Do *Crataegus pinnatifida* capsules sold in Brazil undergo pharmaceutical quality control? Is there a quality control adopted in Brazil?

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ABSTRACT

The Chinese hawthorn, *Crataegus pinnatifida*, Rosaceae, is widely used in Traditional Chinese Medicine (TCM). As there is no Brazilian legislation to carry out the quality evaluation of this drug, this work aimed to carry out the quality control of *C. pinnatifida* capsules (Shan Zha), sold commercially in Brazil. Due to lack of reference standard, an extract of the *C. pinnatifida* fruits (EC) was prepared. The quality control of the fruits presented adequate botanical analysis of the fruits and presented macroscopic characteristics in accordance with Chinese Pharmacopoeia. For the fruits, the sample had no foreign matter and the total ash content was 0.724 ± 0.160%. The capsules showed adequate dose uniformity, moisture content of 14.75 ± 0.476%, total ash content of 1.51 ± 0.199%. The total flavonoid (TF) percentage was 0.012% for the dried extract of fruits and 0.005% for the Shan Zha capsules. This study highlights the importance of carrying out quality control to ensure the authenticity and quality of products that fall within the classification of supplements in Brazilian legislation and guide the dose calculation for *C. pinnatifida* products.

Keywords: Thin layer chromatography, phytopharmaceutical products, total phenolic content.

INTRODUCTION

The adoption of the Traditional Chinese Medicine (TCM) has increased around the world. Herbal medicines, acupuncture, massage, and food therapy are techniques used in TCM to recovery the health (Wang et al. 2018).

Between the herbal list used in TCM, one of the most used species in the Asian region is the *Crataegus pinnatifida* Bunge, popularly known as Hawthorn. It belongs to the Rosaceae family, in which there are around 100 species distributed in North America, Europe and Asia (China 2015; Dehghani et al. 2019). The *Crataegus* species are multi-branched and thorny shrubs, that reach up to 10m (China 2015). The species are commonly cultivated for their fruits production and for ornamental uses because their white flowers (Rigelski and Sweet 2002; Dai et al. 2007; Yang et al. 2012).

In China, the part of interest of the Hawthorn is in the reddish berries (fruits) for therapeutical application (Chang et al. 2006; Wu et al. 2014) and as regular food (Degenring et al. 2003). These fruits are rich in flavonoids, mainly procyanidin B2, epicatechin, quercetin and catechin. The medicinal effects reported are anti-inflammatory properties, inhibition of free radical production and LDL cholesterol oxidation, and also as a stimulator of digestion, improving digestive functioning (Liu et al. 2010; Jurikova et al. 2012; Kumar et al. 2012; Wu et al. 2014; Dehghani at al. 2019).

In some countries, as in Brazil, the TCM products are not marketed as medicines, but as health products, supplements, among others (Lin et al. 2018). In 2014 was launched a Brazilian guide for

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the manufacturing and marketing processes of TCM products, that do not require registration with the National Health Surveillance Agency (ANVISA). The current legislation for quality control of TCM products has similar requirements as the food supplements and they are popularly known as nutraceuticals (Brasil 2014). However, the increasing in the Chinese herbal products uses as a phytomedicine makes necessary the establishment of the quality criteria for the Chinese herbal drug (Wang et al. 2018). Then, the objective of this work was to evaluate the quality of the capsules of Shan Zha (*C. pinnatifida*) sold in Brazil as food supplement.

MATERIALS AND METHODS

Materials

The reference standards was bought from United State Pharmacopoea and the specifications are: hyperoside (Lot R087N0, China), rutin (Lot: R054J0, 91.3% purity, China), epicatechin (Lot F09990, 99.0% purity, China), chlorogenic acid (Lot R001C0, 97.3% purity, China), and quercetin (Lot R120F0, 89.1% purity, China).

C. pinnatifida is commercially available as an herbal drug (fruits) and capsules. The fruits were purchased in the city of Hong Kong, China and they were used for quality control analysis as a reference standard. The *C. pinnatifida* capsules (Shan Zha) were purchased in the Brazilian trade, in March 2019.

