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# Effectiveness of different methods for the extraction of principle actives and phytochemicals content in medicinal herbals

[Efectividad de diferentes métodos para la extracción de principios activos y contenido de fitoquímicos en hierbas medicinales]

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**Abstract:** In this present study, we investigated the influence of various extraction methods including maceration, sonication, infusion, decoction, and microwave extraction, on the chemical and biological potential of phytochemicals extracted from three medicinal plants (*Ageratum conyzoides*, *Plantago major* and *Arctium lappa* L). The results were subsequently analyzed by variance analysis. Our results suggested that sonication is the most effective extraction method among the five methods tested herein, for the extraction of phytochemicals that have a high antioxidant potential and high phenolic content. The three plants employed for this study had a high concentration of flavonoids and phenolics which was compatible with the chemosystematics of the species. All the samples possessed a Sun Protection Factor (SPF) of less than 6. Interestingly, a maximum reaction time of approximately 20 min was noted for the complexation of $AlCl_3$ with the flavonoids present in the phytochemical extract during analyses of the kinetic parameters. We finally identified that the Ageratum conyzoides extract, prepared by sonication, possessed a significant pharmacological potential against hepatocarcinoma tumour cells, whose result can guide further studies for its therapeutic efficacy.

**Keywords:** Medicinal herbals; *Ageratum conyzoides*; *Plantago major*; *Arctium lappa*; Phytocompounds.

**Resumen:** En el presente estudio, investigamos la influencia de varios métodos de extracción, incluyendo maceración, sonicación, infusión, decocción y extracción por microondas, sobre el potencial químico y biológico de los fitoquímicos extraídos de tres plantas medicinales (*Ageratum conyzoides*, *Plantago major* y *Arctium lappa* L). Los resultados se analizaron posteriormente mediante análisis de varianza. Nuestros resultados sugieren que la sonicación es el método de extracción más eficaz entre los cinco métodos aquí probados, para la extracción de fitoquímicos que tienen un alto potencial antioxidante y un alto contenido fenólico. Las tres plantas empleadas para este estudio tenían una alta concentración de flavonoides y fenólicos que era compatible con la quimiosistemática de las especies. Todas las muestras poseían un factor de protección solar (SPF) menor a 6. Curiosamente, se observó un tiempo máximo de reacción de aproximadamente 20 min para la complejación de $AlCl_3$ con los flavonoides presentes en el extracto fitoquímico durante los análisis de los parámetros cinéticos. Finalmente, identificamos que el extracto de Ageratum conyzoides, elaborado por sonicación, posee un importante potencial farmacológico frente a las células tumorales del hepatocarcinoma, cuyo resultado puede orientar nuevos estudios sobre su eficacia terapéutica.

**Palabras clave:** Hierbas medicinales; *Ageratum conyzoides*; *Plantago major*; *Arctium lappa*; Fitocompuestos.

## INTRODUCTION

Data from the WHO (World Health Organization) indicate that nearly 35,000 to 70,000 species of plants have been widely used as medicines since a long time (Farnsworth & Soejarto, 1991; Padulosi & Leaman, 2002; Dutra *et al*., 2016). Remarkably enough, this represents 14-28% of the total 250,000 plant species estimated worldwide, which indicates that approximately two-thirds of all the plant species known so far have been employed for primary health care (Farnsworth & Soejarto, 1991; Padulosi & Leaman, 2002; Dutra *et al*., 2016). These data, therefore, reinforce the necessity of fundamental screening studies for establishing the pharmacological effectiveness of popularly used medicinal plants, as well as, for identifying their curative and toxicological properties (Sofowora *et al*., 2013; Kayser, 2018). By employing this strategy, medicinal plants with the recognized therapeutic potential need further exploration from a current chemical-pharmacological point of view. Although several species of medicinal plants with immense phytotherapeutic importance are known to mankind, studies identifying novel active principles and investigating the effects of different extraction methods on the properties of plant-derived biomolecules are scarce (Padulosi & Leaman, 2002; Dutra *et al*., 2016).

*Ageratum conyzoides* (AC), *Arctium lappa* (AL) and *Plantago major* (PM) are medicinal plants of enormous phytotherapeutic importance. AC, for instance, is widely used in several cultures as a remedy for a wide range of diseases, possibly due to its excellent anti-inflammatory and antinociceptive properties (Bahtiar *et al*., 2017). AL, popularly known as "Bardana", is a vegetable that has been widely used in diverse cultures since a long time as a diuretic and for the treatment of hypertension, gout, hepatitis and others inflammatory disorders (Predes *et al*., 2009; Silva *et al*., 2013). Likewise, several species belonging to the genus *Plantago* (especially PM) has been widely used for decades in the treatment of various ailments, including inflammation and cancer (Piyaviriyakul *et al*., 2017; Heravi *et al*., 2018).

