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Seasonality and hydrodistillation time effects on the yield and chemical composition of leaves essential oil of *Piper mikanianum* (Kunth) Steudel

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Resumo: *Piper mikanianum*, proveniente de Santa Catarina, sul do Brasil, é uma conhecida fonte de safrol, um arilpropanóide precursor de várias moléculas bioativas. Este trabalho determinou a influência da sazonalizade e do tempo de hidrodestilação no rendimento e composição química do óleo essencial (OE). Folhas desidratadas foram hidrodestiladas com e sem fracionamento por um período total de 240 min. Os rendimentos do OE variaram de 0,66-0,84% com os maiores valores sendo observados na estação da primavera e verão. A maior concentração de safrol foi obtida no óleo do inverno (81%). No experimento de fracionamento a concentração de safrol variou de 75-80% na F1 (óleo destilado durante 60 min) e 42-64% na F2 (óleo destilado entre 60-240 min).

Palavras-chave: óleo essencial, fracionamento, *Piper mikanianum*, safrol, sazonalidade.

Abstract: *Piper mikanianum* from Santa Catarina, southern Brazil, is a well known source of safrole, an arylpropanoid precursor of several bioactive molecules. This work determined the seasonal and hydrodistillation time influence on the essential oil (EO) yield and composition. Dehydrated leaves were hydrodistilled with and without fractioning during a total period of 240 min. The EO yields varied from 0.66-0.84% with the highest values been observed during spring and summer seasons. The highest concentration of safrole was found in the winter oil (81%). In the fractioning experiments the concentration of safrole varied from 75-80% in F1 (oil distilled during 60 min) and 42-64% in F2 (oil distilled between 60-240 min).

Keywords: essential oil, fractioning, *Piper mikanianum*, safrole, seasonality

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INTRODUCTION

The synthesis and storage of secondary metabolites are energetically expensive and several biotic and abiotic factors can interfere in the metabolism of a plant throughout its life cycle [1]. The seasonality is a factor that leads to alteration of biosynthetic pathways, which changes the content of practically all classes of secondary metabolites, including essential oils [1,2].

Piperaceae have been widely studied and distinguished by high chemical diversity and remarkable biological activity [3]. Many plant families have global distribution, but few have ethnobotanical and ethnopharmaceutical representation like Piperaceae [4,5]. *P. mikanianum* is traditionally used to emmenagogue, acute and chronic metrorrhagias, “stomach cramps”, “it neutralizes snake venom effects”, amenorrhea, leukorrhea, uterine hemorrhage and also for losing weight [6].

Among the species of *Piper* rich in safrole *P. hispidinervium* (long pepper), can also be mentioned, since its essential oil composition is almost exclusively safrole (> 90%), *P. marginatum* with 64% of safrole, *P. cavalcantei* with 69% and *P. callosum* with 64%. These species exist in the Brazilian Amazon Rainforest and the oils are extracted from the leaves [7-9]. In the south of the Brazil we found *Piper mikanianum* (Kunth) Steudel occurring in Santa Catarina [10], with the essential oil from the leaves consisting of terpenes and the arylpropanoid safrole, and its concentration reaches 82%, becoming a new source of this natural metabolite. When compared to previous studies with the same species collected in Rio Grande do Sul [11,12] and Parana [13], only in Santa Catarina *P. mikanianum* presented this arylpropanoid in the essential oil.

Several studies have demonstrated the importance of safrole as a precursor of bioactive substances such as prostaglandin analogues, of the non-steroidal anti-inflammatory related to indomethacin, piroxicam and etodolac [14]. Anti-thrombotic agents from the class of O-benzylether of oximes, selective inhibitors of the sulfonamide PGHS-2 and N-acylhydrazine and hydrazones with analgesic properties [14-16]. From piperonal, derived from safrole, was synthesized (±)-epiasarinin, an aryl furofuran associated with several biological properties, including antioxidant, anticancer, antiviral and immunosuppressive [17] and chalcones protease cruzain inhibitors, targeting the synthesis of new trypanocidal agents [18]. Prototypes compounds with potential antitumor activity were also synthesized from safrole incorporating a peptide subunit [19].

Given the economic potential associated with *P. mikanianum* it becomes a priority to produce effective knowledge which would provide economic and ecological subsides for the adoption of better management strategies of this species [20].

Therefore, this is the aim of the current study when it evaluate the influence of seasonality and the time of hydrodistillation on the yield and chemical composition of the essential oils of *P. mikanianum* from Santa Catarina.

