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Moringa oleifera: phytochemical detection, antioxidants, enzymes and antifugal properties

Moringa oleifera: detección fitoquímica, antioxidantes, enzimas y propiedades antifúngicas

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Abstract. *Moringa oleifera* is a plant with a high biotechnology potential due to its high content of proteins, carbohydrates and lipids, and secondary metabolites. Some of their secondary compounds have been advocated for traditional medicinal uses. This work shows an overview of the histological organization and the composition of some biochemical components (e.g.: enzymatic inhibitors, phytochemicals, enzymatic profiles and antifungal potential) of different plant tissues. These components could be associated with the physiology and defense mechanisms of the study plant species. These activities were detected using synthetic substrates by conventional strategies based on spectrophotometrical and zymography assays. Moringa oleifera presented a typical histological array for dicotyledonous plants. According to the phytochemical assay, leaves showed the highest antioxidant activity, which could be related with their contents of phenolic compounds. Also, trypsin inhibitors were detected in different parts of the plant, indicating possible implications as defensive traits. The presence and abundance of the detected compounds could be associated with a general status of the physiology of the plant. It should be noted, however, that all components detected could be involved in plant defense mechanisms.

Keywords: *Moringa oleifera*; Phytochemicals; Plant defense; Trypsin inhibitors; Proteinases.

Resumen. *Moringa oleifera* es una planta que presenta un alto potencial biotecnológico debido a su alto contenido de proteínas, carbohidratos y lípidos. Por otra parte, algunos de sus metabolitos secundarios han sido promovidos para su uso en medicina tradicional. En el presente trabajo, se muestra una revisión de la organización histológica y la composición general de algunos de los constituyentes bioquímicos (inhibidores enzimáticos, fitoquímicos y perfiles enzimáticos) de diferentes tejidos de la planta. Dichas actividades fueron efectuadas con sustratos sintéticos por métodos convencionales basados en ensayos espectrofotométricos y zimografías. *Moringa oleifera* presentó un arreglo histológico típico de una dicotiledónea. De acuerdo a los ensayos fitoquímicos, las hojas mostraron la actividad antioxidante más alta, la cual podría estar asociada con sus altos contenidos de compuestos fenólicos. También se detectaron inhibidores de tripsina en diferentes partes de la planta, lo que podría tener implicancias en la formación de elementos defensivos. La presencia y abundancia de los componentes detectados podría estar asociada con un estado general de la fisiología de la planta. Los componentes detectados también podrían estar involucrados en los mecanismos de defensa de la planta.

Palabras clave: *Moringa oleifera*; Fitoquímicos; Defensa de plantas; Inhibidores de tripsina; Proteinasas.

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INTRODUCTION

Moringa oleifera is a widely cultivated tree considered as a multi-purpose plant. It includes its use as functional food, cleaning water material, oil extraction for biofuel production, and other applications. In a traditional way, it is used with medicinal purposes around the world due to empiric observations. These benefits have been associated with metabolites such as phenolic compounds, vitamins and proteins (Fahey, 2005; Goyal et al., 2007; Adedapo et al., 2009). Nevertheless, the complete knowledge about what kind of metabolites are present in each organ, and their ecological and biological roles, are poorly elucidated. Some studies about macromolecular characterization have been made in the last years, including a protein with the ability to agglutinate, proteinase inhibitors, lectins, carbohydrates and lipid contents (Olayemi & Alabi, 1994; Santos et al., 2005). Plant parts, including leaves, stem, roots, seeds and flowers have been reported as source of different biochemical compounds with anticarcinogenic, antiinflammatory, antidiabetic, antioxidant, and antimicrobial effects (Fahey, 2005; Anwar et al., 2006; Goyal et al., 2007; Chumark et al., 2008; Peixoto et al., 2011). *Moringa oleifera* contains essential amino acids, carotenoids in leaves, and components with nutraceutical properties, supporting the idea of using this plant as a nutritional supplement or constituent in food preparation. Some nutritional evaluation has been carried out in leaves and stem. Studies have shown that conventional antinutritional factors like trypsin and amylase inhibitors, glucosinolates, cyanogenic glycosides, saponins and tannins were not detected, at least with the used methodology (Makkar & Becker, 1997). However, a comprehensive study including the root system is still missing. Secondary metabolites from *M. oleifera* leaves have shown antimicrobial effects against various human pathogenic bacteria, including the genera *Shigella*, *Pseudomonas*, *Salmonella* and *Bacillus*. These secondary metabolites appear to be involved in plant defense mechanisms (Clark, 1981, Chuang et al., 2007; Doughari et al., 2007; Mashiar et al., 2009). Recently, the presence of a trypsin inhibitor has been reported in leaves. This indicates an important activity against serine proteinases. This inhibitor has been stable in a wide range of pH, detergents, reducing agents and high temperatures, which coincides with the high stability reported for some proteins involved in plant defense (Bijina et al., 2011). Despite its potential applications, a comprehensive knowledge of *M. oleifera,* including its responses at the biochemical and biological levels, is still limited. Also, the biochemical implications on human health of the bioactive compounds present in this plant, their relation with the plant defense strategies against biotic and abiotic agents, as well as the physiological roles of proteins, enzymes and secondary metabolites are not well understood when ecological interactions are considered.

This report presents a brief histological and biochemical description of leaves, stem and roots of *M. oleifera*, and the distribution of metabolites often associated to plant defense.

