Abstract
The Atlantic Forest is regarded a rich source of aromatic plants with a broad spectrum of biological properties. The leaves of seven Piper species were collected from the Atlantic Forest domain in the coastal region of Paraná state in winter (W) and spring (S). The volatile oils were extracted from the leaves through hydrodistillation method. The volatile oils were characterized by means of GC-FID and GC-MS. Sesquiterpenes were among the major compounds in all species studied, with many dozens of representatives. However, very low concentrations of monoterpenes were observed; α-pinene in *Piper mosenii* and *P. cernuum*, δ-3-carene in *P. rivinoides*, camphene and β-pinene in *P. cernuum* are exceptions. The arylpropanoids myristicine and elemicin were found in considerable amount in *P. diospyrifolium* (26.2%/S) and *P. mosenii* (16.4%/W), respectively. These oils were also screened for inhibitory activities against acetylcholinesterase (AChE) and α-glucosidase (AG). For AChE the most active samples were *P. diospyrifolium*, *P. aduncum* and *P. cernuum*, with inhibiting level > 93%. For the less sensitive AG, the most promising candidates were *P. diospyrifolium* and *P. mosenii*, with inhibiting level > 65%. These results highlight the importance of native plants as renewable source of new inhibitors for AChE and AG. Further investigation is required to identify the most active constituents or fractions from the selected volatile oils.

Key words: α-glucosidase, acetylcholinesterase, chemical composition, *in vitro* assays, *Piper* spp., volatile oils.

Resumo
A Floresta Atlântica é considerada uma rica fonte de plantas aromáticas com amplo espectro de propriedades biológicas. Na busca de novos inibidores da acetilcolinesterase (AChE) e α-glicosidase (AG), folhas de diferentes espécies de Piper localizadas na Floresta Atlântica no litoral do Paraná - Brasil, foram submetidas a hidrodestilação e seus óleos voláteis caracterizados por CG-DIC e CG-EM. A coleta ocorreu em duas estações distintas, inverno (I) e primavera (P), de 7 diferentes espécies. Sesquiterpenos foram identificados entre os compostos majoritários em todas as espécies estudadas, com dezenas de representantes. No entanto, foram observadas baixas concentrações de monoterpenos; α-pinene em *Piper mosenii* e *P. cernuum*, δ-3-carene em *P. rivinoides*, camphene e β-pinene em *P. cernuum* são exceções. Os arilpropanoides miristicina e elemicina foram identificadas em quantidades significativas em *P. diospyrifolium* (26.2%/P) e *P. mosenii* (16.4%/I), respectivamente. Para a AChE os amostras mais ativas foram *P. diospyrifolium*, *P. aduncum* e *P. cernuum*, com níveis de inibição > 93%. Para a AG, que se mostrou menos sensível às amostras, os candidatos mais promissores foram *P. diospyrifolium* e *P. mosenii*, ambos com níveis de inibição > 65%. Esses resultados
Essential or volatile oils are accessible plant extracts usually possessing desirable biological properties, with applications in various branches of industry from the perfumery and cosmetics as well as medicines, where there is a constant demand for new products and therapies (Shaaban et al. 2012; Lesgards et al. 2014; Carneiro et al. 2020).

The genus *Piper* is of pantropical occurrence and abundant in the Atlantic Rainforest biome, where numerous species are known to provide essential oils; therefore, it is a potential source of biological active compounds. A recent review well describes the properties and applications of *Piper* species secondary metabolites, providing useful insights for further studies that includes essential oil targeting enzymes (Salehi et al. 2019).

Inhibiting key metabolic enzymes on a pathologic process has been a research subject in several medicine fields, highlighting acetylcholinesterase and α-glucosidase inhibitors, providing useful information for the development of new treatments for diseases such as diabetes and Alzheimer’s (AD) (Souza 2011; Ganeshpurkar et al. 2019).