Regarding the chemodiversity of the species evaluated, e.g., the *AC* particularly present pyrrolizidine alkaloids (Faqueti *et al*., 2017), phenolic acids, coumarins, benzopyrones, chlorogenic acid, coumaric acid, tannins, polymethoxyflavones (Sultana *et al*., 2012; Faqueti *et al*., 2017;), sesquiterpene lactones (Chagas-Paula *et al*., 2015), glycosylated flavonoids (Munikishore *et al*., 2013), terpenoids, chromenes, and also phytol (Vera, 1993). In the case of *AL* species are described flavonoids (Ionescu *et al*., 2014), phenylpropanoids (Gao *et al*., 2013), and phenolic acids derived from caffeic acid such as dicaffeoylquinic acid (Carlotto *et al*., 2015). Other bioactive aromatic compounds such as arctiin (Coulerie *et al*., 2016), free sugars or heterosides such as the glycosylated phenolics arctiisesquineolignan B and arctiiphenolglycoside A (He *et al*., 2016), glycosylated lactones (Yang *et al*., 2015), butyrolactone lignans (Chagas-Paula *et al*., 2015), terpenes, steroids and fatty acids such as the amide-derived fatty acids (Yang *et al.*, 2016) have been reported. In PM has widely been reported flavonoids and phenolic acids derived from hydroxycinnamic, chlorogenic and neochlorogenic acids (Maksyutina, 1971a; Maksyutina, 1971b), aliphatic acids (e.g., such as tartaric acid, citric acid, malic acid, malonic acid and succinic acid) (Olennikov *et al*., 2005), flavonoids such as glycosylated flavanone (Endo *et al*., 1981) and flavones luteolin 7-glucoside and luteolin 7-glucuronide (Lebedev, 1976), glycosylated phenolic acids such as verbascoside (Egorov *et al*., 2004), terpenes and steroids, fatty acids such as stearic acid, pentadecanoic acid, oleic acid, eicosapentaenoic acid and docosahexaenoic acid (Ringbom *et al*., 2001).

Studies aiming to establish a standard method for the rapid and selective extraction of bioactive phytochemicals from vegetable matrices still face challenges, primarily arising from the inherent limitations of various conventional extraction methods. In this regard, the choice of the extraction method depends on the characteristics of the target active principle that is to be extracted, among other factors. Since the extraction strategy plays a crucial role in the outcome of the extraction, including the yield, phytochemical content, and stability of the biomolecule extracted, and also the following tests performed. This study, therefore, evaluated the effectiveness of different methods (maceration, sonication, infusion, decoction, and microwave) for the extraction of principle actives and phytochemicals content in AC, AL and PM medicinal herbals. Herein, were also evaluated and compared the chemical composition, antioxidant capacity, and bioactivity of these plants.

## MATERIAL AND METHODS

### *Plant material*

The dried plant material marketed in sachets for the

production of medicinal tea was obtained from the company of herbal medicines *Nature Ervas* (Teófilo Otoni City, Minas Gerais State, Brazil).

***Extraction methods***

The extracts have prepared following the legislation from Brazilian Pharmacopoeia, in which recommend that the crude extracts (hydroalcoholic 70% extract) were made in a ratio of 1:5. For the preparation of the crude extract by the maceration method, in particular, the plant material (5.0 g) passed through the process of maceration in 25 mL of 70% hydro alcoholic solution for a period of three days at nearly room temperature. For the crude extract by sonication method, the plant material (5.0 g) was extracted in (25 mL) of 70% hydroalcoholic solution on ultrasound-assisted using ultrasonic bath (Unique®) for a period of about 20 min, three times. In case of other desired phytopreparations (such as infusion, decoction, and microwave extraction), the respective ratio of 1:50 was used for these all cases (i.e., about 5 g of plant material was added in 250 mL of water). Finally, the resulting filtrate was evaporated under vacuum at 40°C until the solvent was effectively removed. Then, all materials obtained were dried and the yield (%) of these extractions was calculated. Particularly, the samples were extracted by infusing the plant material containing (5 g) of the plant into (250 mL) of distilled water and deionized for 10 min of infusion at 85°C. For the decoction phytopreparation, (5 g) of plant material in (250 mL) of water was submitted to heating until the water boiled and, after cooling, the decoct was filtered for further analysis. For microwave extraction using microwave oven (Philco), (5 g) of vegetable material in (250 mL) of water was heated to the microwave for a period of 5 min at medium power.

All five phytopreparations obtained were conditioned in a refrigerator to avoid degradation/oxidation of the metabolites, and the chemical tests were performed one day later. As already explained, preliminary tests using different extraction methodologies were carried out to evaluate extraction methods on phytochemical content and pharmacologycal properties.

***Analysis by RP-HPLC-PDA and $^{1}$H NMR***

RP-HPLC-PDA analysis of all the phytopreparations was carried out in a Shimadzu Chromatograph equipped with two Shimadzu LC-$_{10}$AD pumps, Shimadzu SIL $_{10}$A auto-injector, UV-Vis array detector model Shimadzu SPD MX AVP. Briefly, the data acquisition and processing have treated on the Shimadzu Classlc$_{10}$ software (version 1.64A). For these experimental measured at room temperature, were used a Phenomenex C18-Hydro (250 x 4.6 mm, 4 μm) with a flow of 1 mL min$^{-1}$, the injection volume was 30 μL. In addition, the $^{1}$H-NMR (500 MHz) spectra of the samples were then obtained on the Varian Inova 500® Spectrometer. For comparison of micro molecule extraction, all samples were analyzed at the same concentration (dissolving 10 mg of the dried material in 300 μL deuterated water).

***Statistical analysis***

The efficacy of these extraction methods was compared between the groups studied in this study by analysis of variance (ANOVA) with at least three independent experiments and are presented as the mean ± SEM. In this case, the differences were then considered statistically significant at $p<0.05$.

