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Effect of Extraction Type on Bioactive Compounds and Antioxidant Activity of *Moringa oleifera* Lam. Leaves

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Abstract: The objective of this study was to determine the extraction yield, the content of bioactive compounds and in vitro antioxidant activity of different extracts from *Moringa oleifera* (MO) leaves, and their potential use as a natural feed additive for ruminant diets. For the preparation of MO extracts, three treatments were used: (1) MO extract in distilled water (MOEW, 0%), (2) ethanol-water (MOEEW, 50%) and (3) absolute ethanol (MOEE, 100%). The extraction yield and the antioxidant activity measured with the DPPH assay in the MO extracts were higher for MOEW and MOEEW. From all treatments, MOEEW had the highest antioxidant activity evaluated with the ABTS assay and showed a higher content of bioactive compounds. On the other hand, the principal component analysis showed that the first two principal components explained 96.5% of the variability of the data. The variables that contributed to the greatest variation were condensed tannins (CT), total phenolic compounds (TPC), total flavonoids (TF), and extraction yield. A high correlation ($p \leq 0.001$) was observed between TPC and extraction yield with $r^2 = 0.989$. The content of bioactive compounds and antioxidant activity was higher in the MOEEW extract; therefore, its inclusion in ruminant diets can be suggested to potentially improve their productivity and product quality.

Keywords: extract; natural alternatives; natural feed additives; polyphenols; ruminants

1. Introduction

Medicinal plants have been used in animal nutrition to decrease animal stress, improve feed efficiency, provide nutrients, and improve health and quality of livestock products [1,2]. Due to their relative low cost of production and their potential to use them as alternatives to synthetic supplements, medicinal plants have been used as additives on animal feed in distinct forms [3,4]. These additives, mostly based on their high number of polyphenols (phenolic acids, flavonoids, and tannins) have the ability to strengthen immune systems, combat reactive oxygen species, and in many cases reduce methane (CH_4) production from ruminants [4].

The extraction of these compounds depends on different factors such as the type of solvent and extraction times used to obtain different degrees of quality and quantity of

bioactive compounds from raw extracts. Today, the most common extraction method for pharmaceutical and/or nutraceutical products is the use of ethanol [5].

Moringa oleifera is a tree-foliage that has an extraordinary range of beneficial effects harnessed in nutrition, medicine, and for other industrial purposes [6]. This tree has been used in animals and humans because it is one of the richest plants in nutrients discovered so far. It is abundant in digestible protein (20–35% dry mater [DM] includes all essential amino acids), crude fiber (7–35% DM), crude fat (7–20% DM), minerals (8–11% DM), and vitamins [7,8]. In this sense, the leaves are the most important source of bioactive compounds, and its medicinal use has been attributed to the high content of polyphenols (10–82 mg/g DM), phenolic acids (0.40–1.03 mg/g DM), flavonoids (1.29–86 mg/g DM), tannins (5–27 mg/g DM), alkaloids, saponins (2–81 mg/g DM) and carotenoids (17.6–39.6 mg/g DM) [9,10]. Additionally, young and old leaves possess oxidation resistance to protect major biomolecules from reactive oxygen species and are a source of vitamin A (15.99 mg/100 g DM), vitamins B1, B2, and B3 (0.05, 0.80 and 220 mg/100 g DM, respectively), vitamin C (245 mg/100 g DM), iron (49 mg/100 g DM) and several essential (35.40 mg/100 g DM) and non-essential (41 mg/100 g DM) amino acids [7,11,12].

In recent years, leaf extracts from MO have been used as a dietary supplement in goats [13], sheep [14,15], cows [16], and other animals as a strategy for improving production performance and quality of animal products [17]. Some authors [15,18] have reported that dietary supplementation of lactating sheep with herbal extracts obtained from MO leaves and other plant components (i.e., rosemary, acacia, chestnut and quebracho) improves the functional properties of milk and meat from small ruminants and in many cases, they are inexpensive local products.

