Identificación, establecimiento *in vitro* y análisis fitoquímico preliminar de especies silvestres de ñame (*Dioscorea spp.*) empleadas con fines medicinales

Identification, *in vitro* establishment and preliminary phytochemical analysis of wild yam (*Dioscorea spp.*) used for medicinal purposes

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Resumen

Tubérculos del género *Dioscorea* comercializados con fines medicinales, fueron recolectados con el propósito de lograr su establecimiento a condiciones *in vitro*. Previamente se lograron identificar taxonómicamente las especies y por medio de análisis fitoquímicos demostrar su potencial farmacéutico. El material recolectado fue identificado como *Dioscorea coriacea, D. lehmannii, D. meridensis, D. polygonoides* y una especie comestible *D. trifida*. Tubérculos recolectados de centros de acopio y traídos de campo fueron lavados, desinfectados, asperjados con Ácido Giberélico (AG_3) y sembrados en sustrato BM-2®, en invernadero a 18°C día y 10°C noche. Los tubérculos completos o por secciones fueron almacenados en bolsas herméticas a temperatura ambiente. Posteriormente se desinfectó material vegetal de las especies *D. coriacea, D. lehmannii, D. meridensis* y *D polygonoides,* seleccionando explantes de brotes sanos (*D. coriacea* / laboratorio) para su establecimiento. Se evaluaron tres medios de cultivo para establecimiento, el que presentó los mejores resultados fue Medio Murashige & Skoog (1962) suplementado con BAP 1 mL/L, AG_3 1 mL/L y Putrescina 2 mL/L. Para la extracción y análisis de metabolitos secundarios se utilizaron tubérculos de *D. coriacea, D. lehmannii* y *D. polygonoides*, empleando como solvente de extracción metanol. Se encontró mayor concentración de extracto vegetal en *D. coriacea* (54%), y mediante cromatografía en capa delgada (CCD), se confirmó la presencia de saponinas, que resultó mayor en comparación con *D. polygonoides* especie reconocida por su alto contenido de saponinas. Estos resultados permitirán realizar análisis más avanzados de los compuestos presentes y plantear su propagación masiva en condiciones *in vitro*.

Palabras clave: diosgenina, micropropagación, ñame silvestre, cultivo de tejidos vegetales, saponinas, fitoquímica.

Abstract

Wild tubers of the genus *Dioscorea* sold for medicinal use were collected for the purpose of achieving its establishment under *in vitro* conditions. First we taxonomically identified the species and through phytochemical analysis demonstrated pharmaceutical potential. The material collected was identified as *Dioscorea coriacea*, *D. lehmannii*, *D. meridensis*, *D. polygonoides* and the edible species *D. trifida*. Tubers collected from wholesale distributors and from the field were washed, disinfected, sprayed with Gibberellic Acid (GA_3) and planted in substrate BM-2®, in a greenhouse at 18 ° C during the day and 10 ° C overnight. Whole tubers or sections thereof were stored in sealed bags at room temperature. Subsequently plant material of the species *D. coriacea, D. lehmannii, D.* meridensis and *D. polygonoides* was disinfected and healthy buds (*D. coriacea* / laboratory) were selected for *in vitro* establishment. Three different culture media were evaluated for establishment; that which presented the best results was the Murashige & Skoog (1962) medium, supplemented with BAP 1 mL / L, GA_3 1 mL / L and Putrescin 2 mL / L. For the collection and analysis of secondary metabolites, tubers of *D. coriacea*, *D. lehmannii* and *D. polygonoides* were used, using methanol as the extraction solvent. The highest concentration of plant extract, 54%, was found in *D. coriacea*, a higher value than that of *D. polygonoides,* which had been reported previously; the

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presence of saponins was confirmed by thin layer chromatography (TLC). These results will enable more advanced analysis of the present compounds and enhance their mass propagation under *in vitro* conditions.

Key words: diosgenin, micropropagation, wild yam, tissue culture, saponins, phitochemistry.

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Introduction

The *Dioscorea* genus groups together 600 herbaceous, climbing, liana or reed plant species, mostly distributed around the tropics. Numerous wild species of this genus serve as food for different countries in Africa, Asia, Latin America and Australia, primarily in times of shortage or poverty. Others are used in Asia, Europe and North and Central America for medicinal purposes to treat rheumatism, intestinal spasms and cramps, and pains, or as abortifacients. Some are used in phytotherapy and homeopathy, and popularly as piscicides, pediculicides and insecticides; to make shampoos and soaps; or to poison arrows for hunting (Waizel, 2009).

