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Pharmacognostic and Physicochemical Studies of *Theobroma cacao* bean husk in Cuba

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Abstract:

Pharmacognostic, Physicochemical and Microscopical studies of *Theobroma cacao* bean husk was carried out. The microscopic evaluation revealed characters that are of diagnostic value and useful in authentication of the plant. The Physicochemical analyses reveals values for moisture content, alcohol extractive, water extractive, total ash, water soluble ash and acid insoluble ash which are within the World Health Organization (WHO) standards for crude drug from medicinal plants. Extracts were prepared by Soxhlet extraction with ethanol at 90 %. Phenol and flavonoid content of the extract were measured by Folin-Ciocalteu and $AlCl_3$ assays. Information obtained from these studies can be used as markers in the identification and standardization of this plant as a herbal remedy and also towards monograph development on the plant.

Key Words: *Theobroma cacao*, bean, husk, pharmacognostic, physicochemical, phenolic content.

Introduction:

Commercial exploitation of cocoa (*Theobroma cacao* L. Malvaceae) generates a volume of husk that could be used in the production of pectins on an industrial scale. Cacao is one of the agroalimentary products from tropical origin with a high impact in international market and its grain exportation representing more than 71 % of produced volume, derived from the high aggregated value promoting for chocolate industry and its derives (Cartay, 1999). In commercial exploitation of cocoa bean only the seeds are economically used, representing approximately 10 % of fresh fruit weight. This circumstance has promoted serious environmental problems such as the appeal of fetid smell, landscape deterioration and problem of disposition. The generated waste is principally constituted by the husk, considered a focus for the propagation of *Phytophora* spp, which is the main cause for economical loses in this activity (López et al., 1984). In Venezuela, cacao seeds production during the period of 2001–2005, was 16.000 ton/year, generating 160.000 ton/year of husk in the mentioned period. Even though it is not the biggest producer, the finest cacao is produced in Ecuador with the variety called "Cacao Nacional" (FAOSTAT, 2017). Native to lowland rainforests of the Amazon and Orinoco river basins, cacao is grown commercially in the New World tropics as well as western Africa and tropical Asia. Its seeds, called cocoa beans, are processed into cocoa powder, cocoa butter, and chocolate (Fig. 1) (Cook, 2018). In the cocoa bean industry, some by-products go underutilized. Some of these components could provide other innovative products, and such is the case with the husk of the cocoa bean. Previous studies have attributed the husk with a high antioxidant capacity, which added to its relative low cost, makes it an attractive ingredient for the production of infusions. However, prior to promoting it as such, its quality needs to be guaranteed (Sangronis et al., 2014).

Figure 1. Cacao beans and seeds

This situation has motivated the develop of a lot of studies with the finality of increase the commercial value and to diversify the use of cacao husks, that traditionally have been utilized as animal food and soil's recovery. In our country, the bean husk is underutilized generating a big number of by-products. The aim of this research was to evaluate the pharmacognostic and physicochemical parameters of the husk of *Theobroma cacao* L. towards standardization and monograph development.

Material and Methods:

Sample collection and processing:

The sample was the husk of cocoa bean after its separation from the fruits. It was supplied in 2018 by the Chocolate Factory located in Baracoa, Guantánamo Province, Cuba. After the collection the husks were packet in nylon bags without elimination of foreign matters. The material was grounded in a high-speed hammer mill. The sample keeps its brown color (Fig. 2) and a very nice chocolate's smell.

Figure 2. Toasted cacao beans, husk and powered drug

Pharmacognostic analysis:

Microscopic analysis of husk:

Microscopic analysis was carried out on the powdered sample using a light microscope NOVEL (China) with 10x microscope objective lens, and coupled to HDCE-50B digital camera (China) and Scope Image Dynamic Pro software. Ground powder was cleared for some minutes in sodium hypochlorite solution. It was washed in water and then stained in glycerinated gelatin. Some physiochemical reactions were done, such as Sudan red III at 5% in ethanol at 70% to determine oil globules, Lughole solution (iodine at 1% with potassium iodide at 2% in water) to determine starch (Gattuso M y Gattuso S. 1999; Miranda y Cuéllar, 2000), and eosin assay to determine proteins (Johansen, 1940).

Physicochemical Analysis:

Physicochemical analyses were carried out on the powdered sample following standard methods of WHO, 1998 and Miranda & Cuéllar, 2001. Moisture content, alcohol extractive value, water extractive value and total ash value were tested for.

Extract preparation:

The extracts were prepared with the ground material (60 g) without screen extracted in a Soxhlet apparatus with 675 mL of ethanol at 90% for 20 hours. The ethanolic extracts were concentrated and evaporated under vacuum to 200 mL at 120 rpm, a temperature of 70˚C and 500 mbar.