EXPERIMENTAL PROCEDURES

Plant material

Leaves of *P. mikanianum* without any attacks by herbivores or other injuries were collected in Atalanta, Santa Catarina, Brazil ($27^{o}28´$S; $49^{o}48´$W, 548 m). The vegetation in this region is characterized as Dense Ombrophile Forest, being the climate of the city, according to the Koppen classification, is the type mesothermal humid. The period of greatest rainfall is in September and October [21].

The collections were made every three months for a total of four collections during the four seasons, and these are: autumn (April/2008), winter (July/2008), spring (October/2008) and summer (January/2009). Samples were randomly collected in the same area and in the morning.

One specimen, which served as a comparison for species identification, is deposited in the Herbarium Dr. Roberto Miguel Klein located on the campus I of the Universidade Regional de Blumenau -FURB under registration n^{o} 7590.

Extraction of the essential oil

The fresh leaves were subjected to hydrodistillation in the same very day of collection using modified Clevenger apparatus for 4 hours under nitrogen atmosphere and constant heating. The essential oil (EO) was collected and dried by adding anhydrous magnesium sulfate (Dinâmica, Diadema, SP, Brazil), determining the extraction yield by the ratio of the mass of oil and the mass of the plant material used (w/w). The EO obtained was stored under inert atmosphere at 4 ºC.

Fractional hydrodistillation of the essential oil

Leaves of *P. mikanianum* were dried in the shade and at room temperature until constant weight and subjected to hydrodistillation (60 g of dried leaves and 500 mL of distilled water). The experiment was performed in triplicate using plant material of the spring, experiments A and B, and summer, experiment C. The fraction 1 (F1) was obtained by removing the EO of the Clevenger after 60 min of hydrodistillation. Without interrupting the process, the hydrodistillation was continued until completing 240 min, thus producing the second fraction (F2) of EO. For each fraction, the mass, its relative percentage to the total weight of essential oil (mass F1 + mass F2) and the contents of safrole, mono and sesquiterpenes were determined.

Chemical composition of essential oil

Analyses of the essential oils diluted in tridistilled hexane 1% (w/v) were performed by a combination of techniques employing Gas Chromatography Flame Ionization Detector (GC-FID) and Gas Chromatography-Mass Spectrometry (GC-MS).

The analysis by GC-FID was performed using a Shimadzu Gas Chromatograph model GC-14 B with a fused silica capillary column (Simplicity-1/Supelco, low polarity) with 30m x 0.25 mm x 0.25 µm (length x diameter x thickness of stationary phase). The FID was maintained at 280 ºC, injector at 250 °C and helium carrier gas at a flow rate of 1 mL min^{-1}. The sample injection of 0.5 µL was conducted in split mode 1:50, using the following temperature program: initial temperature 60 °C for 4 min, followed by heating at 5 °C min^{-1} up to 190 ºC, remaining at this temperature for 2 min, followed by heating at a rate of 10 °C min^{-1} up to 250 °C, kept at this temperature for 15 min, which leads to a total analysis time of 53 minutes.

A representative sample was selected for the identification of essential oil components, this being the oil collected in the period of hydrodistillation between 50-240 min using fresh leaves collected in the winter. The analysis by Gas Chromatography/ Mass Spectrometry (GC-MS) was made using a gas chromatograph Varian® CP-3800 coupled to mass spectrometer Saturn® 2000, together with the management software Saturn® GC/MS Workstation 5.51.

In the analysis by GC-MS were given the same analytical conditions described for GC, and an ionization energy of 70 eV. The mass spectra of the constituents detected were compared with the spectra of the library NIST 02.

The relative percentages of the constituents identified were calculated based on the areas of corresponding chromatographic peaks obtained by GC, without correcting for response factors. Complementary identification of the essential oil was made from the retention index (RI) determined experimentally for a homologous series of linear hydrocarbons (C_8 - C_{19}) according to Dool and Kratz [22] equation, together with comparative visual analysis with mass spectra using the text of Adams [23].

RESULTS AND DISCUSSION

Yield of essential oils and seasonality

The period of lower yield in the oil was achieved during the winter (0.66%) being the highest in spring (0.84%), as seen in Figure 1

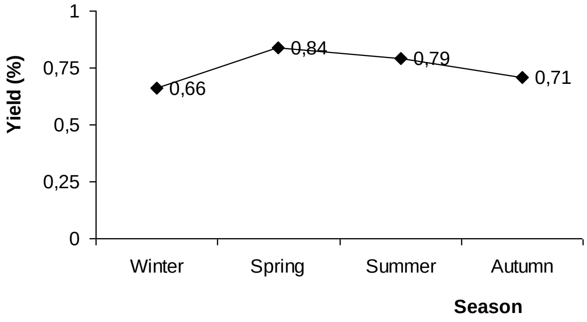

Figure 1. Yield of essential oil from leaves of *Piper mikanianum* (Kunth) Steudel collected in Atalanta, SC, Brazil, in different seasons of 2008-2009

This result was higher than the ones from Rorig & Von Poser [11] who obtained only 0.25% yield of fresh leaves collected in summer, being however lower than the results by Leal et al. [13] which yielded 3.1% for fresh leaves also collected in the summer. The latter results is at least surprising since such high yields in the genus *Piper* are only associated with dried plant material [24].

