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Research article

IN VITRO TISSUE CULTURE, CHEMICAL COMPOSITION AND BIOLOGICAL ACTIVITY OF *PLUMBAGO SCANDENS* L.

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ABSTRACT: A protocol was developed for the micropropagation of *Plumbago scandens* L. from the shoot tip and node explants.The best response of shoot elongation (10.18±2.01 mm) was observed on MS basal medium supplemented with 0.02 mg/L IAA – 0.02 mg/L GA_3. The maximum number of root induction (10.0±2.21) and shoot elongation (8.24±3.24 mm) was observed on medium containing 0.01 mg/L IBA and 0.01 mg/L GA_3. The *in vitro* propagated plants were transferred to soil with 80% survival rate. Profuse compact callus was induced and proliferated from several explants (cotyledons, internodes, hypocotyls and roots) cultured on MS medium supplemented with all the combinations of 2,4-D – GA_3 or 2,4-D alone and combinations of IAA – BAP or IAA alone, and the highest percentage of friable callus (90%) were induced in the sections of compact callus using 2.0 mg/L IAA – 0.02 mg/L BAP – 0.5 mg/L GA_3.The qualitative determination of chemical constituents in the extracts was evaluated by a gas chromatography coupled to a mass spectrometry, and it was verified the presence of plumbagin only in root extracts but not in *in vitro* plantlets.The antibacterial activity of root extracts against various pathogenic bacteria, and the minimum inhibitory concentrations (MICs) was determined. Chloroform extracts showed good antibacterial activity against *Neisseria gonorrhoeae* between 0.4 to 1.0 mg/L with 20.4 to 30.0 mm (diameter zone of inhibition); inhibition against *Staphylococcus aureus* was moderate, and lower against *Escherichia coli*. Chloroform extracts had the lowest MICs for *N. gonorrhoeae* (<0.1 mg/mL per disc), and the activities against *S. aureus* (MIC 0.2 mg/mL) and *E. coli* (MIC 0.4 mg/mL) were less pronounced.

Key words: Biological activity, callus induction, Plumbaginaceae, plumbagin, shoot elongation.

INTRODUCTION

Plumbago scandens L. belong to the family Plumbaginaceae, order Plumbaginales. The order consists of the single family Plumbaginaceae, with about a dozen genera and perhaps 400 species, according to Cronquist classification (Cronquist, 1988); however, according to the system proposed by the Angiosperm Phylogeny Group, the family Plumbaginaceae es placed with Polygonaceae, Droseraceae, Amaranthaceae, and others, in the order Caryophyllales, Core Eudicots (APGIII, 2009). *Plumbago*, with about 20 species, is the largest genus of the Plumbaginoideae, and in Peru has been reported four species: *P. auriculata* Lamarck, *P. coerulea* H.B.K., *P. indica* L. and *P. scandens* L. (Brako and Zarucchi, 1993). *Plumbago scandens* is a subshrub with white flowers quite widespread in the seasonally dry forest in Peru and Ecuador, and popularly is known as "pega-pega", because the flowers are characterized by having a tubular calyx with glandular trichomes (hair) secreting a sticky mucilage. It can also be found in Southern Florida, Texas, and Arizona, Mexico, Central America, the west Indies and South America (Verhoek-Williams, 1970). In Brazil is a native species found in a typical vegetation of "restinga", which es characterized by high luminous intensity, sandy soil and water restriction (Paiva et al., 2011). The *Plumbago* species, specially *P. zeylanica* as the best studied species, contains a variety of important chemical compounds as naphthaquinones, glycosides, triterpenoids, alkaloids, coumarins, flavonoids, phenolic compounds, tannins, saponins, carbohydrates, fixed oil and fats and proteins (Ravikumar and Sudha, 2011; Ming et al., 2011; Pant et al., 2012). The pharmacological importance of this perennial shrub lies in its ability to produce a naphtoquinone, called plumbagin (5-hydroxy-2-methyl-1,4-naphtoquinone-$C_{11}H_8O_3$), the principal active compound; this compound is primarily present in roots in higher amounts with only about 1% in the whole plant (Krishnaswamy and Purushothaman, 1980), and in *in vitro* plants (Moncada et al., 2011). A wide range of medicinal properties of *P. zeylanica* are attributed to plumbagin and other secondary metabolities.

Plumbagin have a potential as anticarcinogenic, antioxidant, cardioprotective, antibacterial, antifungal, antimalarial, antifertility, and anti HIV activity. Information about dose range tested, type of extract used, the model used, controls, duration of the study as well as their pharmacological result has been recently reviewed (Dhale and Markandeya, 2011; Pant et al., 2012; Kishore et al., 2012; Ganesan and Gani, 2013; Borhade et al., 2014).