MATERIALS AND METHODS

Histological analysis. *Moringa oleifera* plants were obtained from greenhouses of the Agronomy Faculty of Universidad Autónoma de Nuevo Leon (UANL) in Nuevo Leon, México. Each plant organ was separated and fixed in a solution of formaldehyde, ethanol and acetic acid in distillated water (5:25:10:60 v/v) during 24 h. Then, samples were rinsed with distillated water for 2 h and sections of fixed organs were handmade using razor blades. Adequate sections were selected and exposed to sequential dehydration in ethanol (90%) and stained with 0.1% Safranin O solution during 4 h. These samples were thereafter washed to eliminate excess of stain with ethanol (70%) during 15 min twice. Sections were exposed to dehydration up to absolute ethanol for 10 min, and stained by immersion in 0.05% Fast Green solution for 1 min. The excess of colorant was washed away with ethanol (95%) during 5 min, and dehydrated with absolute ethanol during 15 min. This was followed by sequential immersion in ethanol-xylene solutions by gradually increasing xylene concentration up to reach 100%. Finally, preparations were mounted using Canada balsam.

Extract preparation. Four plants (≈ 9 month-old) were obtained, and parts were separated and cleaned (Fig. 1 A). The cortex was cut off with knifes removing the central woodhard tissue. Samples of 10 g were prepared and rinsed with distillated water for 5 min and dried. Each separated part was ground using water for the aqueous extract, and ethanol at 70% for the ethanolic extract. Macerated samples were immersed in each solvent (1:2 ratio) during 20 min and clarified by centrifugation at 10000 *g* for 5 min to 4 °C. The liquid fraction was obtained and kept at -70 °C for subsequent analysis.

Phytochemical assay. Both extracts were exposed to phytochemical assay using color reactions according to several protocols as follows:

Polyphenol detection with $KMnO_4$. To 100 µL of each sample, 100 µL of distilled water were added. Reactions were carried out by the addition of 20 µL of $KMnO_4$. The reaction was considered positive when color change was in the yellowish or greenish tones. Distilled water was used as control.

Polyphenol detection with $FeCl_3$. To 100 µL of each sample, 100 µL of distilled water were added and the reactions were started with addition of 20 µL of $FeCl_3$. The reaction was positive when dark-greenish, blue to dark-blue or even blackish tones were presented in the mixture. Distilled water was used as control.

Flavonoid detection. To 100 µL of extract some fragments of metallic magnesium and three drops of 25% HCl solution were added. A positive reaction was considered when color of the solution changed to pink, yellow or orange.

Tannin detection. Detection was based on gelatin precipitation in saline solution. Ten tubes were prepared with 2 mL of that solution; 3 tubes were filled with a 10% solution of

NaCl; 3 with a 10% solution of NaCl and a 2% solution of gelatin, and 3 with a 2% solution of gelatin. Five hundred µL of the extract were added to each tube. Reaction was positive when whitish precipitation or coagulum formation was evident. Distilled water was used as control.

Alkaloid detection. Samples of 600 µL of each extract were heated at 95 °C, and 100 µL of HCl (10%) were added. Tubes were left at room temperature for cooling them and then 20 µL of Wagner reagent were added and mixed. The reaction was considered positive when maroon precipitation was formed.

Saponin detection. Three hundred µL of each extract and 300 µL of boiling water were mixed, then cooled, and thereafter tubes were vigorously agitated. Foam formation during more than 10 min was considered a positive reaction for saponin presence.

DPPH' scavenging capacity. DPPH' assay was carried out following Martínez-Avila et al. (2011). Briefly, 2.9 mL of 60 µM DPPH' radical were added to 100 µL of each extract. Then, samples were located in a dark place, and after 30 min the absorbance (Abs) was measured at 517 nm. Controls contained 100 µL of distilled water. The radical-scavenging capacity of the extracts was calculated with the following equation and expressed as DPPH' percent of inhibition:

$$Inhibition\ (\%) = \left[(Abs_{control} - Abs_{sample}) / \mathrm{Abs}_{\mathrm{control}} \right] * 100$$

Electrophoresis and zymography. For enzyme detection, the extracts were concentrated by precipitation using cold acetone (-70 °C). Three volumes of acetone were added to each extract, which were then incubated at 4 °C. Then, they were centrifuged at 8000 *g* for 5 min. The resulting pellets were dried, and then resuspended in a minor volume of distillated water.

To visualize protein profiles and obtain separation of proteins, the extracts were separated using polyacrylamide electrophoresis following an adapted protocol of Laemmli (1970). Run conditions included 20 min at 80 V and 40 min at 120 V in 10% polyacrylamide gels. Protein profiles were stained with Commassie blue. Zymography was carried out with the same conditions described above. For amylase detection, starch with low amylase content (SIGMA-ALDRICH) was copolymerized with polyacrylamide. Once electrophoresis was stopped, the gels were obtained and incubated in buffer 0.25 M Tris-HCl pH 6.8 at 37 °C during 30 min. A iodine-potassium solution was used for amylase visualization. Amylase activities were related as clear bands in a dark background; clear bands resulted from starch hydrolysis. Porcine skin gelatin was copolymerized with polyacrylamide for proteinases detection. Samples were then separated using electrophoresis with the same condition described above. Gels obtained were incubated at different pH conditions during 1 h at 37 °C. The buffers used for proteinase activity were for pH 5 (48.5 mL of 0.1 M Citric acid with 51.50 mL of 0.2 M Na_2HPO_4), pH 6 (36.85 mL of 0.1 M Citric acid with 63.15 mL of 0.2 M Na_2HPO_4), pH 7 (17.65 mL of 0.1 M Citric acid with 82.35 mL of 0.2 M Na_2HPO_4) and pH 8 (0.1M Tris-HCl). After incubation, gels were stained with Commassie blue solution; clear bands in a dark background indicated gelatin hydrolysis, which was related to the pH conditions.

