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Full Length Research Paper

High performance liquid chromatography (HPLC) of *Hoodia gordonii* commercial powder

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***Hoodia gordonii* is traditionally used by the San people in South Africa due to its properties of appetite and thirst suppression, and it is scientifically proven and explained by the presence of active glycoside P57. The scarcity of the plant due to the difficulty of cultivation coupled with the growing demand for products antiobesity on *H. gordonii*, created a setting in which the adulteration of other species or even other genera is a real possibility. In this context, the use of high performance liquid chromatography (HPLC) is presented as a fundamental tool to certify the authenticity of products sold as *H. gordonii*. The aim of this study was to determine the authenticity of commercial samples of *H. gordonii* powder by means of qualitative analysis to confirm the presence or absence of active glycoside P57 in the samples. The tests were performed on C18 column (250 × 4.6 mm, 5 µm) using a gradient elution of water and acetonitrile, both containing 0.1% acetic acid and using geranyl tiglate default. Qualitative analysis showed that commercial samples of *H. gordonii* powder present in its constitution species of the genus *Hoodia*, due to confirmation of the active glycoside P57 by recovery experiments. However, considering the quantitative aspect, the contents of the active glycoside in the samples are below the limit of quantification, suggesting that they are unable to produce inhibition of appetite at the doses recommended for human consumption.**

Key words: *Hoodia*, high performance liquid chromatography (HPLC), commercial powder.

INTRODUCTION

Hoodia gordonii is traditionally used by the San people in South Africa due to its properties of appetite suppression. There are about 13 species described to date (*Hoodia alstonii, Hoodia currorii, Hoodia Dreger, Hoodia flava, H. gordonii, Hoodia juttae, Hoodia mossamedensis, Hoodia officinalis, Hoodia parviflora, Hoodia pedicellata, Hoodia pilifera, Hoodia ruschii, and Hoodia triebneri*) belonging to the genus *Hoodia*. However due to the proven anorectic activity attributed to the active glycoside P57 (MacLean and Luo, 2004), *H. gordonii* is a specie that has attracted more interest in marketing (Avula et al., 2006; Van Heerden et al., 2007). However, considering that *H. gordoni*, a native of the Kalahari Desert in southern Africa, its growth outside the natural environment is extremely difficult. Due to the limited area of cultivation and its slow maturation cycle the supply of *H. gordonii* becomes very limited (Avula et al., 2006). Therefore, *H. gordonii* is currently listed as an endangered species and their export is strictly controlled by the government of South Africa and the convention on international trade in endangered species of wild flora and fauna in danger of extinction (CITES[1]) (Avula et al., 2008).

At the same time, demand for products for weight loss containing *H. gordonii* has grown sharply in recent years, reflecting the current availability of more than 100 products marketed in the U.S. market in different presentations (Avula et al., 2007, 2008). Despite the great commercial interest by *H. gordonii*, as evidenced by the

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[1] CITES is one of the most important international environmental agreements for the preservation of species, with most countries of the world as signatories, including Brazil, which joined the convention in 1975. It regulates the import, export and reexport of animals and plants, their parts and derivatives, through a system of issuing permits and certificates that are issued when they meet certain requirements. One of the requirements for issuance of licenses is whether a particular type of trade or otherwise affect the survival of the species (IBAMA, 2010).

large consumption in the form of capsules, there are few reports about the plant (Pereira et al., 2010).

Thus, it sets up a scenario of great imbalance between supply and demand for products based on *H. gordonii*, causing the adulteration of other species or even other genera then become a possibility (Avula et al., 2008; Rader et al., 2007; Van Heerden , 2008). In this context, the development and / or optimization techniques such as high performance liquid chromatography (HPLC) for rapid analysis of products marketed as *H. gordonii* is crucial to determining its authenticity and safety for human consumption (Avula et al., 2006, 2007, 2008).

Therefore, the aim of this study was to determine the authenticity of commercial samples of the powder H. gordonii using qualitative and quantitative analysis to confirm the presence or not of the active glycoside P57, employing for this purpose, HPLC techniques described in the literature.

MATERIALS AND METHODS

Analyses were performed in the Laboratories of Biochemistry and Organic Chemistry of the Department of Chemistry (DQI), Federal University of Lavras (UFLA - MG).