Quality control of fruits of C. pinnatifida and capsules sold in Brazilian market

In order to verify the authenticity of the capsules content, the organoleptic and macroscopical analysis was performed. For both analyses no standard protocol or pharmacopoeia monograph was found. Then the specifications for the appearance of *C. pinnatifida* extract were made by comparison of capsules sold in Brazilian market and the dried lyophilized extract of the fruit of *C. pinnatifida*, produced by our research group.

For the quality control of the samples, the determination of foreign material, macroscopic and microscopic description, specifically, were carried out for the fruits and specifically the average weight for the capsules. The determinations of humidity, total ash and chromatographic profile and total flavonoids determination were performed for both samples.

Determination of Foreign Matter of fruits

About 100 g of dried *C. pinnatifida* fruits were weighed; the material was spread on a surface and visually analyzed. The separation of dirt was conducted, according to Brazilian Pharmacopoeia 6th Ed. (Brasil 2019).

Macroscopic and microscopic Inspection of Fruits

The organoleptic characteristics of the plant material were analyzed. For microscopic analysis, the desiccated sample went through the process of comminution and sieving to obtain powder (#35 mesh). Histological slides of the powder were prepared following general methods for plant histology, cited in the Brazilian Pharmacopoeia 6th Ed. (Brasil 2019). This analysis was performed in an Olympus® CX31 optical microscope coupled to a Motic® 2300 camera with an image capture system and the result found was compared with the description contained in the Chinese Pharmacopoeia (China 2015).

Preparation of the standard dried extract from *C. pinnatifida* fruits (EC)

From the fruits of *C. pinnatifida* standard dry extract (EC) was produced. The extract was prepared using 80% ethanol (w/w) as solvent and using 1/10 (w/w) of dry extract/solvent proportion. The extraction method adopted was the turbolysis, with 15 min of agitation and 5 min rest intervals. Sequentially, the extract was filtered using filter paper (Whatman, Sigma-Aldrich®). The liquid extract was evaporated at 40 °C and reduced by half, using the Büchi R-200 rotaevaporator. The extract was then lyophilized using the lyophilizer (Alpha 1-4, Christ). The dry extract was stored in a freezer at -25 °C.

Determination of and humidity level, total ashes and chromatographic profile of fruits and Shan Zha capsules

For the tests humidity level and total ashes, the samples used were the fruits of *C. pinnatifida* and the total content of Shan Zha capsules.

The determination of total ash was performed according to the gravimetric method described in Brazilian Pharmacopoeia 6th Ed. (Brasil 2019), but the sample weight was modified to 1 g for the fruit samples and Shan Zha capsules. All analysis were carried out in triplicate.

For the chromatographic profile, the dried extract of the fruits and the total content of capsules were considered. Approximately 1.0 g of each material were weighed and mixed with 4 ml of methanol in separate tubes. All mixtures were taken to ultrasound for 15 min, then filtered on a 0.45 µm PVDF membrane and transferred to an amber vial, for later application on the chromatoplate.

Five different standards were used: hyperoside (1), rutin (2), epicatechin (3), chlorogenic acid (4), and quercetin (5). The chosen standards were based on the description by Jurikova et al. (2012). Standards were prepared separately in methanol, in the following proportions standard:methanol

- hyperoside (1:2), rutin (1:2), quercetin (1:5), chlorogenic acid (1:5), and epicatechin (1:1).

The standards were applied on a chromatographic plate containing silica gel 60 F254 Merck, using the Automatic TLC Sample 4 (CAMAG), in which a fixed volume of the standards was 10 µl, and the samples were applied at different volumes of 5, 10, 20, and 30 µl. At the end of sample application, the plate was placed in a bipartite chromatographic chamber (CAMAG), previously saturated for 30 min, with the aid of 20 ml of mobile phase on each side of the chamber. The mobile phase was prepared with ethyl acetate, methyl ethyl ketone, formic acid, and water (20:12:4:4, v/v). After the elution of the chromatoplate, for the visualization of the compounds, two steps were followed. First, a mixture of diphenylboric acid (1%) in methanol, and polyethylene glycol (5%) in methanol was sprayed on the plate and analyzed under UV light at 365 nm. After that, the same plate was taken to the oven for five minutes at a temperature of 105 °C and then again observed under UV light 365 nm, this being the second observation.

