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INFLUENCE OF THE SEASONAL CHANGES AND OF THE EXTRACTION METHOD ON FLAVONOID CONTENT IN *PEPEROMIA PELLUCIDA* L. (H.B.K.)

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ABSTRACT: During the obtainment of herbal products, standardization of raw plant materials is ensured by the quality of herbal drugs, of the extraction conditions, followed by the determination of the content of one or more components or of the most representative compounds of their composition, which are adjusted to values previously defined, resulting in greater uniformity of plant derivatives, important for the production of safe and effective medicines. This study aimed to analyze the marker (3', 4', 7-tri-O-methoxyflavone) content of *Peperomia pellucida* L. (HBK) in three periods of collection in order to assess the seasonal changes and different extraction methods (maceration and percolation) with different solvent concentrations. The highest incidence of rain over a given period does not influence the marker content present in the sample. The maceration using 70% of ethyl alcohol obtained the highest marker content value.

INTRODUCTION: The obtainment of standardized herbal products depends on the quality and uniformity of raw plant materials. Regarding the herbal drugs, standardization is subject to adequate control of planting, drying and subsequent handling conditions.

For extracts, the responsible for much of the market of raw plant materials in Brazil and worldwide, standardization is ensured by the quality of herbal drugs, the extraction conditions, followed by the determination of one or more components or of the most representative compounds of their composition, which are adjusted to values previously defined, resulting in greater uniformity of plant derivatives, important in the production of safe and effective medicines [1].

The time at which a plant is collected is one of the most important factors, since the amount and sometimes even the nature of the active constituents, is not constant during the year. There are reports, for example, of seasonal variations in the content of all classes of secondary metabolites, such as essential oils, sesquiterpene lactones, phenolic acids, flavonoids, coumarins, saponins, alkaloids, tannins, epicuticular grease, iridoids, glucosinolates and cyanogenic glycosides [2, 3, 8].

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This study aimed to analyze the content of the marker 3', 4', 7-tri-O-methoxyflavone in *Peperomia pellucida* L. (H.B.K.) in three periods of collections in order to assess the seasonal changes and different extraction methods (maceration and percolation) with different solvent concentrations.

MATERIAL AND METHODS

Raw Plant Material: *P. pellucida* was collected in the district of Icoaraci, through Ver-as-ervas® Association, located in Belém, Pará – Brazil. The sample was identified by Prof. Dr. Milton Hélio Lima da Silva, from Museu Emilio Goeldi – Pará, and was registered under the following number: MG.191457.

Assessment of the Seasonal changes: *P. pellucida* was collected on March 30th, May 31th and July 30th, 2010 and rainfall indices were surveyed for each period of collection.

The content was analyzed by High Performance Liquid Chromatography. A calibration curve was prepared, with the concentrations of 2, 10, 20, 40 and 100 ppm, using acetonitrile as solvent and 3', 4', 7-tri-O-methoxyflavone as marker, which was isolated and identified by Nuclear Magnetic Resonance technique[4].

The analyses were performed in triplicate by weighing 30 mg of each sample. The mass was transferred to a test tube, 3 mL of acetone was added and then it was sonicated for 10 min, using Branson® model 2510 ultrasonic bath. Subsequently, the obtained solution was transferred to glass jars with the aid of a Pasteur pipette and a piece of cotton used as a filter. This solution was called as the first volume of extraction. 3 ml of acetone were added to the mass retained in the test tube and transferred to the ultrasonic bath to be sonicated for 10 min. After the completion of sonication, the solution was pipetted, filtered, and added to the first volume of extraction. The solutions were homogenized.

The obtained solution was transferred to a chapel so that all the solvent was evaporated. After drying, the mass from the extraction was treated by Solid Phase Extraction (SPE) for retaining the interfering substances, especially chlorophyll. The mass was solubilized in 500 µL of acetonitrile with the aid of ultrasonic bath for 1 min. 500 µL of ultrapure water was added and the solution was sonicated for 1 min. Then, the solution was transferred to a SPE Strata Phenomenex® C18 E 100 mg/mL cartridge, previously conditioned with 1 ml of acetonitrile and 1 ml of ultrapure water.

