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Quantitative standardization of pharmacologically active components from antiplasmodial plant *Solanum nudum* Dunal (wild and *in vitro*)

[Estandarización cuantitativa de componentes farmacológicamente activos de la planta antiplasmodial *Solanum nudum* Dunal (silvestre e *in vitro*)

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Abstract: *Solanum nudum* Dunal (Solanaceae) is most commonly known and used by the population of the colombian Pacific coast as an antimalarial treatment. This article study into optimization and quantitative analysis of compounds steroidal over time of development of this species when grown *in vitro* and wild. A new steroidal compound named SN6 was elucidated by NMR and a new method of quantification of seven steroidal compounds (Diosgenone DONA and six steroids SNs) using HPLC-DAD-MS in extracts of cultures *in vitro* and wild was investigated. Biology activity of extracts was found to a range of antiplasmodial activity in FCB2 and NF-54 with inhibitory concentration (IC_{50}) between (17.04 -100 µg/mL) and cytotoxicity in U-937 of CC_{50} (7.18 -104.7 µg/mL). This method creates the basis for the detection of seven sterols antiplasmodial present in extracts from *S. nudum* plant as a quality parameter in the control and expression of phytochemicals.

Keywords: Antimalarial; *Solanum nudum*; Micropropagation *in vitro*; Phytochemicals; HPLC-DAD-MS.

Resumen: *Solanum nudum* Dunal (Solanaceae) es el más conocido y utilizado por la población de la costa del Pacífico colombiano como tratamiento antipalúdico. Este artículo estudia la optimización y el análisis cuantitativo de compuestos esteroides a lo largo del tiempo de desarrollo de esta especie cuando se cultiva *in vitro* y en forma silvestre. Un nuevo compuesto esteroideo llamado SN6 fue dilucidado por RMN y se investigó un nuevo método de cuantificación de siete compuestos esteroides (Diosgenone DONA y seis esteroides SN) usando HPLC-DAD-MS en extractos de cultivos *in vitro* y silvestres. La actividad biológica de los extractos se encontró en un rango de actividad antiplasmodial en FCB2 y NF-54 con concentración inhibitoria (IC_{50}) entre (17.04 -100 µg/mL) y citotoxicidad en U-937 de CC_{50} (7.18 - 104.7 µg/mL). Este método crea la base para la detección de siete esteroles antiplasmodiales presentes en extractos de planta de *S. nudum* como parámetro de calidad en el control y expresión de fitoquímicos.