Acetylcholinesterase inhibitors are among the major drugs for AD treatment. A therapeutic approach to treat AD is to increase the availability of acetylcholine in the brain via the inhibition of the main catalytic enzyme acetylcholinesterase. Restoring acetylcholine levels improves the cognitive function of many patients (Santos et al. 2011). Following this approach, Volcho et al. (2018) have summarized the use of monoterpenes, monoterpenoids and their derivatives as promising agents for the treatment of neurodegenerative diseases (NDDs).

On the other hand, diabetes is a metabolic disorder characterized by chronic hyperglycemia and changes in the metabolism of carbohydrates, fats and proteins (Ali et al. 2006). It is caused by inherited or acquired deficiency in insulin secretion, resulting in increased blood glucose level (Bhandari et al. 2008). One of the therapeutic approaches to mitigate or treat diabetes is to reduce postprandial hyperglycemia, delaying the absorption of glucose by inhibiting the hydrolysis of carbohydrates (Araujo 2015). Inhibitors of α-glucosidase exert their antidiabetic effects by slowing down and reducing the rate of glucose absorption mediated by inhibiting the breakdown of complex carbohydrates in the gastrointestinal tract (Conceição et al. 2017).

Thus, the investigation of new inhibitors of acetylcholinesterase and α-glucosidase enzymes by the *in vitro* screening using several volatile oils from *Piper* species was conducted as a preliminary work. The chemical composition of the samples is also described.

Samples of 2 seasons, winter (W) and spring (S), from 7 Piperaceae species, were obtained and characterized by means of chromatographic techniques (GC-FID and GC-MS) using analytical conditions described by Bezerra et al. (2020). The percentage composition [%] was determined by electronic integration of the FID signal by dividing the peak area of each component by the total area. Their linear retention indexes (AI) were calculated from the injection of a linear homologous series of hydrocarbons (C7-C26) and compared with data from literature (Adams 2007). AI and [%] are the mean values of triplicate analysis. The results are presented as supplementary material (Tab. S1, available at <https://doi.org/10.6084/m9.figshare.15224274.v1>). A large variety of structurally related compounds were identified, with terpenes as the most abundant class of compounds. They are also among the major compounds in all species studied for both seasons, namely, E-caryophyllene (*P. diospyrifolium* Entry 27), α-murolene (*P. rivinoides* Entry 47), α-pinene (*P. mosenii* Entry 2), germacrene B (*P. gaudichaudianum* Entry 63), β-dihydroagarofuran (*P. cernuum* Entry 49), caryophyllene oxide (*P. arboreum* Entry 66) and spathulenol (*P. aduncum* Entry 65). The sesquiterpenes spathulenol, caryophyllene oxide, E-caryophyllene, α-copaene (Entry 22) and α-caryophyllene (Entry 35) were the only compounds to be found in all 14 samples and in amounts superior to trace. Caryophyllene oxide, an epoxide derived from E-caryophyllene, was present in high concentration in *P. arboreum*,...
indicating a pronounced epoxidation activity in this species.

Arylpropanoids were also found in some species, especially in *P. diospyrifolium* and *P. rivinoides*. Myristicine (Entry 55) and elemicin (Entry 62) are their main representatives, although safrole (Entry 18), dillapiole (Entry 74) and apiole (Entry 90) have also been identified. This class of metabolites was not found in *P. gaudichaudianum* and *P. cernuum* and in very low concentration (<1.0%) in *P. aduncum*.

The only two compounds identified that do not belong to these two classes of secondary metabolites are two aliphatic ketones (Entries 5 and 72).

The seasonality affected the chemical composition of the essential oils. This effect is more pronounced upon some compounds than others and may follow different pattern, for instance, myristicine (Entry 55) in *P. dispopyrifolium* is present in 6.6% (W) and 26.2% (S), contrasting with bicyclogermacrene (Entry 46) which in *P. mosenii* is present in 15.5% (W) and 8.3% (S), on the other hand no significant variation was observed for caryophyllene oxide, 31.5% (W) and 30.5% (S).

It was observed that very few compounds have been identified associated to a single species, therefore candidates as chemical markers. Only those constituents present at a concentration of at least 10% are herein mentioned: elemicin (Entry 62) in *P. rivinoides*, β-dihydrofuran (Entry 49) and 10-epi-γ-eudesmol (Entry 76) in *P. cernuum*, muurola-4,10(14)-dien-1-β-ol (Entry 78) in *P. arboreum* and pogostol (Entry 86) in *P. diospyrifolium*.