Yams in the United States are actually sweet potatoes and therefore, the US Department of Agriculture requires that the "yam" label is always accompanied by the "sweet potato" label. Real yams are a species of *Dioscorea* (USDA, 2012). Real yams have been commonly used in the pharmaceutical industry to treat greatly diverse conditions, such as inflammation, joint pain, diabetes, infections and dysmenorrhea. The pharmacologically active components of the *Dioscorea* species include diosgenin, which is a steroid saponin, and dioscin, a form of diosgenin with sugars.

The diverse methods for yam micropropagation include the cultivation of meristems, effective for the elimination of viral infections and the conservation of germoplasm. Another one of the mechanisms used for propagation is microtuberization, which allows the conservation of basic genetic material of mother clones free from disease in a reduced space without the need for costly operations of collecting yam in the field (Perea & Buitrago, 2000).

The Research Group on Yam Cultivation of the Biotechnology Institute of the Universidad Nacional de Colombia (IBUN, for the Spanish original) focuses its research on wild yam, as well as on farmed species and varieties. This work has developed methodologies for *in vitro* cultivation, generating knowledge on crops considered to be orphans (FAO 2006), nationally and internationally, with a clear approach to have a positive impact on the quality of life of yam producers in Colombia.

This work aimed to establish the conditions of *in vitro* cultivation of wild and commercialized yam species in Bogotá marketplaces, contributing to future work with these species to ensure availability of vegetable material. Additionally, their taxonomic identification was proposed, as well as a first phytochemical analysis to identify their phytotherapeutic potential.

Materials and Methods

Selection of Material

Tubers known as sarsaparrille were acquired at collection centers in Bogotá in sections and as complete tubers. Additionally, there was material donated (tubers with stems, leaves and roots) by the Natural Sciences Institute and Research Group on Phytochemistry and Pharmacognosy of the Universidad Nacional, and material collected in different municipalities of the Cundinamarca department.

Study and Determination of the Species

To determine the species of tubers collected in their natural habitat from the field, the morphological features that define the characteristics of the genus and the species were considered, using the Herbario Nacional Colombiano (National Colombian Herbarium - COL) and botanical literature as a reference (Knuth, 1924). The altitude, location and description of the plant and species were recorded. For the other tubers, the morphological features that define the characteristics of the species of the *Dioscorea spp*. genus; the color and diameter of the young and mature stem; the presence or absence of thorns at the base or throughout the stem; the presence or absence of stem tubers and the shape of the root tuber, as well as the width, epidermis color, parenchyma color, presence or absence of cracks in their surface and organoleptic properties of the tubers were reported (IPGR/IITA, 1997).

Adaption of Wild Yam in the Greenhouse

The tubers acquired in collection centers were previously washed with detergent and water and planted in flowerpots. Gibberellic Acid (GA_3) was previously applied to them by spraying to break dormancy. Tubers donated in sections that were found in airtight bags with the development of shoots were washed and disinfected for establishment. Tubers from the field were washed and stored in airtight bags for one month to break dormancy and then planted in the greenhouse.

Once washed with plenty of soap and water, the tubers were planted in flowerpots with BM2® (mix for germination) and sand (2:1), taken to the greenhouse, watered every two days, and kept at a temperature of 20 °C daytime, 10 °C nighttime. The planted tubers were checked daily and the shoots and roots were checked weekly, recording at 8, 15, 30 and 60 days. At 30 days, they were kept at room temperature (approximately 18 °C daytime and 10 °C nighttime).

Introduction of Vegetable Material to in Vitro Conditions

Nodal cuttings were each cut at 2 cm and washed with plenty of water and Extran®. Then they were submerged in a solution of Tween 20.8 ml / 100 ml of sterilized distilled water, Isodine® 3.5% for 30 minutes, which has the active ingredient of povidone-iodine, and in sodium hypochlorite 2.5% for 15 minutes. They were rinsed three times with sterile distilled water between each step for 5 minutes.

In Vitro Explant Culture

Single-node, 15 mm-long segments without leaves from the stems of the donor plants were used. The explants were planted individually in 20 ml of medium per container, 12 containers per treatment were used, and the contamination was assessed at 3, 5, 8 and 15 days from planting. The following media were used.

i. M&S medium (Murashige and Skoog 1962), thiamine 3 ml/L, BAP 2 ml/L, activated carbon 3 ml/L, sucrose g/L, and agar g/L.

ii. M&S medium (Murashige and Skoog 1962), morel vitamins 2 ml/L, folic acid 1 ml/L, L-asparagine 4 ml/l, cysteine 10 mg/L, sucrose 30 g/L and agar 5 g/L.

iii. M&S medium (Murashige and Skoog 1962), thiamine 3 ml/L, BAP 1 ml/L, GA_3 1 mL/L, putrescine 2 ml/L, sucrose 30 g/L and agar 5 g/L.

