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Phytochemical Screening, Secondary Metabolites Total Contents and *In vitro* Total Antioxidant Capacity of Extracts of *Boerhavia diffusa* Linn Growing in Kinshasa (DR Congo)

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ABSTRACT

Criblage Phytochimique, Contenu Total des Métabolites Secondaires et Capacité Antioxydante Totale *in vitro* des Extraits de *Boerhavia diffusa* Linn Poussant à Kinshasa (RD Congo).

Le criblage phytochimique et l'estimation quantitative des métabolites secondaires des extraits des feuilles, des tiges et des racines de *Boerhavia diffusa* Linn de la RD Congo ont été effectués et la capacité antioxydante totale correspondante évaluée. Les extraits au dichlorométhane (DCM), au méthanol (MeOH), au mélange dichlorométhane-méthanol (MeOH/DCM) et de l'eau ont été préparés. Le criblage phytochimique a révélé une large gamme de métabolites secondaires et lors de l'extraction, les racines ont fourni un rendement le plus élevé, suivi des tiges et ensuite des feuilles. Quantitativement, les polyphénols ont été les métabolites secondaires majoritaires (15,54 ± 3,42~62,42 ± 3,25) µgAG/g dans DCM, MeOH et MeOH/DCM suivis par les flavonoïdes (8,23 ± 0,33~15,72 ± 0,28) µg QE/g dans l'eau. Les tannins et les alcaloïdes sont considérables dans les systèmes de solvants DCM, méthanol et mélange dichlorométhane-méthanol. Les concentrations les plus élevées ont été observées dans les extraits de la tige. Les extraits de feuilles ont présenté la capacité antioxydante totale la plus élevée. Ceci justifie l'utilisation de la plante en médecine traditionnelle pour traiter les affections liées à l'oxydation.

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INTRODUCTION

Plants and plant-based drugs are the basis for the majority modern pharmaceuticals used for different ailments. Pharmacotherapy started with the use of plant in the treatment of various diseases [GAUTAM et *al.*, 2016]. In Africa, it is known that about 80 % of the population relies on plants for healing purposes [BURTON et *al.*, 2015].

The exploration of chemical, pharmacological and phytochemical screening of the plant extracts would provide a basis for the development of the new lead molecules for the discovery of drugs. In addition, knowledge about the chemical constituents would be valuable in discovering the actual value of folkloric remedies [GAUTAM et *al.*, 2016; NGOUPAYO et *al.*, 2016].

The Democratic Republic of the Congo (DRC) is reputed for the extraordinary richness of its biodiversity and possesses a wide variety of plant species of ethno-medicinal relevance. These plant species represent an enormous reservoir of secondary metabolites with biopharmaceutical potential for modern

industries [TSHILANDA et *al.*, 2015; 2016]. One of these plants growing in Kinshasa not phytochemically studied so far is *Boerhavia diffusa* Linn.

Boerhavia genus is a set of 40 tropical and subtropical species from Asia, Africa, America and Australia. *Boerhavia diffusa L.* is one of species of *Nyctaginaceae* family named after Hermann Boerhaave, a famous Dutch physician of the 18th century and is characterized by its cotyledons and opposite leaves of different sizes. The plant has a long history of uses by the indigenous and in traditional medicine [APU et *al.,* 2012; MISHRA et *al.*, 2014].

The literature reported that in India and Nepal, *B. diffusa* is used in traditional medicine for the treatment of diabetes, stress, dyspepsia (dyspepsia), abdominal pain, inflammation and jaundice, enlargement of spleen, heart diseases, bacterial infections and impotence [MALHOTRA et *al.*, 2013]. In Nigerian folk medicine, this plant species is widely used for the treatment of epilepsy, infertility and menstrual pain [APU et *al.*, 2012]. In Democratic Republic of Congo (DR Congo) the plant is used in traditional medicine. A decoction of the leaves is used in DR Congo to treat gonorrhea and to calm pain [MUZILA, 2006]. The roots are used in particular in Kongo Central as antidote of snake bites as well as aphrodisiac [LATHAM et KONDA, 2007].

