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Exhaustive Extraction of Bioactive Compounds and Antioxidants from Ataulfo Mango Seed

Extracción Exhaustiva de Compuestos Bioactivos y Antioxidantes de la Semilla de Mango Ataulfo

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Abstract
Mango (*Mangifera indica* L.) is an important fruit worldwide, with high production that allows the production products such as juices and jams. Mango processing generates wastes, such as peels and seeds, which have been explored for their phytochemical profile and bioactivity. However, mango seed extraction processes generate waste sediment that has not been analyzed to determine whether polyphenols or antioxidants are present. Therefore, the objective of this study was to perform exhaustive extraction of mango seeds and evaluate their polyphenolic content (PC) and antioxidant activity (AA). The results showed a strong relationship between the content of the extracted material, PC, and AA. Extraction 1 showed a PC of 24.79 ± 1.41 mg GAE/g (hydrolysable) and 12.38 ± 1.70 mg CE/g (condensed) and AA was DPPH: 334.65 ± 18.71 mg TE/g, ABTS: 576.50 ± 16.91 mg TE/g and FRAP: 325.20 ± 14.09 mg TE/g, these results were significant concerning extractions 2, 3, 4, and 5. However, the second extraction was significant in hydrolysable tannins, ABTS, and FRAP against extractions 3, 4, and 5. Extractions 4 and 5 showed the lowest results, with no significant differences between them in all assays. Therefore, it is possible to make at least two extractions of mango seed and obtain significant values of polyphenols and antioxidants in a second extraction, which allows further use of the seed and waste sediment from the first extraction.

Keywords: Agro-industrial waste, Polyphenols, Trolox, Yield.

Resumen
El mango (*Mangifera indica* L.) es una fruta importante a nivel mundial, con una elevada producción, que permite su uso para la obtención de productos como jugos y mermeladas. Su procesamiento genera residuos como las semillas, que han sido exploradas por su perfil fitoquímico y bioactividad. Sin embargo, los procesos de extracción de semillas de mango generan sedimentos residuales que no han sido analizados para determinar si aún se encuentran presentes polifenoles o antioxidantes. Por lo tanto, el objetivo de este trabajo fue realizar una extracción exhaustiva de semillas de mango y evaluar su contenido polifenólico (CP) y actividad antioxidante (AA). Los resultados mostraron una alta relación entre el contenido del material extraído con el CP y la AA. La extracción 1 mostró un CP de 24.79 ± 1.41 mg GAE/g (hidrolizables) y 12.38 ± 1.70 mg CE/g (condensados) y la AA fue DPPH: 334.65 ± 18.71 mg TE/g, ABTS: 576.50 ± 16.91 mg TE/g y FRAP: 325.20 ± 14.09 mg TE/g, estos resultados fueron significativos en relación a las extracciones 2, 3, 4 y 5. Sin embargo, la extracción 2 fue significativa en taninos hidrolizables, ABTS y FRAP frente a las extracciones 3, 4 y 5. Las extracciones 4 y 5 obtuvieron los resultados más bajos, sin diferencias significativas entre ellas en todos los ensayos. Por lo tanto, es posible hacer al menos dos extracciones de la semilla de mango y obtener valores significativos de polifenoles y antioxidantes en la segunda extracción, lo que permite un mayor aprovechamiento de la semilla y del sedimento residual de una primera extracción.

Palabras clave: Polifenoles, Rendimiento, Residuo agroindustrial, Trolox.

INTRODUCTION

Mango (*Mangifera indica* L.) is one of the most important tropical fruits worldwide, in 2019 world mango production was 55.9 million tons (Medina-Rendon et al., 2021) and it is expected to be 84.0 million tons by 2030 (OECD/FAO, 2021). The main producers include India, China, Thailand, Indonesia, Pakistan, Mexico, and Brazil (Altendorf, 2019). In Mexico, several varieties of mango are produced, such as Manila, Kent, Tommy Atkins, Haden, Criollo, and Ataulfo, with the last being the most important variety, which even has an Appellation of Origin. According to data from the "Secretaria de Agricultura y Desarrollo Rural (SADER)", mango production in Mexico in 2021 was 2 million 156 thousand tons, 30% of which corresponded to Ataulfo mango (SADER, 2023).

Mangoes are consumed fresh or processed into products, such as juices, jams, dehydrated pulp, or pulp in syrup. However, mango processing generates a large amount of waste, approximately 14 million tons (Torres-León et al., 2021a). Although the Ataulfo variety is exported more for fresh consumption, several companies in Mexico use it for processing into various products (Espinosa-Palomeque et al., 2023). It has been estimated that a medium-sized plant that can process 200 tons of mangoes per day can generate 84 tons of waste, consisting of peel (15-20%) and seed (20-45%) (Torres-León et al., 2021b).

Mango processing wastes have attracted attention for their chemical composition, as they have been shown to be rich in bioactive compounds such as polyphenols, which have potential benefits for human health. In addition, the mango seed has been described as the part of the fruit with the highest concentration of bioactive compounds (Nicolás García et al., 2023). Torres-León et al. (2021c) conducted extensive research on Ataulfo mango seed, showing its potential as an antioxidant and antimicrobial agent.

Extraction processes that have been used for Ataulfo mango seeds include conventional methods such as maceration and the use of emerging technologies such as microwaves and ultrasound (Cárdenas-Hernández et al., 2021). Additionally, the potential of fermentation to recover bioactive compounds has been explored (Torres-León et al., 2019). All the techniques used have been optimized to obtain the highest amount of bioactive compounds possible, considering zero waste and circular bioeconomy strategies; however, in most cases, at the end of the extraction processes, sediment remains as a final waste that is usually discarded. Therefore, it is necessary to evaluate the residual material to determine the possibility of performing another extraction process to obtain a greater amount of bioactive compounds, considering that many of these compounds are bound to the plant matrix. Therefore, the objective of this study was to perform an exhaustive extraction process on mango seeds and evaluate the polyphenol content and antioxidant activity to determine the number of times mango seeds could be extracted to obtain bioactive compounds with antioxidant potential.

MATERIALS AND METHODS

Material and reagents

Mango seed was obtained from processing mango (*Mangifera caesia* Jack ex Wall) purchased in Saltillo, Coahuila. The seeds were dried in an oven at 40 °C for 48 h, reaching a constant moisture content of 5.37%. The dried seeds were ground in a blender and passed through a 40-mesh sieve. Mango seed powder was stored in plastic bags at room temperature (25 °C).

Chemicals

2,2-diphenyl-1-picrylhydrazyl (DPPH), 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), Folin-Ciocalteu's reagent, sodium carbonate, and gallic acid (3,4,5-trihydroxybenzoic acid) were purchased from Sigma Aldrich.