The essential oils obtained from seven different species of *Piperaceae* in two distinctive seasons were tested against anticholinesterase and α-glucosidase enzyme at a concentration of 1,000 mg mL\(^{-1}\). Neostigmine and acarbose were used as positive controls, respectively. The inhibition assay of α-glucosidase (from Saccharomyces cerevisiae, Sigma-Aldrich®) was performed as described by Kim *et al.* (2004) and the anticholinesterase (from Electrophorus electricus - electric eel, Sigma-Aldrich®) activity was determined using the spectroscopic *in vitro* method of Ellman, according to Mata *et al.* (2007). A detailed procedure is available in the supplementary material. The inhibition percentages of each essential oil samples with the corresponding statistic treatments are shown in Table 1.

In the inhibitory assay for acetylcholinesterase, the activity was not affected by the collecting season only for *P. arboreum* and *P. mosenii*. On the other hand, a significant variation was observed for *P. diospyrifolium*, changing from an effectiveness of 98.5%/W to 68.4%/S.

Yilmazer-Musa *et al.* (2012) reports in their work that a promising sample is one that is able to inhibit at least 50% of the enzymatic activity at the concentration used in this work, therefore, the most active samples are associated to the winter season obtained from *P. aduncum*, *P. cernuum* and *P. diospyrifolium*, with average values superior to 90%. A second effective group in inhibiting this enzyme is formed by *P. arboreum* and *P. mosenii*, with average values in the range of 75–77%. At this point is difficult to make any rationalization in terms of inhibitory effect and a specific chemical feature, however few observations can be drawn from these preliminary results regarding the most active samples, as follow, myristicine (Entry 55) in *P. diospyrifolium* cannot be responsible itself for the inhibitory activity or even to be associated with this activity; small variation in chemical composition, as for *P. cernuum* and *P. aduncum*, might affect in a considerable manner the activity and finally, the acetylcholinesterase enzyme is less selective towards sesquiterpenes, since different representatives are among the major compounds in these 3 species.

Contrasting results in relation to acetylcholinesterase have been found in the inhibitory assay for α-glucosidase, which showed a lower number of effective samples. An outstanding exception was *P. diospyrifolium* (W), followed by *P. rivinoides* (W) (Tab. 1). *P. diospyrifolium* presented a very high level of inhibition, 88.2%, against 65.1% for *P. rivinoides*. In terms of chemical composition, both samples have some amount of monoterpenes, very few identical major compounds, such as E-caryophyllene (Entry 27) and myristicine (Entry 55) and more uncommon major compounds, pogostol (Entry 86/10.5%) in *P. diospyrifolium* and δ-3-carene (Entry 8/12.6%), bicyclogermacrene (Entry 46/5.3%), α-muurolene (Entry 47/19.9%) and elemicin (Entry 62/16.4%) in *P. rivinoides*, thus making it difficult any correlation between chemical constituents and the biological activity with the current results.

In conclusion, this study showed that native species from phytogeographical regions of the Atlantic Forest can be considered a promising renewable source of a variety of
chemicals almost exclusively belonging to terpenoid and arylpropanoid classes. Moreover, some of their essential oils are inhibitors of acetylcholinesterase and α-glucosidase. Three species have presented remarkable results therefore they have been selected as candidates for further investigation, 
P. diospyrifolium
and 
P. cernuum
for acetylcholinesterase and 
P. diospyrifolium
and 
P. rivinoides
for α-glucosidase. Fractioning of essential oils, isolation of major compounds and combination of the most active compounds and fractions can be envisaged to potentiate and modulate these desired properties. This work values plant biodiversity in the State of Paraná with a focus on the identification of new chemical substances of biological relevance.

### Supplementary material (Material and Methods, Table with detailed chemical composition, and corresponding references) are available online at <https://doi.org/10.6084/m9.figshare.15224274.v1>.

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