Pharmacological studies have demonstrated that *B. diffusa* is known to possess anticonvulsant, diuretic, anti-inflammatory, antifibrinolytic, antibacterial, anti-hepatotoxic, anthelmintic, febrifuge, anti-leprosy, antiasthmatic, antiurethritis, antilymphoproliferative, antimetastatic, immunosuppressive, antidiabetic, antioxidant, immune-modulation, hepatoprotective, anti-nociceptive, nephroprotective, bacteria induced ulcer and diarrhea and antiurolithiatic activities [GAUTAM et *al.*, 2016].

In addition *B. diffusa* is known to contain numerous phytochemicals that include flavonoids, alkaloids, triterpenoids, steroids, lipids, lignins, tannins, phlobaphenes [MALHOTRA et *al.*, 2013]. Rotenoids are reported to be the most encountered compounds in this species [MUZILA, 2006]. However, to the best of our knowledge the chemical composition of *B. diffusa* growing in DR Congo has not yet been fully chemically characterized and its antioxidant activity has not been studied so far. Since a long time, rotenoids are used as fishing poisons in many countries [BAIRWA et *al.*, 2013]. Searching to use DR Congo's biodiversity for the benefit of the local populations, this study was initiated. Therefore, the aims were to conduct phytochemical screening, to estimate secondary metabolites amounts for standardizing purposes and to evaluate *in-vitro* antioxidant potential of different parts of *B. diffusa* growing in Kinshasa, DR Congo.

MATERIAL AND METHODS

Plant material

B. diffusa plants used in this study were harvested in three sites in Kinshasa: Ecole de Navigation (Kauka area in the commune of Kalamu), Archidiocèse de Kinshasa (Funa area in the commune of Limete) and in the bush of Kinkole (Kinkole area in the commune of the N'sele) in Democratic Republic of the Congo on September 2020. They were authenticated at Herbarium of INERA (Institut National d'Etudes et Recherches Agronomiques), Voucher specimen herbal 320 was deposited at the Herbarium, Faculty of Sciences, University of Kinshasa. Plants were mixed and dried in the shade. Before grinding and submitted to extraction, different plant parts (leaves, stems and roots) were separated and grounded separately to powder at room temperature. After the grinding process of different parts ,we obtained respectively 210 grams of stem powder, 210 grams of root powder, and 310 grams of leaf powder.

Extraction and chemical screening

As previously reported [TSHIBANGU et *al.*, 2016], 100 grams of stems, 100 grams of roots and 150 grams of leaves of each powder were repeatedly submitted to decreasing polarity extraction by cold percolation with methanol (MeOH) (700 mL×2) for 48 hours and dichloromethane (DCM) (1000 mL×2) for 48 hours. In addition, a mixture of MeOH/DCM (1/1) was also used for other samples powders. Chemical screening was performed on these organic extracts and an aqueous extract (10g of leaves, stems, roots and the whole plants powders in distilled water (W) (100 mL x 2) for 48 hours in order to obtain organic and aqueous extracts, respectively) to investigate the presence of alkaloids (Dragendorff's test), saponins (Frothing test), total polyphenols (Burton's test), flavonoïds (Shinoda's test), tannins ($FeCl_3$ test), anthocyanins (HCl test), leuco-anthocyanins (Shinoda's test), quinones (Borntrager's test) and triterpenes (Lieberman's test) according to the standard protocol [APU et *al.*, 2012].