The solution collected in this extraction (V1) was discarded, leaving the analyte retained on the cartridge. Through this cartridge, 0.5 ml of a acetonitrile:water (1:1) solution was passed and the collected solution (V2) was separated in a glass jar. Then, we passed through this cartridge more 2 ml of a acetonitrile: water (7:3) solution. The solution collected (V3) was added to the previously obtained (V2), resulting in a total volume of 2.5 ml, and transferred to the chapel to evaporate the solvent. The residual mass was resuspended in 200 µL of acetonitrile, filtered using a nylon syringe filter with 13 mm and 0.45 µm, and analyzed by High Performance Liquid Chromatography, using an isocratic method with 54% of acetonitrile.

We used a Shimadzu® High Performance Liquid Chromatography, composed of two pumps (model LC-10AD), detector with a single sign of absorbance in the ultraviolet region, operating at a wavelength of 270 and 400 nm (model SPD-10AV), membrane degassing (model DGU-14A), Rheodyne 7752i sample injector, with 20 µL sample loop, Shimadzu® communication interface (model CBM-10A) coupled with a Pentium II microcomputer and Class LC-10A integration software.

The chromatographic parameters used were Gemini Phenomenex® C18 column (250 x 4.6 mm, 5 µm, 110Å) Phenomenex® C18 pre-column (4.0 x 3.0 mm, 5 µm), acetonitrile:water (54:46) as the mobile phase, flow of 1 mL/min., injection volume of 20 µL and wavelength at 342 nm.

Assessment of the Extraction Method:

1. **Sample Processing :** After collection, the fresh plant material (whole plant) was washed with water and 70% of ethyl alcohol and, then, dried at room temperature for 2 days. *Peperomia pellucida* L. (H.B.K.) was placed in a circulating air oven for 7 days at a temperature between 42 and 45°C [5]. After removing the plant material from

the oven, this was triturated in a knife mill and sieved (30 mesh), obtaining dry powder.

2. **Preparation of tinctures of *Peperomia pellucida* L. (H.B.K.):** Tinctures of *P. pellucida* were prepared using maceration and percolation as extraction methods, at ambient temperature (27°C). For the maceration, three tinctures were prepared using the proportion 1:10 (raw plant material/ solvent), just modifying the concentration of ethyl alcohol (50, 70 and 90%). In a stainless steel reactor, the plant drug was in contact with the solvent for 7 days [6].

   For percolation, a tincture was prepared using the proportion 1:10 (raw plant material/70% of ethyl alcohol). In a percolator, the plant drug was in contact with the solvent for 7 days. The content was analyzed by high performance liquid chromatography using the same chromatographic parameters described above for the evaluation of the seasonal changes. The analyses were performed in triplicate by weighing 20 mg of each sample.

RESULTS AND DISCUSSION:

Assessment of the Seasonal changes: Samples were collected at predefined months once there is a higher frequency of *P. pellucida* in the district of Icoaraci, due to a higher incidence of rainfall in the region during March and May and, in July, it becomes more difficult to collect this material, due to the rainfall decrease.

The chromatograms obtained with one of the replicates of the samples and the calibration curve (concentration 40 ppm) are shown in **Figures 1, 2 and 3**.

3', 4', 7-tri-O-methoxyflavone, used as the marker, has a retention time of approximately 15 min.