Determination of total phenolic compounds and total flavonoid content:

Total phenols were measured in triplicate from an ethanolic extract of the husks, according to the method of (McDonald y col., 2001; Pourmorad y col., 2006; Memnune y col., 2009; Chlopicka y col., 2012) with slight modifications, using the Folin-Ciocalteu reagent and gallic acid as standard. To a sample of 200 µL were added10 mL of Folin-Ciocalteu reagent 1:10, 1.8 mL of distilled water (shaken and waiting 5 min) and after that were added 8 mL of sodium carbonate at 7.5% shaken again and left 2 hours in the dark at laboratory temperature. Absorbances were read at 765 nm on spectrophotometer Rayleigh UV-1601 (China). Quantification was done using a gallic acid standard curve and the results were expressed as gallic acid equivalents mg/mL. The standard curve was prepared using 500 mg of gallic acid dissolved in 20 mL of ethanol at 96%. The solution was transferred to an afforded matrass of 50 mL and completed with distilled water. From this solution were realized dilutions to 10, 20, 30, 40 and 50 mg/100 mL of gallic acid, built a standard curve of absorbance vs. concentration. Flavonoid content of the extract was determined by following colorimetric method according to Chang y col., (2002) y Pourmorad y col. (2006). Briefly, 0.5 mL solution of extract (at 10% w/v) in ethanol at 96% were separately mixed with 1.5 mL of methanol, 0.1 mL of 10% aluminum chloride, 0.1 mL of 1 M potassium acetate, and 2.8 mL of distilled water, and left at room temperature for 30 min. The absorbance of the reaction mixture was measured at 415 nm on spectrophotometer Rayleigh UV-1601 (China). The calibration curve

was prepared by preparing quercetin solutions at concentrations 5 to 80 µg/mL in ethanol.

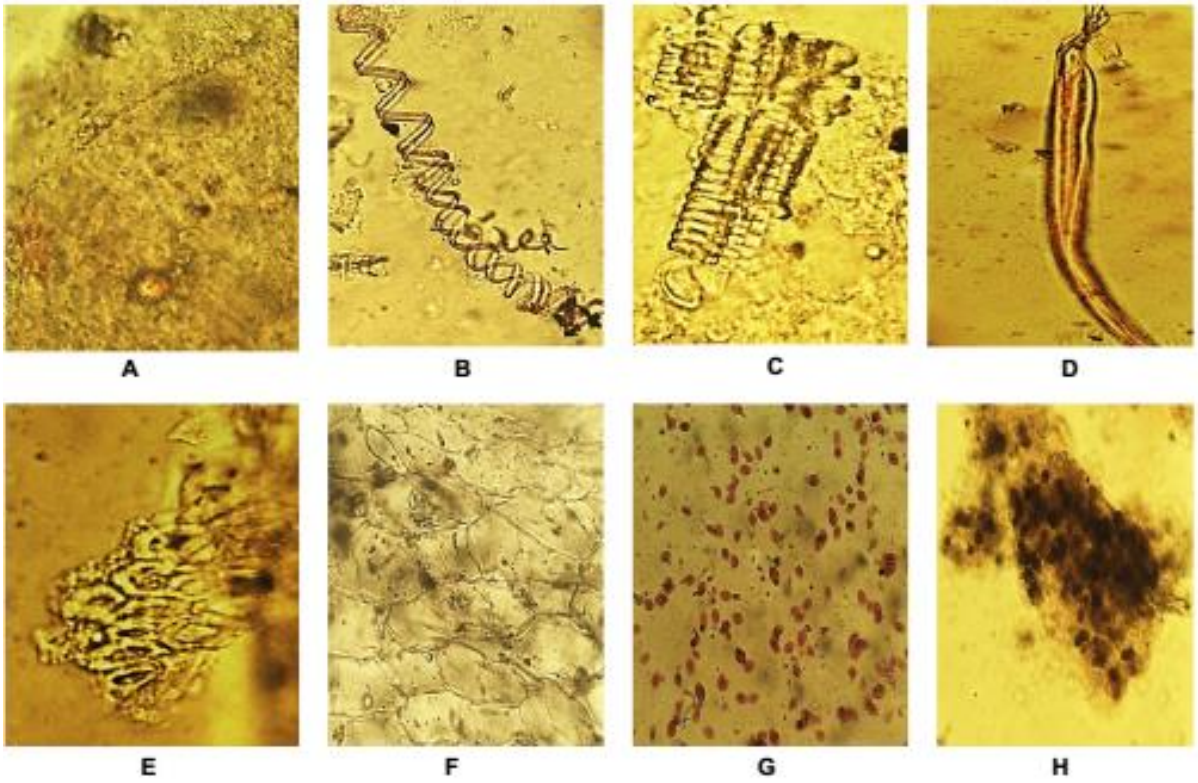
Statistical analysis:

Results are presented as mean ± SD. Statistical analyses were performed by Student's t-test. The values of $p < 0.05$ were considered significant.