Extraction and Analysis of Secondary Metabolites

The collected tubers were washed, cut and dried in a heater with forced air circulation for 48 hours. Subsequently, they were ground in a hammermill and the extraction was carried out with methanol by exhaustive percolation. The solvent was withdrawn from the extract using a rotary evaporator connected to a vacuum pump at 38 °C. Once the dry extract was obtained, each one of the extracts was characterized by the thin-layer chromatography (TLC) technique on silica gel 60 GF_{254} using the following mobile phases:

i. Methanol: Chloroform: Acetic acid. 6:6:1,

ii. Ethyl acetate: Formic acid: Glacial acetic acid: Water. 100:11:11:27,

iii. Chloroform: Ethyl acetate. 4:1,

iv. Methanol: Chloroform: Acetic Acid. 7:3:1,

v. Ethyl acetate: Methanol: Formic acid: Glacial acetic acid 100: 27: 11:11,

vi. Methanol: Chloroform. 9:1.

The Petri dishes were developed with Godin's reagent (vanillin in ethanol, H_2SO_4 in ethanol) and anisaldehyde/ H_2SO_4.

Table 1. Wild species used for extraction (different collections).

Samples	Selected Tuber
S1	*Dioscorea lehmannii I*
S2	*Dioscorea lehmannii II*
S3	*Dioscorea lehmannii III*
S4	*Dioscorea coriacea I*
S5	*Dioscorea coriacea II*
S6	*Dioscorea polygonoides*

Statistical Analysis

To establish the environment with the best results and to verify the significant differences, an analysis of variance, ANOVA, was conducted with a level of significance of $\alpha = 0.05$, using the Windows® Statistix program.

Results and Discussion

Selection of Material

The material collected in the marketplaces was taxonomically identified as *D. lehmannii,* and the tubers donated in sections were identified as *D. coriacea* and *D. meridensis*. Tubers collected from the field were identified as *D. coriacea, D. polygonoides and D. lehmannii*. *D. trifida* tubers came from the Chocó Region, from a marketplace in Quibdó. *D. polygonoides* tubers were only used to obtain an extract for reference. The *D. coriacea* material corresponded to the tuber donated by the laboratory of the Research Group on Phytochemistry and Pharmacognosy of the Universidad Nacional. The *Dioscorea spp*. tubers were purchased at marketplaces. Prevention and relief of illnesses related to the blood system, by purification or reconstruction, are attributed to them. However, different species of tuber are offered under the same name of sarsaparrille, which generates uncertainty about the attributed therapeutic effect. The collected material is presented in Figure 1 and Table 2.

Pinzón (2011) recorded 72 medicinal plant stands in 26 points of sale distributed among 12 of the 20 locali-

ties of Bogotá D.C.; primary and secondary markets and individual stands, with D. coriacea and D. meridensis (62–60%) being the species with the greatest supply in Bogotá. D. lehmannii was also acquired in several of the city's points of sale.

