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Determination of the main flavonoids in *ojo de gallo* (*Sanvitalia procumbens* Lamarck) yerba extracts from three different sites as potential biomolecules

Determinación de los principales flavonoides en extractos de yerba ojo de gallo (*Sanvitalia procumbens Lamarck*) silvestre de tres sitios diferentes como potenciales biomoleculas

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ABSTRACT

This work focuses on determining the main flavonoids with medicinal effects present in the wild *ojo de gallo* plant endemic to the State of Guanajuato in Mexico. For this purpose, the concentration of kaempferol, catechin, and rutin was determined in the wild *ojo de gallo* grass collected from three different sites in the state of Guanajuato (Comedero Grande, Ex – Hacienda de Márquez, and Tejamanil). The dehydration process of the plant was carried out later to determine the concentration of the metabolites of interest using the high-performance liquid chromatography (HPLC) technique. A statistical analysis was carried out to determine the relationship between the concentration of each metabolite and the various collection sites, also considering the results of the fertility analysis. The results show that the highest concentration for kaempferol was obtained in Ex – Hacienda de Márquez with a value of 24.15 ppm, while for catechin and rutin, it was in Tejamanil reporting values of 26.52 ppm and 46.98 ppm, respectively. The statistical analysis shows no significant difference between Ex -Hacienda de Márquez and Tejamanil for the three metabolites. These results indicate that the site called Comedero Grande favors the presence of kaempferol and rutin while the site called Tejamanil favors the presence of catechin; this opens the possibility of future studies that allow finding the appropriate conditions to maximize the flavonoids of interest in this plant.

Keywords: Biomolecules; catechin; kaempferol; rutin; ojo de gallo grass.

RESUMEN

El presente trabajo se enfoca en la determinación de los principales flavonoides con efectos medicinales presentes en planta silvestre de ojo de gallo endémica del estado de Guanajuato en México. Para tal fin la concentración de kaempferol, catequina y rutina se determinaron en muestras de ojo de gallo recolectadas en tres sitios diferentes del estado (Comedero Grande, Ex – Hacienda de Márquez y Tejamanil). Se realizó el proceso de deshidratación de la planta para posteriormente determinar la concentración de los metabolitos de interés mediante la técnica de HPLC. Se realizó un análisis estadístico para determinar la relación entre la concentración de cada metabolito y los distintos sitios de recolección, considerando también los resultados del análisis de fertilidad. Los resultados muestran que la mayor concentración para kaempferol se obtuvo en Ex - Hacienda de Márquez con un valor de 24.15 ppm mientras que para catequina y rutina fue en Tejamanil reportando valores de 26.52 ppm y 46.98 ppm respectivamente; en este sentido, el análisis estadístico muestra que para los tres metabolitos no existe diferencia significativa entre Ex -Hacienda de Márquez y Tejamanil. Estos resultados indican que el sitio denominado Comedero Grande favorece la presencia de kaempferol y rutina mientras que el sitio llamado Tejamanil favorece a la presencia de catequina, esto abre la posibilidad a futuros estudios que permitan buscar las condiciones adecuadas para maximizar los flavonoides de interés en esta planta.

Palabras clave: Biomoleculas; catequina; kaempferol; rutina; hierba de ojo de gallo.

INTRODUCTION

In 2015, the members of the United Nations approved 17 objectives as part of the 2030 agenda for sustainable development, whose purpose is to end poverty, protect the planet, and improve the lives and prospects of people all over the world (United Nations, 2023). Accordingly, the third objective, called "Health and well-being," focuses on: "Guaranteeing a healthy life and promoting well-being at all ages is essential for sustainable development" (United Nations, 2023), for which the preservation of health is a fundamental aspect of achieving this goal. Empirical evidence has been developed over hundreds of years by many people worldwide about using plants as curative elements for diseases. Mexico has not been the exception since there has been a wide use of plants throughout history in this sense and the culinary. Thus, in 1975, the IMEPLAM (Mexican Institute for the Study of Medicinal Plants) was created to develop multidisciplinary research with plants used to treat common diseases in Mexico. This institute formed the IMSS Medicinal Herbarium, which includes more than 120,000 specimens (Domínguez-Barradas *et al*., 2015). 12% of the terrestrial diversity of the planet is found in Mexico, and it is estimated that there are 23,400 vascular plants, of which 3,000 have medicinal effects and, of this amount, only 450 have been pharmacologically validated; meanwhile, the rest of biological diversity of the country has not been studied or documented (CONABIO - Comisión Nacional para el Conocimiento y Uso de la Biodiversidad, 2019).

