Article

Identification of new secondary metabolites in *Persea americana* Miller variety Drymifolia

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Abstract

Plants have developed various defense strategies under conditions of biotic and abiotic stress, among the main ones, highlights the synthesis of secondary metabolites that act as herbicides, larvicides, repellents, attractants, fungicides, bactericides, insecticides, etc. In the present investigation, the chemical characterization of 54 collections of *P. americana* Miller variety Drymifolia from the avocado producer fringe of the state of Michoacán and conserved in the germplasm bank of the Faculty of Agrobiology 'Presidente Juárez' was raised. The study is part of a comprehensive strategy to evaluate the potential of collections as rootstocks with attributes of resistance to pests and diseases. When analyzing the chemical profile, a total of 47 secondary metabolites were identified, 16 of them, have not been reported in *Persea* leaf tissue. Some compounds such as β-pinene, caryophyllene, estragole, hexadecanoic acid, heptacosane and α-tocopherol were present in the 54 collections analyzed. Estragole with antifungal, larvicidal, insecticidal and genotoxic biological activity predominated with a concentration of 26.53%, with respect to the total. Additionally, significant statistical differences were determined for 12 secondary metabolites. In contrast, no relationship was found between the concentration of the metabolites and the origin of the collections.

Keywords: avocado, secondary metabolites, variability.

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Introduction

Mexico ranks as the world's leading producer of avocado, a place that obtains the 149 185 hectares planted, the state of Michoacán, is placed as the leading producer of this fruit with 195 042 t representing 94.9% of national production (SIAP, 2019). In Michoacán, avocado cultivation is established in a region known as the 'avocado producer fringe of the state of Michoacán' (Gutiérrez-Contreras *et al*., 2010). There is a wide genetic diversity of *P. americana* Miller variety Drymifolia it has been used as rootstock of the Hass variety (Cuiris-Pérez *et al*., 2009).

For being a source of genes for resistance to physical factors, pests and pathogens (Rincón-Hernández *et al*., 2011); however, the enormous phytogenetic wealth is threatened by the destruction of ecosystems and substitution of traditional cultivars by improved cultivars (Lorea-Hernández, 2002). Consequently, during the last years, efforts have been added for the conservation and characterization of these genetic resources (Gutiérrez-Diez *et al*., 2009).

In particular, in the state of Michoacán, work has been done on the characterization of the creole avocado germplasm; these works include genotypic information (Cuiris-Pérez *et al*., 2009) and phenotypic information (Guillén-Andrade *et al*., 2013). The importance of studying the chemistry of plants is that they have developed various defense strategies under conditions of biotic and abiotic stress; for example, the plant synthesizes secondary metabolites (MS) that cause plants to be unpleasant or toxic to some herbivores (Anaya-Lang and Espinoza-García, 2006).

Some with larvicidal biological activity (Senthilkumar *et al*., 2008), bactericidal and fungicidal insecticide (Khokra *et al*., 2008; Moreno *et al*., 2009; Hanamanthagouda *et al*., 2010), in addition to their ecological importance by participating in the plant adaptation processes, attraction of pollinating insects and seed dispersers (Sepúlveda-Jiménez *et al*., 2003).

The variation of the chemical profiles of the Persea genus has been documented in several studies (Quing-Yi *et al*., 2009; Torres-Gurrola *et al*., 2009; Rincón-Hernández *et al*., 2011) in the most (Torres- Gurrola *et al*., 2016) reported a total of 363 identified MS, corresponding to 13 chemical groups, of the total, 258 are related in different tissues of *Persea americana*: 125 have been reported in pericarp, 109 in leaf tissue, 17 in seed, three in bark, two in flower and two in root. Due to the importance of knowing the chemical profiles, as defense mechanisms, of the genetic resources of *P. americana* Miller variety Drymifolia.

With the objective of knowing the foliar chemical variability of the 54 collections of this species, established in the germplasm bank of the Faculty of Agrobiology, dependent on the Michoacán University of San Nicolás de Hidalgo, as an integral part of a strategy to evaluate its potential as rootstocks with characteristics of resistance to pests and diseases, among other attributes additionally, the need to establish the possible relationship of the MS present in the collections with their geographical origin was raised.