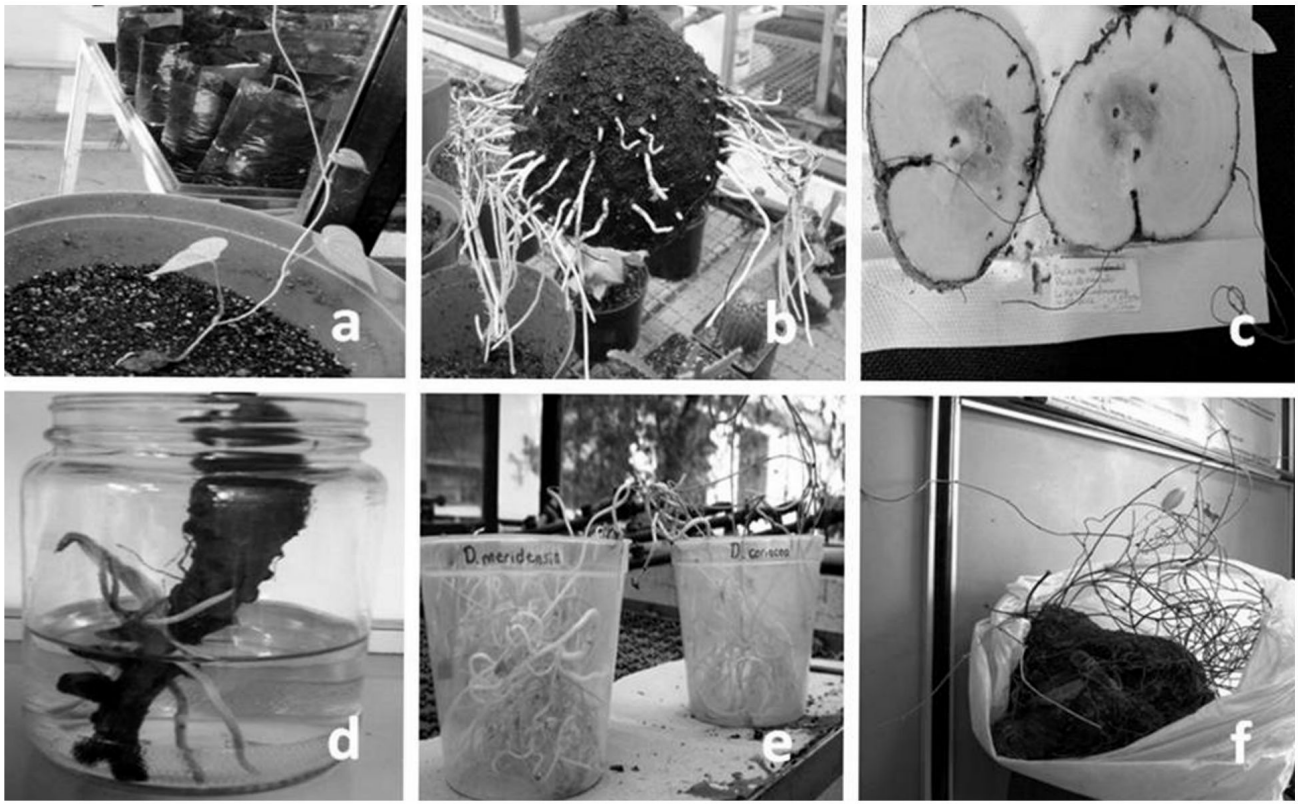

Figure 1. Collected Dioscorea tubers. **a.** *D. lehmannii* from the field, **b.** *D. lehmannii* from the marketplace cut from tip of the base, **c.** *D. coriacea* cut into sections and stored in airtight bags, **d.** *D. trifida,* **e.** *D. coriacea and D. meridensis* donated by the laboratory in sections, **f.** *D. coriacea* donated by the laboratory.

Table 2. Selected tubers.

Tub.	Species	Origin
T1	*D. lehmannii*	Field - Complete tuber
T2	*D. lehmannii*	Marketplace - Cut from base
T3	*D. lehmannii*	Marketplace - Complete tuber
T4	*D. trifida*	Natural Sciences Institute - Complete tuber
T5	*D. coriacea*	Natural Sciences Institute - Cut into sections
T6	*D.meridendis*	Natural Sciences Institute - Cut into sections
T7	*D. coriacea*	GIFFUN - Stems and leaves

When the tubers were put into plastic bags, they abundantly sprouted, with the D. coriacea species (Figure 1f) being the one that developed the greatest number of shoots. Hata et al. (2003) reported similar results, recording dormancy in the period between harvest of the tubers and storage of the D. rotundata tuber material in airtight bags. A greater content of sapogenins and presence of diosgenin was obtained from this material, inferring that the dormancy phenomenon is related to an increase in the content of some sapogenins, possibly because when consuming the tuber's starch to form stems, the proportion of sapogenins increases with respect to the amount of dry material.

The tubers previously placed in airtight bags presented nodal shoots (T1, T5, T6 and T7). As was to be expected, the tubers planted in BM2® and sprayed with GA_3 produced abundant roots (T2, T3 and T4), due to the action of the gibberellins in activating the metabolism, protein synthesis and mobilization of reserve substances to generate the elongation of the germ or in this case, the formation of roots. Tubers of the D. lehmannii and D. trifida species (T2, T3 and T4), which only produced roots and no shoots, were not used in the trials because the material, disinfection designs and establishment were for nodal shoots.

The action of GA_3 is non-toxic for humans or animals and it is easy to manage. The techniques may vary from immersion of the tuber or spraying. This depends on the variety and state of rest in which the tubers are found, causing sprouting in many varieties when the rest period has almost finished. For immersion, Marca (1997) suggests that the recently harvested tubers be washed and infected, air dried and later submerged in a solution of GA_3 (5 ppm) for 10 minutes. After the treatment, the tubers must be dried and placed in a chamber or warm environment between 18 °C and 25 °C to induce sprouting. After 15 days, there is abundant sprouting.