On the other hand, four compounds, the naphthoquinones plumbagin and *epi*-isoshinanolane, palmitic acid and sitosterol, were qualitatively extracted from roots of *P. scandens* with different techniques, resulting the extraction in the Soxhlet apparatus the most efficient extraction technique; however, prolonged heating time promoted plumbagin degradation (Paiva et al., 2004). In addition, the naphthoquinones exhibited their highest content during floration, while the content of the two others components decreased during this stage, revealing an inverse profile (Paiva et al., 2011). Recently, Moncada et al. (2011) reported biological activity of extracts from roots of *P. scandens* on MDR strains of *Mycobacterium tuberculosis*. Likewise, in *P. indica*, also known as *P. rosea*, one of the common plants used in Indian traditional system of medicine, preliminary phytochemical evaluations revealed the presence of steroids, tannins, glycosides, phenols, flavonoids and saponins in the root methanolic extracts, and had substancial antioxidant activities, impliying that *P. indica* roots can be used as a potential source of natural antioxidants (Eldhose et al., 2013), and in the pharmacognostic studies of aerial part of methanolic extract of this species indicate the presence of reducing sugars, alkaloids, steroids, flavonoids and gums (Saha and Paul, 2012). *P. zeylanica* can be propagated by seeds, rooted shoots from the base of the plant or by semi-ripe cuttings, treated with a growth hormone, and seeds germinate in 21 – 30 days; however, prolonged storage over 3 months results in a drastic decline in germination rate (Pant et al., 2012). This situation would be happening to other species of *Plumbago* as *P. rosea* and *P. capensis* (Datta and Mishra, 2012), and *P. scandens* (Farcio, 2003), therefore these conventional propagation methods of the *Plumbago* species are rather difficult and insufficient to meet the large growing demand owing to the poor germination of seeds and death of young seeddlings under natural conditions. In this context, the participation of modern biotecnological techniques as plant tissue culture may be necessary.

In *P. zeylanica* the technique of *in vitro* propagation has been successfully utilized for mass multiplication using nodal explants, axillary buds, leaf, embryos or root explants and callus culture. In the past decade, a protocol for rapid micropropagation of *P. zeylanica* through axillary bud proliferation and establishment of higher plumbagin yielding hairy roots cultures was developed as an essential prerequisite to conduct genetic transformation studies (Verma et al., 2002). In the other hand, rapid clonal propagation was obtained using nodal explants collected from healthy growing plant in MS medium supplemented with 1.0 mg/L BAP –1.0 mg/L NAA (Dohare et al., 2012), and 1.0 mg/L BAP – 1.0 mg/L GA_3 (Chinnamadasamy et al., 2010). In another study, nodal segments and leaf explants were cultured on several basal media supplemented with different concentrations and combinations of diverse auxins (2,4-D and NAA) and cytokinins (BA, KIN and TDZ), sole, as well as in combinations, was observed morphogenic callus and high proliferation of shoots (Partidar et al., 2013); likewise, the embryos and nodal cuttings of *P. zeylanica* were used to evaluate the effect of culture media and growth regulators on the *in vitro* shoot production and growth, was observed that the embryos were significantly viable on NPK basal media (Gbadamosi and Egunyomi, 2010), and axillary shoot multiplication, callus induction and shoot regeneration from callus culture was obtained on MS medium supplemented with different concentrations and combinations of plant growth regulators (Sivanesan and Jeong, 2009). Recently, a rapid protocol for callus induction and indirect organogenesis has been developed in this species, and the plumbagin content in callus was comparatively higher in 2,4-D–BA hormonal combination or 2,4-D–KIN than *in vivo* condition (Lubaina and Murugan, 2012). Aversely, in the only study on *P. rosea*, conducted in Thailand, roots cultured were established from young leaf explants on B5 medium supplemented with combination of NAA and KIN, and the plumbagin production was higher [0.016 0.0030% dry weight (DW)] in this culture medium (Panichayupakaranant and Tewtrakul, 2002). There are no studies in tissue culture of *P. scandens*.

This present work aimed at developing an efficient protocol for plant micropropagation and callus induction, determine the chemical composition from wild plants and *in vitro* plants, and the biological activity of *Plumbago scandens* L. against several bacterial pathogens.

MATERIALS AND METHODS

Plant material and *in vitro* seed germination

Mature seeds were obtained from ripe fruits of wild plants of *Plumbago scandens* L. grown in the Motupe and Chongoyape localities, Lambayeque region (Peru) (Figure 1). Plants were identified by botanists Leopoldo Vásquez Núñez and Santos Llatas Quiroz at the herbarium of the Faculty of Biological Sciences, Universidad Nacional Pedro Ruiz Gallo (Lambayeque) and voucher specimens are deposited therein. Seeds were immersed in 70% (v/v) ethylic alcohol for 1 min, transferred to a 2.5% (v/v) solution from a commercial sodium hypochlorite (4.9% active chlorine; Chlorex®, Peru) with 0.1% (v/v) of polyoxyethylene sorbitan monolaurate (Tween 20) for 10 min, and then rinsed three times in distilled water.

Sterilized seeds were germinated by placing them in 10x120 mm test tubes containing 3 mL culture medium solidified with agar-agar (0.7% w/v). The culture medium consisted of MS salts (Murashige and Skoog, 1962), 1.0 mg/L thiamine.HCl, 100 mg/L myo-inositol and 3% sucrose.