***Evaluation of antioxidant activity and flavonoid content***

***Antioxidant activity:*** For the evaluation of antioxidant activity, the *in vitro* photocolorimetric method of free radical DPPH as described by Mensor *et al*. (2001). For the analysis of the samples, 20 μg mL$^{-1}$ of the methanolic solution of the diluted extracts was added to 220 μg mL$^{-1}$ of a free radical methanolic DPPH solution. After 30 minutes of reaction, the reading was carried out at 518 nm using a UV-Vis Shimadzu UV 1601 spectrophotometer. All readings were then performed in triplicate.

***Colorimetric test with $FeCl_3$ for phenolic detection:*** About 10 mL of phytopreparations were reacted with a methanolic solution of $FeCl_3$ in test tubes and observed the coloration acquired after the reaction. The results obtained in the tests carried out in the phytochemical screening were established according to the following parameters: (–) absent; (+) low in abundance; (++) moderate in abundance; (+++) high in abundance.

***Total flavonoid content:*** Total flavonoid content was subsequently determined using the adapted Dowd method (Arvouet-Grand *et al*., 1994). Briefly, 500 μL of 2% $AlCl_3$ in methanol was mixed with the same volume of sample solution (100 μL of extract to 50 mL of distilled water). Thus, the absorbance at 425 nm was read after ten minutes against a blank, consisting of a solution (500 μL) of methanol with 500 μL of $AlCl_3$. In this case, the total flavonoid content was determined using a standard quercetin curve at four concentration points (5, 10, 20

and 40 μg.mL$^{-1}$). Y = 0.0062x - 0.0048, where y is the absorbance and x is the concentration, ($R^2$ = 0.9999). Finally, the total content of flavonoids was expressed as mg of quercetin equivalents per gram of extract, considering the dry extract content of the extracts.

***Determination of Sun Protection Factor***

According to Mansur *et al.* (1986a), the samples were diluted in ethanol (0.2 mg/mL) as well as the aqueous extract was diluted to the same concentration with distilled water. Afterward, the samples were then analyzed in triplicate (at room temperature) using a spectrophotometer (Genesys 10S UV/Vis). Briefly, the absorbance of the solutions was measured at defined wavelengths (290, 295, 300, 305, 310, 315 and 320 nm). Thus, the SPF has obtained using the mean absorbance (n=3), according to a mathematical model previously reported by Mansur *et al.* (1986b).

***In vitro cytotoxicity evaluation of medicinal herbs obtained by sonication methods***

For the cytotoxicity assay using Sulforhodamine B (SRB), a suspension of HepG2 cell line containing 1.5x104 cells/well, was prepared. In this case, the cells were cultured in 96-well plates for a period estimated of 24 hours. Next, the pure chemicals following a serial 1:3 dilution starting at a concentration (200 μg mL$^{-1}$) were added. After 24 hours of treatment, (50 μL) of a solution 50% of trichloroacetic acid (TCA), was added at low temperature, and the plates were incubated for 1 hour at 4°C. Then, the TCA solution was removed, and the plates were washed with tap water 3 to 4 times. Approximately 50 μL of SRB solution at 0.4% (dilute acetic acid) was added. Afterward, the plates were then incubated for about 20 minutes (at room temperature). After removal of the SRB, the plates were washed 3 to 4 times with 1% acetic acid, dried and dissolved dye with (10 mM) Tris Base (Sigma). About 5 minutes after the incubation, the spectrophotometric reading of absorbance was then performed at a wavelength of 570 nm in the plate reader iMark Microplate Reader (Bio-Rad Laboratories, Hercules, CA, USA). In this case, the tests have performed in three independent experiments, as well as, the percentage of living cells was calculated in relation to the negative control, that is, representing the cytotoxicity of each treatment, as proposed by Zhang *et al.* (2004).

## RESULTS AND DISCUSSION

The initial crude extracts using these methods contain a complex mixture of many plant molecules, such as alkaloids, glycosides, phenolics, terpenoids, and flavonoids. In order to the relevant extract fragments of these species, it is expected that the chemical composition of the product obtained will vary according to the chemical constitution of the product obtained, therefore, influence the pharmacological properties of the medicinal herb.

***Antioxidant activity and content of total flavonoids***

No statistically significant differences in the antioxidant activity were observed among the samples extracted by the decoction, infusion, and microwave methods. The antioxidant activities varied between 78 and 82% and were measured by the inhibition of the 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radical. However, among the three methods evaluated, the highest values of complexing capacity were observed for the samples extracted by the infusion method, which can in principle be explained by the fact that the infusion method is a mild extraction method that prevents the degradation of thermo sensitive compounds that contribute to the increase in antioxidant activity. However, our results suggest that the samples extracted by sonication presented excellent antioxidant activity, being above 90% for the AL extract (Table No. 1).

The results depicted in Table No. 2, suggest a close correlation between the antioxidant activity and the concentration of phenolic compounds in the samples. Additionally, this observation is in good agreement with the data reported in the literature, and therefore suggests that the antioxidant activity could be directly correlated to the content of phenols in this species (Castro *et al*., 2007; Da Silva *et al*., 2013). In that case, the phenolic compounds can intercept the free radical oxidation chain by donating the hydrogen's from their phenolic hydroxyls (Righi *et al*., 2011; Xu *et al*., 2017). Particularly, Mesa-Vanegas *et al*. (2015) in their study to evaluate the antioxidant activity from extracts of different polarities from *AC* by different methodologies (ABTS •+, DPPH •, FRAP and ORAC) confirms the antioxidant property of their extracts, indicating a more significant activity of ethyl acetate extract. Yet, it does not demonstrate the possible compounds responsible for this property.