Study and Determination of the Species

Identified species from the field were recorded according to their characteristics (see Materials and Methods), and compared with specimens of the *Dioscorea* genus in the National Colombian Herbarium (See Table 3). Information about the distribution of the species was consulted in the herbarium's database (www.biovirtual.unal.edu.co). The majority of the *Dioscorea* species is from tropical latitudes with a few exceptions in North America, Europe and East Asia. Wilson (1977) includes the Asiatic species *D. japonica* and *D. opposita* as tolerant to frost. However, for *D. alata* and edible tropical species, temperatures under 20 °C restrict growth, which is promoted with temperatures between 25 °C and 30 °C, limited by the range of latitudes between 20 ° N and 20 ° S and maximum altitudes of 1,000 m.a.s.l.

It can be inferred that wild *Dioscorea* species bear significant differences to edible species in their conditions of adaptation, with the content of diosgenin or saponins derived from it being an important factor. This is proportional to the consumption of starch by the tuber, which is higher in agroecological conditions of forests, *subpáramos* and *páramos*, where the wild species that generally grow without much upkeep from farmers are found.

Adaption of Wild Yam

All of the *D. lehmannii* tubers (T2 and T3), wild species of *Dioscorea* brought from the field (T1) and *D. trifida* tubers (T4) were planted in the same conditions, presenting slower sprouting of roots. In the planting conditions, the tubers simultaneously presented shoots and roots. The material donated by the laboratory was planted in the greenhouse just like the sections of different species donated by the Natural Sciences Institute (T5 and T6).

Figure 2 shows that just the tubers previously placed in airtight bags produced shoots, and those sprayed with GA_3 produced roots, which was not favorable for planting. It is possible that the humidity generated in the bags favors the growth of foliage because of the high respiratory rates, and that the absence of this humidity phase with the addition of GA_3 generates roots and not shoots because of the yam phases designated for rest, vegetable growth and reproductive growth. The sprouting of tubers indicates the end of the rest period and the start of the vegetative phase, characterized by rapid growth of the stems and leaves.

Haynes *et al.* (1967) indicated that for *D. alata*, the reproductive phase starts when tuberization reaches an exponential rate of growth, which coincides with a reduction of the growth of stems and leaves. The yam's reproductive phase coincides with flowering followed by the maturity of the tubers.

The tubers treated with GA_3 produced roots. Taking into account that the type of explant required was shoots or buds, they were not used for the planting stage. Additionally, the tubers cut into sections and stored in bags produced better results, reflected in greater sprouting. This coincides with Fergusson (1977), who used sections of *D. alata* tubers with less time required for the growth of the tubers. These results permit the interpretation that this phase is characterized by accelerated cellular division and expansion of cells that will be later involved in microtuberization.

Table 3. Altitudes recorded for wild species collected (Natural Sciences Institute).

Species	Collections in COL	Min. Alt.	Max. Alt	Average Max. Alt.	Average Min. Alt.
D. coriacea	66	400	3700	2212.5	2317.6
D. lehmannii	16	1650	3600	2521.4	2621.4
*D. meridensis**	17	500	2500	1708.8	1738.8
*D. polygonoides**	53	10	2000	1283.8	1328.9
D. trifida	23	5	1120	244.6	255.4

* Data updated in Raz (2015)

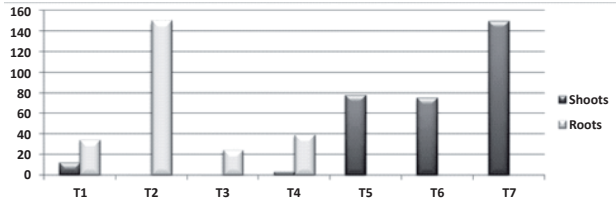

Figure 2. Growth of Dioscorea Shoots and Roots, T1–T4 Greenhouse, T5–T7 Donated.

Disinfection of Vegetable Material

The material used came from conditions with a high presence of microorganisms or contaminating agents. Only the protocol that included Isodine® (PVP Iodine) as a disinfectant had favorable results. The nature of the explant (greenhouse, field or laboratory) and the type of explant (root or stem) were also important factors in planting. Bonafont (2011) states that the solution of Isodine®, which is not active itself, but slowly releases iodine, is the one that possesses bactericidal activity, penetrating the cell wall and combining with different organic substrates through reduction-oxidation reactions.

The best results were obtained from young buds from the tips of the stems where possible endogenous contaminants had not invaded the plant's vascular bundles.

Planting Explants

Nodal explants of Medium iii reported shoots 30 days after being planted (0.2 – 0.5 cm). At 60 days, they still were not ready for multiplication (1 – 1.5 cm). In Medium i, growth was reported at 45 days after being planted due to the absence of regulators, and Medium ii did not present greater development of shoots either (0.8 – 1 cm).