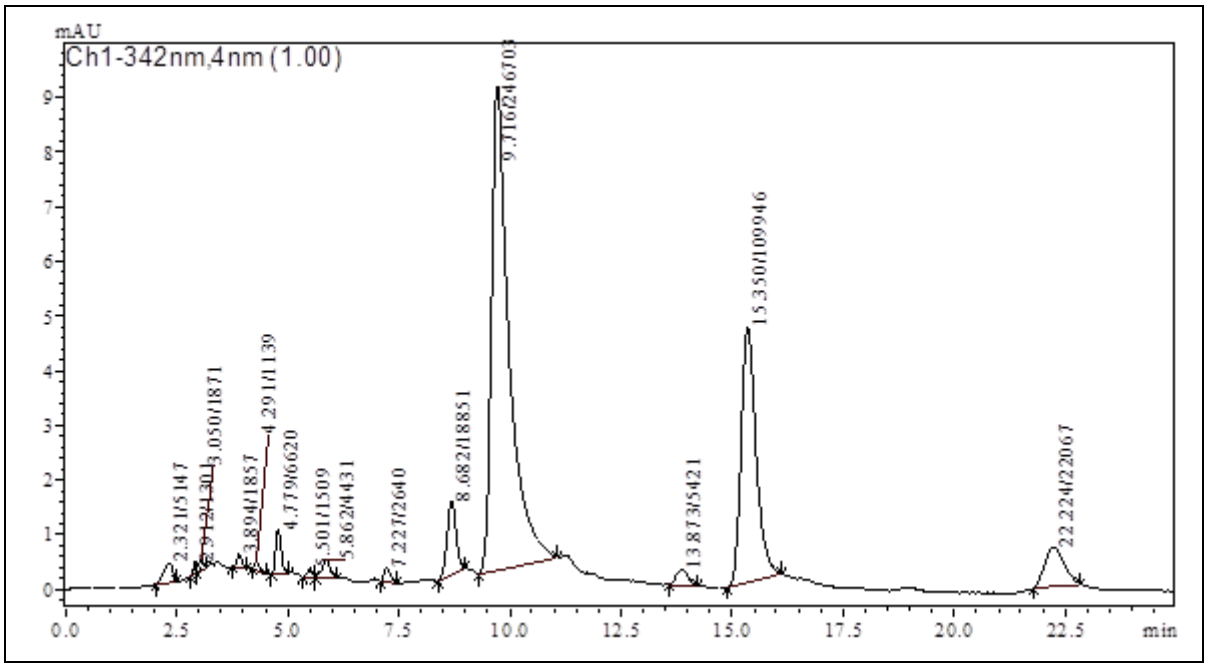

FIGURE 1: CHROMATOGRAM FOR THE SAMPLE OF *PEPEROMIA PELLUCIDA L.* (H.B.K.) COLLECTED IN THE DISTRICT OF ICOARACI ON MARCH 30TH, 2010. 3', 4', 7-TRI-O-METHOXYFLAVONE PRESENTED A RETENTION TIME OF 15.35 MIN