Shoot elongation, roots formation and callus induction

Elongated shoots and nodes (average, 10–15 mm) obtained after 40–45 days of culture in seed germination medium were placed in 18x150 mm test tubes containing 5 mL culture medium supplemented with IAA–GA_3 and NAA–GA_3 combinations. In the rooting of shoot tips and nodes the explants were placed in 25x150 mm test tubes containing 15 mL culture medium supplemented with IAA–GA_3–PP (Plant Prod, NPK fertilizer) combinations, and the callus induction, sections 1 cm side of cotyledons, hypocotyls, internodes and roots were placed in glass flasks (60x50 mm) containing 20 mL culture medium supplemented with 2,4-D–GA_3, IAA–BAP and IAA–BAP–GA_3 combinations. Only in the rooting process in IAA–GA_3–PP treatment, 500 mg/L of antivitrifying agent (Sigma-Aldrich®) was incorporated.

Culture conditions

The pH of all media was adjusted to 5.8±0.1 with KOH and HCl, prior to autoclaving at 121 ºC at 105 kPa for 20 min. One seed and shoot tip or nodes explants were cultured per test tubes and two cotyledons, hypocotyls, internodes and roots were cultured per glass flasks. Each treatment comprised 15 explants and was performed twice. The experiments were evaluated every 15 days, for 60 days. Seed germination and all of the subsequent culture steps (shoot elongation and callus induction) were incubated at 25±2 ^oC. The seeds germination and shoot elongation were incubated with a 16-h photoperiod (36 µmol m^{-2} s^{-1}) and callus induction in the dark.

Phytochemical analysis

Roots, stems, leaves and spikes of *P. scandens* were oven dried at 40 ^oC and powdered (5 to 10 mm of particle diameter). The dried powdered of each plant material were submitted to a dynamic extraction with 150 mL of chloroform (three extractions of 50 mL) during 24 h. In the case of *in vitro* plants the samples were dried at room temperature and then lyophilized. All extracts were evaporated to dryness under reduced pressure.

Instrumentation consisted of a Shimadzu gas chromatograph model GC/17-A equipped with a mass spectrometer model GC/MS QP-5000, as well as a capilar column DB-5MS. In the sample preparation a portion of each crude chloroform extract (2.0 mg) was dissolved in ethyl acetate (1.0 mL) and injected into a gas chromatograph; the injection were performed twice. The solvent used (chloroform and ethyl acetate) were PA grade.

The following chromatographic conditions were used: helium as the carrier gas, mass detector, detector temperature = 250 ^oC, injector temperature = 300 ^oC, flow rate 1.2 mL/min, split of 1:20, injection volumen = 1.0 mL/min, initial temperature = 200 ^oC x 2 min, final temperature = 320 ^oC x 35 min and oven programme from 10 ^oC/min to 290 ^oC followed by an isotherm period of 20 min. The patterns used were plumbagin and some long chain hydrocarbonates (C_{20}, C_{22}, C_{24}, C_{32} and C_{36}).

Biological activity

Bacterial species of Gram positive (*Staphylococcus aureus*) and Gram negative (*Escherichia coli* and *Neisseria gonorrhoeae*) were isolated from clinical samples from Casimiro Ulloa Hospital (Lima, Peru), and identified by microscopic observation and biochemical tests. In the dilutions and inoculum preparations, the extract of wild plants and *in vitro* plants were weighed and dissolved in sterile distilled water to prepare appropiate dilution to get required concentration of 0.4, 0.6, 0.8 and 1.0 mg/mL. The inoculums of bacterial species were prepared in nutrient medium and kept incubation at 37 ^oC for 8 hours. After growth observed, the cultures were stored in the refrigerator at 2–8 ^oC. In the procedure for performing the Kirby-Bauer Disc Diffusion Test 1 mL inoculum suspension was spread uniformly over the agar medium using sterile glass rod to get uniform distribution of bacteria. Sterile discs of 5 mm diameter prepared with Whatman N^o 41 paper filter were loaded with different concentrations of 0.0 (control), 0.4, 0.6, 0.8 and 1.0 mg/L of plant extract. The paper diffuse discs were placed on the medium and incubated at 37 ^oC for 24 hours. The antibacterial activity was recorded by measuring the width of the inhibition zone (mm) with a caliper. In the determination of minimal inhibitory concentration (MIC), the Kirby-Bauer Disk Diffusion Method was utilized with 0.1, 0.2, 0.3 and 0.4 mg/mL concentrations of chloroformic extracts for all bacterial species tested. Equivalent concentrations to tube N^o 5 McFarland nephelometer, corresponding to a density of $1.5x10^8$ bact/cc, was used.

Statistical analysis

Data were processed and analyzed by analysis of variance (ANOVA) and the Tukey HSD multiple range test ($p \leq 0.05$) in order to compare treatment means. All the statistical analysis were carried out the Statgraphics Plus 5.0 software (StatPoint, Warrenton, Virginia, USA).

RESULTS AND DISCUSSION

***In vitro* seed germination**

Data of the *P. scandens* seed evaluation are presented en the table 1. On seed germination, radicle emerges in 8 day and complete expansion of cotyledons between 25 to 30 days.

After 30 days, the seeds germination was 81.8%, and after 45 days the maximum plantlets elongation was 14.3 cm (Table 1). Germination of freshly collected seeds was 100% down to 40% after 45 days of collected.

Table 1. *In vitro* seeds germination of *P. scandens* after 45 days of culture[1].