Nodal segments of *D. coriacea* donated by the laboratory presented better results in Medium iii (1 – 1.5 cm), which contained BAP and GA_3 as growth regulators. The results were favorable in the medium to which GA_3 was added, which is used for the growth of stems and especially for breaking the dormancy in some species (See Figure 3).

The polyamine putrescine is considered to be a plant growth and development regulator because of its demonstrated effect on cellular growth, division and differentiation in low concentrations. Putrescine was included in the design of the environment because of the antioxidant and stabilizing capacity of its membranes (Perea, 2010).

As a result of the statistical analyses, it could be determined that the best treatment was T3 (iii), finding a significant difference of 0.0578 at 30 and 60 days. Significant differences were not found in T1 and T2.

Extraction and Analysis of Secondary Metabolites

The Petri dishes revealed that all the samples contain sapogenins, the color and formation of foam was a qualitative indicator, and the strips revealed greater concentration in the samples that presented a stronger color.

The extraction solvent was methanol, which presented a greater proportion of polar substances. The mobile phase that allowed a greater profile of the extracts to be obtained is formed from a mixture of ethyl acetate: Methanol: Formic acid: Glacial acetic acid 100: 27: 11:11, a mobile phase frequently used to detect flavonoids. (See Figure 4).

With the different chromatographic Petri dishes, it could be established that there is a group of high polarity organic compounds present in the extracts of *D. coriacea, D. lehmannii* and *D. meridensis*, and it could be confirmed that sapogenins are present in all the evaluated species, due to the formation of foam as a qualitative indicator. The thin-layer chromatography showed more polar sapogenin compounds than those present in *D. polygonoides*, which allowed a high degree of glycosylation to be inferred. As Hata and collaborators (2003) had already observed, when analyzing the metabolites present in *D. rotundata,* the concentration of sapogenins obtained for each sample is related to the number of sapogenins detected, concluding that there is a correlation between both results. In other words *D. rotundata* presented a greater number of sapogenins and a greater concentration of these compounds.

The results of the extraction (Table 4) showed that the greatest yield was obtained from *D. coriacea* with a yield of 54% and 21% for each sample processed, followed by *D. polygonoides* and *D. lehmannii*. The Petri dishes developed polar substances (Figure 4e and 4f). However, groups of metabolites other than saponins were detected in the different species, which developed a yellow color with Godin's reagent. These results lead to the proposal of analysis by high-performance liquid chromatography (HPLC), or isolation and identification of the secondary metabolites to determine their chemical nature. Different metabolite groups are detected in different species, not only sapogenins, and other metabolites are detected that present a yellow color and also become a subject of study through more detailed analysis, such as HPLC, or isolation of the metabolites for identification by analysis including nuclear magnetic resonance and infrared.

Studies by Flores (2010), recorded by Reina (2012) in the work document on the regional yam economy, assessed the saponin content in eight species in the Caribbean region, including *D. polygonoides*, to determine their potential use for medicinal and pharmaceutical purposes. The species analyzed were *D. alata*, *D. bulbifera*, *D. cayenensis*, *D. dodecaneura*, *D. esculenta*, *D. polygonoides*, *D. rotundata* and *D. trifida*. The re-