Results:

Pharmacognostic analysis:

Microscopical evaluation of husk showed the following structures: oil bags or fat cavities; different kind of vessel; sclerenchymatous fiber; sclerides; reservation parenchyma with pentagonal cell; presence of proteins and starch grains (Fig. 3).



A: oil bags, oil or fat cavities; **B**: spiral xylem vessel; **C**: annular and lignified xylem vessels; **D**: sclerenchymatous fiber; **E**: Sclerides (Braquiescleride); **F**: reservation parenchyma with pentagonal cell; **G**: proteins (red); **H**: starch grains.

Figure 3. Microscopical structures of powdered husk from *T. cacao* L.

Physicochemical Analysis:

The physicochemical parameters are presented in Table 1.

Table 1. Preliminary physicochemical analyses of husk of *Theobroma cacao*

Parameters (%)	Results $\bar{X} \pm SD$
Moisture content	11.83 ± 0.28
Extractable matter in water	20.85 ± 0.03
Extractable matter in ethanol at 30 %	19.19 ± 0.08
Extractable matter in ethanol at 50 %	15.33 ± 0.03
Extractable matter in ethanol at 80 %	11.08 ± 0.04
Total ash	7.86 ± 0.06
Water soluble ash	2.39 ± 0.01
Acid insoluble ash (HCl at 10 %)	3.02 ± 0.01

Moisture content (11.83 ± 0.28%) was inside the limited index (8%-14%) indicating that the process of dried was the appropriated. Extractable matter in water (20.85 ± 0.03) was higher than extractable matter in ethanol at 30% (19.19 ± 0.08), 50% (15.33 ± 0.03) and 80% (11.08 ± 0.04%) suggesting that this solvent is appropriated to the extraction of active components from the husk and the components are polar. Among ethanolic extraction the higher value was with ethanol at 30% corroborating that the chemical compounds present in the drug have a high polarity. Extractable matter in water is highest than the permitted value of 15% according to COVENIN in 1980 and 2002, respectively.

Total ash (7.86 ± 0.06%) is higher according to the standard allowed (3%-5%), while water soluble ash (2.39 ± 0.01%) and acid insoluble ash (3.02 ± 0.01%) were relatively higher than the standard for medicinal plants (< 2%) according with WHO, 1998. The possible reason is that cacao bean husk was not clean; containing soil and another inorganic impurity, although may be the reached values are the characteristics for this spice. High content

of ashes is indicative that vegetable material has a rich content of minerals (COVENIN, 2002). According to Baena and García in 2012, total ash value is in concordance with previous studies (7-8%).

Total phenolic compounds and total flavonoid content:

Total phenols measured by Folin-Ciocalteu reagent in terms of gallic acid equivalent and total flavonoid content of extracts calculated as quercetin equivalent are shown in Table 2. *Theobroma cacao* husk with 4.12 ± 0.02 mg/mL of gallic acid equivalent and 1.43 ± 0.01mg/mL of quercetin equivalent. Total phenolic content represented the amount of 1373.33 mg/100 g of dried drug, while total flavonoid content represented the amount of 476.66 mg/100 g of dried drug, respectively.

Table 2. Total phenolic content and total flavonoid content

Compounds	Results (mg/mL) $\bar{X} \pm SD$
Total phenolics content*	4.12 ± 0.02
Total flavonoid content**	1.43 ± 0.01

*mg/mL of gallic acid equivalents
**mg/mL of quercetin equivalents

The curve of the average absorbance versus concentrations (Total phenols content) (Fig. 4) is a straight line, which equation is: Y=0.0129X + 0.0293. The correlation coefficient was 0.9996 (≥ 0.99), which is indicative of a good adjustment of model equation of experimental data.

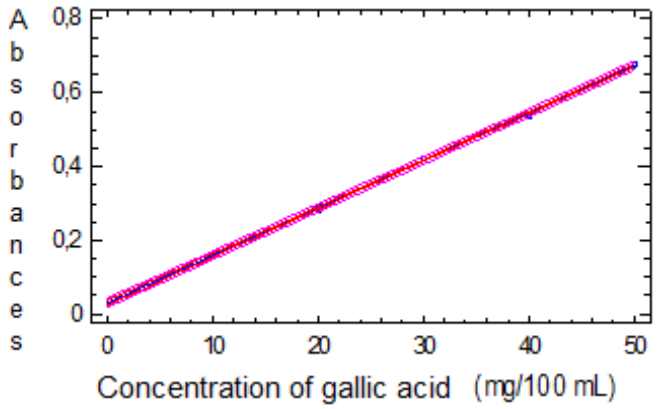


Figure 4. Curve of determination of total polyphenols of the ethanolic dry extract of *Theobroma cacao* L. husk

The curve of the average absorbance versus concentrations (Total flavonoid content) (Fig. 5) is a straight line, which equation is: Y= 0.0037X + 0.0145, with a correlation coefficient of 0.9982 (≥0.99); it is indicative of a good adjustment of model equation of experimental data.