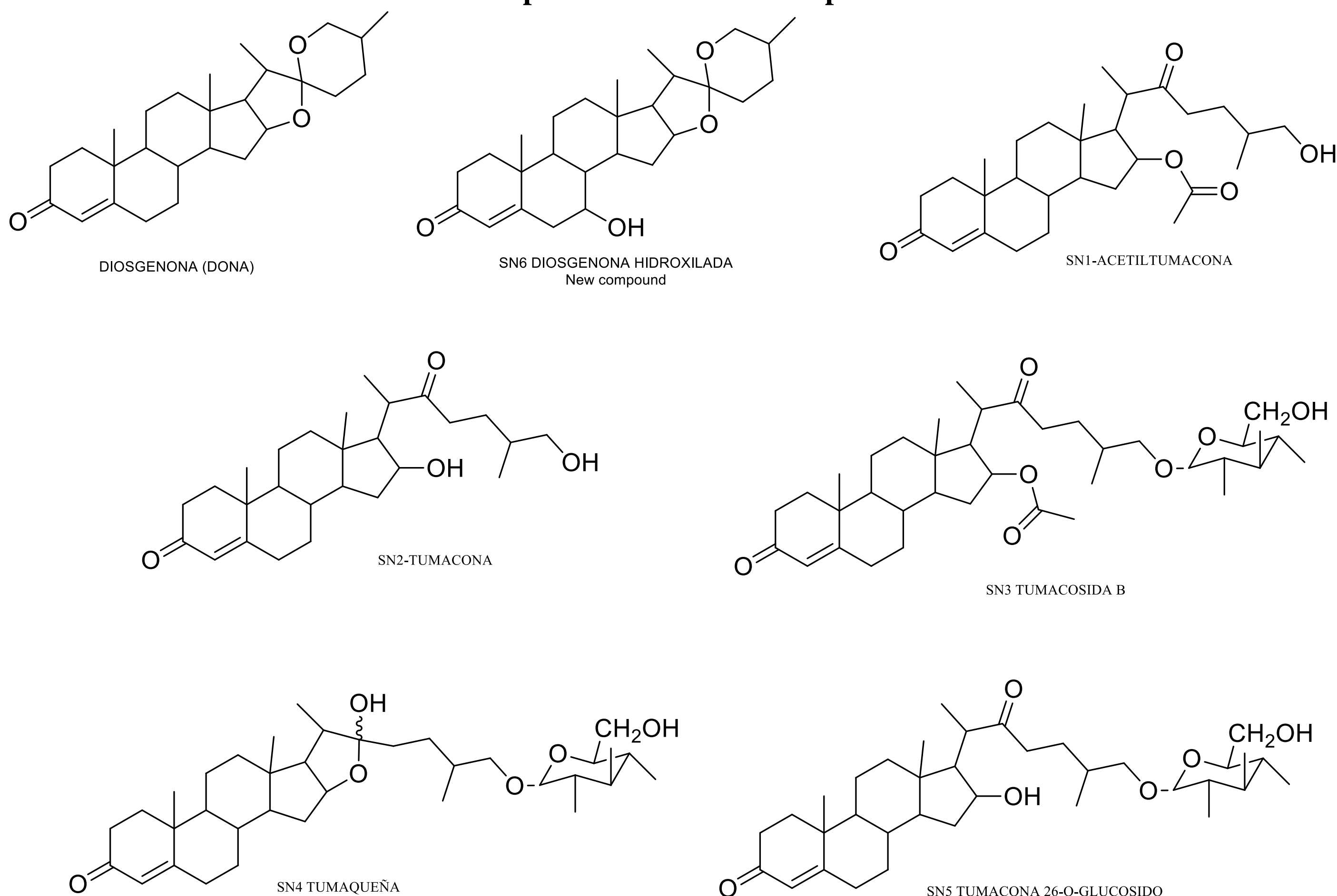
Palabras clave: Antipalúdico; *Solanum nudum*; Micropropagación *in vitro*; Fitoquímicos; HPLC-DAD-MS.

INTRODUCTION

The importance of medicinal plants is now more evident in developing countries (Estupiñán *et al*., 2010). World Health Organization (WHO) encourages, recommends and promotes traditional/herbal remedies in national health care programmes because herbal remedies are easily available at low cost, safe and people have faith in them (Pandey & Tripathi, 2014; INS, 2016). Phytotherapy use has increased in this decade, it consists of using the whole plant to treat illness (Ong, 2004). The new trends in the research of medicinal plants focus on the standardization and quantification of active extracts with specialized processes to obtain pharmaceutical grade extracts from complete plants or specific parts (flowers, leaves, bark, roots, etc.) (Paniego & Giulietti, 1998) However, the active compounds production and their yield can be affected by environmental conditions such as climate, season and soil composition, limiting their exploitation.

Solanum nudum Dunal (Solanaceae) is a plant used in traditional medicine in the Municipality of Tumaco (Department of Nariño, Colombia) where malaria is endemic. It has been reported that malaria cases increased in the country a situation related to the resistance appearance *in vitro* of *Plasmodium falciparum* to multiple drugs (Blair & Madrigal, 2005). Solanum genus has abundant steroidal compounds. Six molecules, five steroids and one sapogenin (diosgenone) have been isolated from *S. nudum,* with a 4-in-3-ona system as a molecular characteristic (Sáez *et al*., 1998; Cadena-González *et al*., 2013;). Pabon *et al*. (2002), reported its antiplasmodial activity, showing its no mutagenic, it's no clastogenic or toxic against mammalian cell lines (Pabón *et al*., 2002; Alvarez *et al*., 2004; Arango *et al*., 2006). Chemical structures corresponding to steroids isolated and evaluated for their antiplasmodial potential are presented in Figure No. 1.