Evaluation (days)	Contamination (%)		Germination seeds (%)		Morphogenic responses	
	Bacterial	Fungal	+	-	Emergency of radicle	Seedlings length (cm)
2	0.0	12.0 a	0.0	0.0		
8	5.5 a	20.7 b	35.0 a	65.5 c	*	
15	10.2 b	31.4 c	62.6 b	44.6 b		4.4 ± 0.4 a
30	10.9 b	33.5 c	81.8 c	24.8 a		9.1 ± 0.9 b
45						14.3 ± 0.5 c

[1]Means followed by the same letters do not significantly differ according to a Tukey test at a 5% probability.

Plant propagation and callus induction

In vitro shoot elongation from the shoot tip and node explants were tested using MS medium supplemented with IAA – GA_3 and NAA – GA_3 in very low concentrations (0.02 mg/L, respectively). The maximum length of shoots (10.18±2.01 cm) was observed on medium containing 0.02 mg/L IAA and 0.02 mg/L GA_3. The highest number of nodes (7.3) and leaves (8.6) induction was also observed in the same combination (Table 2); likewise, a small callus and poor roots formation was observed in the base of explants, and this callus formation reduced the formation of roots. The culture medium devoid of plant growth regulators was used as control and in this case shoot elongation was not observed.

In *Plumbago* genus the micropropagation research has been done mainly on *P. zeylanica*. In this species, in a pioneer work, an efficient protocol was develop for *in vitro* clonal propagation through nodal culture; multiple shoots were induced on MS medium supplemented with BA (0.5 to 1.0 mg/L), and the inclusion of IAA 0.01 mg/L improved the frequency of production of multiple shoots (Rout et al., 1999). In another study, the highest multiplication rate of the explants (2.0±0.81), comprised of nodal cuttings of fresh and healthy plants, was obtained using MS medium supplemented with NAA (0.01 – 0.05 mg/L) and BAP (2.0 – 4.5 mg/L) (Gbadamosi and Egunyomi, 2010); likewise, a rapid shoot proliferation was observed on the nodal explants in MS medium supplemented with 1.0 mg/L BA and 1.0 mg/L GA_3, and the highest length of shoot (5.88±0.44 cm) was achieved after 1 week of incubation (Chinnamadasamy et al., 2010). In addition, MS medium with 4.4 mg/L BA and 1.4 mg/L IAA elicited the maximum number of shoots (12 multiple shoots) from nodal explants, and leaf based callus differentiated into more tan 30 shoots on MS with 160 mg/L adenine sulphate (Chaplot et al., 2006). Other improved protocol was developed for the micropropagation of *P. zeylanica* from nodal explants where the best responses of shoot induction was observed on MS basal medium supplemented with 1.0 g/L TDZ and 1.0 mg/L KIN (Caesar et al., 2013). In another Plumbago species as *P. rosea* roots culture were established from young leaf explants on B5 medium supplemented with NAA – KIN combination in the concentrarion ranges of 0.5 – 2.0 mg/L and 0.1 – 0.5 mg/L, respectively (Panichayupakaranant and Tewtrakul, 2002).

The effect of IAA and IBA on root induction was carried out. The medium without auxins not show any root induction. In contrast, root induction was observed when elongated shoots were cultured on medium with low concentrations of auxins (Figure 2). The maximum number of root induction (10.0±2.21) and shoot elongation (8.24±3.24) was observed on medium containing 0.01 mg/L IBA and 0.01 mg/L GA_3. With increasing of IBA concentration to 0.1 mg/L decreased root formation, and at the highest IBA concentration tested (1.0 mg/L) no roots were formed neither shoot elongation was induced. Of the two auxins tested IBA was found to best followed by IAA (Table 3). These results are in agreement with earlier publish work in *P. zeylanica* when rooting was readily achieved upon transferring the shoots onto half-strenght MS medium supplemented with 0.25 mg/L IBA and 2% sucrose (Rout et al., 1999), and in other studies, regenerated shoots were rooted on half strenght MS medium supplemented with 1.0 mg/L BA and 0.5 mg/L IAA (Chinnamadasamy et al., 2010), with 1.2 mg/L IBA within ten days (Chaplot *et al*., 2006), with either 0.1 mg/L IBA or NAA (Patidar et al., 2013) and with 1.0 mg/L IBA (Caesar et al., 2013). In the other hand, the supplement of 25 mg/L Plant Prod (fertilizer NPK) had no effect on roots formation, while the supplement of 500 mg/L of antivitrifying agent substantially improved the physiological condition of the plantlets.

Rooted plantlets were transferred to plastic pots containing mixture of sterile soil, sand and moss (1:1:1) and were acclimatized in the culture room for 6 weeks with 80% survival rate. The regenerated plantlets were morphologically similar to wild plants, and exhibited normal growing and flowering. In *P. zeylanica*, micropropagated plantlets were hardened in the greenhouse and successfully established in soil with 90 –100 percent survival rate (Rout et al., 1999; Chinnamadasamy et al., 2010; Chaplot et al., 2006; Patidar et al., 2013; Ceasar et al., 2013).