Materials and methods

The research was developed in the Plant Genetic Resources Laboratory (LAREFI) of the Advanced Research Unit in Agrobiotechnology (UIAA) of the 'Presidente Juárez' Faculty of Agrobiology, of the Michoacán University of San Nicolás de Hidalgo, located in the city of Uruapan, Michoacán, Mexico, at the coordinates 19° 25' 10'' north latitude and 102° 03' 30'' west longitude at 1 620 meters above sea level. The process of extraction, identification and quantification of MS was carried out at the Center for Research in Ecosystems (CIEco) of the National Autonomous University of Mexico-Campus Morelia.

The genetic material analyzed consisted of 54 accessions of *P. americana* Miller variety Drymifolia, with four individuals per accession, the accessions come from localities belonging to the municipalities of Ario, Los Reyes, Salvador Escalante, Tacambaro, Tingambato, Uruapan and Ziracuaretiro, all of them located in the Michoacán avocado strip between coordinates 18° 45' and 20° 6' north latitude and 101° 47' and 103° 13' west longitude (Guillén-Andrade *et al*., 2007; Gutiérrez-Contreras *et al*., 2010).

The chemical profile of each of the accessions was determined from leaf tissue of mature leaves and with the use of a gas chromatograph (Agilent HP6890®), provided with a mass detector (Agilent 6890®). The procedures for extracting extracts, the conditions of the gas chromatograph for injecting and analyzing the samples, the identification of MS based on the information contained in the mass spectrum library NIST 05 (National Institute of Standars and Technology) and the Quantification of MS were made based on what was described by Torres-Gurrola *et al*. (2009).

The content and concentration database (mg g^{-1} dry leaf) of each of the identified MS was subjected to a one-way analysis of variance, based on a completely randomized experimental design, with four repetitions, considering to each accession as a treatment. Each observation was represented in the statistical model corresponding to the experimental design, by means of the following expression: Yij= M +Ci + eij. Where: Yij= concentration of MS in collection i and repetition j; M= general mean of the experiment; Ci= effect of the collection i; Eij= experimental error in collection i and repetition j; i= 1, 2, 3, ..., 54 and j= 1, 2, 3, 4.

This analysis was done using the Proc Anova procedure of the statistical package SAS® version 9.4 (SAS Institute Inc., 2012). Based on the analysis of variance, the MS were selected for which there were statistically significant differences ($p< 0.05$). Later, on the list of those selected MS, a discriminant analysis of Stepwise was carried out (Romano and Wolf, 2005) to debug and maintain only that MS that contributed more information to the total variance.

Additionally, a correlation analysis of Pearson and Filom (1898) was carried out to eliminate highly correlated variables, and to avoid problems of collinearity in the matrices. Subsequently, a matrix of averages was obtained to carry out a principal components analysis (PCA), using the matrix of correlations; the Euclidean distance matrix (DE) between accessions was calculated and with it, the cluster analysis was done by means of the Genalex statistical package (Peakall and Smouse, 2006).

Finally, the corresponding dendogram was constructed using the Neighbor-Joinning grouping method. All analyzes were made with the statistical package SAS® version 9.4 (SAS Institute Inc. 2012). The geographical coordinates of the accession source sites were converted to decimals with the Federal Communications Commission program (http: // transition.fcc.gov/mb/audio/bickel/DDDMMSS-decimal.html), with this data was used to construct a Geographical Distances (DG) matrix, which was correlated with a matrix of Euclidean distances, using the Mantel test with the GenAlex statistical package (Peakall and Smouse, 2006; Flanagan, 2006).

Results and discussion

In the analysis of 54 accessions of established in the germplasm bank of the Faculty of Agrobiology, the results obtained indicate the presence of 47 MS of the total, there is a first group of 31 that have been reported in *P. americana* Miller and related species: α-pinene, sabinene, β-pinene, eucalyptus, β-cis-ocimene, estragole, caryophyllene oxide, limonene, α-cubebene, phytol, heptacosane, squalene, chavicol, β-cubebene, caryophyllene, α-humulene, germacrene D-4-ol, tetradecanoic acid, copaene, cubenol, decane, phenyl ethyl alcohol, methylenegolol, nerolidol (E), tetradecanal, γ-elemene, ethyl linolenate, hexadecanoic acid, methyl hexadecanoate, myrcene and oleic acid (Quing-Yi *et al*., 2009; Torres-Gurrola *et al*., 2009; Rincón-Hernández *et al*., 2011; Torres-Gurrola *et al*., 2016).