Figure No. 1
SNs compound of the *S. nudum* plant



For other hand, *in vitro* cultivations along with the method for metabolite quantification allows to establish specific characteristics suitable for commercial crops for this new and potential therapeutic alternative for malaria (Guo *et al*., 2006). In addition to serving as a model for phytochemical studies and as a source of plant material for the genetic improvement of this species through biotechnological methods (Paniego & Giulietti, 1998; David *et al*., 2015). *In vitro* propagated medicinal plants create an immediate source of uniform, sterile and compatible plant material for chemical extraction and characterization (Bernal *et al*., 2001; Lapornik *et al*., 2005). The chemical analysis of *in vitro* cultivated *S. nudum* extracts plays a central role in the development and modernization of this traditional antimalarial herb in Colombia.

However, there aren't studies qualitative and quantitative studies on the composition and expression in plant development *in vitro* of this steroidal compounds and relationship with biological activity. In this context, the objective in this study was to validate the presence and quantify the secondary metabolites with the 4-in-3-ona system of plants wild and *in vitro* from *S. nudum*, and to compare their antimalarial activity with the purpose to obtain uniform plant material free of microbial contamination for production pharmaceutical.

MATERIAL AND METHODS

Collection of plant materials

Seeds and leaves from wild *S. nudum* were collected in Robles and Inguapí del Guadual villages (Latitude: 1.7°42'7"; Longitude: -78.6°41'46") in 2008 (Municipality of Tumaco, Department of Nariño, Colombia). The taxonomic identification was carried out in the Herbarium of the University of Antioquia (HUA), voucher number (162465).

In vitro culture

The *in vitro* micropropagation protocol of the *S. nudum* plant was followed according to Suarez *et al*. (2011). The seeds were extracted and disinfected by washing with surgical soap (Quirucidal®), followed by a fungicide immersion (Colimyl®) 2g/L for 2 hours and then in sodium hypochlorite (NaOCl 0.25%). After each step, the seeds were rinsed 3 times with sterile distilled water and later taken to a culture medium. The manipulation of the vegetal material was carried out in a horizontal laminar flow chamber.

Induction and development of organogenic shoots

In vitro and greenhouse cultures were established from plant seeds. *S. nudum* seedlings germinated from greenhouse and *in vitro* micropropagated conditions were obtained and followed at 3, 6, 9 and 12 months. The vegetal material was extracted in ethanol and rotaevaporated to dryness. All extracts obtained were performed on an Agilent Technology 1200 series (Agilent Technologies, USA) HPLC-DAD-MS system for the identification of the SNs steroidal metabolites.

Marking substances

The isolation and characterization of steroidal compounds from extracts of wild *S. nudum* was carried out according to the methodology described by Sáez *et al*. (1998). The steroidal compounds obtained and evaluated by HPLC-DAD-MS were colest-4-ene-3, 22-dione tumacona A (SN1) ($C_{29}H_{44}O_5$), tumacona B (SN2) ($C_{27}H_{42}O_4$), tumacoside A (SN3) ($C_{35}H_{54}O_{10}$), tumacoside B (SN4) ($C_{33}H_{52}O_9$) and diosgenone ($C_{27}H_{40}O_3$). Additionally, a new molecule was isolated, corroborated by spectroscopic techniques, corresponding to a similar molecule with the characteristic system 4-en-3-one. The new compound was called diosgenone B SN6 ($C_{27}H_{41}O_4$). All the compounds were characterized by spectroscopic techniques (^{1}H NMR and ^{13}C NMR) using a Bruker spectrometer (300 and 250 MHz for ^{1}H), 75 MHz for ^{13}C and dissolved in deuterated chloroform ($CDCl_3$) and deuterated methanol (MeOD). The new molecule is show in Figure No. 1.

Spectroscopic data of diosgenone B (SN6)

White amorphous solid. $C_{27}H_{41}O_4$. **^{13}C-NMR-75MHz δ ($CDCl_3$):** 199.42, 171.00, 123.90, 103.40, 76.50, 73.4, 62.20, 53.50, 52.90, 48.9, 38.60, 39.2, 38.60, 35.70, 35.20, 33.90, 32.70, 32.10, 31.89, 30,1, 29.75, 28.6, 20.66, 17.30, 16.60, 16.44, 13.53.