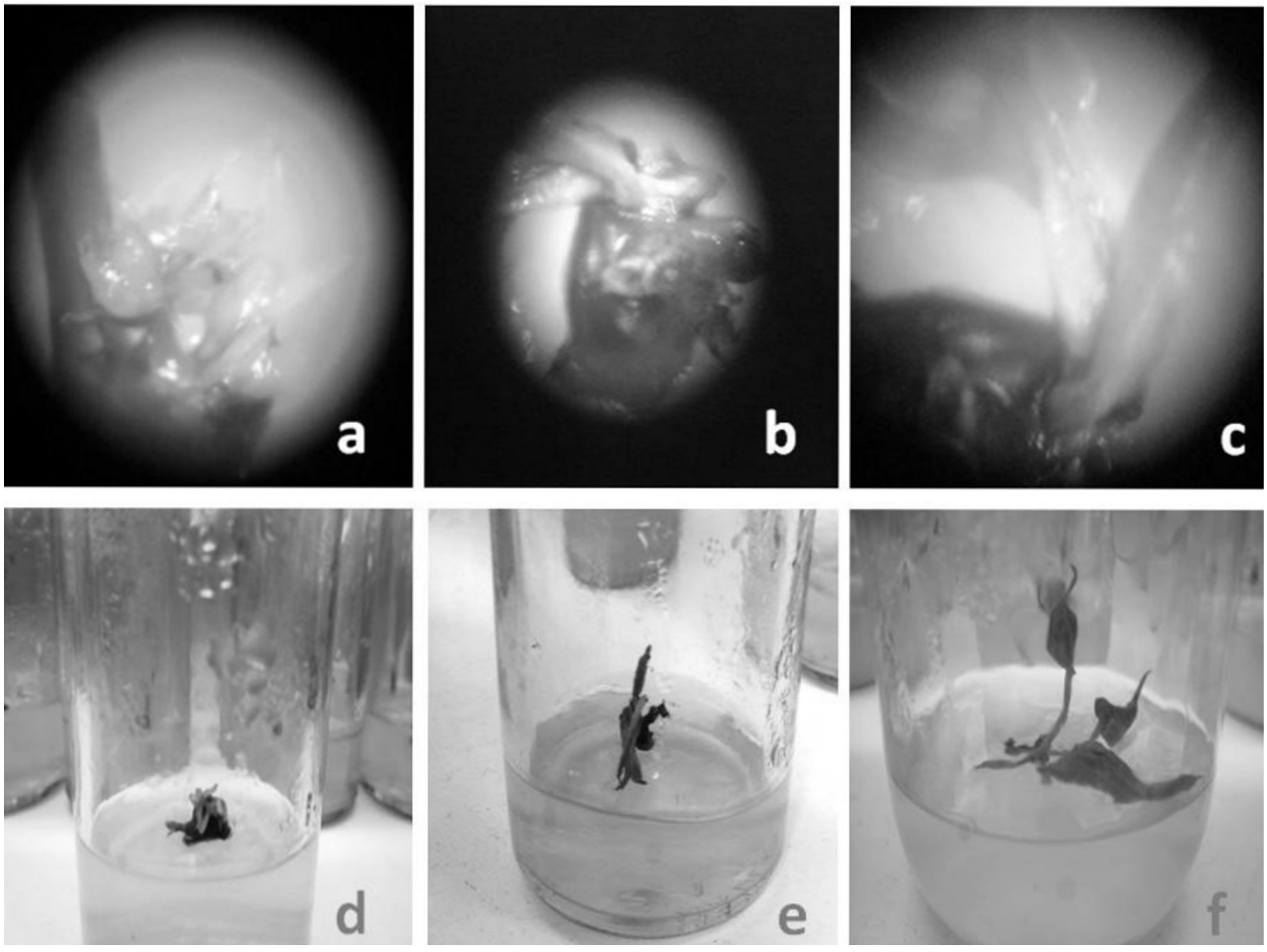

Figure 3. Planting of explants of *D. coriácea*. **a, b.** Shoots observed in stereoscope after 30 days, **c.** Shoots after 60 days, **d.** Nodal buds at 30 days, **e.** Nodal buds at 60 days **f.** Nodal buds 90 days after establishment.

sults showed that the *D. polygonoides* species has the greatest presence of sapogenins.

Similarly, Hata and collaborators (2003) found sapogenins in *D. alata* and *D. rotundata*. These results were based on the hydrolysis of the saponins to obtain the free sapogenins. The study presented a practical way of detecting saponins by thin-layer chromatography of diosgenin/yamogenin and tigogenin/neotigogenin types in the majority of the accessions, using *D. polygonoides* as a reference considering that this species has been widely studied and the polarity and structure of the saponins and terpenes is known.

The *D. coriacea* species presented an extract of 54% dry base and therefore, constitutes a species with an

Table 4. Weights obtained in the samples and yield of sapogenins.

Sample	Dry Material (g)	Dry Extract (g)	Yield (%)
S1 (*Dl* I + *Dl* II)	413.68	72.48	17
S2 (*Dl* III)	487.3	24.101	5
S3 (*Dc* I)	567.9	120.98	21
S4 (*Dc* II)	616.5	330.46	54
S5 (*Dp*)	265	49.66	18