Overall, producing extracts with adequate solvent and extraction methods will result in great amounts of bioactive compounds at a low cost and easy access because MO leaves contain a large amount of these bioactive compounds. We hypothesized that comparing different levels of ethanol would result in an extract that could be potentially used in ruminant supplementation to most improve the quality of the products offered to the consumer without negative consequences on animal health. Therefore, the objective of the present study was to determine the extraction yield, the content of bioactive compounds and in vitro antioxidant activity of different extracts from MO leaves and their potential use as a natural feed additive for ruminant diets.

2. Materials and Methods

This work was carried out in the Food Development Laboratory of the Graduate and Research Department of the Technological Institute of Merida located at 21°00′46.8″ N and 89°39′57.9″ W, Lic. Manuel Berzunza SN, Pedregales de Lindavista, 97219, Merida, Yucatan, Mexico, with a mean of 26 °C of temperature and 1014.1 mm of rainfall per year and a warm sub humid climate with 80% HR [19].

2.1. Moringa Oleifera Extracts (MOE)

Treatments were: (1) MOEW with 0% absolute ethanol and 100% distilled water, (2) MOEWW with 50% absolute ethanol and 50% distilled water and (3) MOEE with 100% absolute ethanol. Fresh MO leaves were obtained from mature trees, which were dried at 40 °C for 72 h in a convection oven, and pulverized in a mill to obtain a particle size of between 0.5 and 1 mm. The extraction of bioactive compounds from dry powdered leaves was performed by magnetic stirring using a solid to liquid ratio of 1:20 (p/v) with absolute ethanol (MOEE), distilled water (MOEW) and ethanol-distilled water (50%, v/v) (MOEEW) for 2 h under continuous stirring (120 rpm) at 25 °C [20]. The samples were centrifuged (1800× g for 10 min at 25 °C) to obtain the supernatant (extract). The sediment was subjected to a second extraction under the same conditions described above. Finally, the supernatants of both extractions were pooled, and the extract was stored at −20 °C until analysis.

To determine the extraction yield, five mL of extract were evaporated to a constant weight in an oven at 80 °C and was calculated as follows:

$$\text{Extraction yield} = (\text{weight of dry extract after solvent removal (g)} * \text{total vol (mL)} / \text{weight of leaf powder (g)} * \text{extract aliquot (mL)}) * 100 \quad (1)$$

The extracts were prepared in duplicate, and all analysis was carried out in triplicate.

2.2. Total Saponins (TS)

The TS content was determined according to the procedure described by Ncube et al. [21]. Aliquots of each extract (250 µL) were mixed with 250 µL of vanillin reagent (8%, *w*/*v* in absolute ethanol) and 2.5 mL of sulfuric acid (72%, *v*/*v*). The reaction mixture was incubated for 10 min at 60 °C (with a water bath) and subsequently, the mixture was allowed to cool for 4 min and the absorbance was measured at 544 nm using a UV-Vis spectrophotometer (Cary 60 Agilent, Santa Clara, CA, USA). TS was expressed as milligrams diosgenin equivalents per 100 mL of plant extract (mg DE/100 mL).

2.3. Condensed Tannins (CT)

For CT determination, the method (vanillin-HCl) described by Selcuk and Erkan [22] was followed. Aliquots of each extract (0.5 mL) were homogenized with 3 mL of vanillin reagent (4%, *w*/*v*, in methanol) and 1.5 mL HCl (36%). The mixture was left to stand in darkness for 15 min at 25 °C. The absorbance of the samples was measured at 500 nm in a spectrophotometer. CT content was expressed as milligrams catechin equivalents per 100 mL of plant extract (mg CE/100 mL).

2.4. Hydrolyzable Tannins (HT)

The HT content was determined according to Çam and Hışıl [23]. The extracts (1 mL) were mixed with 5 mL of KIO_3 (2.5%, *w*/*v*, in distilled water) (Previously exposed for 7 min at 30 °C). The reaction mixture was kept at 30 °C for 2 min and the absorbance at 550 nm was measured in a spectrophotometer. A calibration curve was prepared using tannic acid as standard and the results are expressed as milligrams tannic acid equivalents per 100 mL of plant extract (mg TAE/100 mL).