Therefore, the confirmation of antioxidant activity of these phytopreparations offers a scientific validation of their potential medicinal use, since the use of these herbs as a home remedy merely originated from their habitual use by various communities (Niki, 2010; Xu *et al*., 2017).

**Table No. 1**
**Values % metal chelating activity and sequestering capacity of DPPH in plant samples obtained by different methods of extraction of active principle**

| Extraction | *Ageratum conyzoides* | *Arctium lappa L* | *Plantago major* |
|---|---|---|---|
| **Infusion** | 82 % | 87% | 82 % |
| **Decoction** | 78 % | 85 % | 82 % |
| **Microwave** | 81 % | 86 % | 81 % |
| **Maceration** | 61 % | 75 % | 59 % |
| **Sonication** | 85 % | 92 % | 87 % |

**Table No. 2**
**The concentration of phenolic compounds in plant samples obtained by different methods of extraction of active principle**

| Species | *Phenolic compound concentration* | | | | |
|---|---|---|---|---|---|
| | **Infusion** | **Decoction** | **Microwave** | **Maceration** | **Sonication** |
| **AC** | (+) | (++) | (+) | (++) | (+++) |
| **AL** | (+) | (++) | (+) | (++) | (+++) |
| **PM** | (+) | (+) | (+) | (++) | (+++) |

**(– absent; +low in abundance; ++moderate in abundance; +++high in abundance)**

In order to correlate the content of phenolic compounds obtained from the preliminary iron chloride ($FeCl_3$) screening assay to the observed antioxidant activity, the total flavonoid content was determined by using the phytopreparations extracted by the sonication method, since these samples exhibited a higher antioxidant potential and a high concentration of phenolic substances. The experiment was typically performed by measuring the chelation of aluminium chloride ($AlCl_3$) with the maximum reaction time (sonication) using quercetin as the standard (quercetin mg/100 g). Hence, the total flavonoid content of the AC extract was approximately 298 mg, while the total flavonoid contents of the AL and PM extracts were 154 mg and 122 mg, respectively. These values are within the standards reported by other authors and hence suggest that the amounts of total flavonoids are lower than the amount of total phenolic compounds.

***Complexation kinetics of $AlCl_3$ with herbal extracts obtained by sonication***

The results of complexation kinetics experiments of $AlCl_3$ with the phytopreparations extracted by sonication showed no significant difference after 10 minutes of reaction (Figure No. 1). These results therefore indicated that 10 minutes is enough for the complexation of $AlCl_3$ with the phenolic compounds present in the active principle of the samples considered herein.

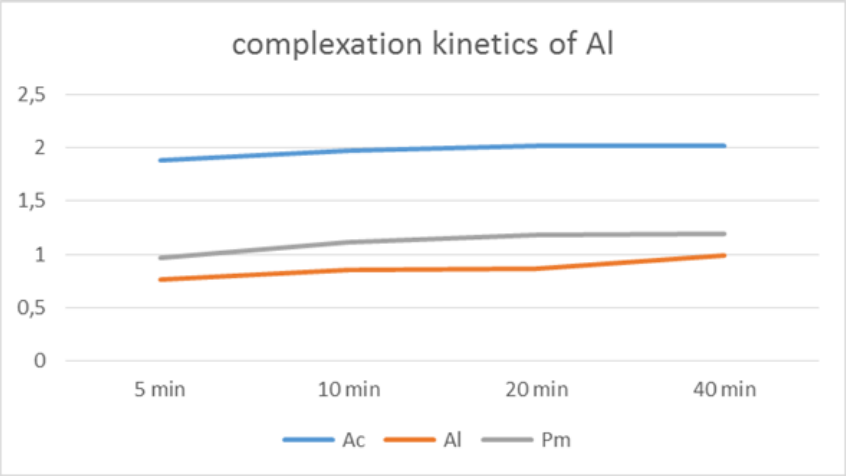


**Figure No. 1**
**Complexation kinetics of aluminum chloride ($AlCl_3$) with phytopreparations obtained by sonication of medicinal herbs: *Ageratum conyzoides* (Ac), *Archium lappa* (Al) and *Plantago major* (Pm)**

Figure No. 2 depicts the UV/Vis absorption spectrum for the AC extract prepared by sonication. The results exhibited a notable difference in the absorption bands, which could indicate the possible presence of phenolic hydroxyl compounds in the samples (that can be seen in A and B).