Exhaustive extraction methodology

Mango seed powder was extracted using 67 % ethanol (solid/liquid ratio: 1 g/23 mL) in flasks. The flasks were then mixed and placed in a heat bath at 60 °C for 30 min. The extract was then transferred to conical tubes and centrifuged at 2000 rpm. Finally, the extracts were stored in light-protected flasks. The sediments in the conical tubes were dried at 40 °C for 24-48 h. Then, the dried sediment was weighed, the volume of ethanol (67 %) was adjusted to maintain the solid/liquid ratio (1 g/23 mL), and the extraction was performed in the same way as the first extraction. The sediment was recovered and extracted again 3 more times. The main extract was obtained at the end of the experiment and four extracts were obtained from sediment extraction.

The percentage of the extracted material was calculated using the following equation:

% extracted material= 100-((dry weight of sediment obtained*100)/(dry weight of the material to be extracted))

Determination of polyphenol content

Hydrolysable Tannins (HT)

HT was determined according to the methodology described by Wong-Paz et al. (2014) using Folin-Ciocalteu reagent. In a microplate well, 20 µL of sample was added. Subsequently, 20 µL of Folin-Ciocalteu's reagent was added and mixed for 5 min. Subsequently, 20 µL of sodium carbonate (10 mM) was added to the mixture and allowed to react for another 5 min. Finally, 125 µL of distilled water was added and the absorbance was read at 790 nm using microplate reader. The results were expressed as milligrams of gallic acid equivalents (GAE) per gram of seed (mg GAE/g seed) according to a gallic acid standard curve (0-1,000 mg/L; R^2 = 0.994).

Condensed Tannins (CT)

CT was performed according to the methodology described by Sepulveda et al. (2020), using HCl-butanol and ferric reagents. Each sample (250 µL) was placed in a test tube. Then, 1,500 µL of HCl-butanol (1:9) was added, followed by 50 µL of the ferric reagent. The test tubes were capped, covered with aluminum foil, and placed in a boiling water bath for 1 h. Next, the tubes were allowed to cool to room temperature (25 °C) and 200 µL were placed in a microplate well. Finally, the absorbance was read at 550 nm using a microplate reader. The results are expressed as milligrams of catechin equivalents per gram of seed (mg CE/g seed) according to a catechin standard curve (0-1,000 mg/L; R^2 = 0.991).

Antioxidant activity assays

DPPH antioxidant activity assay

DPPH assay was performed as described by Torres-León et al. (2017). A total of 193 µL of 60 µM DPPH solution was mixed with 7 µL of sample in each microplate well. After 30 min of reaction in the dark, the absorbance was measured at 517 nm using a microplate reader. The results were expressed in milligrams of Trolox equivalents per gram of seed (mg TE/g seed) according to a Trolox standard curve (0-250 mg/L; R^2 = 0.997).

ABTS antioxidant activity assay

ABTS assay was performed as described by Ordoñez-Torres et al. (2020). For ABTS radical formation, 2.45 mM potassium persulfate ($K_2S_2O_8$) was mixed with 7 mM ABTS solution (1:1 v/v). The mixture was then allowed to stand for 24 h in the dark at 25 °C. Subsequently, the absorbance was measured at 734 nm and the solution was diluted in ethanol until an absorbance of 0.700 nm was reached. For this study, 10 µL of the sample was mixed with 1 mL of ABTS solution and the absorbance at 734 nm was measured using a spectrophotometer. The results were expressed in milligrams of Trolox equivalents per gram of seed (mg TE/g seed) according to a Trolox standard curve (0-250 mg/L; R^2 = 0.994).

Ferric Reducing Antioxidant Power (FRAP) assay

The FRAP assay was performed as described by Torres-León et al. (2017). 10 µL of the sample was mixed with 290 µL of FRAP reagent in a 96-well microplate. The reaction mixture was then incubated at 37 °C for 15 min. Absorbance was measured at 593 nm using a microplate reader. The results were expressed in milligrams of Trolox equivalents per gram of seed (mg TE/g seed) according to a Trolox standard curve (0-125 mg/L; R^2 = 0.999).

Statistical analysis

All experiments were performed in quadruplicate, and the values are presented as the mean ± standard deviation. The experimental design was completely randomized. The data obtained were analyzed using one-way analysis of variance (ANOVA, $p < 0.05$) and Tukey's mean comparison test ($p < 0.05$). Data analysis was conducted using the Statistica 7.0. Pearson's correlation was calculated using Microsoft Excel.

RESULTS AND DISCUSSION

Extracted material

The results of the extraction process are shown in Figure 1. The first (29.09 ± 0.59 %) and second (18.76 ± 1.13 %) extraction had a high percentage of extracted material above 10 %, which were significantly different from each other and against extractions 3 (5.87 ± 1.63 %), 4 (5.41 ± 1.51 %) and 5 (5.09 ± 0.65 %). Extractions 3, 4, and 5 had yields of less than 6 % with no significant differences between them. The results obtained are in accordance with those reported for other agro-industrial wastes such as chestnut wood (15-20 %) (Aimone et al., 2023), grape pomace (7.92 %), peanut shell (15.17 %), mango bagasse (37.07 %) (Braga et al., 2016), and mango seed Chok-Anan (3.31-11.90 %) (Maisuthisakul, 2009).

Mango seeds seem to have a higher extraction yield than different waste varieties, however, factors such as extraction method, temperature, solvent, time, and nature of the sample may influence these results (AL Ubeed et al., 2022). The extraction yield showed that up to a second extraction of mango seed, it was possible to obtain a high amount of extract, however, from the third extraction, the yield was low and there was no significant difference in extractions 4 and 5. Therefore, two extractions of mango seeds may be adequate to obtain a high percentage of the extract.

This is mainly due to the amount of extractable material available although binary ethanol-water mixtures allow the extraction of both polar and less polar compounds (Lim et al., 2019), there is a limit to the amount of material that can be extracted. In agro-industrial wastes such as mango seed there is a certain amount of free phenols, while another part of the phenols are bound in the plant matrix by covalent bonds (Vilas-Franquesa et al., 2023), for this reason, even if more extractions are carried out, it will be impossible to have a greater amount of extract. The largest amount of extract was found in the first two extractions.