Compact callus was observed in several explants (cotyledons, internodes, hypocotyls and roots) cultured on MS medium supplemented with all the combinations of 2,4-D – GA_3 or 2,4-D alone and combinations of IAA – BAP or IAA alone, after 15 days of inoculation. Growth of callus increased significantly and covered the entire surface of the explants (++/+++, empiric scale) within 6 weeks. In cotyledons and internodes, the highest percentage of callus (90 – 100%) was observed using 2.0 mg/L 2,4-D or 0.2 mg/L 2,4-D – 0.2 mg/L GA3 (Table 4), while in hypocotyls and roots, the highest percentage of callus (100%) was observed using 2.0 mg/L IAA – 0.2 mg/L BAP and 2.0 mg/L IAA – 2.0 mg/L BAP (Table 5). Callus induced with 2,4-D and 2,4-D – GA_3 were compact and white and only in IAA – BAP combination higher proliferation of roots was induced in all explants tested. In the other hand, the highest percentage of friable callus (90%) were induced in the sections of compact callus using 2.0 mg/L IAA – 0.02 mg/L BAP – 0.5 mg/L GA_3 (Table 6).

In general, auxins (2,4-D and IAA) were absolutely necessary for callus induction, and morphology of the callus varied with the explant type and plant growth regulators used in the culture medium. In *P. zeylanica* maximum proliferation of callus was obtained with 1.0 mg/L 2,4-D along with 0.5 mg/L BA, and the synergistic effect of 2,4-D with BA or KIN induced creamish white callus from leaf and stem explants (Lubaina and Murugan, 2012); likewise, in this species the higher degree of callus induction was obtained with 2.0 mg/l NAA – 0.5 mg/L BA while the culture medium with 2.0 mg/L BA exhibited higher shoot proliferating efficiency in nodal segment and 1.0 mg/L NA – 0.5 mg/L TDZ in leaf disc (Patidar et al., 2013). Our results are in agreement with that reported in *P. zeylanica*, and are consistent with the current knowledge of hormonal mechanisms involved in induction of callus in plants. In the other hand, callus induction and establishment of cell suspension cultures capable of producing medicinal compounds at a rate similar or higher than intact plants have accelerated over the last few years, mostly due to optimization of the cultural conditions, selection of high-producing strains and employment of precursor feeding, transformation methods and immobilization techniques (Vanisree et al., 2004).

Table 2. Morphogenic responses induction in apical bud and node explants of *P. scandens* after 45 days of culture[1].

Growth regulators (mg/L)			Morphogenic responses		
NAA	**IAA**	**GA_3**	**Shoot elongation (cm)**	**Nodes (N^o)**	**Leaves (N^o)**
0.0	0.02	0.02	10.2 ± 2.0 b	7.3 ± 1.6 a	8.6 ± 1.6 a
0.02	0.0	0.02	6.3 ± 1.3 a	6.1 ± 1.7 a	7.3 ± 1.9 a

[1]Means followed by the same letters do not significantly differ according to a Tukey test at a 5% probability.

Table 3. Shoot elongation and roots formation in apical bud and node explants of *P. scandens* after 45 days of culture[1,2].

Growth regulators (mg/L)					Morphogenic responses	
IAA	**IBA**	**GA_3**	**PP**	**AV**	**Shoot elongation (cm)**	**Roots formation (N^o)**
0.01		0.01	25.0		5.3 ± 1.9 b	2.2 ± 1.2 ab
0.01		0.01	25.0	500.0	5.6 ± 2.1 b	4.4 ± 1.8 b
	0.01	0.01			8.2 ± 3.2 c	10.0 ± 2.2 c
	0.1	0.01			5.8 ± 3.5 b	3.4 ± 4.0 b
	1.0	0.01			0.9 ± 1.9 a	0.2 ± 0.6 a

[1]Means followed by the same letters do not significantly differ according to a Tukey test at a 5% probability.
[2]PP, Plant Prod; AV, antivitrifying agent

Table 4.Effect of 2,4-D – GA_3 for compact callus induction of *P. scandens* after 60 days of culture[1].

Growth regulators (mg/L)		Callus induction (%)							
2,4-D	**GA_3**	**Cotyledons**				**Internodes**			
		-	+	++	+++	-	+	++	+++
0.2	0.0	-	40	60	-	-	30	70	-
2.0	0.0	-	10	60	30	-	20	80	-
0.2	0.2	-	-	100	0	-	70	30	-
2.0	0.2	10	10	60	20	-	10	70	20

[1]-, without callus formation; +, callus covers 1/3 of the explant; ++, callus covers ½ to 2/3 of the explant; +++, callus covers the whole explant.

Table 5. Effect of IAA – BAP for compact callus induction of *P. scandens* after 60 days of culture[1,2].

Growth regulators (mg/L)		Callus induction and morphogenic responses (%)											
IAA	BAP	Cotyledons				Hypocotyls				Roots			
		Callus		Roots		Callus		Roots		Callus		Roots	
		++	+++	++	+++	++	+++	++	+++	++	+++	++	+++
2.0	0.0	70	-	50	30	80	-	90	-	70	-	-	100
2.0	0.2	60	-	20	10	10	90	50	-	70	30	50	-
0.0	2.0	70	-	-	-	70	-	-	-	40	-	-	-
2.0	2.0	40	-	-	-	70	20	-	-	60	40	-	-

[1]-, without callus formation; +, callus covers 1/3 of the explant; ++, callus covers ½ to 2/3 of the explant; +++, callus covers the whole explant.
[2]-, without roots formation; +, 1 – 3 roots formation; ++, 4 – 6 roots formation; +++, > 6 roots formation.