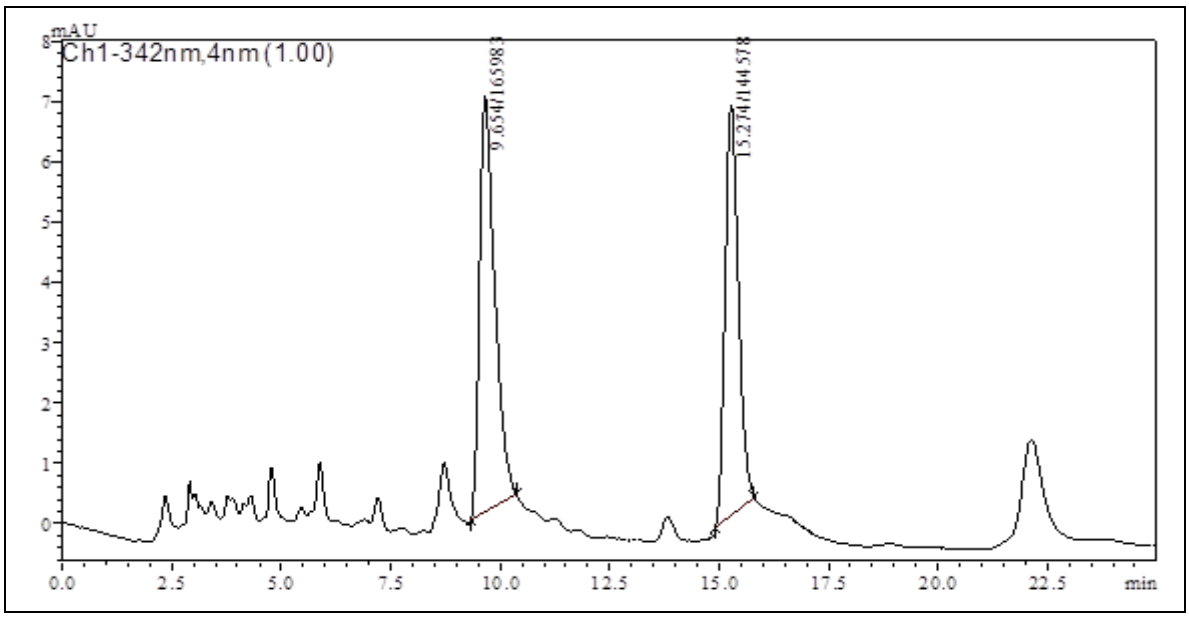

FIGURE 2: CHROMATOGRAM FOR THE SAMPLE OF *PEPEROMIA PELLUCIDA L.* (H.B.K.) COLLECTED IN THE DISTRICT OF ICOARACI ON MAY 31TH, 2010. 3', 4', 7-TRI-O-METHOXYFLAVONE PRESENTED A RETENTION TIME OF 15.27 MIN.

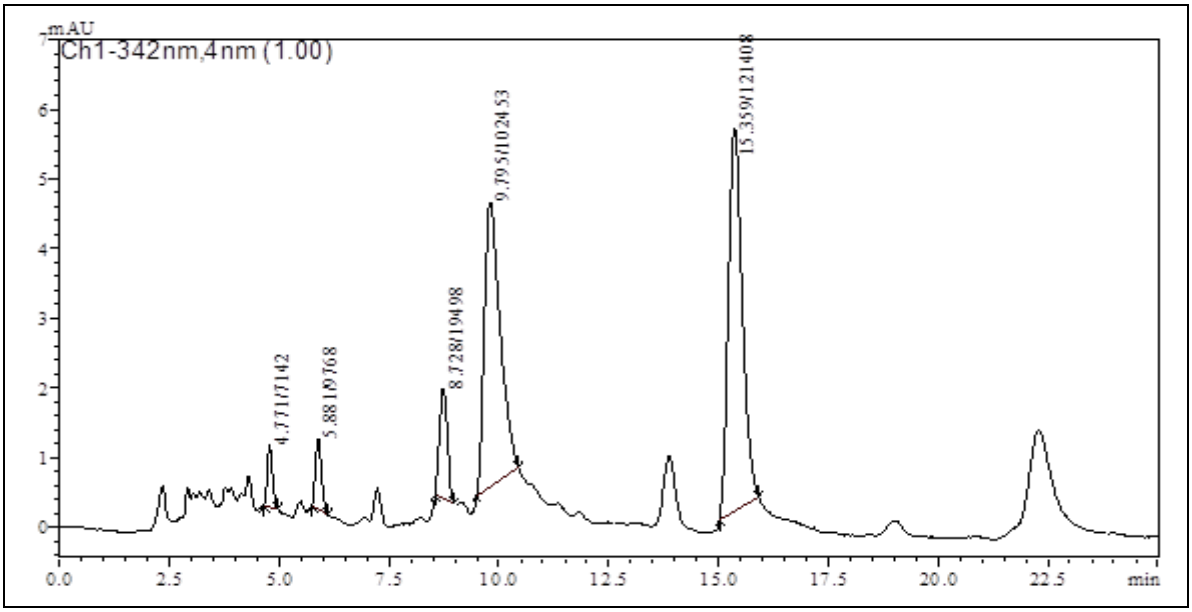

FIGURE 3: CHROMATOGRAM FOR THE SAMPLE OF *PEPEROMIA PELLUCIDA L.* (H.B.K.) COLLECTED IN THE DISTRICT OF ICOARACI ON JULY 30TH, 2010. 3', 4', 7-TRI-O-METHOXYFLAVONE PRESENTED A RETENTION TIME OF 15.36 MIN.

Table 1 presents the values of the content of 3', 4', 7-tri-O-methoxyflavone and correlates them with the indices of rainfall obtained in the district of Icoaraci, located in Belém, in the respective months of collection. The values do not significantly differ. The highest incidence of rain over a given period does not affect the amount of marker present in the sample.