Trypsin inhibitor detection. The trypsin inhibitor detection assay was based on the liberation of *p*-nitroanilide after hydrolysis of the substrate N-Benzoyl D,L argninine *p*-nitroanilide (BApNa, SIGMA MO). A protocol adapted from a previously described method (Erlanger et al., 1961) was followed. The inhibitory activity unit was defined as the decrease in 0.01 units of absorbance per 15 min at 37 °C. Blank reaction was prepared with 1.35 mL of 0.01 M Tris-HCl pH 8, 150 µL of 0.01M of substrate and 300 µL of 30% acetic acid solution. Bovine trypsin control reaction was prepared with 1.275 mL of 0.01M Tris-HCl pH8, 75 µL of proteolytic enzymes with 3.75 µg of trypsin, 150 µL of substrate followed by an incubation of 15 min at 37 °C, and 300 µL of acetic acid to stop the reaction. Final absorbance was measured at 405 nm. To evaluate the trypsin inhibition, the reaction was prepared with 1.225 to 1.175 mL of 0.01M Tris-HCl pH8, 75 µL of proteolytic enzymes, 50 to 100 µL of inhibitory sample followed by an incubation of 5 min. Thereafter, 150 µL of substrate were added and incubated during 15 min, and finally 300 µL of acetic acid were added to stop the reaction. Absorbance was measured at 405 nm. Trypsin Inhibitor Units (TIU) were calculated in relation to the (1) reduction in absorbance when the extracted was added, and (2) total protein concentration, according to the following equation:

$$TIU = [(TAU - TAU + I) / (0.01 * VI] / P$$

Where *TAU* means trypsin absorbance units; *TAU+I* was the absorbance in the inhibitory reaction; *VI* the volume of the inhibitory extract in mL, and *P* the protein concentration in mg/mL.

Antifungal assay. *Phytophthora parasitica*, *Fusarium oxysporum* and *Alternaria solani* growth was evaluated on each plant part in the presence of aqueous and ethanolic extracts. A fragment of mycelia (4 mm^2) was cut for each fungal species culture, and deposited in the center of Petri dishes containing PDA impregnated with the extract. The radial growth was registered at 24 h intervals for 120 h. The experiment was performed using 5 replicates.

Protein quantification. Absorbance of clarified extracts was recorded at 220 nm. Determination of protein content was done by comparison with a standard curve derived from absorbance of BSA in a concentration gradient.

Statistical Analysis. Data were analyzed using ANOVA to detect differences among the treatments. Means were compared using the Tukey test (p=0.05). Analyses were done using the statistical software SAS version 9.0.

RESULTS

Anatomical description. *Moringa oleifera* had a typical histological array of dicotyledonous plants. Leaflet blades were always dorsiventrally compressed. Transverse sections showed a unistratified epidermis with stomata on both sides; they were more abundant in the abaxial side. Epidermal cells were more or less isodiametric, polyhedral and uniformly arranged. Mesophyll consisted of 6 to 8 layers of chlorenchymatous tissue. Two cellular types were observed according to the shape and position of each cell. The first type was a palisade parenchyma on the adaxial face with a compact arrangement of tabular cells. The second type was a spongy parenchyma on the abaxial face, with more or less compactly-arranged cells and scarce intercellular spaces; the cells were irregularly shaped. The central bundle was collateral and usually semi-triangular shaped, with the apex pointing out towards the adaxial face, and subtended by some collenchymatous cells towards the abaxial face (Fig. 1 B and C).

Transverse sections obtained from the middle part of the third apical growing internode presented an epidermal cell layer. It was subtended by two or three rows of collenchymatous cells (identified because of their irregularly thickened walls), followed by cortical parenchyma cells with around 20 layers up to the vascular region. Immersed in the cortical parenchyma, there were fibers arrayed in separate bundles with a cylindrical disposition. The vascular system was an ectophloic siphonostele, with well-developed piths; such array had around six-layers of phloem with a cylindrical distribution surrounding an eight-layer vascular cambium, represented by rectangular, thin-walled cells. Xylem was represented by vascular rows, which increased in diameter from the center to the periphery of the section. They were also distributed in a cylindrical way forming a pith of parenchyma with wide, thin-walled cells (Fig. 1 D and E).

The root showed a multistratified epidermis in transversal section, with wide tabular, thin-walled cells with up to 9 layers in general. There were also extra rows of 5 to 6 cells forming elevations which gave the transversal section a "daisy-flower" shape. There was a cortical region constituted of parenchyma cells, with schlerenchyma cells separated in a cylindrical array reaching the limit with the endodermal region. The casparian band was formed by cells with lignified walls enclosing the vascular region. Xylem was distributed in five poles when the root was young, but no poles could be clearly distinguished in mature roots. Phloem was inserted between xylem poles. No pith was observed (Fig. D and E).