2.5. Total Phenolic Compounds (TPC)

The TPC content was determined using the Folin-Ciocalteu reagent following the procedure described by Moo-Huchin et al. [24]. The extract (50 µL) was transferred to a test tube with 3 mL of distilled water and the solution was homogenized in a vortex and kept at rest for 5 min. To the above solution, 750 µL of $NaCO_3$ (20%, *v*/*v*, in distilled water) and 950 µL of distilled water were added, followed by vortex homogenization. The reaction mixture was incubated for 30 min at 25 °C and the absorbance at 765 nm was measured. TPC was expressed as milligrams gallic acid equivalents per 100 mL of plant extract (mg GAE/100 mL).

2.6. Total Flavonoid Compounds (TFC)

The TFC content was determined through the aluminum chloride method as described by Moo-Huchin et al. [24]. The extract (1 mL) was transferred to a test tube with 4 mL of distilled water and 300 µL 5% $NaNO_2$ and allowed to rest for 5 min. To the above mixture, a methanolic solution of 10% $AlCl_3$ (300 µL) was added, followed by vortex homogenization. Subsequently, 2 mL of 1 M NaOH was added to the reaction mixture and the volume of the mixture was adjusted to 10 mL with distilled water. The spectrophotometer was set to a wavelength of 415 nm and the absorbance of the sample was measured. TFC content was expressed as mg quercetin equivalents per 100 mL of plant extract (mg QE/100 mL).

2.7. Antioxidant Activity

DPPH• and ABTS•+ assays were performed according to Moo-Huchin et al. [24]. The decrease in absorbance was measured quantitatively on the spectrophotometer at 515 nm for DPPH• and 734 nm for ABTS•+. The calibration curve (in both assays) was prepared using Trolox as standard and the results are expressed as mM Trolox equivalents/100 mL of extract.

2.8. Statistical Analysis

A completely randomized design was used where the quality parameters of the extract (extraction yield, bioactive compounds, and antioxidant activity) were the random effects, and the percentage of absolute ethanol was considered as the fixed effect. Data was analyzed using Statistical Analysis System software [25] version 9.0 (SAS Institute Inc., Cary, NC, USA). ANOVA tests were performed using a PROC ANOVA for a completely randomized design, and multiple comparison of means were performed by Tukey's test. A principal component analysis was performed using PROC PRINTCOMP to determine which variables explained the greater variability of the extraction data, as well as its correlation between them. Significance was declared at $p \leq 0.05$.

3. Results

3.1. Principal Component Analysis

Table 1 shows the equations of the principal components, where the values of the variables in the equation have been standardized by subtracting their mean and dividing them by their standard deviations. The first two principal components explained 96.5% of the data variability. The principal component analysis shown in Table 1 shows that CT, TPC, and TFC explained the PC1 variation, and DPPH and the extraction yield PC2. The extraction yield was the fourth variable that mostly explained the variation in the first component and maintained a significant correlation in order of importance with TPC ($p \leq 0.001$, $r^2 = 0.989$), DPPH ($p \leq 0.001$, $r^2 = 0.972$), CT ($p \leq 0.001$, $r^2 = 0.941$), TS ($p \leq 0.01$, $r^2 = -0.824$), TFC ($p \leq 0.05$, $r^2 = 0.745$). This indicated that these variables were found to be strong to moderately correlated to extraction yield. Table 2 shows Pearson correlation coefficients (r^2) between each pair of variables. The correlation coefficients ranged from −1 to +1, and they measured the strength of the linear relationship between the variables. The TS had an inversely proportional correlation ($r^2 = -0.701$ to -0.956) with the other variables. This showed that higher yields led to higher amounts of bioactive compounds extracted and antioxidant activity (Table 2). The strongest correlations, in order of importance found among the variables, were TPC-yield, TPC-CT, DPPH-yield, TFC-TS, CT-TS, CT-yield, and TPC-DPPH (Table 2).