Chromatographic method

Standardization of the analytical method and quantification was performed in high-efficiency liquid chromatography unit coupled to a diode array detector and mass spectrometer (HPLC-DAD-MS Agilent Technology 1200 series). The chromatography system consisted of a quaternary pump with degasser G1354A, autosampler G1313A, column compartment G1316A, UV/Visible detector with diode array and the ChemStation Software 32bit G2170BA HPLC 2D. A Luna Phenomenex column

was used, with a particle size of 5μ, C18 100Å and 250 x 4.60mm, series 564054-31. The solvents used for the separation were water as solvent (A) and acetonitrile as a HPLC grade solvent (B). The injection volume and the flow rate were 20 μL and 1.0 mL/min, respectively. Elusion of the analysis was programmed following the gradient of solvents at a flow rate of 1.0 mL/min of 65% A and 35% B up to minute 10, then 20% A and 80% B up to minute 20 and finally with 10% A and 90% of B for a total 40 minutes. A chemical ionization at atmospheric pressure (APCI) unit, in positive mode, with quadrupole detector was used. The vaporization temperature was 450ºC and the voltage was 3000 V for the capillary. The identification of the marking substance was carried out by comparing the retention time with the corresponding standard. For the total ionic current (TIC), the relative abundance of the molecular ions was observed, and the major peaks were selected as the molecular ion [M + 1]. The total time of the analysis was 40 min. The reading was performed at a wavelength of λ = 254 nm. Compounds were identified by comparison of their retention times (t_R) and relative masses according to Table No. 1. Extracts of *S. nudum* were analyzed to perform quantification in percentage (w/w). Standards and extracts were dissolved in an acetonitrile-water solution (1:1) and filtered by Nylon membrane 0.45 μm (hydrophilic) DISMIC-13 NP; 0.39 MPa ADVANTEC before the injection. 20 μL of the standards and of each extract was injected in triplicate. A relative standard deviation for these five injections was below by 2.0%

Table No. 1
Data of retention times and molecular ions of the marker substances in the extract of *S. nudum*

Marking substance in S. nudum extract	*Retention times (t_{R*})(min)*	*Molecular ions [M+1]*
SN1 ($C_{29}H_{44}O_5$)	16,34	473,0
SN2 ($C_{27}H_{42}O_4$)	15,24	431,0
SN3 ($C_{35}H_{54}O_{10}$)	13,35	635,4
SN4 ($C_{33}H_{52}O_9$)	8,48	593,3
SN5 ($C_{33}H_{52}O_9$)	7,38	593,3
SN6 ($C_{27}H_{41}O_4$)	19,02	430,3
DONA ($C_{27}H_{40}O_3$)	33,36	413,3

$*t_R$ = time retention

Evaluation of antiplasmodial activity
In vitro culture of Plasmodium falciparum strains

Antiplasmodial activity assays were performed on the chloroquine-sensitive (NF-54) and the chloroquine resistant (FCB2) *P. falciparum* strains. The samples were cultured and maintained according to the method of Trager & Jensen, (1976); using a suspension of 5% human type A+ erythrocytes in a RPMI-1640 culture medium (Sigma R6504) dissolved in sterile water with 25 mM HEPES, 5.0% $NaHCO_3$, 10% fresh human serum type A+ (inactivated at 56°C for 30 minutes); incubated in an 5% O_2, 5% CO_2 and 90% N_2 atmosphere. The culture media were changed daily and fresh red blood cells were added twice a week (Trager & Jensen, 1976).

In vitro antiplasmodial activity by the SYBR Green I® method

For the evaluation of *in vitro* antiplasmodial activity the methodology described by Smilkstein *et al.* (2004), was adapted. The tests were carried out in 96-well Falcon® plates (flat bottom). A suspension of parasitized red blood cells was prepared with a hematocrit of 2.5% and a parasitemia of 1%. The culture with the treatments and the positive chloroquine (CQ) control were incubated at 37°C for 48 hours in a 5% CO_2, 5% O_2 and 90% N_2 atmosphere. Subsequently, the content of each well was transferred to Greiner Pro one dishes and the parasites were marked with a solution of SYBR® Green I 2X in lysis buffer. The plates were incubated