Traditional medicine has existed since the oldest civilizations, and its practices are primarily based on the use of medicinal plants, which contain active ingredients that, in sufficient doses, produce curative effects and have pharmacological action (Li *et al*., 2018; Wasana *et al.,* 2022; Julsrigival *et al.,* 2021). Through the knowledge of ethnobotany and herbal medicine, research was carried out on the active principles present in the best-known plants, which gave rise to the branch of science called Phytochemistry (Rivas *et al.,* 2016; Awan *et al*., 2021; Mechaala *et al.,* 2022). The products derived from secondary metabolism in plants are of greater interest since they are considered to be the active principles of medicinal plants due to the effects they produce, among which are antimicrobial, anti-inflammatory, antioxidant, antitumor, antiviral, and anticancer effects, among others (Afrin *et al*., 2015; Rai *et al*., 2017). In this sense, what is known as a phytocomplex is contained in the extract obtained from a plant or a part of it, using several possible extraction methods regarding the ingredient's therapeutic characteristics.

Among the secondary metabolites studied for their various properties are kaempferol, catechin, quercetin, rutin, and myricetin. Kaempferol is a compound whose properties have been studied to improve the absorption capacity of glucose in the body (Moore *et al.,* 2023), be anti-inflammatory (Chang *et al.,* 2023) and be anticancer (Ribeiro *et al*., 2023). In this sense, catechin is a secondary metabolite that has been shown to have beneficial antidiabetic, anticancer, antiviral, and antihypertensive activity (Cai *et al*., 2018; Michel *et al*., 2022; Zare & Noruzi, 2023). In the same way, as in the two previous cases, quercetin has antibacterial, purifying, and antiapoptotic properties in animals (Gong *et al*., 2023; Willian de Alencar *et al*., 2023; Hou *et al*., 2023). Rutin is a flavonoid with anticancer activity in various cell lines, which is a promising aspect in combating this condition, as well as in previous cases, it has nutraceutical properties (Imani *et al*., 2020; Jia *et al*., 2022; Sengupta *et al*., 2023). Finally, myricetin has properties that contribute to the processes of inhibition of proliferation, migration, and invasion of cancer cells, inducing apoptosis in cells where this process is of interest (Gu *et al*., 2023); it also has pharmacological activities, antimicrobial, antithrombotic, neuroprotective, and anti-inflammatory effects (Pan *et al*., 2023; Nie *et al*., 2023).

Ojo de Gallo grass (*Sanvitalia procumbens*) is a plant considered native to Mexico, and it is part of the wild flora of the State of Guanajuato, with an annual cycle that stands out for its flowers with a brown-purple disc-shaped center, which could remind the eye of a rooster, for which its common name stands out. It is distinguished by its elliptic to ovate "petals" (lamellae of ray florets), 5 to 9 mm, shorter than the edges of the fruit, which are purple. In most of the populations, the central flowers (tubular) are brown, but there are some with other colors, such as cylindrical hypocotyl, up to 40 cm long, sometimes reddish; cotyledons broadly elliptic to oblong, 2-5 mm long and 1-3 mm broad, without hairs; epicotyl cylindrical, up to 9 mm long, with strigose hairs; leaves opposite, petioles 1-2 mm long, lanceolate to ovate, 2-8 mm long and up to 3 mm wide, hairless, and with a ciliated edge. That is why the *ojo de gallo* grass turns out to be of great interest for its characterization and to standardize a manufacturing process with low environmental impact and develop a food supplement option (Biblioteca Digital de la Medicina Tradicional Mexicana, 2023); this work is focused on the detection and determination of kaempferol, catechin, and rutin in the *Ojo de gallo* grass collected from three specific sites.

MATERIAL AND METHODS

Plant samples of *ojo de gallo* grass were collected in three different geographical areas of the State of Guanajuato (Comedero, Ex - Hacienda de Márquez, and Tejamanil), where it was found in the wild.