Table 1. Vectors and values of the main principal components (PC), which represent the extraction yield, the bioactive compounds, and the antioxidant activity of different MO extracts.

Parameters	PC1	PC2	PC3
Extraction yield	0.358	0.366	0.032
TS	−0.383	0.124	0.278
CT	0.388	0.103	−0.089
HT	0.284	−0.601	−0.063
TPC	0.378	0.248	−0.054
TFC	0.369	−0.219	−0.476
DPPH	0.324	0.483	0.256
ABTS	0.333	−0.369	0.784
Eigenvalue	5.17	1.10	0.13
Variation explained (%)	80.5	16	2.2
Variation cumulative (%)	80.5	96.5	98.7

Standardized values of the variables. DPPH: 2,2-diphenyl-1-picryl-hydrazyl-hydrate, ABTS: 2,2′-Azinobis-3-ethylbenzothiazoline-6-sulfonic acid. TS: Total saponins; CT: Condensed tannins; HT: Hydrolyzable tannins; TPC: Total phenolic compounds; TFC: Total flavonoid compounds.

Table 2. Pearson's linear correlation matrix between each pair of variables.

	Extraction Yield	TS	CT	HT	TPC	TFC	DPPH
Extraction yield	1						
TS	−0.824 **	1					
CT	0.941 ***	−0.946 ***	1				
HT	0.372	−0.786 *	0.634	1			
TPC	0.989 ***	−0.894 **	0.975 ***	0.502	1		
TFC	0.745 *	−0.956 ***	0.889 **	0.839 **	0.831 **	1	
DPPH	0.972 ***	−0.701 *	0.866 **	0.229	0.938 ***	0.619 *	1
ABTS	0.599	−0.844 **	0.767 *	0.873 **	0.684 *	0.834 **	0.498

* ($p \leq 0.05$): low; ** ($p \leq 0.01$) moderate; *** ($p \leq 0.001$): and high correlation significance between the variables. TS: Total saponins; CT: Condensed tannins; HT: Hydrolyzable tannins; TPC: Total phenolic compounds; TFC: Total flavonoid compounds.

Table 2 shows strong correlations between CT and TPC ($p \leq 0.001$, $r^2 = 0.975$), TFC ($p \leq 0.01$, $r^2 = 0.889$) and DPPH ($p \leq 0.01$, $r^2 = 0.866$). CT was the variable that best explained PC2. In contrast, ABTS showed a negative correlation with TS ($p \leq 0.01$, $r^2 = -0.844$) and was the variable that had a moderately strong to moderate relationship with TFC ($r^2 = 0.834$), HT ($r^2 = 0.873$), CT ($r^2 = 0.767$) and TPC ($r^2 = 0.684$), while DPPH was correlated with extraction yield ($r^2 = 0.972$), TPC ($r^2 = 0.938$), CT ($r^2 = 0.866$) and TFC ($r^2 = 0.619$).

3.2. Extraction Yield

Some differences ($p \leq 0.05$) were found in the extraction yield values. MOEW and MOEEW extracts were those with the highest value (between 26.76 and 26.94%, respectively), while MOEE extract had the lowest (Table 3).

Table 3. Bioactive compounds content and antioxidant activity of *M. oleifera* extracts.

Parameter	MOEW	MOEEW	MOEE
Extraction yield (%)	26.76 ± 0.62^{b}	26.94 ± 0.19^{b}	7.14 ± 0.02^{a}
TS (mg DE/100 mL)	39.25 ± 4.5^{b}	22.66 ± 1.59^{a}	55.30 ± 3.71^{c}
CT (mg CE/100 mL)	11.05 ± 0.19^{b}	13.58 ± 0.79^{c}	4.82 ± 0.71^{a}
HT (mg TAE/100 mL)	112.47 ± 3.85^{b}	183.35 ± 1.82^{c}	120.5 ± 2.75^{a}
TPC (mg GAE/100 mL)	121.60 ± 1.94^{b}	130.57 ± 1.89^{c}	55.29 ± 0.72^{a}
TFC (mg QE/100 mL)	2.66 ± 0.13^{b}	3.68 ± 0.38^{c}	1.95 ± 0.08^{a}
DPPH (mM trolox/100 mL)	32.75 ± 1.72^{b}	30.48 ± 1.08^{b}	21.05 ± 0.36^{a}
ABTS (mM trolox/100 mL)	277.70 ± 22.86^{a}	346.23 ± 19.68^{b}	259.98 ± 12.84^{a}