Reagent

The compound geranyl tiglate standard was purchased from the Sigma-Aldrich and kindly donated by Dr. Hans-Gerd Janssen, professor at the University of Amsterdam (Netherlands) and researcher at the Department of Research and Development, Unilever Food and Health Research Institute (Vlaardingen, Netherlands).

The HPLC analysis was performed using these reagents: methanol and acetonitrile HPLC grade (Merck®), glacial acetic acid HPLC grade (Vetec®) and ultra pure water obtained by Milli-Q system.

Achievement of commercial samples of powdered *H. gordonii*

Two commercial samples of commercial powder *H. gordonii* (PHG), known as HA and HB, were bought from pharmacies in the cities of Lavras and Juiz de Fora, Minas Gerais and two from different suppliers of domestic raw materials, accompanied by technical reports from tests carried out by ourselves, but coming originally from China.

Preparation of extracts from the commercial powder *H. gordonii*

The method of extracting the active glycoside P57 was performed according to Janssen et al. (2008) with modifications, using the two commercial samples of powdered *H. gordonii*. The two commercial samples of PHG were extracted under reflux with methanol in the ratio 1:10 for 1 h in a round bottom flask. After the mixture was allowed to stand at room temperature for cooling and, then centrifuged at 2,500 × g for 10 min.

The precipitate was discarded and the supernatant (extract) used in chromatographic analysis. Before injection in the column an appropriate volume of extract (2 ml) was passed in Millipore microfilter of 0.25 mM, and the first 1.0 ml discarded and the remaining volume collected in vial for HPLC. Each extract was injected in triplicate in the analysis.

Preparation of standard solution and determination of the analytical curve

The compound geranyl tiglate pattern was chosen for its structural similarity to the 12-substituent tigloilpregnano active glycoside present in P57. Thus, Janssen et al. (2008) presented chromatographic characteristics, such as recovery, repeatability and linearity, to qualify it as an external standard on samples of *H. gordonii*. A standard stock solution of geranyl tiglate was prepared at a concentration of 2×10^{-3} mol L^{-1} equivalent to 470 mg ml^{-1} in methanol, from which dilutions were made to prepare the solutions used. Calibration curves were prepared with seven different concentrations of standard in the range of 47 to 1120 ng ml^{-1}, injected in duplicate. From the results of the analytical curves were calculated limits of detection (LOD) and quantification (LQ) for the method.

High performance liquid chromatography

Chromatographic analysis of commercial samples of *H. gordonii* were performed using a chromatograph with detection in the UV region at 220 nm after extraction of samples. All measures of high performance liquid chromatography (HPLC-UV) were made on a Shimadzu UFLC (Kyoto, Japan) equipped with 2 pumps (LC 6AD), with diode array detector (DAD) (SPD M20A), gun with auto sampler (SIL 10AF) and data logger (CBM 20A). The separation was performed on an analytical Nucleosil 120-5 C18 column, 250 × 4.6 mm packed with 5 mm particles (Macherey-Nagel GmbH & Co., Germany) operated at room temperature. The injection volume used was 10 ml. For elution we used acetic acid and acetonitrile HPLC grade and ultra pure water, according to Avula et al. (2006) and Janssen et al. (2008) with modifications presented in Table 1.

Recovery tests

Recovery experiments were performed for identifying the presence (qualitative analysis) of the active glycoside P57 in the samples and to confirm the accuracy of method. To this end, we performed multiple injections of standard solution and samples of preparations containing the HA and HB plus known amounts of standard (spiked samples). The recovery values were obtained by the difference between the peak area of spiked sample and the peak area of the sample divided by the peak area of standard and expressed in percentages.

RESULTS AND DISCUSSION

Determination of the chromatographic conditions

Initially the standard solutions were injected with geranyl tiglate to determine the retention time and optimal concentrations. Figure 1 shows the chromatogram of a standard solution at a concentration of approximately 2×10^{-4} mol L^{-1} is equivalent to 47 mg ml^{-1}.

There is a retention time around 33.9 min for the standard geranyl tiglate close to that found by Janssen et al. (2008) under similar conditions.

To determine the retention time of default, subsequent

Table 1. HPLC gradient of elution*.

Step	Time (min)	Composition	Description
1	0.01	80% A + 20% B	Beginning
2	35	Increasingt until 100% B	Gradient elution
3	40	100% B	Gradient elution
4	45	80% A + 20% B	Reconditioning
5	50	Finish	

*According to Avula et al. (2006) and Janssen et al. (2008) with modifications. Chromatographic conditions: mobile phase, water (A) and acetonitrile (B) both with 0.1% acetic acid), Detection: UV 220 nm, flow: 0.8 ml min^{-1}.