Quantitative estimation of secondary metabolites

Total contents of polyphenols, flavonoïds, tannins and alkaloïds of the differents extracts of *B. diffusa* were determined spectrophotometrically using respectively the Folin-ciocalteu, aluminum chloride, Folin-ciocalteu and Fazel methods with some slight modifications [VIJAY et *al.*, 2014; BENAHMED-BOUHAFSOUN et *al.*, 2015; TSHIBANGU et *al.*, 2016] using GENESYS 10S spectrophotometer. The secondary metabolites were quantified by calibration curves obtained by measuring the absorbance of known concentrations of gallic acid equivalent (GAE) standard solutions (10-150 µg/g of GAE in 80% MeOH) for polyphenols at 765 nm [BENAHMED-BOUHAFSOUN, 2015], quecertin equivalents (QE) standard solution (5-100 µg/g of QE) for flavonoïds at 510 nm [TSHIBANGU et *al.*, 2016], tannic acid equivalent (TAE) standard solution for tannins at 725 nm [VIJAY

et *al.*, 2014] and atropine (AT) standard solution, for alkaloids at 470 nm [VIJAY et *al.*, 2014], respectively. Calibration curves were plotted as variation of absorbance versus concentration of standard and Beer's law was followed over the concentration range of standards.

Content estimations

Polyphenols

Plant extract (200 µL) was mixed with 1 mL of Folin-Ciocalteu reagent previously diluted 10 times with twice distilled water and 0.8 mL of sodium bicarbonate solution (7.5% Na_2CO_3). The mixture was incubated during 30 min at room temperature; the absorbance was measured at 765 nm. The results were calculated as µg gallic acid equivalent (GAE) per one gram dry powder and reported as mean value ± standard deviation (SD) (the standard curve equation:

Y= 0,006x-0,002 ; (R^2 = 0,997) [SINGLETON et ROSSI, 1965; MILICA et *al*, 2012].

Flavonoïds

Aliquot (100 µL) of each plant extract was added to 10 mL volumetric flask containing 4 mL of twice distilled water. Then 0.3 mL of 5% $NaNO_2$ was added to the flask and after 5 min, 0.02 mL of 10% $AlCl_3$ was also added. At 6th min, 2 mL of Na_2CO_3 (1 M) was added and the total volume was made up to 10 mL with double distilled water. The solution was mixed completely and the absorbance was measured versus prepared reagent blank at 510 nm. Total flavonoid content was expressed as µg quecertin equivalents (QE) per one gram dry powder. One mL of standard solution (quecertin: 5-100 µg/mL) was used to construct calibration curve (the standard curve equation: Y = 0.009x + 0.006; (R^2 = 0,999) [NABILA et NASSIMA., 2013].

Tannins

Plant extract (1 mL) was added to 7.5 mL of distilled water and 0.5 mL of Folin-Ciocalteu reagent and 1 mL of sodium carbonate (Na_2CO_3 35%). The absorbance was measured at the wavelength of 725 nm. The tannins content (expressed as µg tannic acid equivalent per one gram dry powder, TAE) was calculated using the following relation: Y = 0,443x - 0,264; (R^2 = 0,720) [SCHANDERL, 1970].

Alkaloïds

The plant extract (1mL) was added to 1 mL of 2 N HCl. The pH of the solution is neutralized with 0.1 N NaOH. Bromocresol Green solution (5 mL) and phosphate buffer (5 mL, pH 4.7) previously prepared were added. The mixture was shaken and the complex formed is extracted with 1, 2, 3 and 4 mL chloroform. Finally, the extracts are collected in a 10 mL volumetric flask and diluted to adjust the volume with chloroform. The absorbance for test and standard solutions were determined against the reagent blank at 470 nm. The total alkaloids (expressed as µg atropine equivalent per one gram dry powder, ATE) was calculated using the following relation: Y = 0.005x - 0.002 (R^2 = 0.998) [FAZEL et *al*, 2008].

Total Antioxidant Capacity

Total antioxidant capacity is a quantitative method to evaluate the water and fat-soluble antioxidant capacity. It is based on the reduction of Mo(VI) to Mo(V) by the sample or extract and the measure of the absorbance of the bluish-green phosphate-Mo(V) complex subsequently formed at acid pH [MOHADJERANI, 2012].