Polyphenol content

The polyphenol content results are shown in Figure 2. In general, mango seeds have a higher content of hydrolysable tannins than condensed tannins (Torres-León et al., 2021b). The content of hydrolysable tannins (24.79 ± 1.41 mg GAE/g) was similar to that reported by Cárdenas-Hernández et al. (2021) for Ataulfo mango seed (29 mg GAE/g), and these results are higher than those reported for red grape seeds (20. 69 ± 0.13 mg GAE g^{-1}) (Di Stefano et al., 2022), pomegranate seed (1.84-4.67 mg GAE g^{-1}) (Falcinelli et al., 2017),

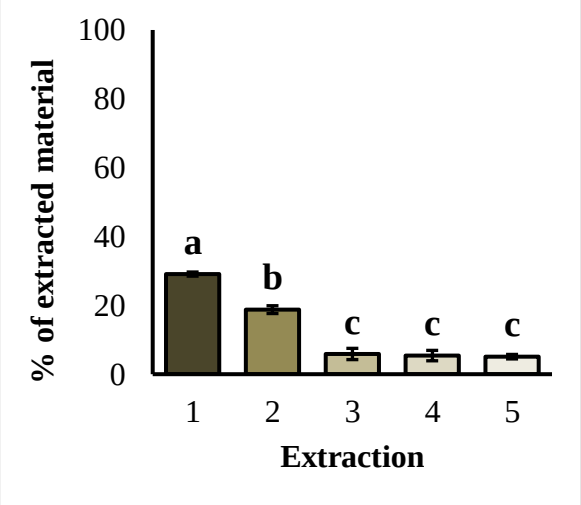

Figure 1. Percentage of material extracted from mango seeds. Different letters indicate significant differences ($p < 0.05$).

and litchi cv. Jizui pericarp (11.79 ± 1.05 mg GAE/g) (Li et al., 2012). Condensed tannins (12.38 ± 1.70 mg CE/g) were higher than those reported in *Anabasis articulata* stems (4.03 ± 0.34 mg CE/g) (Benhammou, N. et al., 2013) and *Passiflora* leaves (0.65 ± 0.04 mg CE g^{-1}) (Pineli et al., 2015).

Statistical analysis shows that the first (24.79 ± 1.41 mg GAE/g) and second (14.18 ± 2.34 mg GAE/g) extraction were significantly different among all extractions for hydrolysable tannins, while for condensed tannins only the first extraction (12.38 ± 1.70 mg CE/g) was significantly different from all others. This indicates that by performing an additional extraction it is possible to obtain a significant amount of hydrolysable tannins, however, for the condensed tannin content the amount obtained (4.97 ± 0.77 mg CE/g) is neither high nor significant with respect to the third extraction (3.94 ± 0.62 mg CE/g).

These results are related to those obtained for the yield of the extracted material, where the most influential factors were the nature of the sample and the solvent used. In general, mango seeds contain more hydrolysable tannins than condensed tannins (Torres-León et al., 2021b); thus, they are predicted to have more hydrolysable molecules. In addition, a large part of the polyphenols is bound, which makes their extraction difficult; thus, after a certain number of extractions, it is no longer possible to obtain more polyphenols. A study by Torres-León et al. (2019) showed that a large part of the polyphenols in mango seeds are bound (~1500 mg GAE/100 g) while free polyphenols represent a content of 713.6 ± 70 mg GAE/100 g. In terms of solvents, ethanol has proven to be a suitable solvent for the

extraction of mango seed compounds, moreover, ethanol is preferred for food applications. In fact, binary ethanol-water mixtures have proven to be better for the recovery of bioactive compounds, and a study by Lim et al. (2019) showed that mixtures of 25-75 % ethanol-water allowed the recovery of the highest amount of polyphenols in mango seed (76.52 - 101.68 mg GAE/g), because in the mixture, water dissolves the polar compounds, and the organic solvent (ethanol) recovers the less polar constituents.

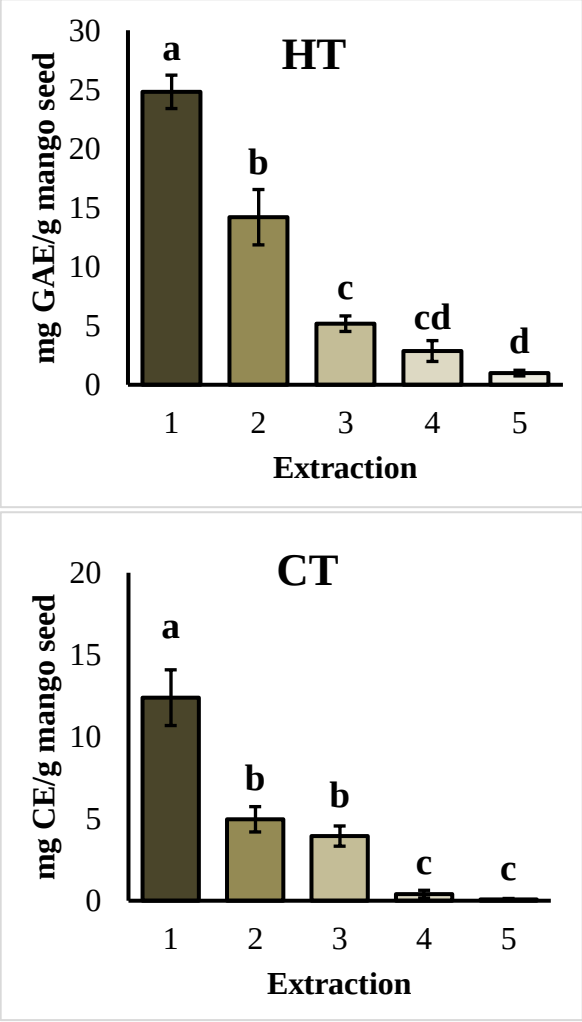

Figure 2. Polyphenol content of mango seed extracts. Different letters indicate significant differences at $p < 0.05$. HT: Hydrolysable Tannins, CT: Condensed Tannins, GAE: Gallic Acid Equivalent, CE: Catequin Equivalent.

Antioxidant activity

The antioxidant activity results are shown in Figure 3. The ABTS assay showed the highest antioxidant activity (576.50 ± 16.91 mg TE/g), followed by DPPH (334.65 ± 18.71 mg TE/g) and FRAP (325.20 ± 14.09 mg TE/g) (Yap et al., 2023). These results are similar to those reported by Torres-León et al. (2017) for Ataulfo mango seed for DPPH (431.1 ± 114.90 mg TE/g), ABTS (499.2 ± 38.43 mg TE/g) and FRAP (455.1 ± 76.53 mg TE/g) and higher than those reported for various Indian legumes (DPPH: 42.9-571.1 mg TE/100 g, ABTS: 4.5-194.9 mg TE/100 g, FRAP: 90.6-2,773.5 mg TE/100 g) (Parikh & Patel, 2018).

All three assays showed that the first extraction was significantly different from the others. Extractions 2, 3, 4, and 5 had much lower values, below 70 mg TE/g, and even between extractions 4 and 5, there was no significant difference in any of the assays. A second extraction allows obtaining a considerable amount of hydrolysable tannins; moreover, extractions 2 and 3 still provide considerable antioxidant activity since extractions 4 and 5 show lower activities of DPPH, ABTS, and FRAP between 3.76 - 5.99 mg TE/g. Although a second extraction is optimal for obtaining a higher amount of polyphenols, it is necessary to evaluate its adequacy, considering that the highest antioxidant activity is obtained in the first extract.