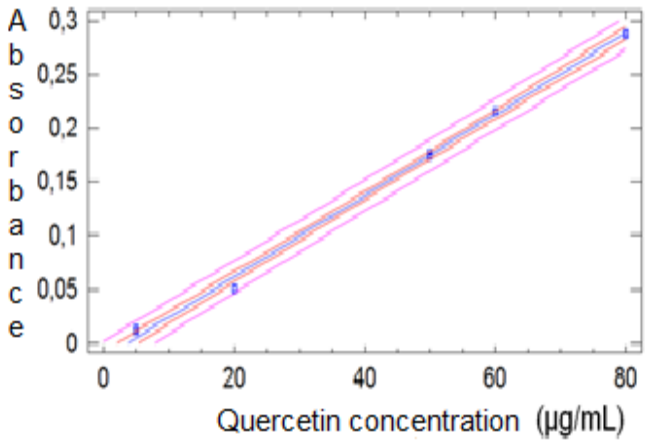


Figure 5. Curve of determination of total flavonoids content of the ethanolic dry extract of *Theobroma cacao* L. husk

Discussion:

From the study, important diagnostic characters that might be useful in determining authenticity and identifying adulteration of the crude drug are observed. These are found in the abundant sclerides (Braquiescleride) and reservation parenchyma with pentagonal cell. Two different kinds of vessel were observed: spiral xylem vessel and annular and lignified xylem vessels. Abundant oil globules seen on drug cells and numerous starch grains can be used to determined authenticity of the plant. The moisture content of 11.83 ± 0.28%, which is within the recommended range of 8–14% for vegetable drug was an indication that the plant can be stored for a long period of time with less probability of microbial attack. The water extractive value of 20.85 ± 0.03% showed that water permeates the cells of the husk and thus, a better extractant compared to alcohol with extractive value of ethanol at 30% (19.19 ± 0.08), at 50 % (19.19 ± 0.08%) and at 80% (11.08 ± 0.04%). Total ash of 7.86 ± 0.06% was low implying that the crude plant has low inorganic components. The aim of ashing was to remove all traces of organic matter (Baena and García, 2012). The total ash value can be used to detect foreign organic matter and adulteration with sand and earth, therefore, reflecting the kind of care that must be taken in preparing the plant for drug. Total phenolic content is highest value comparing with those values reported by Cuéllar and Guerrero in 2012, when starting from a butanolic fraction they found that the content of phenolic compounds was between 3.31µg/mL and 14.32 µg/mL using Ferulic acid as external standard and HPLC. Andueza et al., 2015, in Venezuela, reported that using two different samples of cacao husk (national and imported) the total phenolic content were 2.00 ± 0.220 g GAE/100 g and 1.51 ± 0.102 g GAE/100 g, and total flavonoids content were 0.315 ± 0.087 g RE/100 g and 0.199 ± 0.078 g RE/100 g, respectively, using rutin as standard of reference. In the same way, total polyphenolic content of the cacao husk sample used to elaborate a milky beverage in Ecuador showed a value of 2.56 mg GAE/g of sample. After beverage elaboration

from an aqueous extract at 10 % with cacao husk, coffee husk and orange pericarp that amount was 4.55 ± 0.43 mg GAE/g of sample (Franco and Suárez, 2014). Those results are lowest than the results reached by the Cuban researchers team. Found results suggest a new use for this material that might help with the free radical's inhibition properties which have beneficial effects on the human health.

Conclusions:

The evaluation of a crude drug is an integral part of establishing the correct identification of a plant material. For this, pharmacognostic and physicochemical parameters must be determined. In this regard, the microscopic features of cacao bean husk have been studied. Studies revealed the presence of abundant oil globules or oil bags, reservation parenchyma with pentagonal cell, and abundant presence of protein and starch grains. Studies of physicochemical constants can serve as a valuable source of information and are usually used in judging the purity and quality of the drug. The extractive values give an idea about the chemical constitution of the drug and from the study, the extractive value of water highest followed by alcohol at different concentrations. The ash value determines the earthy matter or inorganic composition and other impurities present along with the drug. For the first time, were develop the determination of total polyphenols and total flavonoid contents in husk bean of *T. cacao* L. in Cuba. To conclude, this study could be used as a diagnostic tool for the standardization of this medicinal plant and will helpful in characterization of the crude drug.

Conflict of interest:

Authors declared not conflict of interest.

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