TABLE 1: VALUES OF CONTENT OF 3', 4', 7-TRI-O-METHOXYFLAVONE AND OF THE RAINFALL AT EACH MONTH OF COLLECTION

Month of collection	Values of content (mg) of 3', 4', 7-tri-O-methoxyflavone in 100 g of sample	Average of rainfall (mm)[a]
March	1.63 ± 0.081	9.54
May	1.88 ± 0.063	unavailable values
July	1.71 ± 0.097	4.37

[a]The values were provided by Centro de Previsão de Tempo e Estudos Climáticos (CPTEC) and Instituto Nacional de Pesquisas Espaciais (INPE) [7].

Assessment of the Extraction Method: The chromatograms obtained with one of the sample replicates are shown in **Figures 4, 5, 6 and 7**.

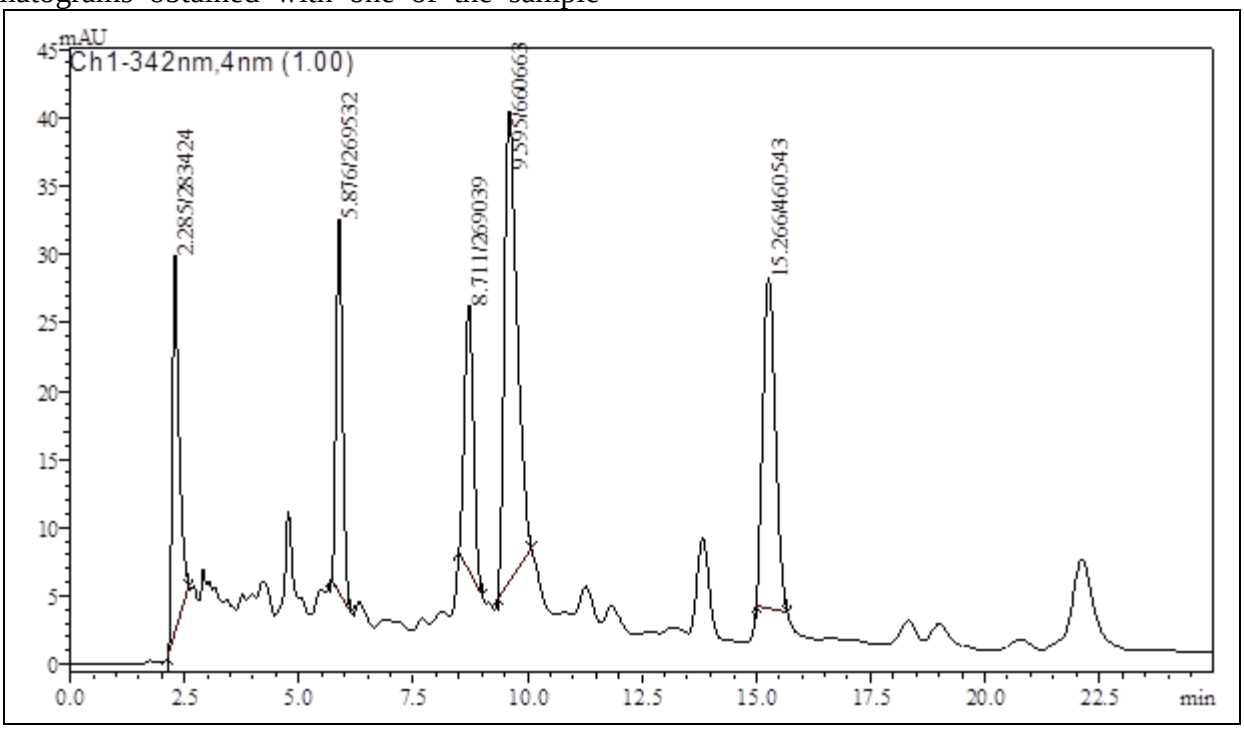

FIGURE 4: CHROMATOGRAM FOR THE EXTRACT OF *PEPEROMIA PELLUCIDA* L. (H.B.K.) OBTAINED BY MACERATION, WITH A CONCENTRATION OF 50% OF ETHYL ALCOHOL. 3', 4', 7-TRI-O-METHOXYFLAVONE HAS A RETENTION TIME OF 15.27 MIN.