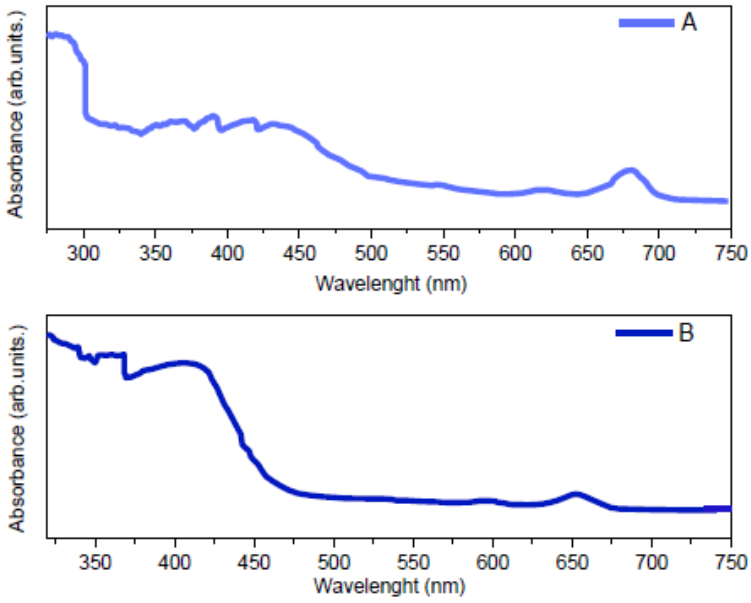


**Figure No. 2**
**UV/Vis spectrum of the species *Ageratum conyzoides* in the absence (A) and the presence of $AlCl_3$ (B).**

***Determination of the Solar Protection Factor (SPF) of extracts with higher phenolic content***

One of the objectives of this work was to determine the *in vitro* SPF of the phytochemicals present in the medicinal herbs, and only those compounds that exhibited a strong absorption in the UVB region were subjected to this experiment. Although the determination of SPF is essentially an *in vitro* experiment, it has been demonstrated that the efficacy of this particular methodology is in good correlation with that of *in vivo* experiments (Breder *et al.*, 1986; Santos *et al.*, 1999; Ferrari, 2002). This is since this

experiment relates the absorbance of the substance in question with the erythematogenic effects of radiation and with the intensity of light at wavelengths ranging between 290 and 320 nm, corresponding to the UVB region of the spectrum (Oliveira *et al*. 2013). Hence, the value of SPF can be easily estimated by UV-Vis spectrophotometry. In this case, the SPF value is a number that indicates the efficacy of a sunscreen or a test compound in terms of the height, width, and location of its absorption curve within the UV spectrum (Breder *et al*., 1986). The SPF value of the three phytopreparations was evaluated in this study. Although the flavonoid contents of the three phytopreparations were high, the resulting SPF values were less than 6, which were inferior to the desired SPF value established by the Brazilian legislation, through the RDC nº 30 on July 1, 2012. According to the Brazilian legislation, the minimum acceptable SPF value is 6. Therefore, none of the plant species tested herein under standardized conditions could be considered to have potential photoprotective effects.

***Comparison of effectiveness of the chemical marker extraction methods of the medicinal herbs by $^{1}H$ NMR and RP-HPLC-PDA***

To give obtain further detailed information on the efficacy of the extraction methods used to extract active principles from medicinal herbs, all the phytopreparations were subjected to analyses by RP-HPLC-PDA and $^{1}H$ NMR (Figure No. 3 and Figure No. 4, respectively) for the subsequent comparison of the efficacy of these methods in phytochemical extraction. Analyses of the $^{1}H$ NMR data revealed that apart from maceration and sonication, the other extraction methods could extract the same group of phytocompounds that differed only in their concentrations. Similar profiles have obtained few changes in phytochemical content and variation in final concentration (e.g., for the same amount of mass has used in these experiments). The phytopreparation obtained by sonication and the presence of other peaks even being the minority by the technique used were verified during analyses of the chromatograms obtained by RP-HPLC-PDA. However, the data obtained from the chromatograms were found to corroborate with the data obtained by NMR, in which the variations were only observed in the concentration of the metabolites.

Notably, the chemical composition of AC is yet to be accurately established. The $^{1}H$ NMR spectrum of the hydroalcoholic extracts of AC, extracted by sonication and maceration, showed signals in the region of 6-7 ppm, indicative of the presence of α-oxygenated aromatic hydrogens (Figure No. 4). Additionally, the presence of aromatic hydrogens was detected at around 8.0 ppm, possibly arising from the peri-carbonylic hydrogens. Singlets were observed at around 9.0 ppm, possibly due to the presence of nitrogen in the aromatic alkaloids, such as the toxic pyrrolizidine alkaloids previously described in this species by Faqueti *et al.* (2017). The chemical composition of AC has not yet been accurately.

Several signals were detected from the alpha-oxygenated aromatic hydrogens, which were present in compounds such as phenolic acids, coumarins, benzopyrones, chlorogenic acids, coumaric acids, tannins, and other phenols previously reported in this species, and also in several aromatic methoxy hydrogen compounds such as polymethoxyflavones (Sultana *et al*., 2012; Faqueti *et al*., 2017). Therefore, the presence of carbinolic, ester, and aliphatic hydrogens observed in the spectra of these samples, suggested the presence of compounds such as sesquiterpene lactones, previously described in this species (Chagas-Paula *et al*., 2015). However, the analysis of free sugars and heterosides, such as flavones and glycosylated flavonoids (Munikishore *et al*., 2013), revealed that this species contains a high concentration of free sugars and heterosides. In general, free sugars and heterosides can be easily detected by analysis of the 1H NMR spectrum (Figure No. 4), indicated by a high signal intensity and the occurrence of multiplets between 3.0 and 4.5 ppm, respectively. Additionally, the presence of aromatic compounds can be detected at 6-8 ppm. These extracts contained terpenes and saponins, as indicated by the signals observed between 0.8 and 2.9 ppm, in addition to other compounds such as several terpenoids, chromenes, and phytol (diterpene alcohol), the presence of which has been previously reported (Vera, 1993). As reported in the literature, the chromenes are compounds having low polarity, which are the main constituents of the leaves of AC.