in the dark and the relative fluorescence units (RFU) were read in a spectrofluorometer at an excitation and emission wavelengths of 485 nm and 538 nm respectively. Treatments from each crude extract were prepared to a standard solution of 10 mg/mL in DMSO. From this solution, 50 µL were taken and adjusted to 1000µL with a complete RPMI-1640 medium, obtaining a final concentration of 0.5 mg/mL. Seven concentrations of each extract were evaluated in a range between 100 -1.56 µg/mL, each concentration was evaluated in duplicate in the dish and three independent tests were carried out. The chloroquine CQ control was evaluated in a range between 150-4.7 nM and the control of the Peruvian quine extract (MeOH: H_2O, 70:30) was evaluated in a range of 0.01-10 µg/mL. Data from three trials were analyzed to find the half maximal inhibitory concentration in µg/mL (IC_{50}). A sigmoidal concentration-response curve with Hill slope (variable slope). The data was analyzed and plotted using GraphPad Prism 4 for Macintosh, version 4.0b that yields the adjustment value (r). (GraphPad Software, San Diego, California, USA). Consensus for the evaluated extracts was: highly active <5 µg/mL, promising 6-15 µg/mL, moderate activity 16-30 µg/mL, low activity 31-50 µg/mL and inactive> 50 µg/mL (Muñoz *et al*., 2000; Fidock *et al*., 2004; Jonville *et al*., 2008; Malebo *et al*., 2009).

Tests of cytotoxic activity in vitro

To evaluate the cytotoxic activity of the extracts, the method of 3- (4,5-dimethylthiazol-2-yl) -2,5-diphenyl tetrazolium bromide (MTT) was used according to Mosmann (1983), which reveals cellular damage at the mitochondrial level.

Cytotoxicity in cell line U-937

U-937 cells are promonocytes of human histiocytic lymphoma, which were maintained in continuous cultures in the Malaria Group Laboratory. These cells were cultured at 37°C and 5% CO_2 in a RPMI medium supplemented with 10% inactivated Fetal Bovine Serum (SBF). The culture media were changed every 48 hours or according to medium pH changes, with centrifugation for 10 minutes at 1000 rpm (Moore *et al*., 1967). Cells from the U-937 line were counted in a Neubauer chamber and plated in 96-well flat bottom plates, 200,000 cells/mL in RPMI 1640 medium with 10% FBS, incubated at 37°C with 5% CO_2 for 72 hours in the presence of each of the seven concentrations of each extract or compound evaluated in a range between 100 -1.56 µg/mL, each concentration was evaluated in duplicate on the plate and three independent trials were conducted. Subsequently, the activity of mitochondrial dehydrogenase was measured, adding 20 µL/well of MTT to a concentration of 5 mg/mL and incubating for 3 hours at 37°C and 5% CO_2. To dissolve the crystals formed, 100 µL/well of a solution of 50% isopropanol and 10% SDS were added and the absorbance was read at 595 nm in an ELISA reader (BioRad). The data obtained from three independent tests was analyzed with the GraphPad Prism 5 program to find the toxic concentration in µg/mL (CC_{50}) using a non-linear logistic regression model. Consensus for the samples evaluated was: highly toxic <10 µg/mL, cytotoxic 10-40 µg/mL, moderately cytotoxic 41-100 µg/mL and non cytotoxic> 100 µg/mL. Additionally, the selectivity index (IS) was calculated, which indicates selectivity towards the parasite and corresponds to the relationship between the cytotoxic activity (CC_{50}) and antiplasmodial activity (IC_{50}) (Reed *et al*., 2002; Koch *et al*., 2005; Kaou *et al*., 2008).

RESULTS AND DISCUSSION

At present due to advancement in the chemical knowledge of active extracts from plants, various methods like botanical, chemical, spectroscopic and biological methods are used for estimating active constituents present in the crude extracts. The protocol established by Suarez *et al*. (2011), was adopted in this work and a follow-up protocol was established for the induction of organogenic sprouts, quality the phenotypic and the phytochemical stability of the reproduced material to establish a constant source for compounds extraction assays and biological tests in addition to facilitating their conservation both ex situ and in situ (Pandey & Tripathi, 2014). An important example of antimalarial plants is *Artemisia annua*.