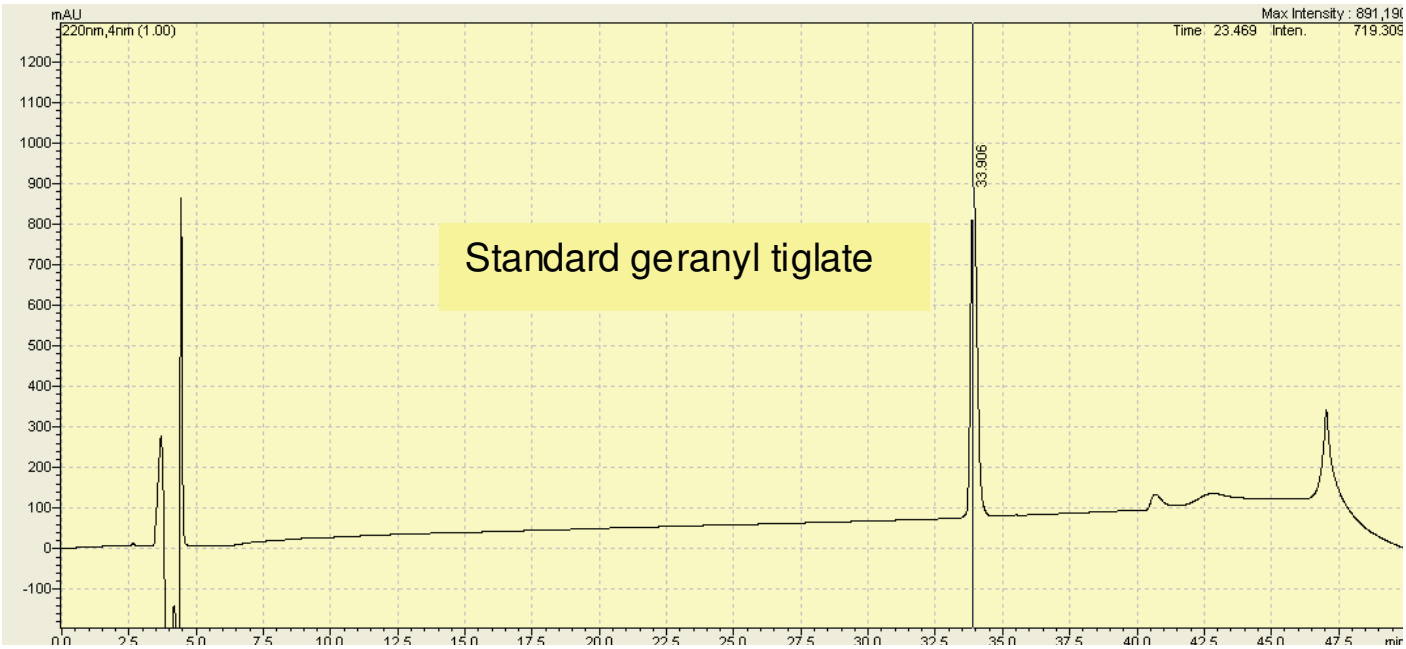


Figure 1. Chromatogram of standard geranyl tiglate concentration of 2 × 10^{-4} mol L^{-1}. (Chromatographic conditions: analytical column 250 x 4.6 mm C18 (5 mm), Macherey-Nagel Nucleosil 120-5; flow: 0.8 ml. min^{-1}, mobile phase: water (A) and acetonitrile (B), both containing 0.1% acetic acid elution, starting with 80% A and 20% B, rising gradually to 100% B in 35 min and maintained until 40 min after the column was reconditioned for 5 min in initial conditions).

injections were made of samples HA and HB to guide the choice of the optimal concentration of standard solution and the analytical curve. Such analysis revealed that the optimal concentration of the standard to be used would be around 2 × 10^{-6} mol L^{-1} equivalent to 0.47 mg mL^{-1}.

Curve analytical and analytical parameters

In defining the ideal chromatographic conditions, samples were prepared following a calibration. The seven points of the analytical curve showed a linear correlation between concentration and peak area (R^2 = 0.9985), indicating the linearity of the detector response for all concentrations of the standard in the range considered.