Average weight of capsules

The average weight was only considered to the capsules, and it was determined according to the methodology described in the Brazilian Pharmacopoeia 6th Ed.

Validation method for determination of total flavonoids

The method was validated for linearity and precision.

For the evaluation of the linearity parameter, a calibration curve with the quercetin (QUE) standard was constructed. Seven concentrations of quercetin solution were prepared in methanol:water (80:20) (1.29, 1.62, 1.94, 2.26, 2.59, 2.91 and 3.23 µg/ml). One ml of $AlCl_3$ solution (2%) prepared in acetic acid (5%) was added to ten milliliters of the QUE solutions and the final volume of 25 ml was completed with acetic acid (5%). The analysis was performed in a Shimadzu UV-1800 spectrophotometer, in the 425 nm wavelength after 25 min of reaction. The spectrophotometer was adjusted using the black solution. The blank solution was prepared as the samples, substituting the 10 ml of quercetin solution for 10 ml of methanol:water (80:20).

In order to verify if the regression data of the equation was statistically significant, some tests were performed for the adjustment check of the linear model with analysis of validation of the regression, and its effectiveness. The residual analysis was applied in order to confirm the significance of the regression model, with the investigation of the difference between the observed y values and the y values estimated. The homoscedasticity was calculated by the Grubbs test and did not present an outlier, presenting itself as homoscedastic.

The precision parameter was evaluated as repeatability, and intermediate precision parameters. For the repeatability, the results were obtained in a same day for three different concentrations analyzed in triplicate. In the intermediate precision parameter, values obtained on three different days of analysis for the same analyst were performed.

Accuracy was evaluated as the percentage of recovery according to the guideline 166/2017 (Brasil 2017) and the robustness was tested for the variation of wavelength from 425 to 420 nm.

Determination of total flavonoids in the dry extract and the Shan-Zha capsules

For the assay, the stock solutions were prepared by the dissolution of the lyophilized extract and the internal content of Shan Zha capsules in methanol:water (80:20) and the final concentration was 24 mg/ml and 40 mg/ml, respectively. These solutions were used to determine the of total flavonoid content according to method described previously. The results were expressed as milligrams of quercetin equivalent per gram of dry mass (mg QUE g^{-1})

RESULTS

The sample of the *C. pinnatifida* fruits analyzed did not have foreign matter. The macroscopic analysis was carried out as shown in Figure 1A and 1B, which reveals the general appearance presented by the sample of the herbal drug consisting of the dried *C. pinnatifida* fruits and in cross-sections measuring about 2 cm in diameter and 2 – 3 mm wide, with a slightly aromatic odor and a sour, slightly sweet taste. The fruits were fleshy, pomid type or berry, with a dark yellow pulp, almost brown, with a grainy consistency when dry and pasty when hydrated. The epicarp is reddish containing lenticels.

The middle region of the fruit (Figure 1B) highlights the septum and the five locules, each containing a pyrene (stone) (mr). The basis and apex of the fruit could also be observed (bs) and (ap). The pyrenes are hard, yellowish in color, slightly brownish and reniform (py) in shape.

The microscopic analysis performed with the powder allowed the identification of some elements, such as sclereids, isolated or in groups, interspersed with parenchymatic, isodiametric and thin-walled cells that constitute the fleshy part of the fruit (Figure 1C), cells with dense and brownish content that make up the outermost region of the fruit, the epicarp (Figure 1D), in addition to idioblasts