Characterization of the essential oils and chemical composition

As observed by our group [10], the chromatographic analysis of oils obtained in four seasons gave chromatograms containing a major constituent. The Figure 2 corresponds to the representative chromatogram related to the autumn sample.

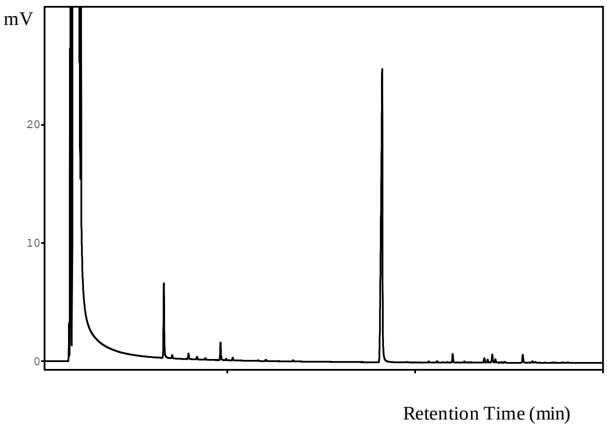

Figure 2. Chromatogram of EO extracted from fresh leaves of *Piper mikanianum* collected during autumn (April 2008).

For the identification of its constituents was used the fraction 2 (F2, hydrodistillation of 50-240 min) of essential oil (EO) from leaves collected in the winter which has higher concentrations of constituents with retention times exceeding 20 min (Figure 3), different from that observed in the total EO (Figure 2).

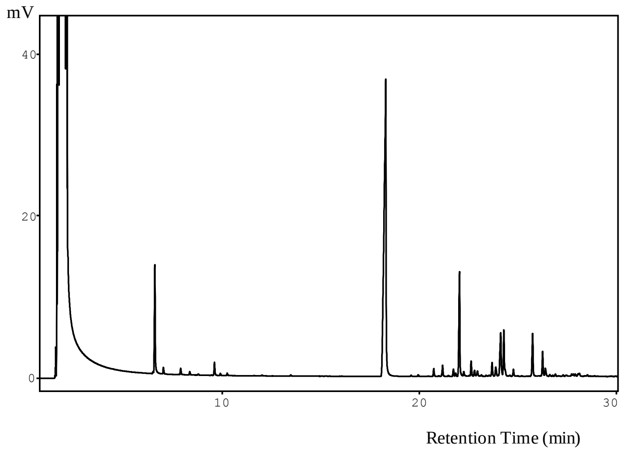

Figure 3. Chromatogram of fraction 2 (F2) of EO extracted from fresh leaves of *Piper mikanianum* collected during winter (July 2008).

The sample was subjected to analysis by Gas Chromatography with Flame Ionization Detector (GC-FID) for the determination of the retention index (RI) and by Gas Chromatography coupled with Mass Spectrometry (GC-MS.) The combination of these techniques allowed the identification of sixteen constituents with a minimum concentration of 0.7%, as shown in Table 1.

The sample showed safrole, α-pinene, valencene and isocaryophyllene as main constituents. These compounds were also identified in our previous work [14]. Parmar et al. [4] in the review of the genus *Piper* refers to the presence of bicyclogermacrene, β-caryophyllene and limonene, among these only limonene was identified in the sample examined here.

Table 1. Constituents of fraction 2 (F2) representative of the essential oil from fresh leaves of *Piper mikanianum* collected in winter in the city of Atalanta – SC (July 2008).