Antioxidant activity studies use the DPPH, ABTS and FRAP assays in a complementary way, the ABTS assay has the ability to interact with hydrophilic and lipophilic type molecules, which allows a greater detection range than DPPH (Munteanu & Apetrei, 2021), this can be corroborated as the assay with the highest antioxidant activity was ABTS, the DPPH assay can only interact with lipophilic molecules which is a disadvantage, however, DPPH is stable, reproducible and need not be generated compared to ABTS (Bibi-Sadeer et al., 2020). The FRAP assay, as well as ABTS, can interact with hydrophilic and lipophilic molecules; however, the mechanism of action is different, since ABTS and DPPH are based on the neutralization of a radical, while FRAP is based on the reduction of the ferric ion (Fe^{3+}) to ferrous (Fe^{2+}) because the results of the assays can be different (Munteanu & Apetrei, 2021). Therefore, a single antioxidant assay does not allow to be broadening the view of antioxidants; therefore, the use of different assays is recommended to distinguish the dominant mechanisms of different antioxidants and their potential.

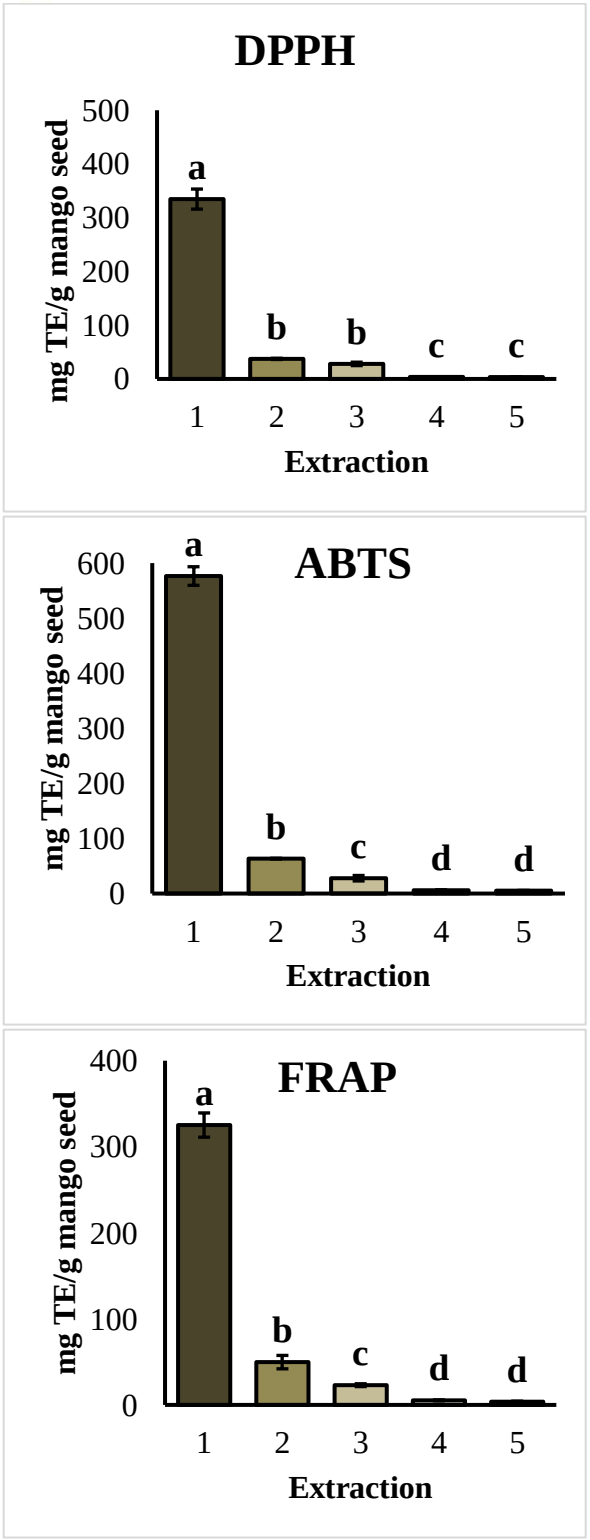

Figure 3. Antioxidant activity of mango seed extracts by DPPH, ABTS and FRAP assays. Different letters indicate significant differences at $p < 0.05$. TE: Trolox Equivalent.

Correlation analysis of Yield with HT, CT, DPPH, ABTS and FRAP

In addition, Pearson's correlation analysis was performed to determine the extraction yield, polyphenol content, and antioxidant activity (Table 1). The results showed a strong correlation of extraction yield with HT and CT, as well as DPPH, ABTS, and FRAP. These results are predictable since, as the amount of extractable material available decreases, the amount of polyphenols that can be obtained is reduced; at the same time, as there are fewer polyphenols, the antioxidant activity is decreased, as shown by strong correlation between HT and CT with DPPH, ABTS, and FRAP. Moreover, the antioxidant assays are strongly and positively related to each other, in fact, DPPH and ABTS have the highest correlation between antioxidants, but FRAP had the best correlation with HT and CT, all these results are similar to those reported by Ribeiro et al. (2013) for exotic and native fruits from Brazil, Muñoz-Bernal et al. (2020) for wine samples, Xiong et al. (2020) for dried yellow bean samples, Tymczewska et al. (2023) for spice extracts and Rumpf et al. (2023) for lignins, therefore although the type of plant material is different the correlations between polyphenols and antioxidants show similar trends.

Table 1. Pearson's correlation of yield against HT, CT, DPPH, ABTS, and FRAP assays.

Test	Yield	HT	CT	DPPH	ABTS	FRAP
Yield	1					
HT	0.9912	1				
CT	0.9322	0.9665	1			
DPPH	0.8829	0.9018	0.9425	1		
ABTS	0.8884	0.9032	0.9364	0.9994	1	
FRAP	0.9037	0.9190	0.9485	0.9988	0.9992	1

Due to the previously mentioned, studies of yield, polyphenol content and antioxidant activity of previously extracted plant matrices can help to determine the viability of exhaustive extraction of the material to obtain high amounts of polyphenols or antioxidants by conventional techniques, because once the content of free phenols is exhausted it is no longer possible to recover more compounds, this can be corroborated by the fact that at least two extractions of the mango seed seem to be adequate to obtain significant values, but more than two extractions is no longer suitable, therefore emerging technologies such as ultrasound, microwaves, pulsed electric fields and supercritical fluids could be more appropriate,

as they have been shown to have the ability to cause more damage to the plant cell wall than conventional techniques, but they have a higher acquisition cost in general (Cristianini & Guillén Sánchez, 2020). In addition, it is necessary to evaluate in these technologies an exhaustive extraction to determine their potential to use the greatest amount of material possible, because some extraction methodologies using emerging technologies perform two or three extractions of the material without previously evaluating whether there is a greater recovery of polyphenols or antioxidants in those two or three extractions or all is recovered in a single extraction (Alañón et al., 2021), which could imply unnecessary cost and energy expenditure.