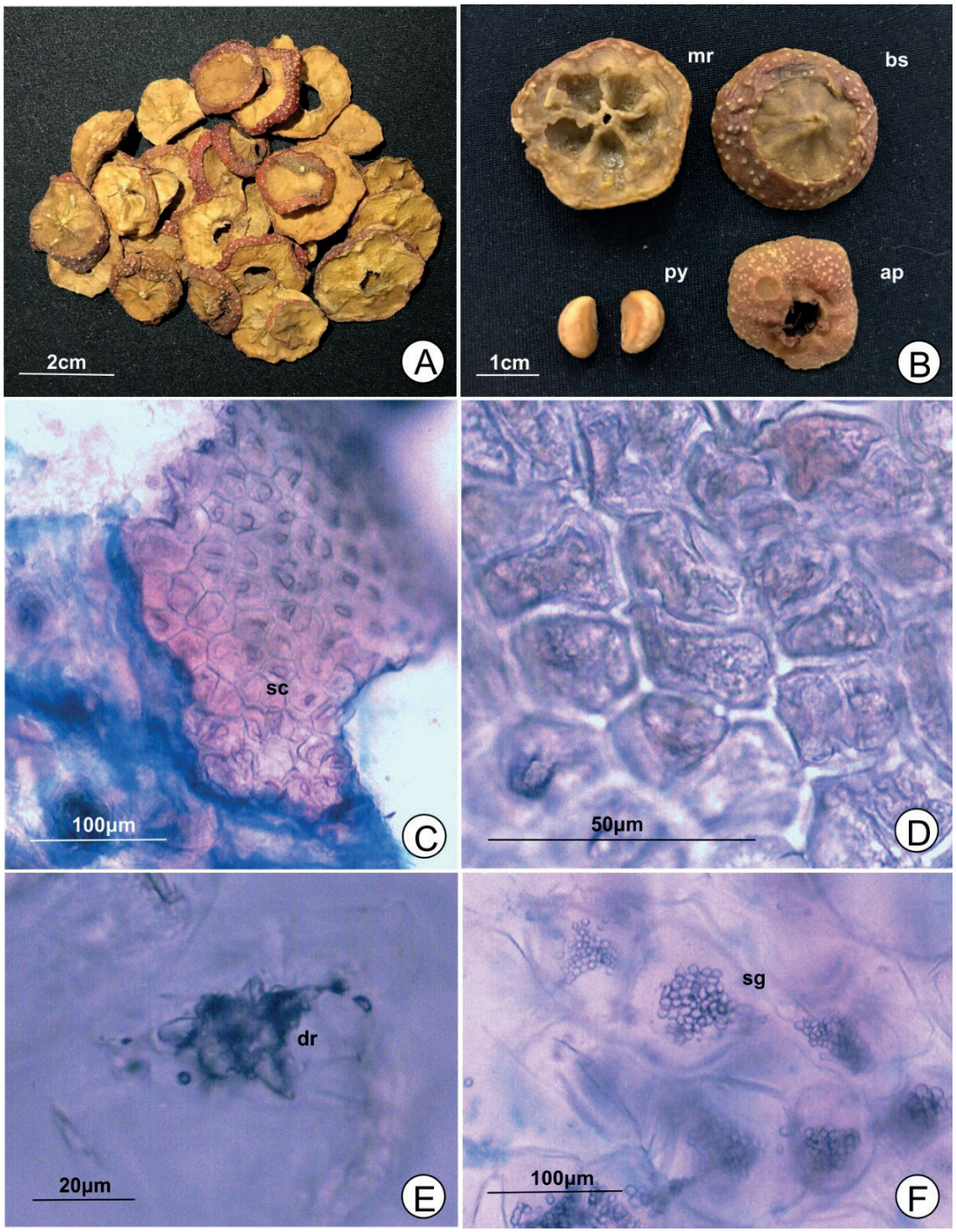

Figure 1. *Crataegus pinnatifida*. **A.** Samples, dried fruit in cross sections. **B.** fruit part: cross section in the median region showing the septa (mr), fruit bases (bs), pyrenium containing the seed (py) and Apex of the fruit (ap). **C.** Sclereids (sc). **D.** Cells with reddish-brown/ yelowish-brown content. **E.** Druses of calcium oxalate (dr). **F.** Starch grains present in parenchimatics cells (sg).

containing druses of calcium oxalate and starch grains (Figure 1E and 1F).

The analysis of the *C. pinnatifida* capsules (Shan Zha) content showed that it had dark brown color powder, composed by the dry extract of the fruits of *C. pinnatifida*, with uniform particles, very similar to the color of the fruits.

The average weight of the capsules was 399.0 mg, with a variation of 0.012%, which was in accordance with the pharmacopoeia specifications.