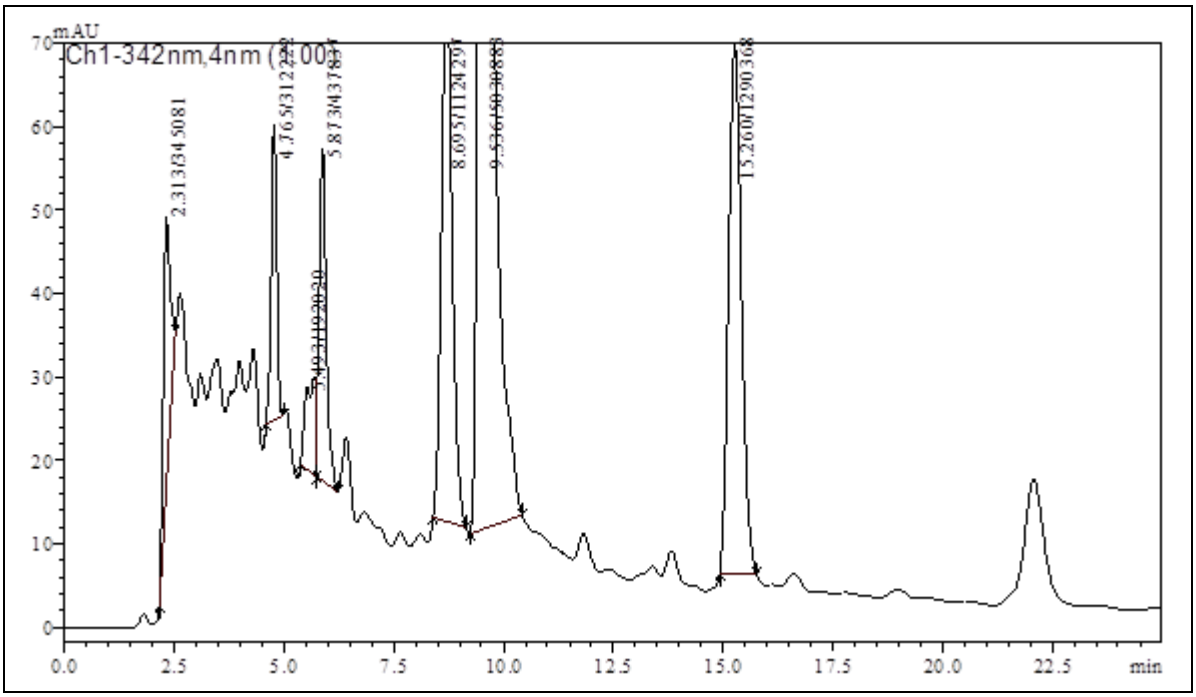

FIGURE 5: CHROMATOGRAM FOR THE EXTRACT OF *PEPEROMIA PELLUCIDA* L. (H.B.K.) OBTAINED BY MACERATION WITH A CONCENTRATION OF 70% ETHYL ALCOHOL. 3', 4', 7-TRI-O-METHOXYFLAVONE HAS A RETENTION TIME OF 15.26 MIN.

