareto analysis. Pareto charts are used extensively by improvement teams all over the world; indeed, the technique has become fundamental to their operation for identifying the really important problems and establishing priorities for action [22]. Thus, the standardized effects of the both independent variables and their interactions on the dependent variables were investigated with a Pareto chart. A simple way to do this test is to add a line in the standardized Pareto chart to the height of a critical value, the effects whose bars exceed that line will be significant; while, the negative or positive sign indicate whether the effect is antagonistic or synergistic, respectively. Fig. 3 shows the Pareto chart of the standardized effects of the measured responses. The doses applied positively influenced all the evaluated responses, except in number of leaves on the shoots 45 d; in addition, significant interactions, generally antagonistic, were detected between dose and solvent factors.

In order to determine the best global conditions to then achieve the objective of the study, the

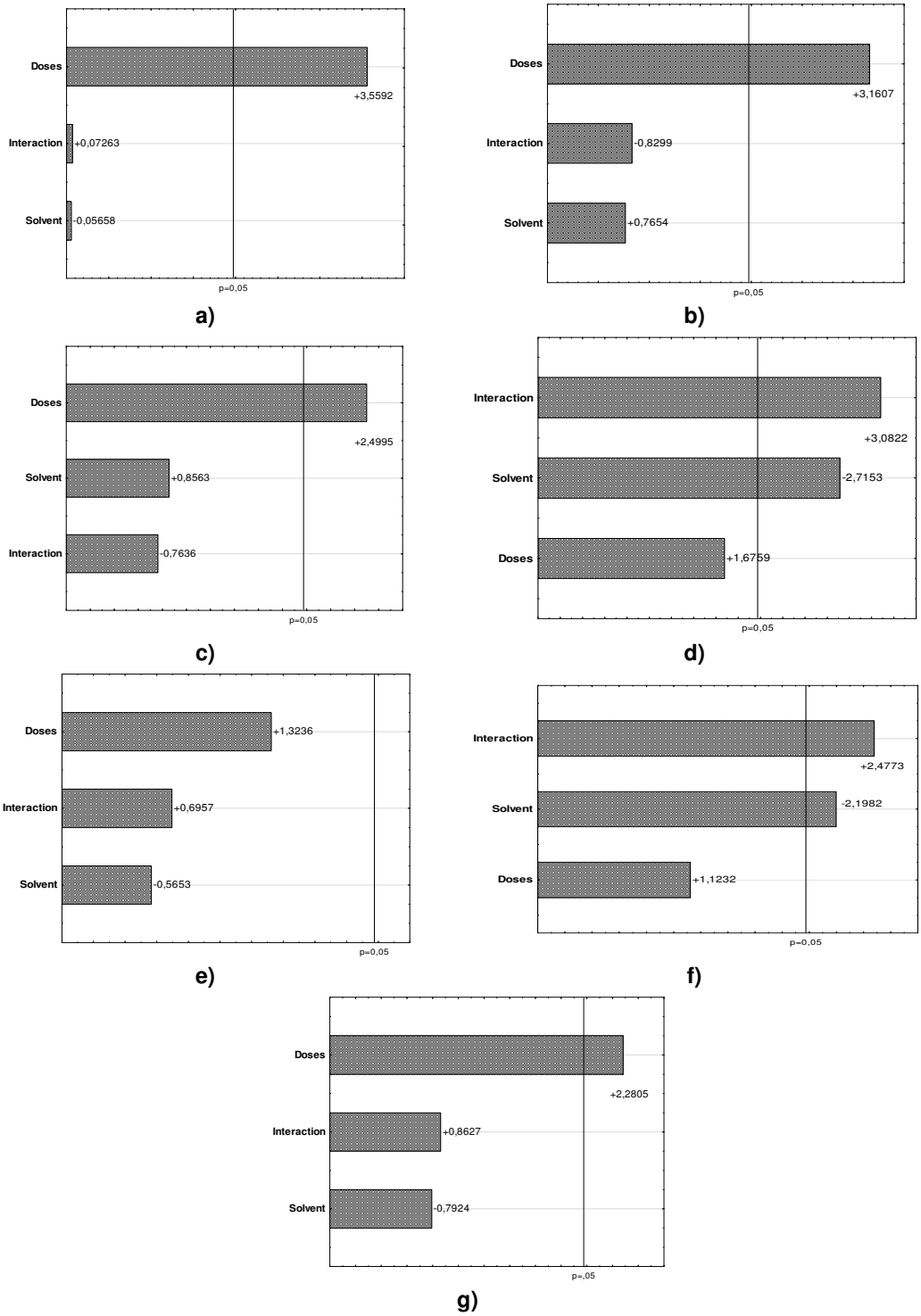

Fig. 3. Pareto chart showing the standardized effect of independent variables and their interactions on the response variables. The length of each bar in the chart indicates the standardized effect of each factor: a) number of sprouted buds; b) length of shoots 15 d; c) number of leaves in the shoots 15 d; d) length of shoots 45 d; e) number of leaves on the shoots 45 d; f) number of roots; and g) length of the first roots formed