Table 6. Effect of IAA – BAP and IAA – BAP – GA_3 for friable callus induction of *P. scandens* after 60 days of culture[1].

Growth regulators (mg/L)			Callus induction (%)			
IAA	BAP	GA_3	-	+	++	+++
2.0	0.02	-	10	30	20	40
2.0	0.02	0.5	-	10	30	60
2.0	0.02	1.0	60	40	-	-

[1]-, without callus formation; +, callus covers 1/3 of the explant; ++, callus covers ½ to 2/3 of the explant; +++, callus covers the whole explant.

Table 7. Antimicrobial sensitivity assay of crude extracts of *P. scandens* roots.

Bacterial species	Strain	Zone of inhibition (mm)			
		Root extract concentrations (mg/mL)			
		0.4	**0.6**	**0.8**	**1.0**
Escherichia coli	S-1	0.0	0.0	0.0	0.0
	S-2	0.0	0.0	0.0	0.0
	S-3	0.0	4.2	8.2	10.4
Neisseria gonorrhoeae	S-1	22.0	24.8	27.2	30.0
	S-2	20.4	23.6	25.6	27.8
	S-3	20.8	23.6	26.2	29.0
Staphylococcus aureus	S-1	12.2	13.6	14.4	16.0
	S-2	12.6	13.6	14.8	15.8
	S-3	12.0	13.4	14.8	15.6

a b

Figure 1. *Plumbago scandens*. a) Wild plant and b) Flower.

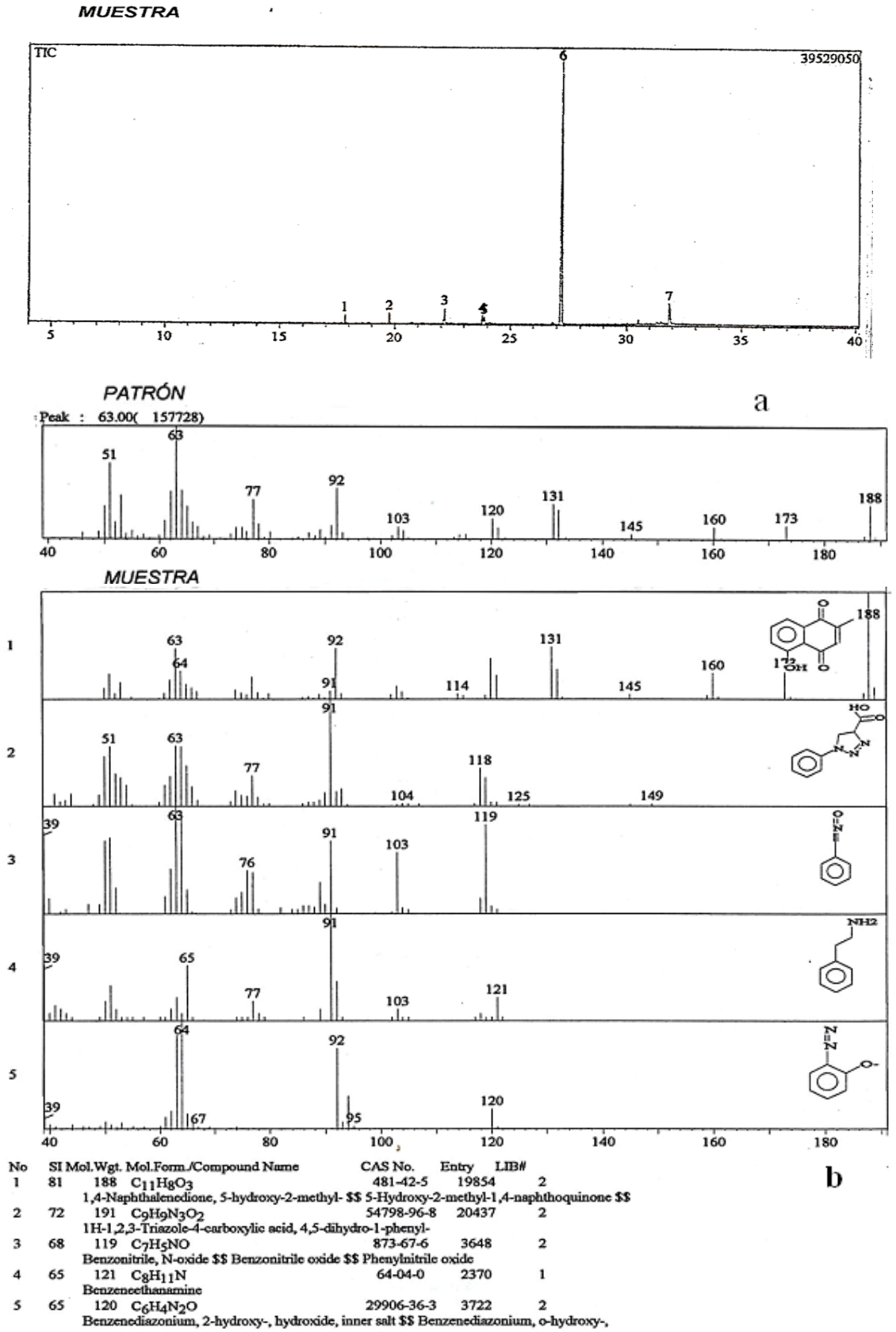

Figure 2. a. Chromatogram peak 1: Plumbagin (5-hidroxy-2-methyl-1,4-naphthoquinone), retention time: 17.83 min; peak 2: aromatic ester; peak 3, 4 and 5: long chain carboxilic acids and peak 6 and 7: low indicative index (not considered) and b. Espectrum GC-MS peak 1 of the crude chloroform extracts from *in vitro* plants of *P. scandens.*