The chromatographic profile of wild *S. nudum* ethanolic extracts is shown in Figure No. 2 and mass spectrum of SN3 and SN4 example compounds in Figure No. 3. The good resolution of the 7 marker substances obtained with this method allowed to identify the ion and quantifying each substance amount within the mix discriminating other components present in a complex sample such as an extract. Table No. 1 reports the retention times of the 7 sterols in the extract and the different molecular ions corresponding to [M+1] and content expressed as percentages of compounds shown in Figure No. 4.

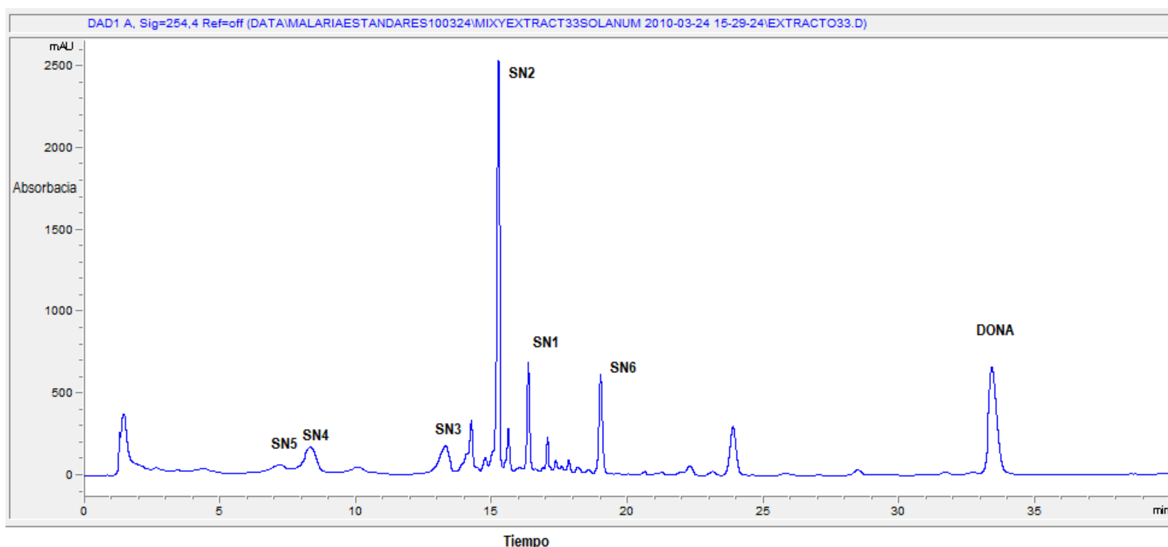


Figure No. 2
Chromatographic profile of the extract of the wild *S. nudum* plant

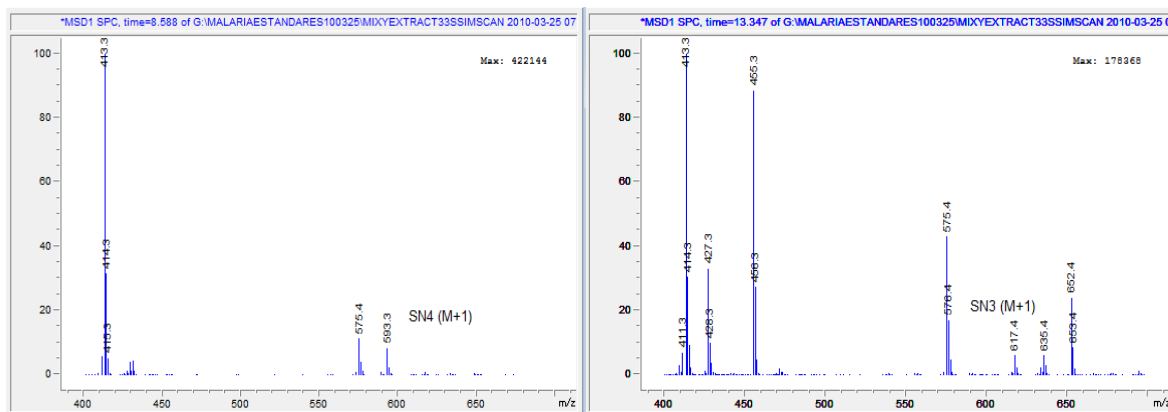


Figure No. 3
Mass spectrum of the compound *SN4 y SN3 to* t_{Rsn4} = 8,48 y t_{Rsn3} = 13,35