Constituent	T_R exp.	T_R lit.	RI exp.	RI lit.	%
α-pinene	5.54	5.85	927	932	19.4
β-pinene	6.69	7.04	970	974	1.1
limonene	8.32	8.69	1022	1024	2.5
safrole	19.33	19.56	1285	1285	44.1
α-copaene	23.14	23.49	1370	1374	1
β-elemene	23.87	24.15	1387	1389	0.9
isocaryophyllene	25.08	24.95	1403	1408	6.8
aromadendrene	26.12	26.27	1438	1439	0.9
γ-muurolene	27.76	27.91	1476	1478	4.3
β-guaiene	28.12	28.51	1484	1492	1.5
valencene	28.45	28.66	1492	1496	8
(E,E)-α-farnesene	28.94	29.03	1503	1505	3
δ-cadinene	29.57	29.72	1518	1522	0.7
E-nerolidol	31.27	31.32	1559	1561	0.7
spathulenol	31.85	31.96	1573	1577	0.8
caryophyllene oxide	32.05	32.16	1578	1582	0.7

T_R exp.: experimental retention time; T_R lit.: literature retention time [22]; RI exp.: experimental retention index; RI lit.: literature retention index [22].

Von Poser et al. [12] identified in the fresh leaves of *P. mikanianum* of Rio Grande do Sul, Brazil, a diversity of constituents, the main being limonene, bicyclogermacrene and β-caryophyllene. Leal et al. [13] obtained as major components in the EO of the leaves of this species collected in Parana, Brazil, the sesquiterpene β-vetivone and the arylpropanoids (Z)-isoelemicin and (E)-asarone, emphasizing the predominance of sesquiterpenes in the sample, unlike what has been observed in the present work, where the sesquiterpenes are present in amounts lower than monoterpenes.

The large differences between the yield and essential oil composition of *P. mikanianum* in the southern states of Brazil may be the consequence of its adaptive history to the biotic and abiotic characteristics of each region, creating different chemotypes for this species. As observed by Andrade et al. [25] when dealing with *P. marginatum*, an Amazonian species, and through the analysis of the EO samples collected at different locations seven different chemotypes were identified, with prominence for the presence of safrole at concentrations between 0.2% and 63.9%.

Evaluation of the effect of seasonality

In all seasons the essential oils of *P. mikanianum* presented in their chemical composition monoterpenes, sesquiterpenes and safrole as major constituent, with a minimum concentration of 72%, with the monoterpenes exceeding the sesquiterpenes (Figure 4).

Insert Figure 4

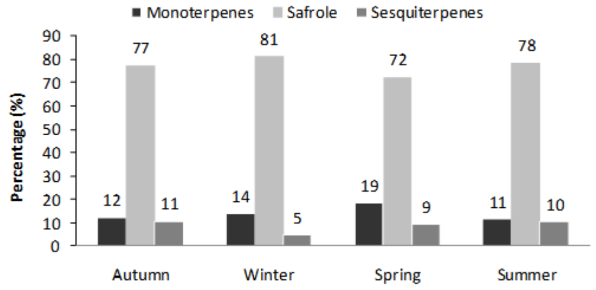

Figure 4. Concentration of the main constituents classes of secondary metabolites in the essential oils from leaves of *Piper mikanianum* according to the seasons of the year.

The highest level of safrole was obtained for samples collected in winter, in this season was also observed a lower percentage of sesquiterpenes and the lowest yield of EO (Figure 1). The spring was the season in which was obtained the highest yield of essential oil. However it was the period when the plant had the lowest content of safrole in the sample.

The low production of oil during the winter can be attributed to the beginning of blooming, in which the oil production could decline due to allocation of the resource for such event. In studies of *Lippia alba* (Mill.) N. E. Brown [26] was found that the oil yield can vary according to the plant phenology, since the highest yield was obtained outside of the flowering period.

Barbosa et al. [27] also found little variation in the essential oils of *Schinus terebinthifolius*, which showed a decrease in oil yield during the flowering and fruiting period. The fruiting period of the *P. mikanianum* is during the summer and this study found that this time the leaves showed levels of monoterpenes and sesquiterpenes similar, and the second highest level of safrole.

Effect of time of the hydrodistillation on the extraction and chemical composition of essential oils

To evaluate the effect of time of the hydrodistillation on the extraction yield and chemical composition of the EOs, three extractions were conducted producing in each two fractions: F1 (oil distilled during 60 min) and F2 (oil distilled between 60-240 min). Two experiments were performed with the leaves of spring (A and B) and one experiment with leaves of summer (C). As shown in Figure 5, it is possible to obtain in the initial phase of the hydrodistillation high percentage of extraction, which accounted for 85-93% of the total mass [F1/(F1 + F2)]. The variation observed might be related to different heating rates provided by the heaters used.