Additionally we calculated the limits of detection (LOD) and quantification (LQ), equivalent to 75 ng ml^{-1} and 250 ng mL^{-1}, respectively.

Chromatographic analysis of samples of commercial powder *H. gordonii*

Preliminary injections of HA and HB samples resulted in peaks with low intensity values that lie in the range between the limits of detection and quantification, but qualitatively revealing the presence of the glycoside P-57 in the samples, which according to Avula et al. (2007), sufficiently guarantee the existence of some species of *Hoodia* in their composition.

These qualitative results show that both samples have commercial species of *Hoodia* in their composition. Besides *H. gordonii* other species of the genus as *H. currorii*, *H. macrantha, H. parviflora* and *H. ruschii*, also have a constitutional glycoside P-57, which in turn is not present in the main contaminant of products based on *H. gordonii*, the cactus *Opuntia ficus* (Avula et al., 2006, 2007).

Additionally, in order to confirm the presence of an active glucoside in commercial samples, and to evaluate the accuracy of the method recovery tests were performed. In these experiments, known amounts of standard samples were added to the HA and HB and these samples (spiked samples) and the pure standard injected below the unit. In Figure 2, chromatograms of recovery assays can be observed.

Moreover, with the aid of analytical curve, calculations were performed to recover the standard in spiked samples (Table 2) that were in the range of 97.0 to 100.6% for the standard compound geranyl tiglate.

It was observed by the analysis of Figure 2 that both the pure standard samples and spiked HA and HB show the same profile after several chromatographic runs (n = 12). Similar trials using fortified samples of *H. gordonii* were effective in confirming the presence of active glycosides in products marketed in different presentations such as capsules, powders, dry extracts and others (Avula et al., 2006, 2007, 2008; Janssen et al., 2008).

In addition, it was found that the samples were spiked HA and HB of the standard recovery values of 97.0 and 99.3% (Table 2), respectively. These recovery percentages are very close to the 99% found by Janssen et al. (2008), using the standard geranyl tiglate on analysis of commercial samples of *H. gordonii*. Therefore, the results for the spiked samples HA and HB provide qualitatively confirm the presence of an active glucoside in P57 samples analyzed to confirm and guarantee the presence of some species of *Hoodia* in its constitution.

Detailed qualitative analysis was used to differentiate species of the genus *Hoodia* from chromatographic profiles of 11 glycosides, including the P57, which is already present in these species (Avula et al., 2007). However, the availability of standards is required for all glucosides.

On the other hand, considering the quantitative aspects, although the amounts have been found active glycoside in HA and HB, the values were below the quantification limit calculated from 250 ng ml^{-1}, which prevents the accurate determination of the percentage of active glucoside in these P57 commercial samples. However, based on the minimum value, measurement would correspond to a concentration of 2.5×10^{-4}% of the active glycoside. This may be as a result of some considerations about the potential appetite suppressant effect of the samples.

It was shown that approximately 7×10^{-5} g of active glycoside purified P57 applied directly into the brain of rats were effective in reducing about 50% in food intake (MacLean and Luo, 2004). Taking into account the recommended adult dose of no more than 2 g/ day of commercial powder, it can be calculated that the amount ingested is less than 5×10^{-6} g of the active glycoside (calculated from the value equivalent to LQ) which is about 10 times less than the amount needed for an effective response in rats. Using some data from the literature, we can make some theoretical calculations and speculations about the amount of commercial powder of *H. gordonii* that would be required to obtain the proposed effects. Therefore, considering a hypothetical level of P57 in the commercial powder (2.5×10^{-4}%), the amount of P57 (5×10^{-6} g) contained in the recommended dose for humans (2 g /day) and effective inhibition of appetite in rats (7×10^{-5} g of active glycoside) extrapolated to humans (10^{-3} g), concluded that it would require the ingestion of at least 400 g / day of commercial samples HA and HB for an effective response in the inhibition of appetite of an adult of 70 kg.

Obviously in practice these intake values should be higher, as in the aforementioned study the effect of inhibition was achieved after direct intracerebral administration of purified active glucoside, not subjected to digestion that causes losses, thus requiring higher amounts of active ingredient. It follows therefore that the maximum recommended dose (2 g / day) of commercial powders analyzed contains quantities of the active glycoside P57 totally insufficient to achieve the supposed effect of inhibition of appetite.