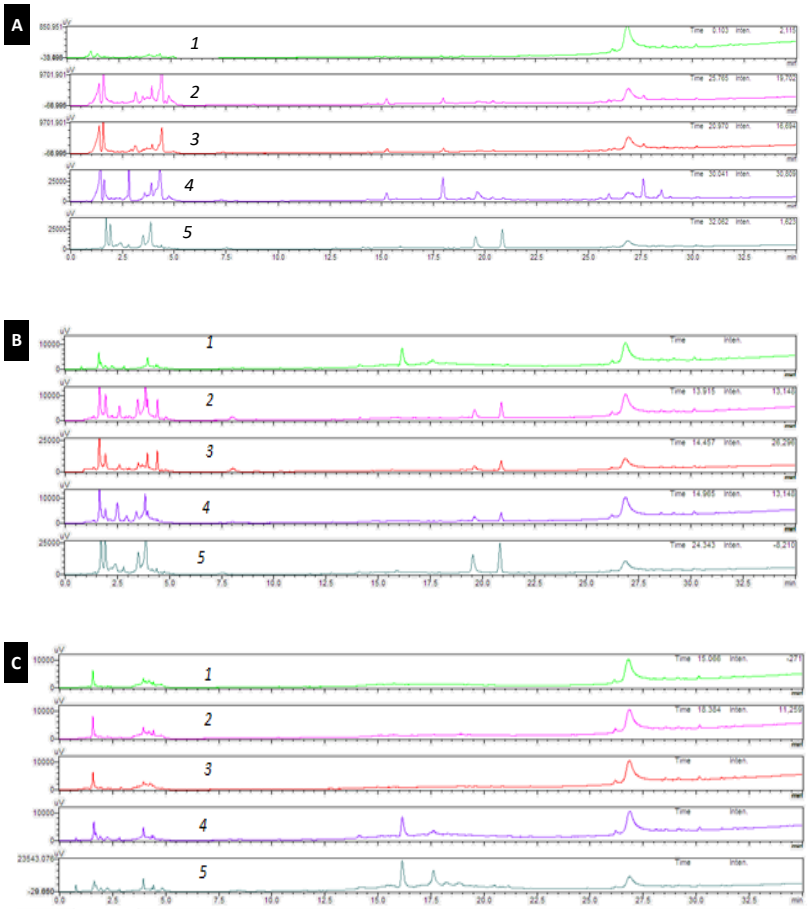


**Figure No. 3**
**Chromatograms of the phytopreparations obtained by different extraction methods: (a) AC, (b) PM and (c) AL, respectively. 1-infusion; 2-decoction; 3-microwave; 4-maceration; 5–sonication**

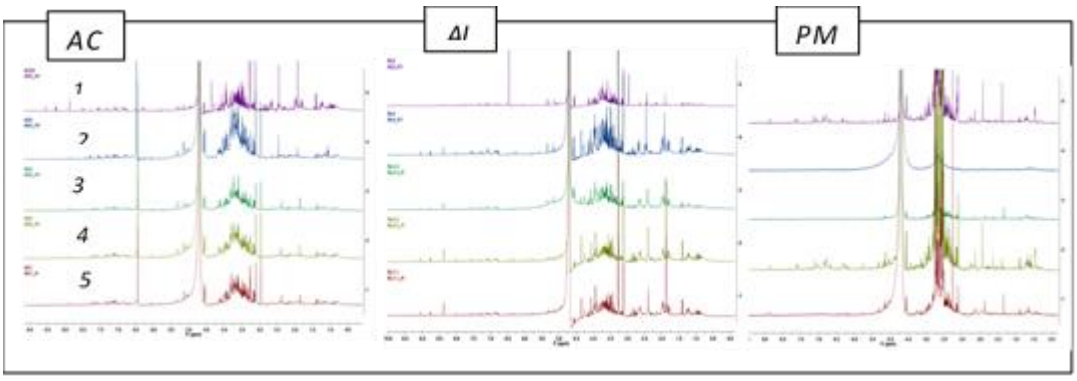


**Figure No. 4**
**$^{1}$H NMR spectra of the phytopreparations obtained by different extraction methods. 1 - infusion; 2-decoction; 3 - microwave; 4 - maceration; 5- sonication**

Analysis of the $^{1}$H NMR spectrum of the AL extract in the region of aromatic hydrogens (around 6 ppm) indicated the presence of phenolic substances with a high substitution pattern, indicated the presence of polyphenols, flavonoids (Ionescu *et al*., 2014), phenylpropanoids (Gao *et al.,* 2013), and phenolic acids derived from caffeic acids, such as dicaffeoylquinic acid (Carlotto *et al*., 2015). This analysis further revealed the presence of other aromatic compounds, such as arctiin, which is a bioactive lignin component of AL (Coulerie *et al*., 2016). The chemical signals detected between 8 and 9 ppm suggest an aromatic system with little oxygen substitution. Analyses of the $^{1}$H NMR spectra suggested a high concentration of sugars, which were indicated by signals detected at 3.1-4.3 ppm and signals produced by anomeric hydrogens at 5.1 and 5.4 ppm, which indicated the presence of free sugars or heterosides such as the glycosylated phenolics arctiisesquineolignan B and arctiiphenolglycoside A (He *et al*., 2016), glycosylated lactones (Yang *et al*., 2015) and butyrolactone lignans (Chagas-Paula *et al*., 2015), previously reported to be present in AL. It is possible that the sugar molecules thus detected were derived from glycosylated flavonoids, saponins, or sugars, and is compatible with the chemosystematics of this species. The signals detected from aliphatic hydrogens could have been derived from terpenes, steroids, and fatty acids such as the amide-derived fatty acids, previously isolated from this species (Yang *et al*., 2016).