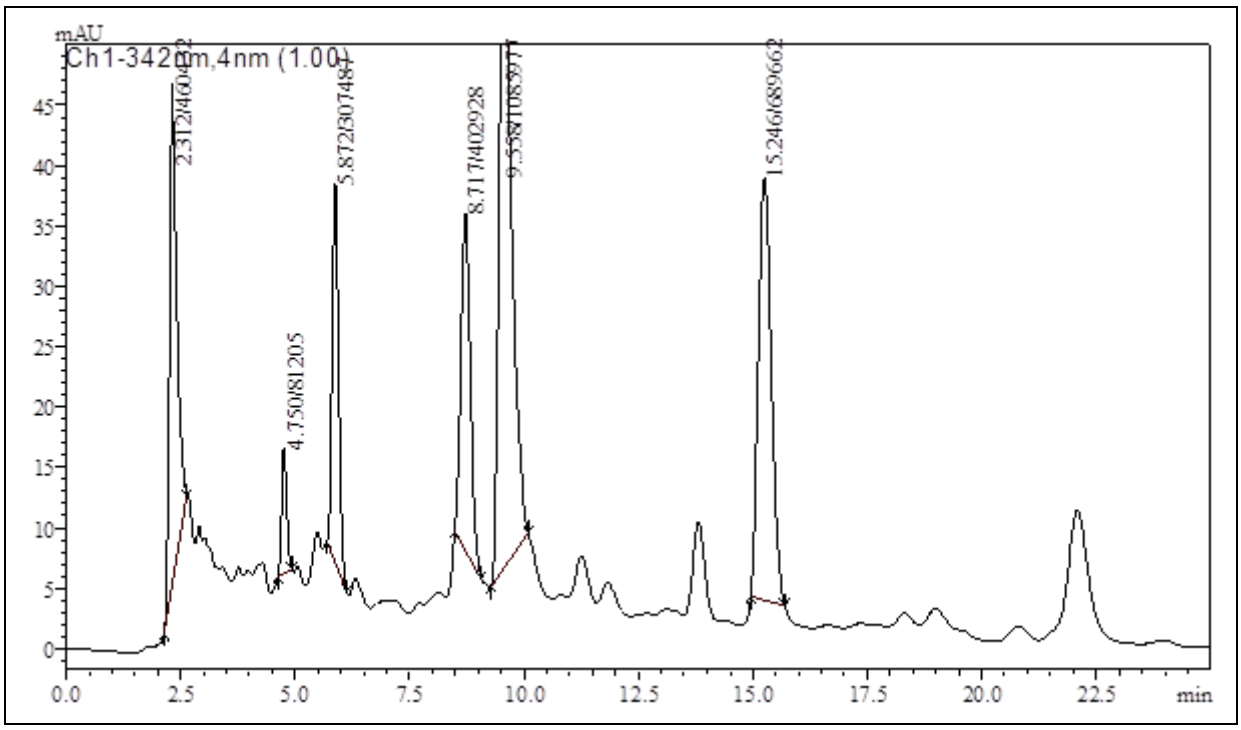

FIGURE 6: CHROMATOGRAM FOR THE EXTRACT OF *PEPEROMIA PELLUCIDA* L. (H.B.K.) OBTAINED BY MACERATION WITH A CONCENTRATION OF 90% OF ETHYL ALCOHOL. 3', 4', 7-TRI-O-METHOXYFLAVONE HAS A RETENTION TIME OF 15.25 MIN.