An aliquot of the extract (0.3 mL) was added to reducing reagent previously prepared as follow: 3 mL of reagent solution (0.6 M sulphuric acid (5.35 g), 28 mM sodium phosphate (16.7 mL) and 4 mM ammonium molybdate (0.35 g) dissolved in 500 mL of distilled water). The mixture was incubated in a boiling water bath at 95°C for 90 min. Then, the samples were cooled to room temperature and the absorbance of the resultant solution was measured at 695 nm against the blank [PHATAK et *al.*, 2015]. Solution of a natural antioxidant, ascorbic acid (AA), a synthetic antioxidant, butylated hydroxytoluene (BHT) and the rotenone sample (Ro) provided by the American museum were also used as standard for comparison. After this analysis rotenones were predominant secondary metabolites in *B. diffusa* [BAIRWA et *al*, 2013; AVIELLO et *al*, 2011].

RESULTS AND DISCUSSION

Extraction and chemical screening

Table 1 shows the yield of extraction of total crude extracts from *B. diffusa* for the three targeted parts of vegetal materials: leaves (L), stems (S) and roots (R).

Table I: Extraction yields

Systems	Yields (%)		
	L	S	R
DCM	0,92	0,19	0,68
MeOH	2,19	3,72	6,32
MeOH/DCM	2,71	4,14	6,38

The results on the extraction yields from Table 1 show that roots contain much more extractable substances than stems, which contain more extractives than leaves. Indeed, the system MeOH/DCM (1:1) is a good extraction solvent for the three parts (R): 6.38%, (S): 4.14% and (L): 2.71% followed by MeOH alone (R): 6.31%, (S): 3.72% and (L): 2.19% and then DCM alone (R): 0.68%, (S): 0.19 and (L): 0.92%. This reveals that the abundant primary and secondary metabolites of *B. diffusa* pass easily through the polar solvents. Recently TSHILANDA et *al.*, 2016, carried out increasing polarity extraction of total crude extracts from *O.*

canum Sims and *O. basilicum* Linn. from DR Congo with MeOH and DCM as extraction solvents and reported a high extraction yields for polar solvent (MeOH) than the non-polar one (DCM) [TSHILANDA et *al.*, 2015; 2016]. The solvents used exhibited the similar behavior; MeOH produced a high yield than DCM. However, the system made of a mixture of the two solvents MeOH/DCM (1/1) yields more slightly superior than each solvent taken separately. Thus, chemical screening (Table 2) was carried out in order to have more insight about the phytochemicals extracted by each solvents and their mixture.

Table 2: Results of chemical screening

Metabolites	W			DCM			MeOH			MeOH/ DCM		
	L	S	R	L	S	R	L	S	R	L	S	R
Polyphenols	+	+	+	+	+	+	+	+	+	+	+	+
Flavonoids	+	+	+	-	-	-	-	-	-	-	-	-
Anthocyanins	-	-	-	-	-	-	-	-	-	-	-	-
Leucoanthocyanins	-	-	-	-	-	-	-	-	-	-	-	-
Tannins	+	+	+	+	+	-	+	+	+	+	+	+
Alkaloids	+	+	+	+	+	+	+	+	+	+	+	+
Saponins	+	+	+	-	-	-	-	-	-	-	-	-
Triterpenes	+	+	+	+	+	+	+	+	+	+	+	+
Quinones	+	+	+	-	-	-	-	-	-	-	-	-

Table 3: Total contents of secondary metabolites in crude extracts.