desirability function was used to determine the best conditions in each of the responses for the desired range. The method consists of transforming the predicted values on a scale of 0 to 1, to indicate how desirable they are: each response variable was placed on the same scale and a desirability function $d_i(x)$ was defined that falls in the interval definite. Fig. 4 show the response plots corresponding to desirability function the seven variables response

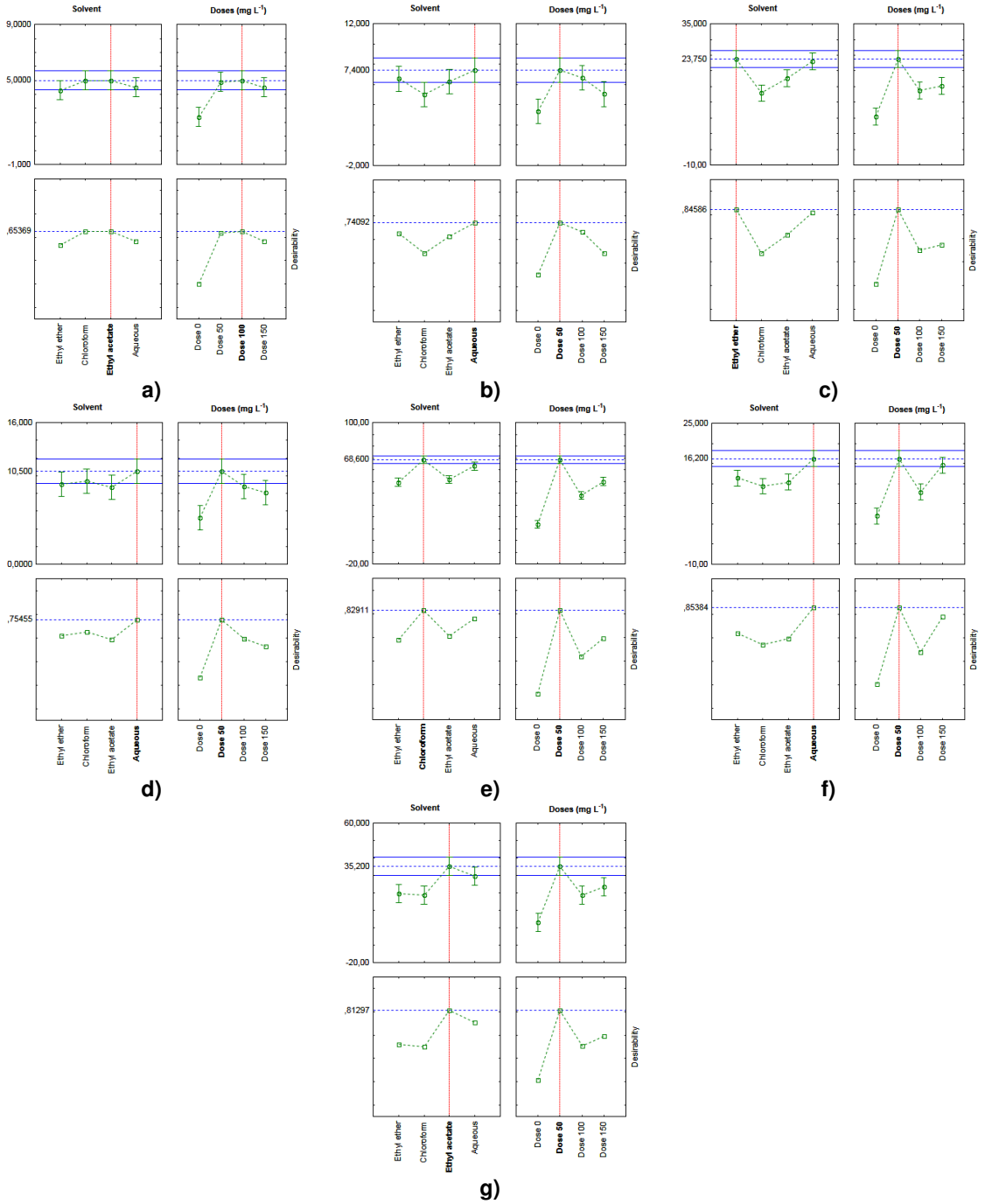

Fig. 4. Desirability function charts: a) number of sprouted buds; b) length of shoots 15 d; c) number of leaves in the shoots 15 d; d) length of shoots 45 d; e) number of leaves on the shoots 45 d; f) number of roots; and g) length of the first roots formed