In addition to emerging technologies, green technologies such as the use of enzymes and fermentations, can help to produce a greater amount of free polyphenols, since the enzymatic processes involved break the plant walls and release the bound phenols, in addition to favoring the hydrolysis of complex molecules (Vilas-Franquesa et al., 2023). However, it is important to consider molecules of interest. For example, pentagalloylglucose (PGG), which consists of central glucose bound to five galloyl groups, and is the major compound in mango seeds, has attracted attention for its biological activities as an antioxidant, antidiabetic and anticancer agent (Mahmoud et al., 2022). Many studies have focused on obtaining mango seed extracts rich in certain compounds, such as PGG; therefore, fermentation processes might not be suitable because PGG is easily hydrolyzed by fungal enzymes into its main components, gallic acid and glucose (Aharwar & Parihar, 2018; Torres-León et al., 2019). As a result, conventional extraction is still a viable, cheap, and safe option for obtaining bioactive compounds from plant matrices such as mango seeds.

CONCLUSIONS

This study determined the polyphenol content and antioxidant activity of mango seeds and waste obtained from extraction using an exhaustive extraction methodology. The results indicated a positive correlation between the amount of extracted material, the polyphenol content, and the antioxidant activity. The first extraction yielded the highest content in all parameters evaluated, while the second extraction yielded a considerable amount of hydrolysable tannins, but not condensed tannins, while the antioxidant activity was still significant for ABTS and FRAP, but not for DPPH. Although the highest antioxidant activity was obtained in the first extraction, the second and third extractions allowed for the recovery of a considerable amount of antioxidants. Therefore, it can be concluded that mango seeds can be extracted at least twice using conventional techniques to obtain a significant amount of polyphenols and antioxidant activity.

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Valorization Of Guava Residues: Evaluation of Substrates and Nutritional Potential in Seed Germination

Valorización De Residuos De Guayaba: Evaluación De Sustratos Y Potencial Nutricional En La Germinación De Semillas

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Abstract
Guava seeds, a byproduct of guava (*Psidium guajava* L.) industrialization, stand out for their high protein content compared to the pulp, which can be further enhanced through germination. In this study, two substrates were evaluated: cotton (CN) and a mixture of peat moss, vermiculite, and perlite (PVP). Under controlled conditions at 30 °C for 30 days, parameters such as sprout height (h), root/shoot ratio, germination speed index (GSI), germination percentage (GP), biomass weight (P), and protein content (PC) were measured. The average height of the sprouts was 5.57 ± 0.15 cm in AL and 5.03 ± 0.15 cm in PVP. The root/shoot ratio varied, with values of 0.52 ± 0.43 for AL and 0.44 ± 0.11 for PVP. GSI was higher in AL with 2.5 ± 0.87 seeds/day, compared to PVP with 1.3 ± 0.4 seeds/day. However, the GP % at the end of the evaluation period was similar in both substrates, at 40 % and 39 % for AL and PVP, respectively. The protein content of the sprouts at the end of the evaluation showed no significant differences (10.39 % ± 0.34 and 11.06 % ± 1.57 for PVP and AL, respectively). Both substrates proved suitable for germination, and the increase in protein content suggests the potential use of these byproducts in both animal and human nutrition.

Keywords: Seed, Guava, Sprouts, Germination, Germination media, Protein.

Resumen
Las semillas de guayaba, como subproducto de la industrialización de la guayaba (*Psidium guajava* L.), se destacan por su elevado contenido proteico frente a la pulpa, siendo este susceptible a incrementarse mediante la germinación. En el presente trabajo, se evaluaron dos sustratos: algodón (AL) y una mezcla de turba, vermiculita y perlita (PVP). En condiciones controladas a 30 °C durante 30 días, se midieron parámetros como altura de los germinados (h), relación raíz/tallo, índice de velocidad de germinación (IVG), porcentaje de germinación (PG), peso de la biomasa (P) y contenido de proteínas (CP). La altura media de los brotes fue de 5.57 ± 0.15 cm en AL y 5.03 ± 0.15 cm en PVP. La relación raíz/tallo varió, siendo 0.52 ± 0.43 para AL y 0.44 ± 0.11 para PVP. El IVG fue mayor para medio AL con 2.5 ± 0.87 semillas/día, mientras que el medio PVP fue de 1.3 ± 0.4 semillas/día. Sin embargo, el % PG al final del tiempo evaluado fue similar en ambos medios con 40 % y 39 % para AL y PVP, respectivamente. El contenido de proteínas de los germinados al final de la evaluación no presento diferencias significativas (10.39 % ± 0.34 y 11.06 % ± 1.57 para PVP y AL, respectivamente). Ambos sustratos resultaron adecuados para la germinación, y el aumento en el contenido proteico sugiere el potencial uso de estos subproductos en la alimentación, tanto animal como humana.

Palabras clave: Semilla, Guayaba, Germinados, Sustrato, Proteína.

INTRODUCTION

Guava (*Psidium guajava* L.), a tropical fruit native to Mexico (Gutiérrez et al., 2008; Vial et al., 2022), is grown throughout South America, Europe, Africa, and Asia (Nivia et al., 2007). Worldwide, its production is around 2,075,000 tons. The main producers are Pakistan, Brazil, India, Mexico, and Egypt, with shares of 22 %, 17 %, 16 %, 15 % and 12 %, respectively. According to 2020 data, Mexico ranks third in the world in production, with an average annual volume of 302,000 tons. India, China, Indonesia, and Pakistan are also important guava producers (Kaur & Ghosh, 2023). Guava is a source of antioxidant compounds, ascorbic acid (vitamin C), phenolic compounds and carotenoids, as well as carbohydrates, dietary fiber and minerals (potassium, calcium, and phosphorus) (Gutiérrez et al., 2008; Kaur & Ghosh, 2023; M. Kumar et al., 2022).

Guava is a raw material for a wide variety of foods such as juices, nectars, sweets, and desserts. After processing a series of residues composed of seeds, peel and pulp are generated, which can represent up to 30 % of the weight of the processed fruit (Gill, 2016; Kaur & Ghosh, 2023; Lima et al., 2019). The final disposal of these wastes is a challenge for the industry, the management and final disposal increase the production cost and can cause serious environmental problems due to microbial decomposition and leachate production (Torres-Leon et al., 2018). Few investigations focus on the use or obtaining of products from guava seeds (Nivia et al., 2007), so it is necessary to develop alternatives that strengthen the production chain and propose possible uses for these agro-industrial residues (Restrepo et al., 2010).