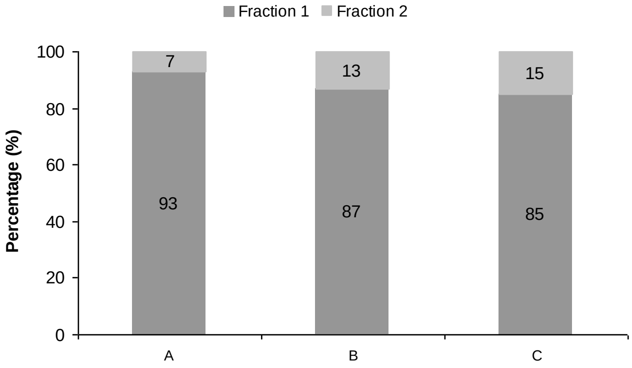

Figure 5. Yields of fractions 1 and 2 obtained as a function of time from hydrodistillations of three experiments using dry leaves of *Piper mikanianum*.

The composition of *P. mikanianum* EOs is strongly influenced by the time of hydrodistillation applied. In Figure 6 it is observed that the concentration of safrole in F1 is higher than those observed for total EO in the same collecting period, that is, in the spring (A and B), from 72% to 75-77%, and in the summer (C) from 78 to 80%. Yet it is noted that in F1 the monoterpenes are in higher concentration than the corresponding total EOs, unlike the sesquiterpenes that have their concentrations reduced from 10 to 4% (average values). The opposite effect occurs on the F2, where the safrole undergoes drastic reduction in their concentration and the sesquiterpenes outweigh the monoterpenes.

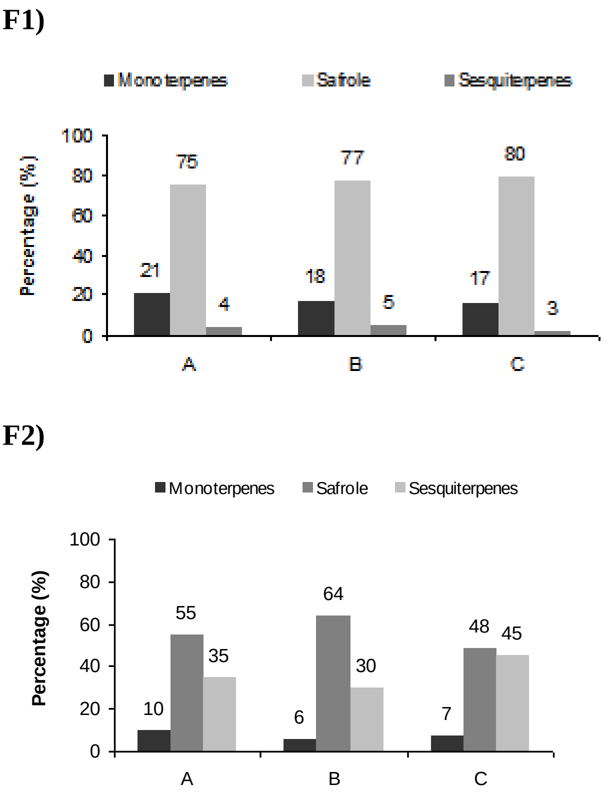

Figure 6. Composition of the fractions of the essential oils (A/B-spring and C-summer) of *Piper mikanianum*: F1 (60 min of hydrodistillation) and F2 (60-240 min of hydrodistillation).

As observed in studies of Barbosa et al. [27] the higher proportion of oil was obtained in the initial phase of hydrodistillation which is accompanied by the more volatile constituents, monoterpene and safrole.

The result obtained in this work follows the same trend observed by the Prins et al. [28] that tested the effect of extraction time by hydrodistillation on yield and composition of essential oil of *Rosmarinus officinalis* and found that different heating rates and extraction time can influence the composition of essential oil, favoring the extraction of compounds with higher water solubility and low molecular weight.

CONCLUSION

The results of the present study indicate that independently of the season, the essential oils from leaves of *Piper mikanianum* have high levels of safrole, being the others constituents of terpenic nature. Despite the fact that the highest yield of oil extraction was obtained during spring, it was also the period with the lowest safrole concentration.

Although the highest yields are obtained with four hours hydrodistillation, more than 85% of the oil is extracted in the first 60 minutes of the process (F1). The hydrodistillation time also affects the chemical composition of the essential oil, where reduced times produce samples with higher levels of safrole and monoterpenes. Since it is possible to influence the essential oil composition in the drying stage of the plant material, high levels of safrole can be achieved combining more vigorous drying conditions and short hydrodistillation time.

It can be concluded that not only the collecting period should be considered for obtaining the best relation for oil production and concentration of safrole, but also the control of the variables of the extraction process.

Due to the unique economical potential of this plant, large-scale cultivation and oil extraction may find potential applications and greatly benefit from the current research.

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