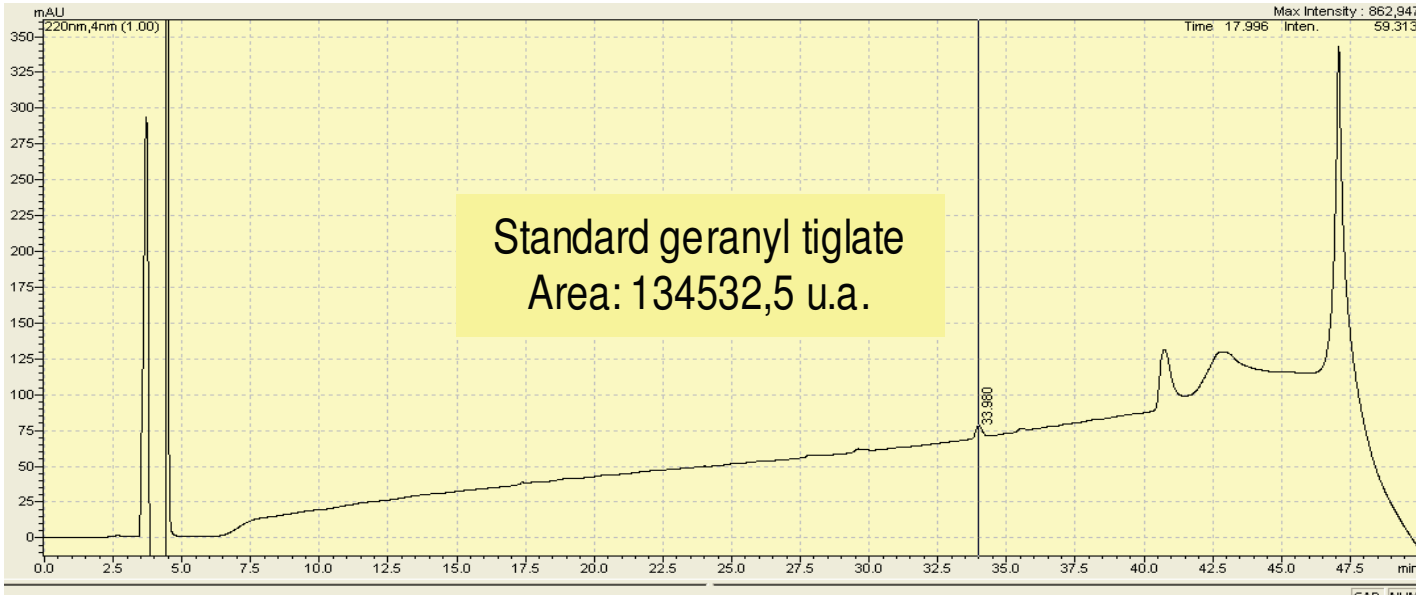
Another comparison can be performed, based on tests of ingestion of *H. gordonii* by rats, which proved to be necessary to consume about 2 g of plant / kg body weight to obtain the ED50 inhibition of appetite (effective dose for achieving a response in 50% of the animals) (Tulp et al., 2001). Such results were found with authentic samples of the plant, thereby suggesting an intake for adults of about 140 g / day plant. In this same study, however, were not analyzed for the content of the active principle in the samples. It is, therefore, used to emphasize the importance of studies with *H. gordonii* involving biological assays of prolonged ingestion associated with determination of the active principle in the samples analyzed todetermine the effective dose, which are so far lacking.

Only recently, studies have shown the levels of P57 in several glycoside samples sold as *H. gordonii*, which include dry extracts, capsules, powders, tablets, dietary supplements, teas and more. According to Avula et al. (2006, 2008), specifically for authentic samples of plants of the genus *Hoodia,* the levels of the active glycoside can vary greatly with values between 0.0051 and 0.218% of dry matter depending on the species. HPLC analysis of plant samples showed levels of P57 of 0.047 and 0.0051%. But analysis of 10 dietary supplements (capsules and tablets), in only 2 were detected with the active glycoside content of 0.17 and 0.0048% (Avula et al., 2006). A similar test, by analyzing 35 commercial products in various formulations (capsules, tablets, powders, teas, gels, juices, and spray bars), showed that in 26 of them the presence of glycoside was not detected, while in the other 9 4 of them were only possible to determine the levels of P57, since in the other five samples, the levels found were below the limit of quantification allowing only qualitative identification (Avula et al., 2008).