The seed represents between 10-12 % of the weight of fresh fruit (Serna-Cock et al., 2013), it is a material with a high nutritional value (Silva-Vega et al., 2017), significant amounts of bioactive compounds such as ascorbic acid (87.44 mg/100 g), total carotenoids (1.25 mg/100 g), insoluble dietary fiber (63.55 g/100 g) (Uchôa-thomaz et al., 2014) and oils, phenols, tannins, vitamins and lecithins (Irshad et al., 2020). Fiber and protein are found in a greater proportion in the seeds than in the pulp, the crude fiber present in the pulp is between 2.8-5.5 % and in the seed between 65-70 %, and the protein present in the pulp is between 0.9-1.0 % while the seed is between 5-10 % (Irshad et al., 2020). Proteins from guava pulp are more digestible than those from soybeans (Silva-Vega et al., 2017), they are rich in glycine, with antimicrobial activity, they are characterized by having a low molecular weight and a three-dimensional structure like antimicrobial peptides from other families; capable of inhibiting the development of bacteria that cause gastrointestinal diseases (Pelegrini & Franco, 2011).

The germination is a very simple, inexpensive, environmental-friendly and safe way to cultivate the germinated seeds and sprouts within a short time (Gan et al., 2017). Seed germination is a low-cost technique that significantly reduces antinutrients, making the consumption of sprouts a nutritionally advantageous option compared to grains or seeds. During this process, enzymes like phytases break down phytic acid, enhancing the absorption of essential minerals. The reduction of enzymatic inhibitors not only improves digestibility, facilitating nutrient assimilation, but also preserves essential nutrients. This approach not only minimizes the presence of antinutrients but also promotes a healthier and more efficient nutritional intake. (Chávez García et al., 2023; Gupta et al., 2015).

Many studies have shown that seed germination induces physiological changes, and the resulting sprouts have a greater number of bioactive compounds. This type of food is increasingly popular, and its consumption is associated with multiple health benefits (Liu et al., 2019). Silva-Vega et al., (2017) have reported that the protein content increases when germinating guava seeds from 8.78 % to 30.5 %. Due to its high vegetable protein content and the relative ease of germination, guava seeds have been considered as a potential food alternative for ruminants (Silva-Vega et al., 2017). However, more focused research is needed to characterize sprouts for their use in human and animal nutrition.

Seed germination is influenced by several factors, mainly temperature and substrate, which can be controlled to improve germination percentage, uniformity, and germination time (Tuan et al., 2019). However, when the guava seeds germinate poorly and unevenly, requiring more time for seedling emergence (Brijwal & Kumar, 2013), this is attributed to the seed dormancy generated by the hard layer and the impermeability to water and gases. Studies on the germination of guava seeds focus on the effects of pretreatments to break the dormancy of the seed and increase the percentage of germination, as well as the ideal conditions for germination. Different pre-treatments (physical and chemical), germination substrate (cotton, filter paper, absorbent paper, sand, vermiculite, among others) and temperatures (15 to 30 ° C, have been evaluated, with the range between 20-30 ° C being those higher germination percentages (Alves et al., 2015; Kumar et al., 2012; Serratos Tejeda, 2012).

The evaluation of guava seed germination and its characterization is essential due to the lack of updated

information. Previous studies have revealed valuable data, including the positive impact of passage through the digestive tract of black howler monkeys on germination and the usefulness of guava seeds in ruminal nutrition (Serratos Tejeda, 2012; Silva-Vega et al., 2017). These findings are fundamental for the conservation and sustainable use of the species, as well as to optimize agricultural production. The economic and nutritional importance of guava underlines the need for updated information on its germination, opening opportunities to improve the quality of human and animal food. Research in these aspects not only enriches scientific knowledge but also has direct and positive impacts on food, agriculture, and the environment.

The objective of this study was to evaluate the effect of cotton, and different substrates mix (peat moss, vermiculite, and perlite) on the germination of guava seeds (*Psidium guajava* L.) under controlled conditions of humidity and temperature. It also explored whether germination increased the nutritional value of the seeds compared to ungerminated seeds. The potential applications of these findings in animal and human nutrition were discussed, along with the potential of germination as a method for valorizing agro-industrial byproducts.

MATERIALS AND METHODS

Raw material

The seeds were obtained from guava fruits (*Psidium guajava* L.) of the "media china" variety. The fruits were acquired at the municipal market in the city of Saltillo, originating from crops in the municipality of Calvillo, state of Aguascalientes (Mexico), and harvested in December 2018. Fruits of light-yellow color, in a state of ripeness suitable for consumption according to the standards set in the NMX-FF-040-SCFI-2002 norm, were selected. Subsequently, the fruits were washed and immersed in a solution of sodium hypochlorite (50 ppm) for 5 minutes. Once cleaned and disinfected, the fruits were cut in half, and the seeds were manually extracted using a spoon. Following this, the seeds were separated from the rest of the fruit, washed with water using a fine mesh sieve, and left to dry on trays at 30 ºC, following the methodology proposed by Alves et al., (2015) (Alves et al., 2015; NMX-FF-040-SCFI, 2002).

Pretreatment

Seeds were submerged in sterile distilled water for 24 hours prior to planting (Núñez-Gastélum et al., 2023). According to the procedure outlined by Silva-Vega et al., (2017), the seeds were then placed in rectangular aluminum trays (185 x 135 x 30 mm), with 100 seeds deposited in each tray (Silva-Vega et al., 2017).

Evaluation of germination substrate

Two germination substrates were evaluated: cotton (CN) (6 grams per tray) and a mixture of peat moss (PVP) consisting of peat moss (60 %), vermiculite (20 %), and perlite (20 %) (20 grams of bed + 5 grams on the seeds). Germination substrates were maintained under consistent humidity and temperature conditions (30 ± 3 °C and 80 % relative humidity) in a bioclimatic chamber (Artificial Climate Chamber - ECOSHEL), as shown in Figure 1.

Figure 1. Evaluating Germination Media for Guava Seeds

Sampling and germination evaluation procedures

Samples were taken at 0, 5, 10, 15, 20, 25, and 30 days. All determinations were conducted in triplicate, and trays were randomly selected at each interval. Germination was considered when the seed exhibited a primary root emission greater than 2 mm in length (Alves et al., 2015; Ramirez et al., 2017).

Measurement and evaluation of growth parameters and germination indices

For root length, the measurement considered the distance from the base of the stem to the apex of the root. Stem length was measured from the base to the apex of the seedling. Additionally, the root/stem ratio was calculated as the relationship between these two lengths (Ramirez et al., 2017).