The humidity level determination in the fruits was 18.92 ± 1.17% and for Shan Zha capsules was 14.75 ± 0.476%.

The total ash determination tests showed

values of 0.72 ± 0.16% for *C. pinnatifida* fruits and 1.51 ± 0.20% for Shan Zha capsules.

The preparation of the dried extract from the fruits of *C. pinnatifida* was conducted using 17.73 g of plant material, that obtained 4.2 g of dry extract, which was later used in some analyses, showing the extraction efficiency of 23.69%.

The chromatography (TLC) technique used allowed the observation of the discrete presence of bands of quercetin, hyperoside and chlorogenic acid could be observed. The fourth standard, the rutin, was not seen in the samples, as well as epicatechin (Figure 2).

For the determination of total flavonoids, the method was validated for linearity and precision.

The linearity was evaluated by calibration curve obtained by plotting peak area against concentration within seven concentrations of quercetin (QUE) reference standard. The equation of the line obtained by regression analysis is presented in the equation 1. The determination coefficient (R^2) was 0.9962. The Figure of the linear regression is in the Supplementary material.

$$y=0.0827 \cdot x+0.0039 \quad \text{Eq. 1}$$

The statistic tests indicated that the regression was statistically significant and all results are presented in the Supplementary material. Results showed that lowest QUE concentration quantified (LOQ) were determined from the calibration curve, and were 0.109 and 0.331 µg/ml, respectively.

The precision analysis was performed and the relative standard deviation (SD) obtained was less than 5%, demonstrating the repeatability and intermediate precision were considered adequate, as criteria described in the Methodology session. The results detailed can be seen in the Supplementary material.

The total flavonoids contents were 0.012% and 0.005% for dried extract of fruits and capsules content, respectively.