Parts	Extracts	Total contents			
		Polyphenols µgAG/g	Tannins µg ATQ/g	Flavonoids µg QE/g	Alkaloids µg AT/g
Leaves	W	ND	ND	10.33 ± 0.11	ND
	DCM	43.67 ± 0.84	1.22 ± 0.01	ND	1.36 ± 0.09
	MeOH	15.54 ± 3.42	0.85 ± 0.01	ND	0.63 ± 0.02
	MeOH/DCM	62.42 ± 3.25	2.82 ± 0.01	ND	1.37 ± 0.09
Stems	W	ND	ND	15.72 ± 0.28	ND
	DCM	32.58 ± 1.08	1.02 ± 0.01	ND	1.73 ± 0.04
	MeOH	28.67 ± 3.67	1.11 ± 0.01	ND	1.37 ± 0.08
	MeOH/DCM	49.50 ± 8.50	2.07 ± 0.01	ND	1.09 ± 0.02
Roots	W	ND	ND	8.23 ± 0.33	ND
	DCM	26.42 ± 1.59	0.99 ± 0.02	ND	0.92 ± 0.24
	MeOH	10.33 ± 0.17	0.78 ± 0.01	ND	0.41 ± 0.01
	MeOH/DCM	34.92 ± 3.59	1.76 ± 0.01	ND	1.01 ± 0.01

ND : not determined

These results from Table 2 show that all chemical groups are similar in aqueous and organic extracts, but polar flavonoids, saponins and quinones glycosides can be found only in the aqueous crude extract (W) while they are not in the organic extracts (DCM, MeOH and MeOH/DCM).

Total contents of secondary metabolites

Table 3 shows the results of total contents of four screened secondary metabolites (polyphenols, flavonoïds, tannins and alkaloïds). The results were calculated by calibration curves obtained by measuring the absorbance of the known concentrations as standard per 1 gram of dry powder.

Results on the total contents of *B. diffusa* secondary metabolites (Table 3) firstly show that, for leaves, polyphenols are concentrated in almost all solvents with high quantity on MeOH/DCM (62.42 ± 3.25) µgAG/g followed by DCM (43.67 ± 0.84) µgAG/g and then MeOH (15.54 ± 3.42) µgAG/g. Tannins are in high concentration in the MeOH/DCM µg ATQ/g systems (2.82 ± 0.01) and with almost slight equivalent quantities in DCM and

MeOH systems. Total contents of flavonoids were determined only in aqueous extract and is 10.33 ± 0.11 µg of quecertin per gram, while alkaloids are more concentrated equivalently in DCM and MeOH/DCM systems than in MeOH. These results reveal that the metabolites in the leaves BDL pass easily through the nonpolar solvents and the amount decrease when the polarity increases, except for alkaloids in methanol/dichloromethane mixture.

Secondly, for stems of *B. diffusa*, like for leaves, polyphenols concentration difference is in almost what all systems, except in MeOH/DCM system where the amount is higher than in DCM. Here also the total contents of polyphenols were not determined in water. Tannins contents were found to be similar in DCM, MeOH and MeOH/DCM systems. Flavonoïds content in aqueous extracts is 15.72 ± 0.28 µg of quecertin per gram, while those of alkaloids are higher in DCM, followed by MeOH and then MeOH/DCM system. One can notice that contrarily to the leaves, a high amount of alkaloids passes in non-polar solvent.

And ultimately for roots of *B. diffusa*, polyphenols are concentrated in MeOH/DCM system followed by DCM and then MeOH, tannins are concentrated in MeOH/DCM system and almost equivalent quantities were found in DCM and MeOH as for leaves. Total content of flavonoids in aqueous extract is the lowest among all the vegetal materials parts and is 8.23 ± 0.33 µg of quecertin per gram. Only DCM and MeOH/DCM system were found to be concentrated with alkaloids. It can be noticed that for polyphenols and tannins the concentrations decrease proportionally with the polarity, while for alkaloids the MeOH yielded a very small amount.

It can be noticed that BDL and many other plants that possess a high antioxidant potential, are known to have high total contents of polyphenols [TSHIBANGU and *al.*, 2016].

Antioxidant Potential of different extracts

Table 4 shows the total antioxidant capacity for each part of the vegetal materials (L, S and R) and Ascorbic acid as a standard (AA) and butylated hydroxytoluene (BHT) synthetic antioxidants, and pure Rotenone (Ro) an other antioxidant isolated and reported in BDL [AVIELLO et *al*, 2011].