Phytochemical analysis

The results indicated that the highest yield of crude extract corresponded to wild plants with 5.1% (leaves 5.9, stems 4.9 and roots 4.3%) while *in vitro* plants was 3.9%. The qualitative determination of chemical constituents in the extracts was evaluated by a gas chromatography coupled to a mass spectrometry. It was verified the presence of plumbagin only in root extracts (Figure 3) but not in *in vitro* plantlets.

In *Plumbago scandens* four compounds were qualitatively and quantitatively detected in root extracts: the naphtoquinones plumbagin and *epi*-isoshinanolone, palmitic acid and sitosterol, and plumbagin was always the major component, independent of the extraction technique; likewise, this napthoquinone is found predominantly on the roots, with low concentrations in aerial parts (Paiva *et al*., 2004).

Figure 3. Plantlets of *P. scandens* with roots formation after 180 days of culture.

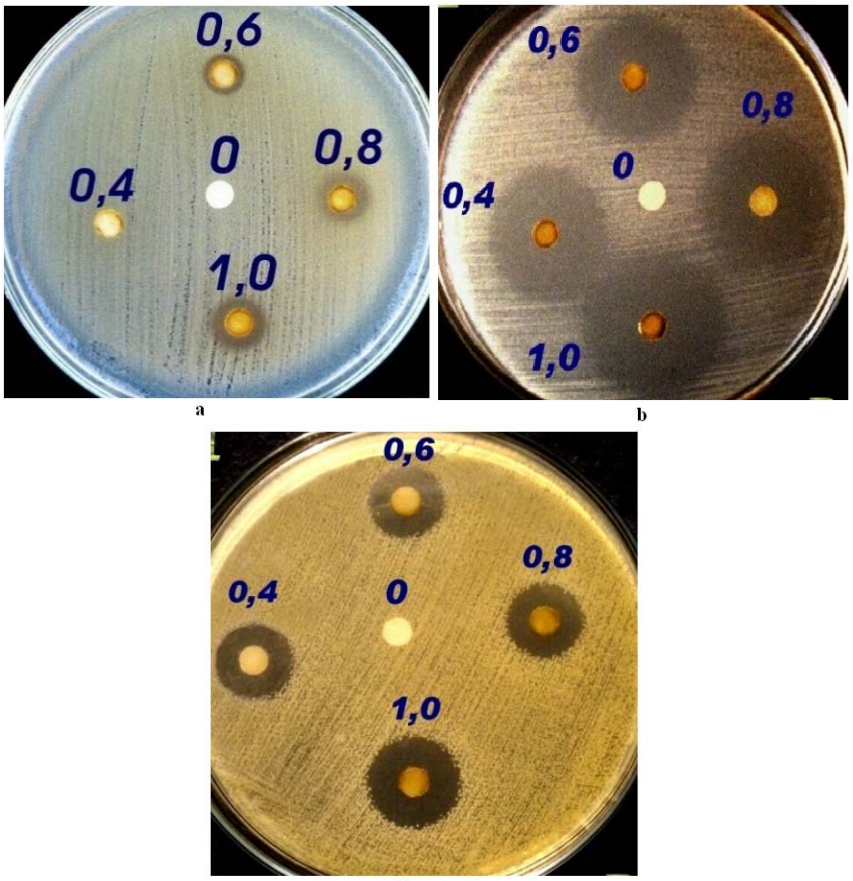

Figure 4. Antimicrobial sensitivity assay of crude extracts of *P. scandens* roots. A. *Escherichia coli,* b. *Neisseria gonorroheae* and c. *Staphylococcus aureus*.

In *P. scandens*, Moncada *et al*. (2011) also reported the presence of plumbagin in the roots of wild plants and seedlings *in vitro* plantlets, and this substance showed antibacterial activity against multidrug-resistance (MDR) strains of *Mycobacterium tuberculosis*. In this study, *in vitro* plantlets of 12 months of age were used, whereas in the present study, the *in vitro* plantlets were smaller than 6 months, therefore plumbagin certainly was not synthesized. In other species of the genus *Plumbago*, such as *P. rosea*, the accumulation of plumbagin in roots also was reported (Panichayupakaranant and Tewtrakul, 2002); however, Kitanov and Pashankov (1994) showed that the flowers of *P. europaea* have high concentrations of this compound. In this context, in root extracts, the naphtoquinones plumbagin and *epi*-isoshinanolone exhibited their highest content during floration, while the content of the two other components, palmitic acid and sitosterol, decreased during this stage, revealing an inverse profile (Paiva et al., 2011). In addition, a protocol for rapid *in vitro* micropropagation of *P. zeylanica* through axillary bud proliferation was develop as an essential prerequisite to conduct genetic transformation with the A4 strain of *Agrobacterium rhizogenes*; the fresh hairy roots produced 2.5 times higher amounts of plumbagin than the fresh, untransformed control roots or the dry hairy roots of the same age (Verma et al., 2002).