The collection areas for *ojo de gallo* grass in the State of Guanajuato are: Feeder with GPS coordinates: Longitude (dec): -101.234167 is located in the southern area of the municipality of Irapuato with an altitude of 1913 m, average annual precipitation between 550-600 mm and an average annual temperature of 19.1 °C; Ex - Hacienda de Márquez is located in the area south of the municipality of Irapuato, at the geographic coordinates 20°46’53” north latitude and 101°21’37” west longitude with an altitude of 1750 m, maintaining similar values of precipitation and average annual temperature with respect to the site described above because they are located in the same geographical area called bajío; and Tejamanil is located in the northwestern part of the municipality, at the geographic coordinates 20°46’34” north latitude and 101°29’41” west longitude with an altitude of 1733 m and as in the two previous sites, it maintains similar values of precipitation and average annual temperature as it is located in the same municipality. The search for the geographical areas where the most significant presence of this plant is found was carried out. In addition, take small farmers as an approach so that environmental conditions are not a variable that interferes with the extraction of metabolites.

Dehydration standardization. In order to dry the entire plant, four removable metal trays made of stainless material were available and placed in a drying channel with 156.40 g of the plant in each tray. Afterward, they were weighed after the indicated time had elapsed to obtain the dry weight of the plant. The trays with the plants to be dried are exposed in the channel to airflow. This flow serves, on the one hand, to heat the plants and, on the other, to evacuate the eliminated moisture content. The convection method was used (Yilmaz & Alibas, 2022), with some modifications at different times (30, 24 and 12 hours) and the following temperatures (45, 65 and 72°C), respectively, to preserve the color of the flower, using an LSIS- B2V/VC 111, VENCEL, USA. The dried plant was obtained and ground to obtain a fine powder, which will be used to obtain the extract, and was also dehydrated for 60 days at room temperature in a dry room, without air inlets, in kraft paper bags with 50 g of complete plant, using this method as a reference to what is usually done in the winery for this type of plants (Banchero *et al*., 2017).

Standardization of the grinding and extraction process. The dehydrated plant (leaf, root, and flower) was ground in an Osterizer blender at speed 4 for two minutes. The extraction was done with 100 mg of dehydrated tissue (leaf, root, and flower) of "Sanvitalia procumbens" in 100 µL of 70% ethanol, shaking overnight at 4°C. It was centrifuged for 15 min at 12,000 rpm, and the supernatant was collected with a 200 µL micropipette to pass it through a 0.20-micrometer millipore filter and placed in 2 mL vials for subsequent HPLC analysis (Sakanaka *et al*., 2005).

Soil analysis. The soil samples were obtained from each of the geographical areas by giving the laboratory 1 kg of sample from each area in triplicate, being a composite sample (for each geographical area, ten extraction points were taken to mix later and obtain the kg of soil). The analyses were carried out in a commercial laboratory specialized in this.

Determination of the profile of metabolites of interest. HPLC on the wild plant determined the secondary metabolite profile using a column (Code) of ZXDB-10. Column description: Agilent column – Zorbax Eclipse XDB-C18, 150 x4.6 mm, 5µm, column temperature (°C) 25° with a minimum flow of 1 ml/min, injection volume of 20 µL, run time of 45 min, U.V. detection at 235, 280 and 360 nm, Post run of 5 min and the mobile phase data shown in table 1. Standards of kaempferol, catechin, and rutin from the Sigma-Aldrich brand at a concentration of 100 ppm were used, creating curves involving the area of the chromatogram that yielded the various concentrations of the measured standards and through this relationship, the concentrations of the samples of interest were calculated. In order to monitor the behavior of the method, the standards were injected at 100 ppm at the beginning, in the middle, and at the end of the analysis, which confirmed that the method was stable, obtaining a variation in the retention times of each compound of 0.03; The retention times being 4,260 min, 6,989 min, and 15,384 min for catechin, rutin, and kaempferol, respectively.

Table 1. HPLC system mobile phase data.

Phase A: Water: Acetic acid: Acetonitrile (88:2:10)	
Phase B: Water: Acetic acid: Acetonitrile (8:2:90)	
Time (min)	**%B**
0	0
5	15
20	50
25	70
30	100
40	100

Statistical analysis. An Analysis of Variance was carried out to evaluate if there was a significant difference in the samples of the metabolites of interest (kaempferol, rutin, and catechin) in three different places. In this study, three samples were taken for each place to be studied, as described in Section 2.1. Indicating a calculated value of

F, comparing it with values reported in tables, and when the calculated value of F is greater than the value in the tables, there is a significant difference.