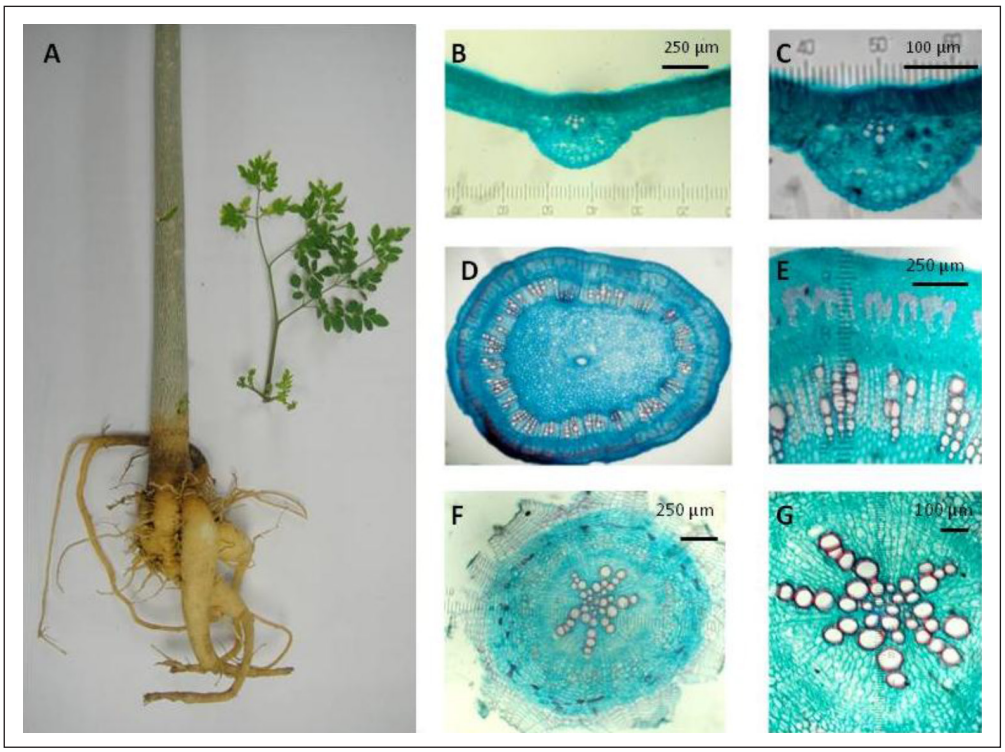

Fig. 1. Anatomical approach of *M. oleifera*. (A) Stem, roots and compound leaves of *M. oleifera*; (B) Transverse section of a leaflet; (C) Visualization at central vein of a leaflet; (D) Panoramic view of the transversal section of the stem; (E) Close view of the cortical and vascular regions; (F) Panoramic view of the transversal section of the root; (G) Close view of the vascular region of the root. Black line: reference for µm.

Fig. 1. Aproximación anatómica de *M. oleifera*. (A) Tallo, raíces y hojas compuestas de *M. oleifera*; (B) Sección tranversal de un foliolo; (C) Visualización a nivel de vena central de un foliolo; (D) Vista panorámica de un sección transversal del tallo; (E) Vista cercana de las regiones cortical y vascular; (F) Vista panorámica de una sección transversal de la raíz; (G) Vista cercana de la región vascular de la raíz. Línea negra: referencia para los µm.

Phytochemical detection. The phytochemical constituents present in the aqueous and ethanolic leaf, stem, root and stem-cortex extracts of *M. oleifera* were mainly detected by basic colored-reactions. The phytochemical assay showed differences among plant parts as indicated in Table 1, confirming the presence of phenolic compounds, alkaloids, flavonoids and the absence of saponins and tannins. The assay showed a more diverse phytochemical composition on leaves, with the presence of alkaloids, $FeCl_3$-reacting-polyphenolic compounds and flavoniods. On the other

hand, a similar phytochemical pattern was observed for the stem and roots, where the predominant group was the $KMNO_4$-reacting polyphenolic compound. No presence of saponins and tannins were detected for both extracts in all plant parts with the methods used. Both types of polyphenolic compounds were assayed under the same conditions. However, the velocity and intensity of reactions, used as indicative of relative abundance, allowed detecting gradual differences among the leaves, stem and roots, where the amount of $KMNO_4$-reacting polyphenolic compounds seemed to be higher. For $FeCl_3$-reacting-polyphenolic compound and flavonoid detection, the reaction time was extended for stem and root and no changes were observed. This indicated that very low amounts (or total absence) of these compounds were present in such organs. Phytochemicals like phenolic compounds are usually related to several biological activities, including antioxidant potential and radical scavenging, which is relevant for therapeutics and nutraceutical applications. In this sense, $DPPH^{\cdot}$ radical scavenging was carried out in all *M. oleifera* parts trying to determine the richer organ in antioxidant potential and the best system for extraction. Our results indicated that activity was present in both extracts, and that higher or minor activities were on leaves or stem and roots, respectively (Table 2).

Enzymatic profiles. An enzymatic profile was established as an indicator for the metabolic state of each organ using zymography techniques. This is because of information about enzymes at each organ could be associated to endogenous roles such as reserve mobilization, protein degradation or plant defense. The proteolytic and amylolytic assay of both extracts included general substrates. For proteinases, the assay was through a wide pH range. Most of the detected enzymes were obtained in aqueous extracts. In general, proteinases showed a greater activity at pH 5 or 8, while a minor proteolytic activity was recovered in the ethanolic extract (Fig. 2). The leaf aqueous extracts showed the highest intensity for proteinases at pH 5; nevertheless, the leaf proteolytic profile was similar to that on the stem, stem cortex and roots. The pattern of proteolytic activities showed a population of two main bands. The first band showed a molecular weight of 40 kDa, and the second one a weight close to 27 kDa (Fig. 2 A and B). In the case of ethanolic extracts, only a band of activity at pH 5 was observed in the stem. The signals corresponding to active enzymes at pH 5 were less visible when incubation was at pH 6. At pH 7 and 8 a different proteolytic pattern was visualized. In aqueous extracts, a band of 80 and another one of 42 kDa were detected at pH 7; bands were more intense at pH 8 as indicated by the arrows (Fig. 2 C and D). In addition, a pattern of three bands of 30, 33 and 39 kDa were detected in the cortex after incubation at pH 7, and the same, but more intense, pattern was observed at pH 8 in all the extracts. The intensity, however, was highest in the ethanolic extracts. Amylase activity was detected in both extracts (Fig. 3); in aqueous extract, amylase activity in the leaf was present as two bands. The more intense band showed a molecular weight around 90 kDa, while it was only 50 kDa in the less intense band. In the case of stem and cortex, the amylolytic activity was intense in the 90 kDa position, showing a continuous hydrolysis throughout the wall. In the root aqueous extract, that activity was less intense in the 90 kDa position; also, a band of 30 kDa was observed in the stem, stem cortex and roots. No other signals were detected in the aqueous extracts. Amylase activity in the leaves, stem and roots was detected with low intensity in ethanolic extracts. This was even increasing the incubation time. In the case of the ethanolic extract for the cortex, a main

Table 1. Phytochemical assay results.
Tabla 1. Resultados del análisis fitoquímico.