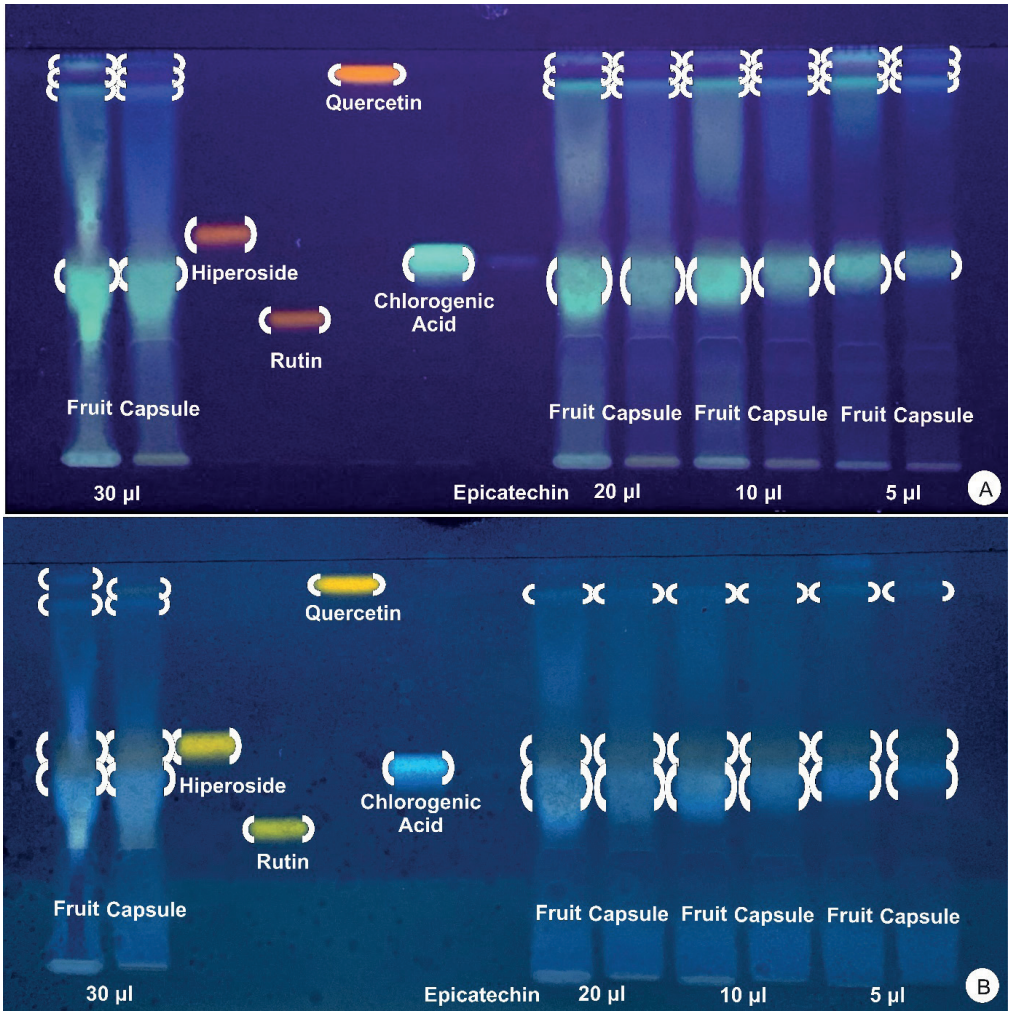

Figure 2. Thin layer chromatography of *Crataegus pinnatifida* fruits and capsules. **(A)** Visualization at 365 nm after staining with diphenylboric acid (1%) in methanol, and polyethylene glycol (5%) in methanol after application. **(B)** Visualization at 365 nm after staining with diphenylboric acid (1%) in methanol, and polyethylene glycol (5%) in methanol, and after placed in an oven for five minutes.

DISCUSSION

The sample of the herbal drug *C. pinnatifida* showed macroscopic characteristics in accordance with those described by the Chinese Pharmacopoeia (2015), among which the appearance of the fragments, similar to fruit slices, as well as the fleshy aspect of the yellowish pulp and surface can be brown or reddish. It is noteworthy that the sample also had pits (pyrenium) described in the literature by Rohrer et al. (1991), characteristic of pommes, or pomid type fruits. In this succulent fruit, the fleshy part refers to the hypanthus, information that is different from the literature, especially that which deals with chemical constituents that are often considered to be the drug made up of berries. The same authors state that the fruits belonging to this genus are characteristic of the Rosaceae (Rohrer et al. 1991).

The analysis of the powder from the capsules content reveled striking elements that correspond to those described in the Chinese Pharmacopoeia (2015), and which include cells with brownish content, belonging to the outermost region of the fruit; the pericarp, isolated or grouped sclereids, in addition to calcium oxalate druses that occur in the pulp parenchyma, as well as starch grains, highlights also mentioned by Rohrer et al. (1991).

When evaluating the Shan Zha capsules content, it was possible to observe the extract of *C. pinnatifida* fruits, with some excipients (that were not analyzed in this work). In addition, it was identified that the inner extract of the capsules had a very similar color to the EC. It has a sweet odor and slightly bitter taste. For these analyses, it was not found in the literature or in the researched pharmacopoeias any monograph that informs the specifications or the appearance of this extract, therefore, it was compared with EC, produced in this work.

Dosage uniformity is a parameter of quality control for commercial capsules. The 6th Brazilian Pharmacopoeia (2019) establishes that hard capsules with an average weight greater than 300 mg, in this way the capsules analyzed were suitable according to Brazilian Pharmacopoeia (2019).

The average weight was carried out for the *C. pinnatifida* capsules and the results revealed that the internal contents had similarities to each other, indicating that there was a uniformity of dose, with a correct filling of the capsules. This result provides an indicator of the technique of preparation of the capsules, showing good manufacturing process (Brasil 2019).

Regarding the percentage of humidity, both samples are above what is described in the Chinese Pharmacopoeia (2015), which is 12%. This can be explained by handled process during the tests, by opening the original flask and incorporating moisture. The determination of moisture is a great importance test because the excess of water in plant drugs can promote the development of microorganisms, in addition to enabling enzymatic activity and hydrolysis. Consequently, these interferents can deteriorate the drug's constituents (Farias 2004). The moisture data of *C. pinnatifida* capsules was not found in the literature, and showed an acceptable moisture value was not found in the literature. The humidity of organics can be related to their hygroscopicity, as well as to the packaging, that will protect the capsules against external factors, such as moisture (Gallo et al. 2015; Yang et al. 2020).