RESULTS AND DISCUSSION

Kaempferol. Table 2 shows the concentrations of kaempferol obtained in the various samples taken (extracts obtained from the entire plant). In this table, it can be seen that sample M5 obtained the highest concentration, corresponding to the Ex - Hacienda de Márquez site, and the lowest concentration was in sample M1 from the Comedero Grande site. The analysis of variance (Table 3) indicates a significant difference in the kaempferol values of the samples in the different study places; subsequently, the Fisher pairwise comparison test (Table 3) was performed to determine where the differences exist. The result shows that there is no significant difference in the concentration of kaempferol in the Tejamanil and Ex - Hacienda de Márquez sites, giving in both cases the highest values; in the case of the Comedero Grande, there is a significant difference concerning the other two sites studied.

Table 2. Kaempferol concentration findings in the sampled sites.

Place	Sample	Concentration (ppm)
Comedero Grande	M1	4.92
	M2	13.32
	M3	13.44
Ex - Hacienda de Márquez	M4	20.10
	M5	24.15
	M6	23.34
Tejamanil	M7	22.56
	M8	14.42
	M9	23.34

M1-M3 Samples were collected in the Comedero Grande community, M4-M6 Samples in the Ex - Hacienda de Márquez community, and M7-M9 Samples in the Tejamanil community.

Table 3. Analysis of variance and Fisher's LSD method with 95% confidence for kaempferol.

Variance Analysis					
Fountain	**GL**	**SC Adjust.**	**MC Adjust.**	**Value F**	**Value p**
Factor	2	240.2	120.12	6.82	0.029
Error	6	105.8	17.63		
Total	8	346.0			
Fisher's LSD method with 95% confidence					
Factor	**N**	**Half**	**Group**		
Ex - Hacienda de Márquez	3	22.53	A		
Tejamanil	3	20.11	A		
Comedero Grande	3	10.56		B	

According to these results, an analysis of the fertilization parameters corresponding to the site that reported the best concentration of kaempferol, which is M5, was carried out, obtaining the following data: Soil with a sandy loam texture, % Organic matter (% M.O.) equal to 1.65, concentrations of $N\text{-}NO_3$, K, Ca, Mg, Na, B and $S\text{-}SO_4$ of 3.80, 310, 4500, 210, 150, 0.31, and 5.36 ppm, respectively. Similarly, the site that presented the lowest concentration of the studied metabolite corresponding to M1 was analyzed, giving data on soil with a sandy loam texture, % of M.O. equal to 3.79, concentrations of $N\text{-}NO_3$, K, Ca, Mg, Na, B and $S\text{-}SO_4$ of 32, 790, 3400, 510, 98, 0.6, and 18.27 ppm. These data indicate that the parameters for site M5 are lower than those recorded for M1 except for Ca, which is indicative that kaempferol is favored in soils with characteristics close to those reported by M5 and, therefore, is a point of reference for future studies of reproduction intensive of the plant studied.

Catechin. Table 4 shows the concentrations of catechin obtained in the various samples taken (extract obtained from the entire plant). The table shows that the highest concentration was obtained by sample M8, corresponding to the Tejamanil site, and the lowest concentration was in sample M6 from the Ex - Hacienda de Márquez site. In this sense, it can be observed that the site called Comedero Grande did not register the presence of this metabolite. The ANOVA analysis (Table 5) shows a significant difference in the catechin values of the samples in the three different study places where the samples were taken. Fisher's pairwise comparison test (Table 5) was performed to determine where there are differences. The result shows that there is no significant difference in the catechin in Tejamanil and Ex - Hacienda de Márquez, given that in the case of Ex - Hacienda de Márquez, the value of the average is the highest, while for Comedero Grande, not registering the presence of catechin confirms the significant difference concerning Tejamanil and Ex - Hacienda de Márquez.

Table 4. Catechin concentration findings in the sampled sites.

Place	Sample	Concentration (ppm)
Comedero Grande	M1	0
	M2	0
	M3	0
Ex - Hacienda De Márquez	M4	7.36
	M5	14.13
	M6	5.89
Tejamanil	M7	15.02
	M8	26.52
	M9	17.60

M1-M3 Samples were collected in the Comedero Grande community, M4-M6 Samples in the Ex - Hacienda de Márquez community, and M7-M9 Samples in the Tejamanil community.

Table 5. Catechin concentration findings in the sampled sites.