Sample	Aqueous extract						Ethanolic extract					
	P1	P2	F	A	T	S	P1	P2	F	A	T	S
Leaves	+	+	+	+	-	-	+++	+	+	+	-	-
Stem	-	++	-	-	-	-	-	++	+	-	-	-
Cortex	-	+++	-	-	-	-	-	+++	-	-	-	-
Roots	-	+	-	-	-	-	-	+++	-	-	-	-

These results were obtained by color reactions and qualitative comparison. P1, $FeCl_3$-reacting-polyphenolic compounds; P2, $KMNO_4$-reacting polyphenolic compounds; F, flavonoids; A, alkaloids; T, tanins; S, saponins. Negative detection is indicated by -, positive reaction is indicated by +, ++ and +++. All reactions were done by triplicate on at least three plants.

Estos resultados fueron obtenidos por reacciones colorimétricas y por comparación cualitativa. P1, compuestos polifenólicos reactivos con $FeCl_3$; P2, compuestos polifenólicos reactivos con $KMNO_4$; F, Flavonoides; A, Alcaloides; T, Taninos; S, Saponinas. La reacción negativa es indicada por -, reacción positiva es indicada por +, ++ y +++. Todas las reacciones se hicieron por triplicado en al menos tres plantas.

Table 2. DPPH' scavenging capacity.
Tabla 2. Capacidad secuestradora del radical DPPH.

	$DPPH^{\cdot}$ scavenging capacity (%)	
Sample	Aqueous extracts	Ethanolic extracts
Stem	20.47 ± 0.455	30.91 ± 1.77
Leaves	85.20 ± 0.001	89.67 ± 0.75
Roots	14.27 ± 0.344	29.48 ± 1.55

The capacity was expressed as the percentage of $DPPH^{\cdot}$ inhibition for each analyzed part and extract. Values are the mean ± 1 standard deviation of n=3.

La capacidad secuestradora fue expresada como el porcentaje de inhibición de DPPH para cada parte vegetal y extracto analizado. Los valores son el promedio ± 1 desviación estándar de n=3.

band of amylolytic activity was observed at 90 kDa position, and no other signal was detected.

Trypsin inhibitor detection. Trypsin inhibitor content as the main proteinaceous antinutritional factor was determined in aqueous and ethanolic extracts as indicated in Table 3. The inhibitory activity was spread throughout all the plant, but higher inhibitory unit content was detected in ethanolic extract of roots, and lower contents were presented by stem and cortex in aqueous extract. The specific activity was related to the amount of protein in each extract.

Antifungal effect. Micelial growth of *A. solani* did not present significant differences in both aqueous and ethanolic extracts; all treatments and the control had the same growth after 120 h of inoculation (Fig. 4 A). In the case of *F. oxysporum*, the average micelial growth after mycelium inoculation did not show significant differences ($p>0.05$) when comparing treatments at 120 h. Micelial growth presented a variation of 4.45 to 5 cm in diameter as observed in Fig. 4 B, where aqueous leaf extract had 5 cm and aqueous stem extract showed 4.8 cm; both presented more growth when compared to the control (water). Aqueous root extract showed minor growth but no significant differences from the control. When analyzed the effect on *P. parasitica*, the ANOVA test detected significant differences ($p>0.05$) when comparing the treatments. Mean comparison showed that growth in treatments was 27.23 to 56.84% of that in control (Fig. 4 C). The greatest