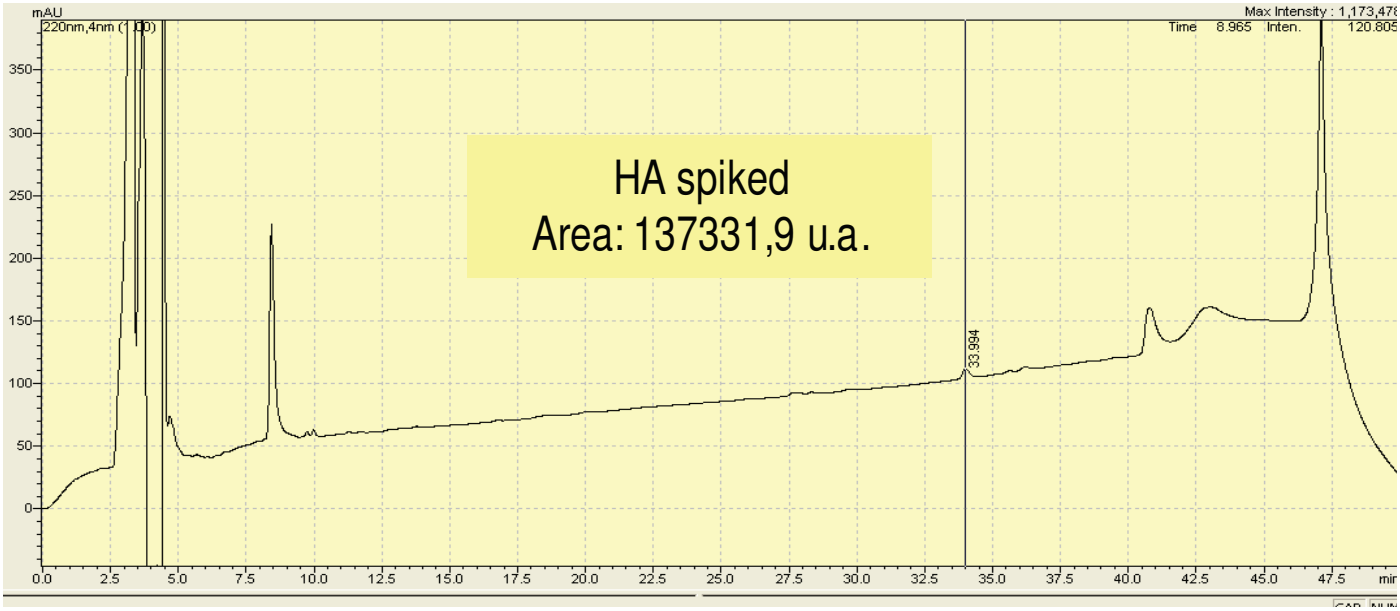
Table 2. Recovery of the pattern in the two spiked samples of *H. gordonii*.

Samples	n	Recovery*(%)	Retention time (minutes)
Standard	6	99.9 ± 0.5	33.980 ± 0.14
HA spiked	6	97.0 ± 0.5	33.994 ± 0.17
HB spiked	6	99.3 ± 1.3	33.957 ± 0.22