The chemical profile of PM clearly exhibited the presence of highly oxygenated phenolic compounds indicated by signals in the 5.8 and 6.2-6.9 ppm regions. In particular, these signals are likely to have arisen from flavonoids and phenolic acids derived from hydroxycinnamic acids, such as chlorogenic and neochlorogenic acids, previously isolated from this species (Maksyutina, 1971a; Maksyutina, 1971b). These results further revealed the presence of a low concentration of aliphatic compounds, such as terpenes, steroids, and steroid derivatives, in addition to a high concentration of sugars. Additionally, few signals were detected from anomeric hydrogens, which suggested the presence of a mixture of sugars and oxygenated aliphatic compounds such as tartaric acid, citric acid, malic acid, malonic acid, and succinic acid, which have been previously reported in this species (Olennikov *et al*., 2005). The phenolic heterosides detected in this study suggests the presence of flavonoids such as glycosylated flavanone (Endo *et al.,* 1981), the flavones luteolin 7-glucoside and luteolin 7-glucuronide (Lebedev-Kosov, 1976), or glycosylated phenolic acids such as verbascoside, which have been previously isolated from this species (Egorov *et al*., 2004). Therefore, the detection of a few signals in the aliphatic region can in principle be attributed to the presence of terpenes and steroids, further suggesting the presence of fatty acids such as stearic acid, pentadecanoic acid, oleic acid, eicosapentaenoic acid, and docosahexaenoic acid, which are commonly found in this species (Ringbom *et al*., 2001). No signals were detected from aromatic alkaloids. The leaves of this medicinal plant have been found to contain the iridoid glycoside aucubin, vitamin K, ascorbic acid, and polygalacturonide.

***Analysis of the variance***

In order to provide a direct comparison of these extraction methods and to employ the differences among theirs, in the present study has also been realized an analysis of variance (ANOVA). These results are summarized in Tables 3 and 4. Fundamentally, the initial algebraic decomposition of the deviation observed in the produced response can initially be decomposed into two parts (Barros Neto *et al*., 2001):

$$(yi - y) = (yi - y) + (yi - yi) \qquad (1)$$

Thus, in this equation, the first part, $(\hat{y}_i - \bar{y})$, denotes the deviation of the forecast made by the model for the point $\hat{y}_i$ associate to the global average $(\bar{y})$. While the second part demonstrates, in fact, the difference between the observed and the expected value (Barros Neto *et al.* 2001). Now, note that it is, of course, necessary to express the first equation in its quantitative terms. Hence, for this, it is usually applied to the sum of the square of all points. This is:

$$\sum (yi - y)^2 = \sum (yi - y)^2 + \sum (yi - yi)^2 \qquad (2)$$

As can be seen, the second equation represents the regression equation described as one part of the total variation of the observations around the average. Being the quantitative terms of this equation usually express by:

$$R^2 = \frac{\sum (\hat{y}_i - \underline{y}_i)^2}{\sum (y_i - \underline{y}_i)^2}$$

where $R^2$ denotes the squared correlation coefficient of the desired relation to each term observed with the predicted values. The third equation is also called SQ (quadratic sums). In Table No. 3, the highest and lowest values found for sum and average has observed for both sonication and maceration methods, respectively. In addition, we also can note that the most significant discrepancies found in this analysis are for the samples prepared by the maceration method.

On the other hand, this first exploratory analysis suggests that all other extraction methods, in general, exhibit a trend of an apparently similar behavior regarding respective values of the sum, average, and variance, as well (Table No. 3). This result corroborates with our preliminary experimental analyzes and, therefore, may indicate a greater variance observed between these extraction methods mentioned above. To end, the coefficient of determined $R^2$ for these data is equal to 0.798. In general, the typical $R^2$ value represents the fit quality of the observed responses and, in this case, also could be applied to the validation of the test set (i.e., $R^2 > 0.6$) – all predictions – coincide exactly with experimental responses.

**Table No. 3**
**Values found the sum, average, and variance for each method under investigation**

| *Method* | *Count* | *Sum* | *Average* | *Variance* |
|---|---|---|---|---|
| **Infusion** | 3 | 2.51 | 0.837 | 0.0008 |
| **Decoction** | 3 | 2.45 | 0.817 | 0.0012 |
| **Microwave** | 3 | 2.48 | 0.827 | 0.0008 |
| **Maceration** | 3 | 1.95 | 0.650 | 0.0076 |
| **Sonication** | 3 | 2.64 | 0.880 | 0.0013 |