The Germination Speed Index (GSI), germination percentage (GP) and biomass weight (PB) were calculated using the formulas and methodology outlined by Alves et al. (2015) (Alves et al., 2015). The GSI was determined at the same time as the germination percentages. It was calculated by dividing the germination percentage by the number of days the seeds were in

the germinator using Equation 1.

Eq 1. GSI = (G1/N1) + (G2/N2) + (Gn/Nn)

Where: GSI is germination speed index; G1 is the percentage of seeds germinated at time 1; N1 is the time (days) since the experimental units were placed in the trays.

Figure 2. Guava seed germination cycle

Determination of protein content

The protein content and weight of seeds (germinated and non-germinated) were determined in triplicate at times of 0, 5, 10, 15, 20, 25 and 30 days. The sample was taken from the 100 seeds sown in each tray; the samples were ground in an Analytical Mill electric trademark Cole Colemer. Protein content was determined by the "Kjeldahl Method" (AOAC, 1999) and a factor of 6.25 was used for the conversion of nitrogen to protein.

Statistical analysis

All determinations were performed in triplicate, and results were expressed as the mean ± standard deviation (SD). Statistical analysis was conducted using one-way analysis of variance (ANOVA), followed by Tukey's test with a significance level of 5 %, using INFOSTAT software version 2018 for Windows (Córdoba, Argentina). A factorial treatment with 2 factors was employed, where factor 1 was the type of culture substrate with two levels (CN and PVP), and the second factor was the follow-up time with seven levels (0, 5, 10, 15, 20, 25, 30 days). The response variables included germination percentage (GP), protein content, and biomass weight (W).

RESULTS AND DISCUSSION

Height of the sprouts

The germination process typically begins between 5 and 7 days after sowing, characterized by the rupture of the seed's outer coat and the emergence of an initial root (Núñez-Gastélum et al., 2023). Figure 2 depicts the various stages of development of guava seed sprouts. By the end of the evaluation period, the sprouts had exceeded 5 cm in height.

Table 1 presents the germination indicators for the evaluated substrate. The sprouts in the CN substrate exhibited an average height of 5.57 ± 0.15 cm, while those in the PVP substrate measured 5.03 ± 0.15 cm. Both values surpassed those reported by El-Deeb et al., (2024), who reported the height of the *Psidium cattleianum* Sabine sprouts, for 28 days, between 1.53 cm and 3.56 cm (El-Deeb et al., 2024). Like Méndez et al., (2009), who documented germination heights of 2.43 cm and 2.68 cm at 37 and 47 days, respectively (Méndez et al., 2009). No statistically significant differences were observed in the parameters of height and root/stem ratio of the sprouts. The ratios were 0.52 ± 0.43 and 0.44 ± 0.11 for CN and PVP, respectively, values similar to those reported by Ramírez et al. (2017) for guava seeds (0.59) (Ramirez et al., 2017).

Table 1. Germination indicators in cotton and peat moss media

Germination media	Cotton (CN)	Peat moss (PVP)
Height (cm)	5.57 ± 0.15[a]	5.03 ± 0.50[a]
Root / Stem	0.52 ± 0.43[a]	0.44 ± 0.11[a]
Germination Speed Index (Seeds / day)	2.50 ± 2.42	1.40 ± 3.60

The variation in GSI values in the present study may also be attributed to the exposure of seeds to the substrate and temperature. In the PVP substrate, seeds were covered by a thin layer of germination substrate, reducing the impact of temperature on the seed coat. Temperature directly influences water absorption and the metabolic reactions necessary for germination (Alves et al., 2015).

Germination percentage (GP)

The germination of guava seeds, including both the initiation and the percentage of seeds that germinate (GP), is influenced by several factors. These factors include temperature, the type of substrate the seeds are placed on, and any pretreatment processes the seeds receive (Serratos Tejeda,

2012). Figure 3a shows the germination percentage (GP). The results suggest that initially, the CN substrate promoted higher sprout growth compared to the PVP substrate. After 5 days of starting the evaluation, the CN substrate showed a significant advantage with 20 % GP compared to 6 % for PVP. However, at the end of the evaluation period, no significant differences were observed between substrates in terms of GP. Furthermore, it was observed that the CN substrate maintained higher GP levels throughout the study period, reaching its maximum of 40 % on day 15 of evaluation. In comparison, the PVP substrate reached its maximum of 39 % on day 30. This suggests that the CN substrate may provide more favorable conditions for initial germination, but in the long term, both substrates may be equally effective in promoting germination.

Although few studies reporting the GP of guava seeds, the reviewed studies show differences in germination times and percentages, mainly due to the conditions of the experiment, such as temperature, substrate, and pretreatment to which the seeds are subjected before germination. Quintero et al., (1999) noted germination starting between 14 and 45 days (Quintero et al., 1999). Méndez et al., (2009) reported germination onset at 13 days post-sowing in different substrates, with a maximum GP of 78.75 % achieved after 27 days (Méndez et al., 2009). Alves et al., (2015) reported an average germination time of 17 days at an alternative temperature range of 20-30 °C (Alves et al., 2015). Ramírez et al., (2017) observed guava seed germination starting on day 7, with a germination percentage (GP) ranging from 1.0 to 5.2 % after pretreatment and specific conditions. By the end of the 30-day evaluation period, GP reached values between 91.4 % and 100 % (Ramirez et al., 2017).

The germination percentages obtained in the present study are similar to those reported El-Deeb et al., (2024), evaluated different sterilization treatments and culture media to determine their effect on the germination and development of seeds of *Psidium cattleianum* Sabine, during 28 days and obtained germination percentages between 4.76 % and 63.46 %, corresponding to the treatments T1 (10 % Clorox® commercial bleaching/15 min) and T4 (15 % Clorox® commercial bleaching, soaking in 15 % HCl/24 h, then 10 % H_2O_2/48 h), respectively (El-Deeb et al., 2024). On the other hand, Gentil et al., (2018), evaluated the effect of temperature on the germination percentage of *Psidium friedrichsthalianum* (O. Berg) seeds using a paper towel as germination substrate, for 90 days. Germination of *P. friedrichsthalianum* seeds was greater than 80 % at the temperatures of 15, 20, and 25 ºC (Gentil et al., 2018).