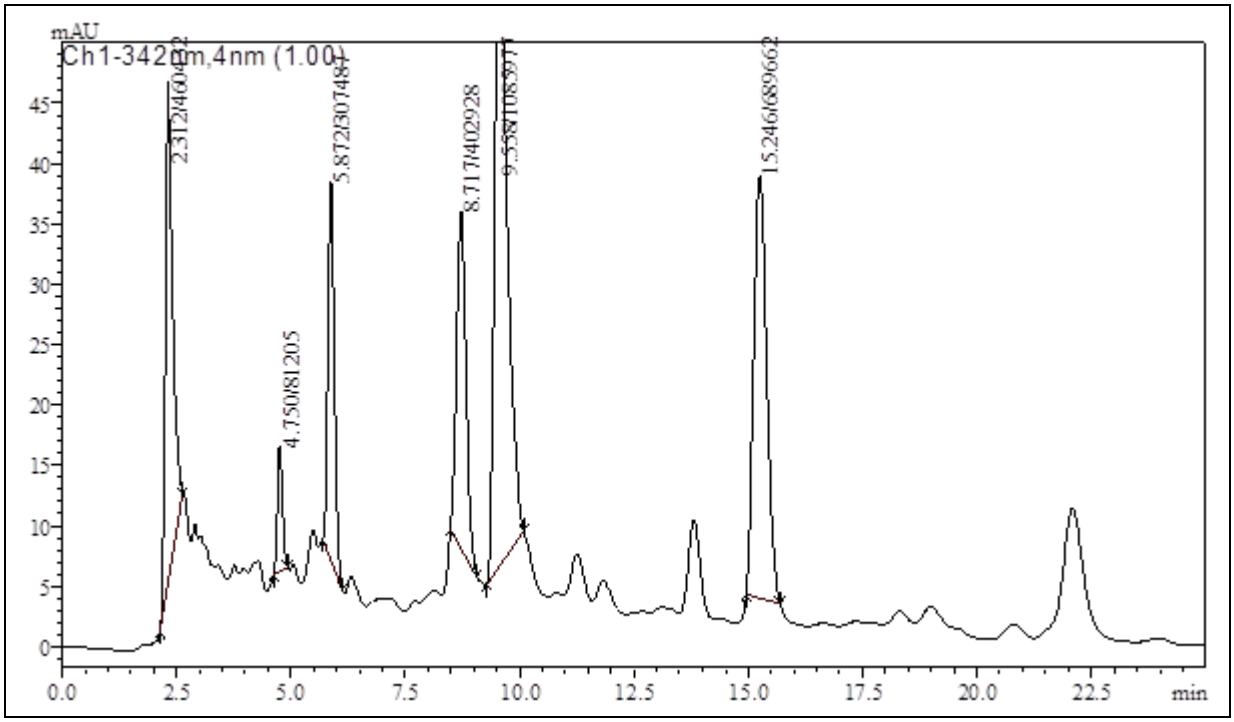

FIGURE 7. CHROMATOGRAM FOR THE EXTRACT OF *PEPEROMIA PELLUCIDA* L. (H.B.K.) OBTAINED BY PERCOLATION WITH THE CONCENTRATION OF 70% OF ETHYL ALCOHOL. 3', 4', 7-TRI-O-METHOXYFLAVONE HAS A RETENTION TIME OF 15.34 MIN.

The values obtained for the content of 3', 4', 7-tri-O-methoxyflavone in each 100 g of sample for maceration, with different concentrations of the solvent, are shown in **Table 2**. A comparison between the values obtained from the marker from the maceration and percolation using 70% of ethyl alcohol is shown in **Table 3**. The best results were obtained by maceration at 70%.

TABLE 2: VALUES OF THE CONTENT OF 3', 4', 7-TRI-O-METHOXYFLAVONE AT DIFFERENT CONCENTRATIONS OF ETHYL ALCOHOL IN THE MACERATION OF *PEPEROMIA PELLUCIDA* L. (H.B.K.).

Concentration (%) of ethyl alcohol in the maceration	Values of the content (mg) of 3', 4', 7-tri-O-methoxyflavone in each 100 g of sample
50	7.26 ± 0.211
70	17.66 ± 0.726
90	10.26 ± 0.440

TABLE 3: VALUES OF THE CONTENT OF 3', 4', 7-TRI-O-METHOXYFLAVONE IN THE MACERATION AND PERCOLATION OF *PEPEROMIA PELLUCIDA* L. (H.B.K.) USING 70% OF ETHYL ALCOHOL

Extration Methods	Values of the content (mg) of 3', 4',7-tri-O-methoxyflavone in each 100 g of sample
Maceration	17.66 ± 0.726
Percolation	11.58 ± 0.548

The maceration and percolation used were made using the same ratio raw plant material/solvent (1:10), the same processing time without occasional stirring, differing only in the type of equipment used in the extraction. In maceration, a reactor was used, and in the percolation, a percolator, both of stainless steel.

The technique proposed and used in the percolation is quite efficient, used worldwide, versatile and simple, allowing the easy use of this technique for small, medium and large-scale industrial process.

The geometry of the equipment is crucial in choosing the best extraction method, yielding better results when using the reactor, as shown in Table 03. There was significant difference when the equipment was changed.

CONCLUSION: The evaluation of the seasonal changes indicated that there is no influence of the rainfall indices on the content of the marker, 3', 4', 7-tri-O-methoxyflavone, for *P. pellucida*, , in samples collected during the following months: March, May and July.

The proposed technique for percolation presents numerous advantages in the development of herbal medicines. However, in the extraction of *P. pellucida* with 70% of ethyl alcohol, maceration is more efficient than the percolation.

In the variation of the concentration of the solvent in the maceration (50, 70 and 90%), there was a higher content of the marker using the concentration of 70% of ethyl alcohol.

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