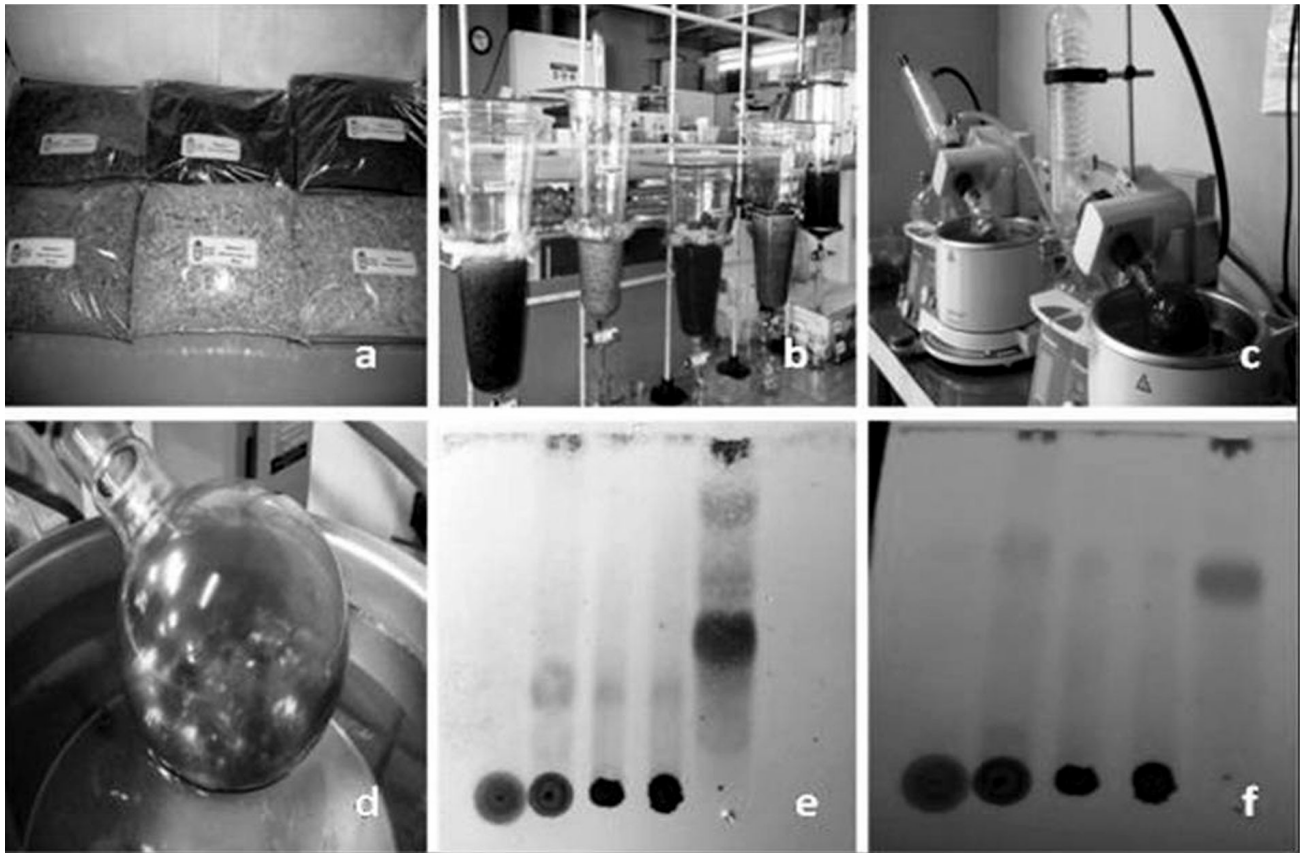

Figure 4. Extraction of secondary metabolites. **a.** Ground and packed yam tubers**, b.** Assembly in percolators, **c, d.** Extraction of methanol solvent in rotary evaporator **e.** Development of the Petri dish with Godin's reagent **f.** Development of bands with ultraviolet light.

apparent high potential of saponin content. If we consider the phytotherapeutic value that it is recognized for in its traditional and popular use as treatment for different health conditions such as hypercholesterolemia and diabetes, its potential increases. As already mentioned by Hata and collaborators (2003), "*un mejoramiento de estas especies puede convertirlas en especies promisorias para la obtención de sapogeninas esteroidales*" [an improvement in these species could make them promising species for obtaining steroid sapogenins].

Conclusions

The best conditions for sprouting were achieved by placing the material in temperature conditions of 18 °C daytime and 10 °C nighttime for 30 days.

The culture environments with a dosage of 1 ml/L of GA_3 were more suitable for the formation of shoots in *D. coriacea* under the experiment conditions. Culture environments that only contained BAP induced the formation of shoots. Similarly, the poylyamine putrescine was effective as an antioxidant.

The wild species of *Dioscorea* that were collected presented a high content of saponins, qualitative indicators such as the surfactant activity developed by the formation of foam, and the presence of bands by TLC that these metabolites manifest as positive.

As a result of this work, a procedure is proposed for the *in vitro* establishment of the *D. coriacea* species, which could be implemented for other species and will also permit the design of culture media for the multiplication, pretransplant and microtuberization stages.

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