considered. The results also show that the highest stimulating activity was concentrated in the most polar fractions (ethyl acetate and aqueous) at the lowest dose, except for the variables number of leaves in the shoots 15 d and 45 d, although no significant differences were observed with respect to the more polar fraction (aqueous).

Table 2. Results of phytochemical screening of the ethanolic extract

Metabolites	Presence
Resins	-
Alkaloids	+
Saponins	+
Free amino acids	++
Coumarinas	+
Cardiotonic glycosides	-
Phenolics and / or tannins	+
Quinones	-
Flavonoids	-
Anthocyanidins	-
Reducing sugars	-

not found (-); presence (+); abundant (++)

Phytochemical screening of the stem ethanolic extract of *C.gynandra* revealed the presence of alkaloids, saponins, free amino acids, coumarins, phenolic compounds and quinones (Table 2). On the other hand, Table 3 shows the chemical composition of the different fractions considered, where the presence of free amino acids, coumarins, saponins, phenolic compounds, alkaloids and flavonoids is evidenced. By separating the fractions, it is shown that free amino acids and coumarins have different polarities, since they were found in all of them; although with greater abundance in the aqueous fraction for the case of free amino acids.

Saponins were only found in the most polar fractions (ethyl acetate and water). While, flavonoids were found in the ethyl ether fraction; these were not identified in the ethanol extract, probably due to its low concentration. Meanwhile, in the chloroform fraction, no phenolic compounds were detected. For the case of alkaloids and flavonoids were only present in the less polar fraction.

Free amino acids are components of proteins in plants, whose presence is essential. These activate the synthesis of chlorophyll, which is very important in photosynthetic activity and, therefore, for the growth and development of plants. They are applied as invigorants and stimulants in critical periods such as transplantation and early stages of plant growth [23]. In seeds of *C. gynandra* the presence of 15 free amino acids has been reported; among the most reprentative compounds are glutamic acid, alanine, glycine and proline [24]. Glycine and proline favor the growth of plants. Free amino acids are not a single type of nutrient; they are also an important factor regulating growth, being able to reach all parts of the plant, including the roots, in a few days.

In a phytochemical study performed on fractions of stem of *C. gynandra*, tannins and saponins were found only in the most polar fraction (ethyl acetate). The results of the reported trial were negative for cardiotonic glycosides and flavonoids [25]. These results coincide partially with those obtained in the present investigation.Tannins have a higher concentration in aerial parts of the plant, because they fulfill defense functions against herbivorous animals [26]. The presence of phenolic compounds and flavonoids

Table 3. Phytochemical composition of the different fractions

Metabolites	Ethyl ether fraction	Chloroform fraction	Ethyl acetate fraction	Aqueous fraction
Resins	-	-	-	-
Alkaloids	+	-	-	-
Saponins	-	-	+	+
Free amino acids	+	+	+	+++
Coumarinas	+	+	+	+
Cardiotonic glycosides	-	-	-	-
Phenolics and / or tannins	+tannin	-	+ phenolic	+phenolic
Quinones	-	-	-	-
Flavonoids	+	-	-	-
Anthocyanidins	+	-	-	-
Reducing sugars	-	-	-	-

not found (-); presence (+); abundant (++); very abundant (+++)

in different organs of *C. gynandra* (leaves and fruits) has been demonstrated. These chemical constituents are responsible for a strong antioxidant activity, which can be used for therapeutic purposes [27].

However, the results obtained in this research are indicative and not conclusive, since in the presence or absence of a metabolite in a phytochemical screening it may be influenced by aspects such as: the time of collection, vegetative state of the plant, concentration and interferences of the metabolites, as well as their solubility in the solvent used.

4. CONCLUSIONS

In the fractions of *C. gynandra*, some non-polar metabolites (alkaloids, flavonoids and coumarins) that stimulate the various growth variables evaluated in *D. erecta* were obtained. However, the best results of stimulating activity were concentrated in the more polar fractions, with the presence of some other secondary metabolites, such as free amino acids, phenolic compounds and coumarins, with a tendency to have a higher activity when the lower doses are applied.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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