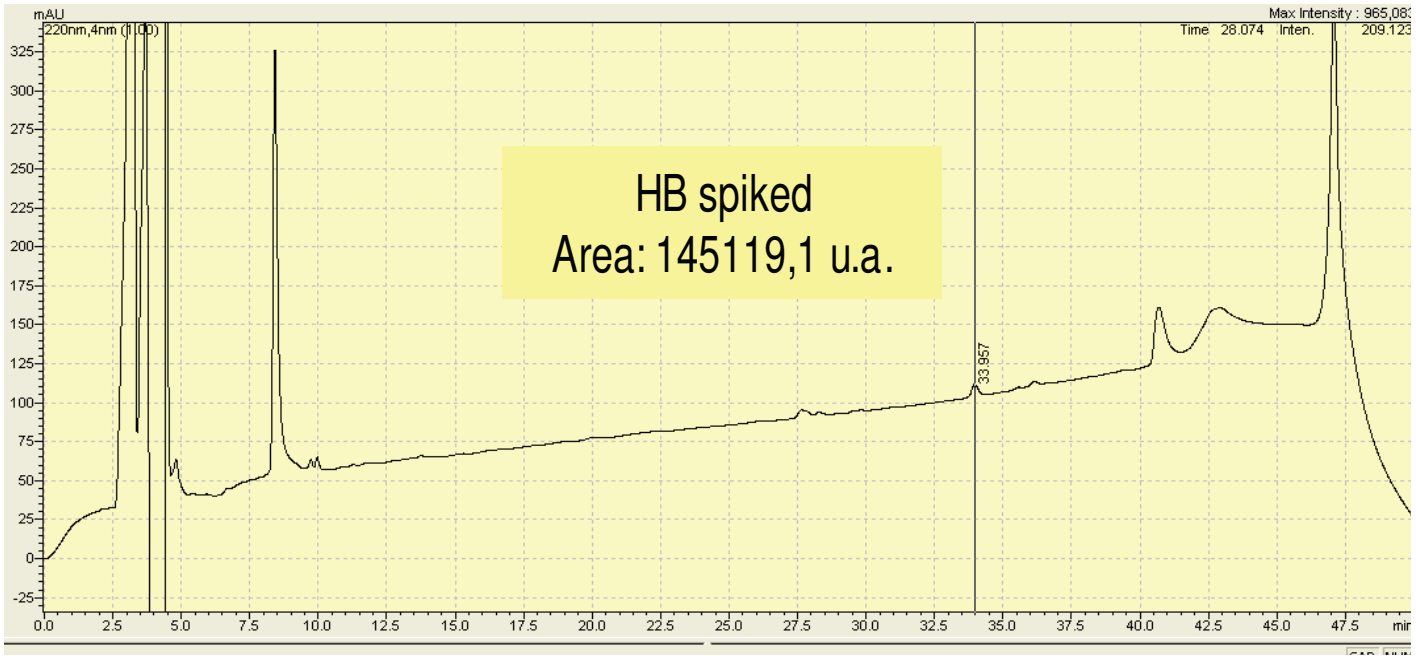
*Calculated by difference between the peak area of spiked sample and the peak area of the sample, divided by the peak area of standard.



(a)



(b)



(c)

Figure 2. Chromatogram of recovery assays: (a) Standard geranyl tiglate 2 × 10^{-6} mol L^{-1} equivalent to 0.47 mg ml^{-1} (b) Sample HA spiked with geranyl tiglate standard 2 x 10^{-6} mol L^{-1} (c) Sample HB spiked with the standard geranyl tiglate 2 × 10^{-6} mol L^{-1}. (u.a.: units of area) (Chromatographic conditions: analytical column 250 × 4.6 mm C18 (5 mm), Macherey-Nagel Nucleosil 120-5; flow: 0.8 ml. min^{-1}, mobile phase: water (A) and acetonitrile (B), both containing 0.1% acetic acid elution, starting with 80% A and 20% B, rising gradually to 100% B in 35 min and maintained until 40 min after the column was reconditioned for 5 min in initial conditions).

These results are, therefore, similar to that found for HA and HB samples analyzed in this study, which was possible only qualitative determination of the active glycoside P57. Moreover, it becomes explicit by the results of this study and others cited the lack of standardization and monitoring of products marketed as *H. gordonii*, suggesting the need for quality control tests before they are released for consumption. The implementation of protocols for analysis of quality control for commercial products based on the *Hoodia* is justified because of the presence of active glycoside P57 in various species of the genus (*H. currorii, H. macrantha, H. parviflora and H. ruschii*) and not only in *H. gordonii.* Thus, such actions should aim at ensuring their authenticity and identity, the definition of minimum levels of acceptable active glycoside P57 commercial samples, and at the same time serve as prerequisites for the release of the marketing and consumption.

These literature data allied to levels of P57 found in our work and the lack of information from suppliers regarding the origin and preparation of the extracts suggest that the samples analyzed HA and HB when consumed at recommended doses (2 g / day) are unable to cause inhibition of appetite.

Conclusion

Qualitative analysis by HPLC showed that commercial samples of the powder of *H. gordonii* present in its constitution species of the genus *Hoodia*, evidenced by the confirmation of the presence of active P57 glycoside. However, the quantitative point of view, the contents of the active glycoside in the samples are below the limit of quantification, suggesting that the doses recommended for human consumption are not able to cause inhibition of appetite.

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