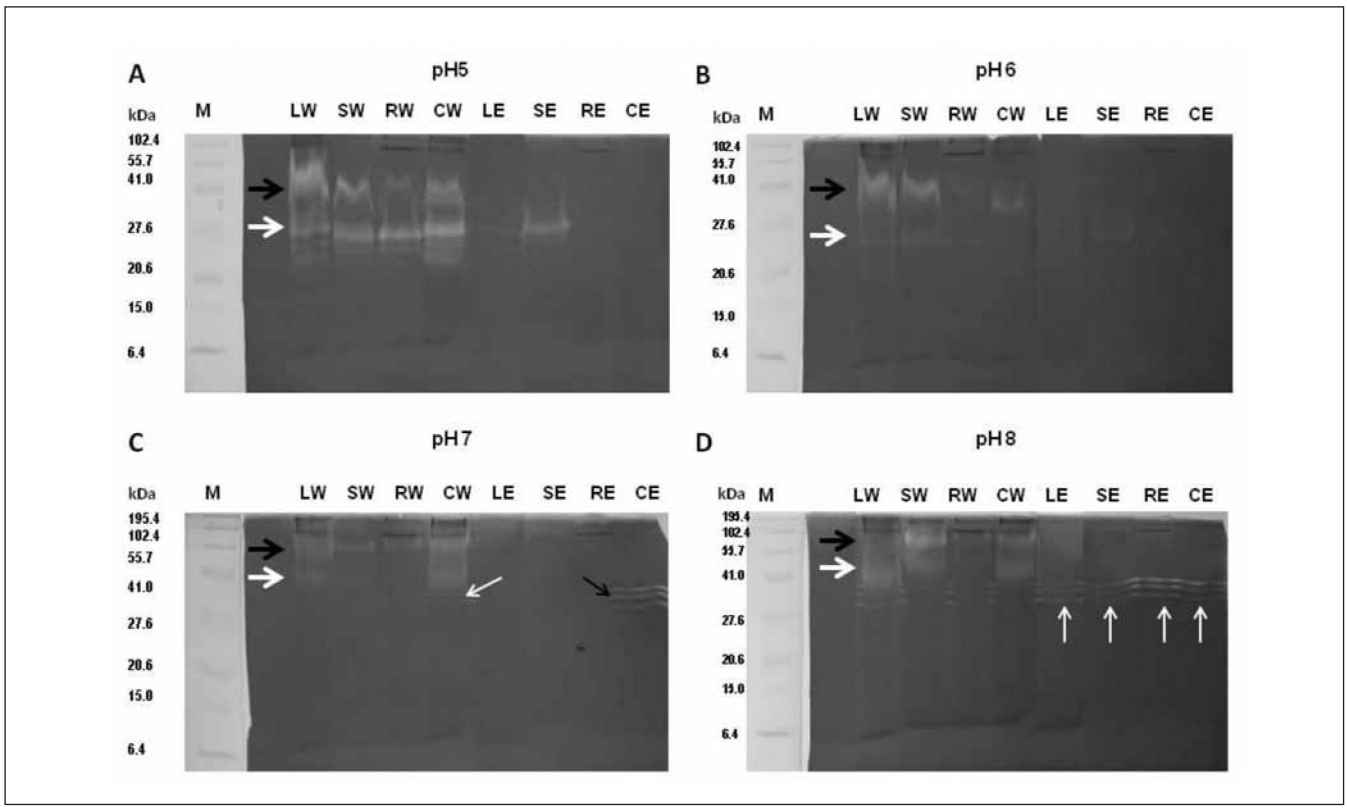

Fig. 2. Proteolytic profiles of extracts from *M. oleifera* vegetative plant parts. Clear bands indicate the proteolytic activity. (A) Profile of enzymes active at pH 5, black arrow indicates a 40 kDa band, and white arrow points out a band of 27 kDa; (B) Profile at pH 6, black and white arrows indicate positions for 40 and 27 kDa bands, respectively; (C) Profile at pH 7, broad black arrow indicates 80 kDa band, and broad white arrow points out at 42 kDa band, both notoriously active at the cortex water extract (CW), thin black and white arrows showed the pattern of 30, 33, 39 kDa bands of activity from the cortex in CW and CE; (D) Profile at pH 8, broad arrows indicates the same pattern as in (C), but more intense at this pH, the pattern of 30, 33 and 39 kDa bands was present in all at pH 8. L: leaf, S: stem, R: root and C: cortex. The solvents are indicated by W (water) and E (ethanolic). M, molecular weight standards.

Fig. 2. Perfiles proteolíticos de los extractos de las partes vegetativas de *M. oleifera*. Bandas claras indican la actividad proteolítica. (A) Perfil de enzimas activas a pH 5, la flecha negra indica la banda de 40 kDa y la blanca apunta hacia la de 27 kDa; (B) Perfil a pH 6, las flechas negra y blanca indican las posiciones de las bandas de 40 y 27 kDa, respectivamente; (C) Perfil a pH 7, la flecha negra ancha indica la banda de 80 kDa y la flecha blanca ancha señala la de 42 kDa, ambas activas en extracto acuoso de corteza (CW). Las flechas delgadas negra y blanca muestran bandas de 30, 33 y 39 kDa en ambos extractos de corteza en CE y CE; (D) Perfil a pH 8, la flecha ancha indica el mismo patrón que en (C) pero más intenso en este pH, el patrón de 30, 33 y 39 kDa estuvo presente en todas las muestras a pH 8. L: hoja, S: tallo, R: raíz y C: corteza. Los solventes están indicados por W (agua) y E (etanólicos). M: estándares de peso molecular.