Figure No. 4

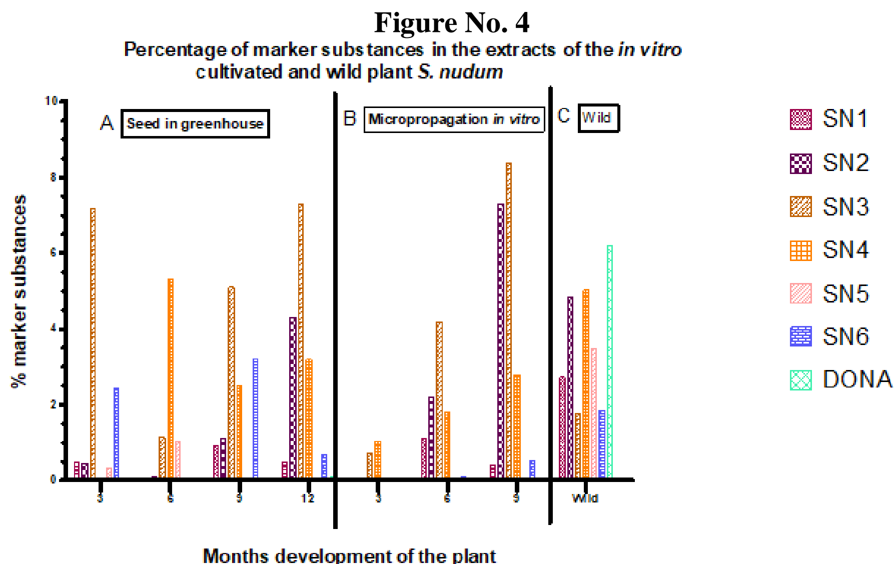


Figure No. 4

(A) Content in percentage marker substances SNs in extracts obtain from seed in greenhouse of *S. nudum* quantificated until 12 month; (B) Content in percentage marker substances SNs in extract obtain from plants of micropropagation *in vitro* quantificated until 9 month; (C) Content in percentage marker substances SNs in extracts obtain fron planta wild adult no quantificated month

Table No. 2

Percentage of marker substances in the extracts of the *in vitro* cultivated and wild plant obtained from plant material *in vitro* and planted in the greenhouse

	Months	SN1	SN2	SN3	SN4	SN5	SN6	DONA	Strain of *P. falciparum* NF-54**	Strain of *P. falciparum* FCB2**	Cellular Line U-937
Seed in greenhouse	3	0.48	0.46	7.16	ND	0.35	2.45	---	24.32	31.65	104.7
	6	---	0.10	1.14	5.31	1.02	---	---	24.14	26.55	14.7
	9	0.90	1.10	5.10	2.5	0.001	3.2	0.05	67.87	100	87.23
	12	0.50	4.30	7.30	3.2	---	0.7	0.08	41.87	73.38	39.85
Micropropagation *in vitro*	3	0.01	0.03	0.72	1.04	---	0.07	---	48.72	49.64	12.3
	6	1.09	2.20	4.20	1.8	0.02	0.11	0.06	66.93	79.98	93.73
	9	0.42	7.30	8.40	2.8	---	0.52	0.06	52.48	68.32	34.47
Wild	ND	2.73	4.86	1.79	5.03	3.5	1.85	6.22	17.04	17.52	7.18

ND = Not determined
--- = not observed in the detection limits
*** X (Medium) ± SD (Standard deviation)**
****Chloroquine positive control IC_{50} = 26,10 nM strain NF-54; Chloroquine IC_{50} = 271,2 nM strain FCB2**
******vehicle DMSO at 5%: no active strain of *P. falciparum* and celluar line U-937**

This is a new method with high sensitivity that allows to detect the molecular ions of the 7 steroidal compounds (DONA and SNs) in the extract by means of HPLC-DAD-MS. Validation parameters were checked with desirable acceptance criteria such as linearity (r^2 = 0.9999), accuracy (R = 90.25 – 101.82%), precision (CV = 0.45 to 4.87%), limits of detection and quantification (0.001 to 8.40%). Phytochemical analysis of the percentage of marker compounds of interest and reports of their antiplasmodial and cytotoxic activity are shown in Table No. 2. The extract of the wild plant shows the presence of all the SNs compounds, DONA being the most abundant (6.22%) noting that it is expressed in very low concentrations in the first stages of development of the plant.