Biological activity

Among the extracts assayed, the chloroform root extracts of *P. scandens* exhibit good activity against *Neisseria gonorrhoeae* (strains 1, 2 and 3) between 0.4 to 1.0 mg/mL (20.4 to 30.0 mm diameter zone of inhibition, and this was followed by *Staphylococcus aureus* with 12.0 to 16.0 mm); the least activity was observed with *Escherichia coli* at 1.0 mg/mL (10.4 mm) (Figure 4). Chloroform extracts had the lowest MICs for *N. gonorrhoeae* (<0.1 mg/mL per disc), and the activities against *S. aureus* (MIC 0.2 mg/mL) and *E. coli* (MIC 0.4 mg/mL) were less pronounced. In this species, the inhibitory effect of both metanolic and ethyl acetate crude extracts and MIC of both roots and *in vitro* plantlets against MDR strains of *Mycobacterium tuberculosis* were determined (Moncada et al., 2011).

In *P. zeylanica* have been realized numerous works, for instance, ethyl alcohol leaf extract exhibited good activity against *Pseudomonas aeruginosa* at 100 mg/L with 17 mm recorded as diameter zone of inhibition, and moderate and poor activity against *E. coli* and *S. aureus* with 16 and 10 mm, respectively (Dhale and Markandeya, 2011).The concentrations used in this study (50 and 100 g/mL) were extremely high compared to the work presented. This may be due to the lower concentration of plumbagin in leaf extracts. In other study, chloroform root extracts (20 µg/disc) exhibited good activity against *E. coli* (16.7 mm), *Salmonella typhi* (14.3 mm) and *S. aureus* (12.0 mm), and had the lowest MICs for *E. coli* (<6 µg/disc) and *S. aureus* (<8 µg/disc) (Jeyachandran et al., 2009). Likewise, ethanolic root extract exhibited good antibacterial and antifungal activities, and among the test bacteria, *Vibrio cholerae* was found to be most sensitive to the extract showing the highest diameter of zone of inhibition (30 mm) and lowest MIC value (200 µg/mL), and the extract was also very effective against *E. coli* and *P. aeruginosa* showing 26 and 24 mm, respectively, of diameter of zone of inhibition and MIC value of 250 µg/mL (Rahman and Anwar, 2007). In the other hand, leaves of *P. zeylanica* extracted with hydro alcohol by hot decoction method were active against Gram positive bacteria (*B. subtilis* and *S. aureus*) and Gram negative bacteria (*E. coli* and *P. aeruginosa*); the maximum zone of inhibition was determined against *S. aureus*at 500 µg/mL (1.2 mm; activity significantly higher than standard antibiotic), and very less zone of inhibition was against *P. aeruginosa* (1.6 mm), *E. coli* (0.9 mm) and *B. subtilis* (1.5 mm) (Raj et al., 2014). In this experiment, although the extract showed activity against all the tested bacteria, the zone of inhibiton was much lower compared to the study presented.

In general, the anti-bacterial properties of the *Plumbago* species may be attributed due to the secondary metabolites present in them, specially, plumbagin. In this sense, plumbagin (20 µg/disc) isolated from the root of *P. zeylanica* showed good activity against *E. coli* (25.6 mm) and *S. aureus* (21.6 mm) among other bacterial species, and the activity of plumbagin was significantly higher than that of streptomycin (10 µg/disc) (Jeyachandran et al., 2009). Likewise, the results of these studies reveals the fact that the organic solvent extracts (chloroform and metanol) exhibited greater antimicrobial activity because the antimicrobial principles were either polar or non-polar and they were extracted only through the organic solvent medium (Raj et al., 2014); soxhlet was the most efficient extraction technique of plumbagin however, prolonged heating time promoted plumbagin degradation (Paiva et al., 2004), and most plumbagin quantification was performed on root of *P. scandens* (Paiva et al., 2011; Moncada et al., 2011) and *P. zeylanica* (Jeyachandran et al., 2009; Rahman and Anwar, 2007; Lemma et al., 2002).

Chloroform extracts had the lowest MICs for *N. gonorrhoeae* (<0.1 mg/mL per disc), and the activities against *S. aureus* (MIC 0.2 mg/mL) and *E. coli* (MIC 0.4 mg/mL) were less pronounced.

CONCLUSIONS

From this study, the best response of shoot elongation and maximum number of root induction were observed on MS basal medium supplemented with 0.02 mg/L IAA – 0.02 mg/L GA_3 and 0.01 mg/L IBA and 0.01 mg/L GA_3, respectively.

Profuse friable callus were induced in the sections of compact callus using 2.0 mg/L IAA – 0.02 mg/L BAP and 0.5 mg/L GA_3. Root extracts have potential to developed as antimicrobial agents, in particular against *N. gonorrhoeae* and *S. aureus*, due to the presence of plumbagin and other secondary metabolites. The protocol developed in the present study will help in the mass production and cellular suspension establishment of *P. scandens* and other medicinally important species.

Conflict of Interests

The author (s) have not declared any conflict of interests.

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