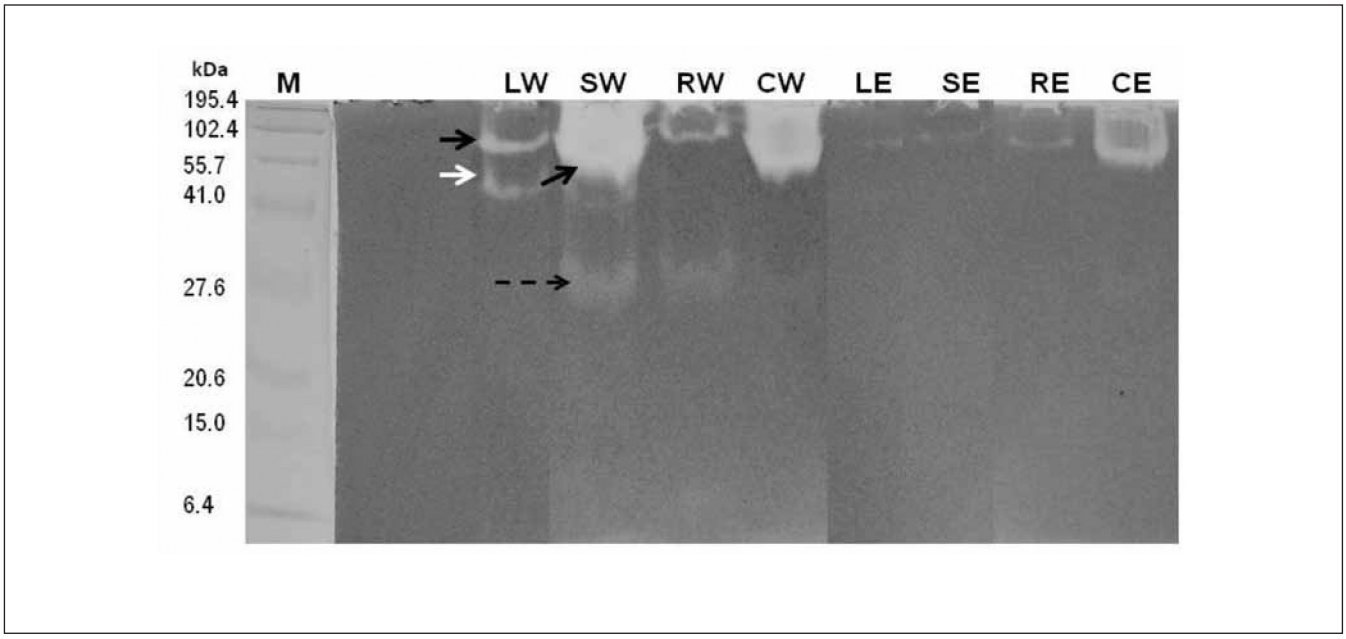

Fig. 3. Amylase activity assay in the extracts. Broad black arrow indicates the position of a 90 kDa band of amylase activity, broad white arrow indicates the position for a 50 kDa band present in the leaf and stem, black dotted arrow points out the position of a 30 kDa band. L: leaf, S: stem, R: root and C: cortex, and W (water) and E (ethanol). M: molecular weight standards.
Fig. 3. Ensayo de actividad de amilasa en los extractos. La flecha ancha y negra indica la posición de una banda de 90 kDa, la flecha ancha y blanca indica la posición de una banda de 50 kDa presente en la hoja y tallo, la flecha negra punteada señala la posición de una banda de 30 kDa. L: hoja, S: tallo, R: raíz y C: corteza, y W (agua) y E (etanólicos). M: estándares de peso molecular.

fungal growth inhibition was caused by the root extract (Fig. 4 C). No differences were detected between the stem and leaf extracts. No growth inhibition effect was detected in the case of the ethanolic extracts.

Table 3. Trypsin inhibitor content.
Tabla 3. Contenido de inhibidores de tripsina.

Sample	TIU/mg protein
LW	712 ± 24
SW	318 ± 6
RW	578 ± 96
CW	352 ± 7
LE	432 ± 10
SE	718 ± 178
RE	1112 ± 248
CE	432 ± 10

Trypsin inhibitory units (TIU). Plant parts: L, leaf; S, stem; R, root; C, cortex. Solvents: W (water) and E (ethanolic) extracts. Values are the mean ± 1 standard deviation of n=3.
Unidades inhibitorias de tripsina. Partes vegetales: L, hoja; S, tallo; R, raíz; C, corteza. Solventes: extractos de W (agua) y E (etanólicos). Los valores son el promedio ± 1 desviación estándar de n=3.

DISCUSSION

Moringa oleifera plants are used as a food source with valuable properties in humans (Anwar et al., 2007; Goyal et al., 2007; Adedapo et al., 2009). Therefore, knowledge about bioactive components through the whole plant, and their relative abundances their potential biological effects could help to design strategies focused on the appropriate utilization of this resource. Current descriptive studies contribute to support the ideas of alternative uses, genetic improvement, and the study of defense strategies to infer ecological interactions and adaptations. In this work, a general histological description for *M. oleifera*, a phytochemical content analysis, and some enzymatic profiles give a perspective about the accumulation and distribution of some metabolites in some parts but not in others, leading to relate their distribution with possible physiological and defensive roles.

There were plenty of non detected metabolites in this work; differences in phytochemical constitutions were observed. In the case of leaves, there was an exclusive detection of $FeCl_3$-reacting-polyphenolic compounds together with alkaloids. Also, the presence of $KMNO_4$-reacting polyphenolic compounds and flavonoids was registered. These compounds could contribute to protect the foliage area from abiotic and biotic stresses; the last phenolic group was shared with the stem but