The seedlings showed morphological characteristics similar to those of the parent plants; the presence of compounds SN1, SN2, SN3, SN4 and SN6 was observed in all the extracts obtained from the seedlings propagated *in vitro* as well as a low concentration of SN5 and DONA. The presence of compounds SN2, SN3, SN4 and some DONA was observed in the extracts of plants germinated in the greenhouse, SN5 and SN6 decreased their concentration. SN1 showed a constant concentration over time and was absent at 6 months.

The extracts were found to have moderate antiplasmodial activity over FCB2 and NF-54 strains (IC_{50} between 17.04 -100 μg/mL) and cytotoxicity on the U-937 cell line (CC_{50} between 18 -104.7 μg/mL). The most active extract, obtained at month 3, had antiplasmodial activity expressed by $IC_{50\ NF\text{-}54}$ = 24.32 μg/mL, $IC_{50\ FCB2}$ = 31.65 μg/mL and no cytotoxic activity $CC_{50\ U\text{-}937}$=104.7 μg/mL whit sterol % of SN1 = 0.48, SN2 = 0.46, SN3 = 7.16, SN5 = 0.35, SN6 = 2.45, without DONA or SN4. In this study, the presence of DONA in the plant was shown to increase its toxicity in U-937 cells, therefore it was the wild plant extracts' higher amounts of DONA could be associated with a more toxic effect and not their antiplasmodial activity.

The basic parameters influencing the quality of an extract are plant part used, solvent and procedure for extraction and this depends the variations in different extraction methods that will affect quantity and secondary metabolite composition of an extract in addition to activity biological and cytotoxicity. For example, Sholikhah & Wijayanti (2014), reported the obtaining standardized extract of *Eurycoma longifolia* jack. root with high therapeutic index which indicated that standardized extract has low toxicity and *in vivo* antiplasmodial activity (Nardos & Makonnen, 2017). Likewise, Lucantoni *et al*. (2010), evaluated transmission blocking activity of a standardized neem (*Azadirachta indica*) seed extract on the rodent malaria parasite *Plasmodium berghei* in its vector *Anopheles stephensi* quantify compounds in the extract and their relationship with activity biological. Likewise, Mesa *et al*. (2019), reported chemical constituents and standardization of *Piper piedecuestanum* Trel & Yunck. with antiplasmodial and cytotoxic activity as a selection criterion to choose an active extract. This the development of analytical method and the biological standardization for extracts enable fulfills the biological specifications to choosen active extracts and evaluate them in future studies of the therapeutic efficacy in murine models.

In this work we report a simple, fast and reliable method of phytochemical analysis that will help control the composition and stability of a phytotherapeutic product from this species, providing a greater knowledge about the therapeutic effects of this medicinal plant. This new method of quantification creates the basis for the detection of sterols present in *S. nudum* extracts as a quality parameter in the phytochemical control of extracts obtained from different selections for production pharmaceutical. It also provides foundation for future studies of pharmacokinetics and pharmacodynamics through the monitoring of small amounts of these substances in animal models given the high sensitivity and specificity of the technique.

CONCLUSION

This analytical methodology permits the identification of the bioactive compounds SN1, SN2, SN3, SN4, SN5, SN6 and DONA in the extracts of the *in vitro* cultivated *S. nudum* plants with samples obtained from the first stages of development, using high resolution chromatography coupled to mass spectrometry. This method showed variations in metabolite concentration thought the different developmental stages of the plant. It is recommended to continue monitoring these compounds over time in order to design a model optimizing the expression of compounds and their concentration compared to their biological potential.

This work is the first study reported about the establishment of a model for studies of physiological and phytochemical type of the *S. nudum* plant, as well as allowing the propagation of new agronomically desired characteristics for Solanaceae

commercial crops of elite material with stable and desired characteristics. In addition, it sets the basis for creating a complement to biotechnological improvement tools such as genetic transformation, guaranteeing both the presence of bioactive compounds, effective against *Plasmodium* and industrial scalability with low cost extraction methods for the production of phytotherapeutic preparations derived from *S. nudum*.

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