Variance Analysis					
Fountain	**GL**	**SC Adjust**	**MC Adjust**	**Value F**	**Value p**
Factor	2	916.7	458.35	8.04	0.020
Error	6	342.2	57.04		
Total	8	1258.9			
Fisher's LSD method with 95% confidence					
Factor	**N**	**Half**	**Group**		
Ex - Hacienda de Márquez	3	22.77	A		
Tejamanil	3	19.72	A		
Comedero Grande	3	0		B	

According to these results, an analysis of the fertilization parameters corresponding to M8 was carried out, obtaining the following data: Soil with a sandy clay loam texture, % of M.O. equal to 1.34, concentrations of N-NO_3, K, Ca, Mg, Na, Cu, B and S-SO_4 of 47.9, 230, 3600, 410, 250, 4.80, 1.4 and 23.58 ppm, respectively. Likewise, the site that recorded the lowest concentration different from 0 of this metabolite was M6 with the following data: Loamy soil texture, % of M.O. equal to 1.39, concentrations of N-NO_3, K, Ca, Mg, Na, Cu, B, and S-SO_4 of 39.6, 260, 3900, 300, 250, 5.3, 1.4 and 19.19 ppm. As can be seen, the fertilization data from both sites are close to values, which indicates that the amount of catechin is not substantially affected by the fertilization parameters studied, which leaves open the possibility of studying to determine factors that allow favoring the presence of this metabolite.

Rutin. Table 6 shows the concentrations of rutin obtained in the various samples taken (extract obtained from the entire plant). This table shows that the highest concentration was obtained by sample M7, corresponding to the Tejamanil site, and the lowest concentration was in sample M1, from the Comedero Grande site. The ANOVA analysis (Table 7) indicates a significant difference in the routine values of the samples in the three different places of study. When performing the comparison test in Fisher's pairs, the result shows no significant difference between Tejamanil and Ex - hacienda de Márquez, obtaining that the mean value is higher in the case of Tejamanil. At the same time, for Comedero Grande and Ex - Hacienda de Márquez, there is no significant difference, but concerning Tejamanil, there is.

Table 6. Rutin concentration findings in the sampled sites.

Place	Sample	Concentration (ppm)
Comedero Grande	M1	11.52
	M2	20.63
	M3	21.30
Ex - Hacienda de Márquez	M4	21.60
	M5	34.91
	M6	11.79
Tejamanil	M7	46.98
	M8	35.33
	M9	35.10

M1-M3 Samples were collected in the Comedero Grande community, M4-M6 Samples in the Ex - Hacienda de Márquez community, and M7-M9 Samples in the Tejamanil community.

Table 7. Analysis of variance and Fisher's LSD method with 95% confidence for rutin.

Variance Analysis					
Fountain	**GL**	**SC Adjust**	**MC Adjust.**	**Value F**	**Value p**
Factor	2	747.2	373.59	5.32	0.047
Error	6	421.5	70.26		
Total	8	1168.7			
Fisher's LSD method with 95% confidence					
Factor	**N**	**Half**	**Group**		
Ex - Hacienda de Márquez	3	39.14	A		
Tejamanil	3	22.77	A	B	
Comedero Grande	3	17.82		B	

Based on these results, an analysis of the fertilization parameters was carried out for the site that showed the highest concentration of rutin, which corresponds to M7, obtaining the following data: Loam texture soil, % of M.O. equal to 1.43, concentrations of N-NO_3, K, Ca, Mg, Na, Cu, B and S-SO_4 of 39.3, 260, 3800, 300, 260, 5.4, 1.5 and 15.98 ppm, respectively. Likewise, the site that reported the lowest amount of this metabolite, M1, was analyzed, whose values were reported in the section where kaempferol is analyzed. These results show that M7 has higher values in N-NO_3, Ca, Na, and B, while for the other parameters, M1 has the highest values; this opens the possibility of generating more specialized studies on fertilization protocols that allow finding the appropriate conditions to favor the presence of this metabolite.

CONCLUSIONS

The results of this study show that for kaempferol, catechin, and rutin, the highest concentration was found in the sites called Ex - Hacienda de Marquez for the former and Tejamanil for the latter two. In this sense, the Comedero Grande site reports kaempferol and rutin's presence. However, it does not favor their presence concerning the other sites. Likewise, by knowing the sites that maximize the concentration of the metabolites of interest, it was possible to determine their fertility and compare them with the fertility data of the site that showed the lowest concentration of each metabolite and thus know the starting nutritional conditions to generate studies of fertilization protocols that allow the plant to be produced in intensive cultivation.

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