Data were represented as mean ± SEM of three measurements. DE: Diosgenin equivalent; CE: Catechin equivalent; TAE: Tannic acid equivalent; GAE: Gallic acid equivalent; QE: Quercetin equivalent. Means in the same row with different superscripts (a, b, c) are different ($p \leq 0.05$). TS: Total saponins; CT: Condensed tannins; HT: Hydrolyzable tannins; TPC: Total phenolic compounds; TFC: Total flavonoid compounds.

3.3. Bioactive Compounds

Table 3 shows the results of the bioactive compounds of MO extracts. According to the results, the type of solvent used for extraction affected the content of bioactive compounds from MO extracts. MOEEW extract had the highest value for CT, HT, TPC and TFC, while MOEE had the highest TS content.

3.4. Antioxidant Activity

The in vitro antioxidant activity of MO extracts was affected by the type of solvent used for extraction. The MOEEW extract obtained higher antioxidant activity with the ABTS assay than the other treatments, but the antioxidant activity evaluated with the DPPH assay was higher in MOEW and MOEEW.

4. Discussion

4.1. Extraction Yield

In the quantification of extraction yield, Vongsak et al. [26] and Saleem et al. [27] suggested considering the solvent type, time, temperature, extraction methods, polarity of solvent, amount of antioxidants in plant tissue, cultivars, geographic location and other aspects, to optimize the extraction conditions of bioactive compounds. The extraction yield results differ with those of Safdar et al. [28], who reported that the extraction yield was 13.44% (MOEEW). The addition of water in MOEE resulted in a higher extraction yield in MOEEW (26.94%), which was attributed to an increase in the polarity and viscosity of the solvent mixture [29]. This shows the potential of different MO leaf extracts to be included in the diet of ruminants to improve their productivity and consequently improve the functional properties of different animal food products.

4.2. Bioactive Compounds

Comparing our results with scientific reports is difficult as bioactive compounds contained in the MO extracts were based on the sample's dry extract or dry powder. However, the TS observed in liquid samples had the highest extraction with MOEE and the lowest with MOEEW, with values of 55.30 and 22.66 mg/100 mL, respectively. It should be highlighted that these compounds have been used as a natural feeding additive in ruminants in proportions of 100 mg/kg of DM intake and have shown to be positive for productivity and health [30].

The most important bioactive compounds found in MO were TPC and HT. In this sense, Du Toit et al. [31] reported 24 to 48, and 16 to 38 mg/g DM, respectively of these compounds contained in fresh and dry, mature, and immature leaves of the MO. In this sense, similar values of TPC were reported in this work in 1 g of MO extracted with 20 mL of solvent, in MOEW, MOEE and MOEEW (24.32, 11.05, 26.11 mg/g, respectively). The differences in the quantification of tannins explained by Du Toit et al. [31] are due to the state of physiological maturity of the plant and the solvent used. Even so, they are within the values reported by them. When using tannins in ruminant feed, one must consider that they should not exceed 4.0% of the DM consumed by the animal or it will become toxic to them. According to Soldier et al. [32] the inclusion of plant extracts rich in tannins to ruminant diets improves animal antioxidant status in addition to obtaining products for human consumption with greater oxidative stability. To avoid toxicity using hydroalcoholic extracts, one should use no more than 50 mL of ethanol per day per sheep [14].

On the other hand, the highest TPC content was observed in MOEEW with 130.57, followed by MOEW and MOEE with 121.60 and 55.29 mg GAE/100 mL, respectively. *Moringa oleifera* extracts in the study had a higher concentration of TPC than that reported by Coz-Bolaños et al. [33] in moringa infusion (24.33 mg/100 mL), and similar to those found by Povolo et al. [34] in leaf extract (values between 77.52 and 158.04 mg GAE/100 mL).