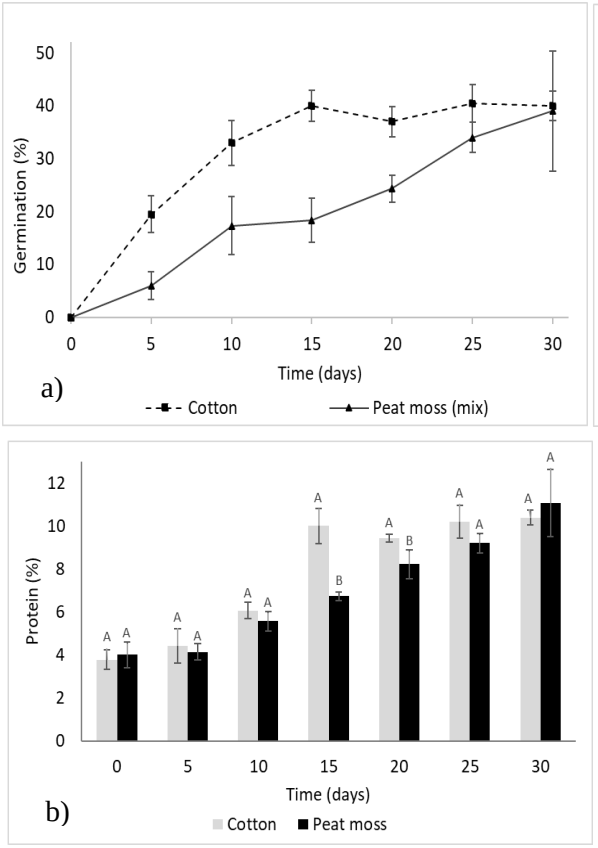

Figure 3. a) Germination percentage of guava seeds. b) Percentage of protein in guava seed sprouts.

Serratos (2012) documented germination values of 5.87 % for seeds directly obtained from the fruit (untreated), and 95.5 % for guava seeds recovered from feces. The passage of seeds through the digestive tract of monkeys acts as a chemical scarification process, weakening the seed coat with gastric juices. This facilitates water penetration and induces germination (Serratos Tejeda, 2012). Méndez et al., (2009) evaluated various germination substrates including sand, soil, bagasse, sand + soil, sand + bagasse, soil + bagasse, and sand + soil + bagasse. They observed the highest germination percentage in the sand substrate, occurring 15, 16, and 17 days after planting with percentages of 12.5 %, 30 %, and 47.08 %, respectively. However, after 27 days, germination reached its peak at 78.75 % (Méndez et al., 2009).

The variation in germination initiation is directly associated with the Germination Speed Index (GSI). Low germination

percentages are attributed to seed dormancy caused by the hardness of the seed coat, which restricts water and gas exchange (Rocas et al., 2002). Additionally, the presence of tannins in guava plants can inhibit seed germination, leading to poor, uneven, and delayed germination (Butt et al., 2013). Pre-treating seeds to facilitate seed coat breakage and interaction with the medium accelerates germ development and emergence compared to untreated seeds. High germination percentages are linked to temperature fluctuations, which can cause small cracks in the seed coat due to expansion and contraction of the integument, facilitating water penetration (Bewley & Black, 1982). Alves et al., (2015) studied the impact of temperature on the germination of guava seeds without pre-treatment. They found that germination percentages at 30 °C ranged from 1 % to 33 %. Alternating temperatures between 20 °C and 30 °C resulted in germination percentages between 94 % and 100 %. They used paper rolls and sand as germination substrates, achieving germination within 23 days (Alves et al., 2015). For their part, Pereira and Andrade (1994) not only evaluated the effect of temperature but also the germination substrate for untreated guava seeds. They found that at 30 °C, the germination percentage was 33.6 %. When temperatures varied between 20-30 °C and 15-35 °C, germination percentages increased to 55.9 % and 53.6 %, respectively. There were no statistically significant differences among the different germination substrates evaluated (vermiculite, filter paper, paper towel). The total evaluation period was 46 days (Pereira & Andrade, 1994). However, some research reports different germination percentages for the same temperature, due to variations in light intensity and water holding capacity provided by the germination substrate (Alves et al., 2015).

Determination of protein content

The observation that guava seeds shed their covering upon germination, thereby reducing their weight, aligns with biological processes known for many seeds. This shedding allows for easier penetration of water and nutrients essential for growth. However, the lack of a direct relationship between seed weight and protein percentage suggests that variations in weight observed during the experiment, particularly with the peat moss substrate, are likely influenced by substrate adherence rather than changes in protein content. Initial substrate adherence can temporarily increase seed weight, affecting early measurements.

The protein percentage did not show statistically significant differences ($P \leq 0.05$) across the evaluated germination times and substrates (Figure 3b). The highest protein content was observed in the peat moss substrate at 20 days (11.06 % ± 1.32), showing a positive correlation with the germination percentage at the same time point. In contrast, the cotton substrate at 15 days had a protein content of 10.38 % ± 1.48 but did not show a positive correlation with germination percentage, which reached its peak at 30 days with 39 %. This discrepancy may be due to the higher standard deviation observed for the cotton substrate at 30 days. The variation in determining protein content can be attributed to the inclusion of all germination material in the analysis, regardless of its germination status. During periods of lower germination percentages, such as at the onset of germination trials, the concentration of ungerminated seeds can skew the average protein percentage towards values typical for untreated seeds. This phenomenon underscores the importance of accounting for the germination state when analyzing seed composition.

In time 0, the protein content of guava seeds was 6.59 %, consistent with values reported in the literature 8.78 % (Silva-Vega et al., 2017), 11.19 % (Uchôa-thomaz et al., 2014) and 7.71 % (Serna-Cock et al., 2013). Literature corroborates the wide range of protein percentages observed in guava seeds. For example, Silva-Vega et al., (2017) reported significantly higher protein values of 30.46 % for guava seed sprouts, which indicates the potential nutritional benefits of germinated guava seeds in animal nutrition contexts (Silva-Vega et al., 2017). This contrasts with the initial protein content of untreated guava seeds, suggesting a significant biochemical transformation during germination that enhances nutritional value.

CONCLUSIONS

The study found that guava seed germination did not significantly differ in terms of germination percentage, sprout weight, and protein percentage between cotton and the mixture of peat moss, vermiculite, and perlite substrates. However, the cotton substrate showed higher values for germination indicators such as height, root/shoot ratio, and germination rate throughout the evaluation period. These results suggest that cotton may provide more favorable conditions for guava seed germination and growth. Further research is needed to understand the underlying mechanisms and long-term effects of different substrates on guava seed germination.

The germination substrate is not a significant factor for the germination speed index (GSI). One of the most crucial factors, as reported, is the pretreatment of seeds before germination. These pretreatments facilitate the breaking of the seed coat, allowing the exchange of substances between the seed and the substrate, thereby promoting the germination process.

Seed germination is an accessible, economical, environmentally friendly, and safe way to cultivate nutrient-rich foods. Further research is needed to establish optimal conditions

for guava seed germination and to increase their protein content, which can enhance the utilization of this byproduct and potentially integrate it into both human and animal diets.

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