The percentage of ash for both the fruits and the capsules had appropriate parameters according to Chinese Pharmacopoeia (2015), which establishes a maximum 3% for total ash. The determination of ash content was also evaluated to verify the determination of non-volatile inorganic substances that could be present as constituents or contaminants in the plant drug, such as stone, sand, earth and mineral constituents (Farias 2004), that was considered satisfactory.

The TLC analysis showed the bands markers in the samples with weaker shades, when compared to the reference standards, and this may be related to the way of preparing the samples, solvent used and even the extraction method. The improvement of the TLC technique still needs to be done to obtain a method that allows a better visualization of the chemical components of the samples, being also able to compare them with the reference standards.

Quercetin is one of the major flavonoids present in the composition of *C. pinnatifida* fruits (Jurikova 2012), thus quercetin was adopted as a marker for the determination of the extract. The analytical method for total flavonoid content showed adequate validation parameters and was applied to quantify the samples of dried extract of fruits and capsules content.

The TF result obtained from the EC demonstrated that 101.3 mg of plant herbal drug were needed for the preparation of 24 mg of extract. The data showed that the TF content of the plant herbal drug was 0.69% (0.0069 mg QUE/g herbal drug).

In dried extract of fruits, 0.012% of TF were quantified, while for Shan Zha capsules the value was 0.005%. For the comparison, the Shan Zha capsules have the half of TF content of the EC. This difference can possibly be justified by the conditions of fruit cultivation, collection, drying, storage, factors that are directly linked to the quality of the product, which can lead to significant changes in its secondary

metabolites (Morais 2009). However, the main cause could be the presence of pharmaceutical excipients commonly used for capsules manufacturing. The actual proportion of excipients is unknown, then it is no possible evaluate how the TF content in the dried extract used for the Shan Zha is closer to the EC.

In Brazil, the Chinese herbal drugs are being sold as food supplements, as commented previously, and this classification allow the companies to follow the quality control for supplements, which are less strict when compared to phytomedicines.

This work brought botanical and analytical techniques which could be used for quality control of Shan Zha capsules or other *C. pinnatifida* derivates marketed in Brazil. For a quality control monography, be established is necessary to know the range of impurity and biomarkers contents acceptable for the raw herbal drug and/or product derivate. Besides that, analysis for many batches and material origin need to be done in different laboratories to understand the regular variability over the results. This work does not have the intention to propose a monography, but all methodology presented here can be considered a starting point for that.

The quality control tests for Shan Zha capsules marketed in Brazil showed good results compared to the dried extract of fruits of *C. pinnatifida* attesting to its identity. However, there are not quality reference parameters for this product in Brazil.

To achieve the same amount intake of TF, the Shan Zha need to be taken in a double amount of EC extract.

CONCLUSION

The botanical and analytical methodology were able to characterize the fruits of *C. pinnatifida,* as well one phytopharmaceutical product containing the same herbal drug, showing an acceptable quality compared to the standard. This study does not guarantee that all TCM products or products containing *C. pinnatifida*, marketed in Brazil, have the same quality profile, but it reaffirms the importance to establish the quality control criteria to harmonize the quality of products that fall under the classification of supplements under Brazilian legislation. Furthermore, the data of total flavonoid content in both products could guide the rational intake of *C. pinnatifida* by dose calculation in each case.

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AUTHORS' CONTRIBUTIONS

Conceptualization, F.B.B.P., M.Y., A.L.M.A., and A.D.; methodology, B.G.S. and W.N.C.; validation, B.G.S. and W.N.C.; formal analysis, F.B.B.P.; investigation, B.G.S. and A.L.M.A.; resources, M.Y. and A.D.; data curation, F.B.B.P., A.L.M.A., and A.D.; writing original draft preparation, F.B.B.P., A.L.M.A., and A.D.; writing review and editing, F.B.B.P. and A.D.; visualization and supervision, A.D.; project administration, F.B.B.P and A.L.M.A.; funding acquisition, A.D. All authors have read and agreed to the published version of the manuscript.

DECLARATION OF CONFLICT OF INTERESTS

The authors have no conflicts of interest to declare.

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