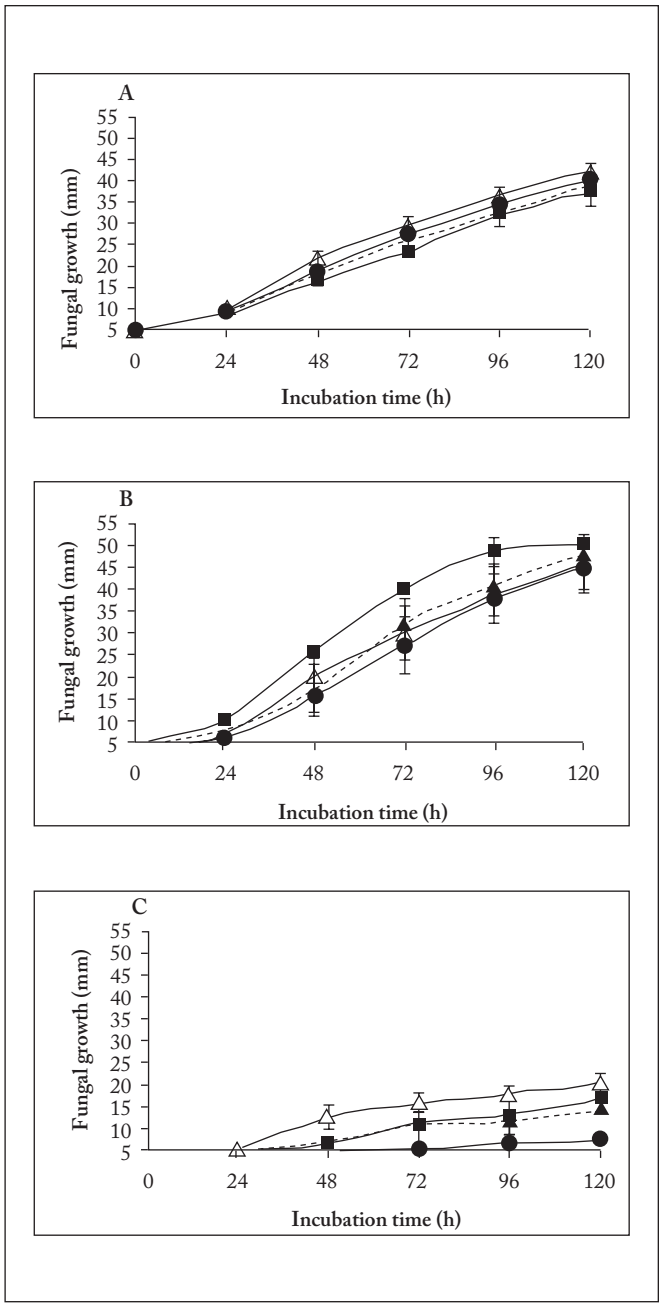

Fig. 4. Effect of *M. oleifera* aqueous extracts on the growth of some phytopathogenic fungi. There was no significant effect on *A. solani* (A) and *F. oxysporum* (B) growth. Growth was affected by all the aqueous extracts in *P. parasitica* (C); the root extract inhibited around 70% of growth. White triangles indicate the control, and black squares, black triangles and black circles indicate leaf, stem and root aqueous extracts, respectively.

Fig. 4. Efecto de los extractos acuosos en el crecimiento de algunos hongos fitopatógenos. No hubo efecto significativo sobre el crecimiento de *A. solani* (A) y *F. oxysporum* (B). El crecimiento fue afectado por todos los extractos acuosos en *P. parasitica* (C); el extracto radical inhibió alrededor del 70% del crecimiento. Los triángulos blancos indican el control, cuadros negros, triángulos negros y círculos negros indican extractos acuosos de hoja, tallo y raíz, respectivamente.

not with the root. Also, other differential accumulation was registered in the case of the relative detection for $KMNO_4$-reacting polyphenolic compounds with increasing amounts from leaves to stems and more intense signal in roots. Such distribution could obey to either the need or not of those compounds for ecological interactions. For example, the presence and accumulation of alkaloids and polyphenols in foliage is related to their deterrent and potential toxic effects to herbivores and insects (Coley, 1988; Macel et al., 2005). In the case of $KMNO_4$-reacting polyphenolic compounds, they showed a gradual accumulation according to the intensity of the signal reaction, increasing it towards the root where the presence of these compounds was the only one detected. Thus, the variation in the profile of compounds among different plant parts probably corresponds to their chemical nature, function and signals perceived from the environment (Flores et al., 1999).

The enzymatic profiles for proteinases and amylases also corresponded with variations among plant parts, with more intensity in signals in stem and roots than in leaves, which could be related with the translocation or utilization of reserves. For example, amylases seemed less abundant in leaves. This very likely indicates the leaves as a source organ, actively producing sugars which are then translocated to sink organs (i.e., stem, roots) where they are stored as starch, and breakdown by amylases, to provide energy to the sinks (Göttlicher et al., 2006).

Usually, antioxidant properties of plant extracts have been attributed to the presence of polyphenolic compounds, which have great potential as antimicrobial agents. Measurement of antioxidant activity is the most widely accepted analysis to attribute the several benefits of phenolic compounds Yemis et al. (2008). Leaves showed the highest antioxidant potential, which coincided with the positive signals obtained for polyphenolic and flavonoid compounds. They are probably responsible for such antioxidant activity. Similar findings have been reported by Gardner et al. (2000) on juices from vegetables, pineapple and apple. As a result, the leaf could be considered the most relevant part of *M. oleifera* as antioxidant source in comparison to other parts of the plant. The fact of its high antioxidant activity is according with findings in previous reports (Chumark et al., 2008; Sreelatha & Padma, 2009).

Usually, trypsin inhibitors are associated to endogenous regulation of proteinases. Their role as constitutive or inducible proteins in complex responses for plant defense has been reported; trypsin-like proteinase inhibitors seem to be a conserved and widely dispersed defense strategy in plants (Ryan, 1990; Cipollini & Bergelson, 2001). In a previous report, proteinase inhibitors with activity against serine proteinases including thrombin, chymotrypsin, elastase and some cysteine proteinases were obtained from seeds, leaves, stem, flowers and bark; this assay was focused in proteinases of therapeutic and industrial importance (Bijina et al., 2011). In the present work, bovine trypsin inhibitor activity was detected in leaves,

stem, roots and cortex in aqueous and ethanolic extracts. This would indicate that *M. oleifera* is a potential source for inhibitors with different properties in all vegetative plant parts. Nevertheless, if the inhibitory activity is involved in plant defense or in endogenous regulation is still unknown.

Evidently, more studies on the general biochemical and molecular biology for this plant species will lead to new and directed applications for this resource, and a more comprehensive study of metabolites, enzymes and other compounds in *M. oleifera.* Their variation through time, developmental stage, or as a particular metabolic state in each organ could lead to improve understanding of plant responses with physiological and ecological implications, looking for a better use of this resource.

Numerous studies have been conducted on different parts of *M. oleifera*, but there is a direct need to isolate and identify compounds associated with biotechnological applications. Nevertheless, it is worth mentioning that endogenous roles of these compounds are still poorly understood. Since this plant has been proposed as a crop, it is planted around the world, which implies it needs to grow under different environmental conditions. Thereafter, it is expected that there will be some variation in the presence or concentration of certain metabolites in different parts of the tree. Despite it is a studied plant, changes in the general physiology and biochemical composition among populations adapted to particular environmental conditions are not known. Here, the presence of some compounds considered as defensive elements has been shown, and their distribution in vegetative plant parts has been established. However, detailed studies for examining the endogenous roles of the study compounds are yet required.

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