It can be seen from the results of total antioxidant capacity from Table 4 that all extracts exhibited a lower total antioxidant capacity relative to that of AA and BHT, however, those activities are slightly close to the one of rotenone solution. This observation could be a confirmation of the presence of rotenoid compounds in the *B. diffusa* as encountered in the literature [BAIRWA et *al*, 2013]. Thus, these extracts can be used instead of rotenone in case of necessity like fishing poison.

$Y = 3.436x - 0.208$ ($R^2 = 0.950$)

By comparing the activities exhibited in the different parts of *B. diffusa*, there is a higher total antioxidant capacity in leaves

Table 4: Total Antioxidant Capacity of total crude extracts

	Extracts	Conc (mg AA/g)
AA	-	0.17 ± 0.01
BHT	-	0.18 ± 0.03
Ro	-	0.10 ± 0.01
	DCM	0.13 ± 0.01
Leaves	MeOH	0.08 ± 0.01
	MeOH/DCM	0.12 ± 0.01
	DCM	0.09 ± 0.00
Stems	MeOH	0.10± 0.01
	MeOH/DCM	0.10 ± 0.01
	DCM	0.07± 0.02
Roots	MeOH	0.13 ± 0.01
	MeOH/DCM	0.09 ± 0.03

followed by stems and then in roots. Indeed, leaves and steams showed high total contents of polyphenols known to possess high antioxidant capacity [NARENDER et *al.*, 2012; NABILA et NASSIMA, 2013]. The toxicity of B. *diffusa* extracts are under evaluation in our research team and will be the subject of a future publication.

CONCLUSION

Owing to its valuable importance, *Boerhavia diffusa L.* is used in phytotherapy in many countries and cultures. This study aimed to carry out phytochemical screening, quantitative evaluation of secondary metabolites and total antioxidant of *B. diffusa* growing in DR Congo. Different parts of the plant: leaves, stems and roots were screened for their phytochemicals and their total antioxidant capacities were evaluated.

Phytochemical screening provided a wide range of secondary metabolites and roots produced the highest yield of extraction. Quantitatively, polyphenols are the most abundant secondary metabolites, including flavonoids. Secondary metabolites have, in general, a highest affinity with MeOH/DCM system than DCM and MeOH meaning that screened metabolites are more apolar compounds though polar solvent system yield large crude extracts. The highest concentrations of secondary metabolites are observed in stems extracts while leaves extracts exhibited the highest total antioxidant capacity.

This plant is already known in phytotherapy in DR Congo traditional medicine. This study highlights that *Boerhavia diffusa L*. total content is rich in apolar polyphenols compounds and possesses an antioxidant potential similar to pure rotenone,

pending the use of *B. diffusa* extracts as the fishing poisons. Since rotenones are used for their hemolytic activity on fishes, the evaluation of *B. diffusa* extracts hemolytic and anti-hemolytic activities are on the way to unfold its mechanism of action.

ABSTRACT

Phytochemical screening and total contents of secondary metabolites extracted from leaves, stems and roots of *Boerhavia diffusa* Linn from DR Congo in dichloromethane, methanol, a dichloromethane-methanol mixture and water were evaluated together with the related total antioxidant capacity. The phytochemical screening revealed a wide range of secondary metabolites and when extracting, roots have provided high extraction yield followed by stems and then leaves. Quantitatively, polyphenols are the highest secondary metabolites (15.54 ± 3.42~62.42 ± 3.25) µgAG/g in DCM, MeOH and MeOH/DCM, including flavonoids (8.23 ± 0.33~15.72 ± 0.28) µg QE/g in water. Tannins and Alkaloids contents is considerable in DCM, methanol and dichloromethane-methanol mixture systems of solvents. The highest concentrations are observed in the extracts stem. Leaves' extracts exhibited the higher total antioxidant capacity. This justifies the use of the plant in traditional medicine in oxidation's related ailments.

Mots clés

Boerhavia diffusa Linn., Phytochemical screening, total contents and total antioxidant capacity.

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CONFLICT OF INTEREST

The authors declare that this work has no conflict of interest with anyone.

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