Total flavonoids showed a higher concentration in MOEEW (3.68 mg QE/100 mL) than in MOEE (1.95 mg QE/100 mL) but both values were higher than those reported by Coz-Bolaños et al. [33] in decoctions, infusions and methanolic extract of moringa leaves. However, the values observed in this study were also lower than those observed by Saleem et al. [27] in MOEW and in extracts of MO with methanol as solvent.

These results agree with Moo-Huchin et al. [20], who showed that the mixture of organic solvents with water allowed the greatest extraction of bioactive compounds in ramon nut (*Brosimum alicastrum*), compared with the extraction using individual solvents. This can be explained because water improves the solvation property and consequently there is a better mass transfer by molecular diffusion.

The findings found in the study conducted by Olagaray and Bradford [35] showed that the aqueous ethanolic extract of MO (50%, in distilled water) can be used as a valuable source of compounds with bioactive potential, which can be used in ruminant nutrition. In fact, several studies reported that flavonoid supplementation to dairy cows can attenuate postpartum inflammation, endoplasmic reticular stress, and hepatic lipid accumulation.

4.3. Antioxidant Activity

The observed values in the assay DPPH range from 21.05 to 32.75 mM Trolox/100 mL (210.5 to 327.5 µM/mL) in the MO extracts and were higher than those reported by Coz-Bolaños et al. [33], which were 0.704, 0.691 and 1.17 µM Trolox/mL, extracted with decoctions, infusions and methanolic, respectively. Furthermore, the work of Saleem et al. [27] reports an DPPH inhibition percentage of 88.50 and 84.46%, in methanolic and aqueous extracts of MO with a concentration of 5 mg/mL. The moderate correlation between bioactive compounds and antioxidant activity reported in this study suggests that the phenolic compounds present in the MO extract probably contribute greatly to antioxidant activity, which was also reported by Polumackanycz et al. [36] in *Morus alba* L. and *Morus nigra* leaves. Similarly, Moyo et al. [37] reported that using acetone or water as a solvent in MO extracts at a concentration of 1 mg/mL of DM used, showed a percentage of inhibition in the ABTS assay of 95.3 and 72.9%, respectively. In this study, a concentration of 259.98 to 343.26 mM Trolox/100 mL was observed, which demonstrates great antioxidant activity in the MO extracts and has the potential to be used as animal or human supplement.

Due to the high content of bioactive compounds and antioxidant activity that were found in MO extracts at 50 % of ethanol (Table 3) it could be suggested to use it as dietary supplements in ruminants at doses that do not exceed 5% of the animal's total DM intake to reduce stress, improve productivity and quality of the final product.

The differences between the responses observed in the antioxidant activity assays of plants (as additives for animal feed) are explained because the methods shown differ in their mechanisms of antioxidant action (electron transfer or hydrogen atom transfer) [1]. On the other hand, the use of MO as a feed additive for ruminant diets is getting attention as they can improve animal's health and product's quality [7,18,38,39]. In this sense, Kholif et al. [13] reported that supplementing with 20 and 40 mL of MOEW per day to lactating Nubian goats can improve feed intake in addition to improved nutrient digestibility and ruminal fermentation parameters. Likewise, Kholif et al. [16] supplied a mixture of phytogenic additives added to the feed at a concentration of 3 g per cow per day (the principal compounds identified in the mixture were menthol, levomenthol, b-linalool, anethole, hexadecanoic acid and p-menthane) and reported improved feed efficiency, milk production and milk contents of total solids, protein, lactose, and fat.

5. Conclusions

The higher polyphenol extraction from *Moringa oleifera* leaves was greater with 50% ethanol. Overall, the variables that were correlated with the antioxidant activity of MO extracts were the extraction yield, amount of condensed and hydrolyzed tannins, content of phenolic compounds, and total flavonoids. More studies are needed to confirm the pharmacological effects of hydroalcoholic extracts in ruminants.

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