Also, the number of parameters (5) and observations (15), lead a 14-total degree of freedom (df) for these experiments. According to these considerations, we now can show that MQs follow one F distribution through the equation below (Barros Neto *et al*. 2001):

$$\frac{MQ_R}{MQ_r} \approx F_{b,w}$$

Hence, *b* and *w* are the numbers of equivalent df of the quadratic average, i.e., because of the regression ($MQ_R$) and the residual quadratic average ($MQ_r$), respectively. On the other hand, is well-known that the regression will be statistically significant if

$$\frac{MQ_R}{MQ_r} > F_{critic}$$

(Barros Neto *et al*., 2001). By the statistical parameters determined, in general, a practical rule that in turn is well-known and can easily be employed to prevent the effect of the response from being masked (in principle by the extent of the experimental error), involves checking if the determined value of the $\frac{MQ_R}{MQ_r}$ is about ten times the point value of the F distribution (evidently at the confidence level chosen and with an appropriate total

number of the df) (Box *et al*., 1969; Box *et al*., 1973). In the case of the ANOVA test, as shown in Table 4, the significance of the regression has been verified by the comparing the regression component of the total variance with to residual part. Thus, considering that the 5% of F distribution, in this case, the result found for the $F_{critic}$ = 3.478 was significant for the observed response. This result indicated that the obtained model fits well the experimental data due to the value obtained gives rather low residues. Likewise, the ANOVA analysis (by linear regression) reveals a *p*-value (0.00165) < 0.05. Therefore, the source of variation was statistically significant for the test set evaluated.

**Table No. 4**
**Parameters obtained by analyzing ANOVA**

| *Source of Variation* | *SQ* | *df* | *MQ* | *F* | *P-value* | *F critic* |
|---|---|---|---|---|---|---|
| **Between the methods** | 0.0936 | 4 | 0.0234 | 9.919 | 0.00165 | 3.47805 |
| **Inside the methods** | 0.0236 | 10 | 0.0024 | | | |
| **Total** | 0.1172 | 14 | | | | |

Overall, the result of the statistic ANOVA analysis could also be used to significant improvement of the extraction procedure of active principles found in diverse medicinal plants, considerably reducing the time and number of experiments performed to optimize these processes routinely used in the field of natural products (Gutt *et al*., 2016; Nunes *et al*., 2016; Pan *et al*., 2017; Francisco *et al*., 2018; Guizellini *et al*., 2018), and hence have an enormous benefice for diverse medicinal purposes.

***Bioactivity of medicinal herbs obtained by the sonication method***

The cytotoxic potential of the extracts of the medicinal herbs, AC, PM, and AL, extracted by sonication, were evaluated against the human hepatocellular carcinoma cell line, HepG2; ATCC HB-8065. Sonication is be the most effective method for the extraction of phenolic molecules and other compounds of pharmacological interest, which explains its widespread use. The results of the experiments on evaluating the cytotoxic potential of the herbal extracts are depicted in Figure No. 5. It is clearly seen at figure 5 that AC has a high cytotoxicity in the HepG2 hepatocellular carcinoma cells at a concentration of 200 µg $mL^{-1}$, wherein 3% of the cells were viable. On the other hand, the two other plants, PM and AL, exhibited low cytotoxicity, with more than 80% viable cells.

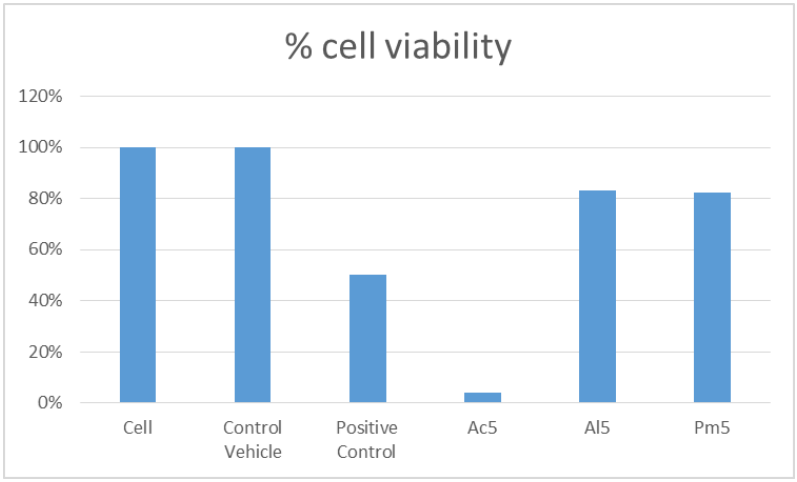


**Figure No. 5**
**% Cell viability from herbal medicines. Ac5 - *Ageratum conyzoides* sonication, Pl5 - *Plantago major* sonication; Al5- *Archium lappa* sonication**

Although this study has been performed at a preliminary level, it provides novel information on vegetable species marketed as medicinal herbs. The information reported herein requires further detailed investigation, which may result in technological innovations in the fields of cosmetology and pharmacy. The species of plants selected in this study are globally used for medicinal purposes; however, their biological properties and metabolic profiles are yet to be elucidated. Understanding the bioactivities and metabolic profiles of these plants is essential for ensuring the safety of human consumption. Since there are different extraction methods for preparing phytotherapeutic preparations, studies evaluating the efficacy of such methods are necessary for identifying the most effective method for extracting the active principles or pharmacologically-active biomolecules from medicinal plants. The results of this study revealed that sonication was the most effective extraction method for extracting phytocompounds. The samples prepared by this method exhibited a pronounced antioxidant potential and contained a high content of the phenols, both of which are essential characteristics of therapeutically important natural compounds. Several plants containing phytochemicals have been found to be beneficial for the improvement of overall health. Research studies such as the present one progressively characterizes the bio availabilities of plant products and extend their use in health care.

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