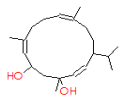
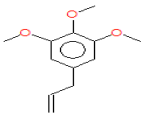
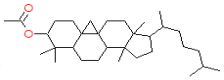
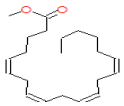
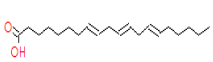
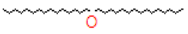
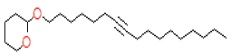
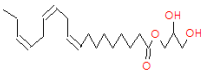
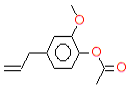
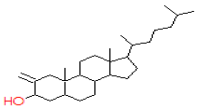
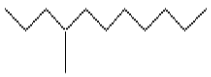
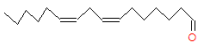
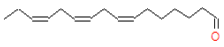
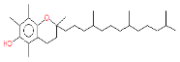
A second group, consisting of 11 MS, have not been reported for *P. americana* and related species; however, for these there are reports of their presence in other plant species. 4,8,13-Duvatrien-1,3-diol has been identified in tobacco as growth inhibitor 8,11,14 eicosatrienoic acid (z,z,z)- in *Melittis melissophyllum* (Velasco-Negueruela *et al*., 2004), eugenol acetate, has been reported as the main component in the essential oil of *Syzigium aromaticum* and with antioxidant activity; cis, cis, 7,10-hexadecadienal in *Euphorbia heterophylla* with cytotoxic activity, antioxidant and antimicrobial cis,cis,cis-7,10,13-hexadecatrienal in *Azadirachta indica* and *Allamanda cathartica* with larvicidal activity.

The elemicin in *Myristica fragrans* and *Daucus carota* with toxic and antibacterial activity and in *Syzigium aromaticum* with antioxidant activity palmite in *Annona diversifolia* acting as an anticonvulsant (Cano-Europa *et al*., 2010), α-tocopherol in *Elaeis oleifera* and *Vaccinium meridionale* with antioxidant activity cycloartenol acetate in propolis with antimicrobial activity 2H-pyrene, 2- (7-heptadecinyloxy) tetrahydro- me was identified in the medicinal plant *Andrographis paniculata* 2-methilenecholestan-3-ol, was reported as a component of the essential oil of the flowers of *Artemisia austro-yunnanensis* with antioxidant activity (Chen-xing *et al*., 2014) and in *Alstonia scholaris*: plant used in herbal medicine for its medicinal properties (Islam *et al*., 2013).

Finally, a third group of five MS were identified for which there are no reports of their presence in plant species: undecane and tridecane have been reported as defensive compounds in *Loxa deducta* and *Pellaea stictica* methyl arachidonate as a growth factor in animals. α-glyceryl linolenate, is used as an industrial solvent, undecane 4-methyl has been identified in wastewater and crude oil.

Based on the works, which have been published on this topic, in this research a total of 16 new metabolites were present in foliar tissue of *P. American* Miller Drymifolia variety and belonging to nine chemical groups: esters (4), alcohols (2), phenolic compounds (1) acids; (1) ether; (1) alkanes; (3) aldehydes; (2) acetone; (1); and polyterpenes (1). In Table 1, the name of the metabolite and its chemical structure are presented.

Table 1. Chemical structures of 16 new secondary metabolites identified in foliar tissue of *Persea americana* Miller variety Drymifolia.

4,8,13- Duvatrien-1,3-diol[c]	Elemicin[3]	Cycloartenol acetate[1]	Methyl arachidonate[1]
			
8,11,14- eicosatrienoic acid (z,z,z) -[4]	Palmitone[8]	2H-pyrene, 2- (7-heptadecyloxy) tetrahydro[5]	α-glyceryl linolenate[1]
			
Eugenol Acetate[1]	Undecane[6]	2-methylenecholestan-3-ol[2]	Undecane, 4-methyl[6]
			
Cis,cis-7,10-hexadecadienal[7]	Cis,cis,cis-7,10,13-hexadecatrienal[7]	α- tocopherol[9]	Tridecane[6]
			

[1]= esters, [2]= alcohols, [3]= phenolic compounds, [4]= acids, [5]= ether, [6]= alkanes, [7]= aldehydes, [8]= ketones and, [9]= polyterpenes.

When analyzing the concentration and frequency at which the 47 MS were identified in the 54 accessions, it was determined that β-pinene, estragole, caryophyllene, hexadecanoic acid, heptacosane and α-tocopherol were identified in all the accessions and, of these last, estragole stood out for presenting the highest percentage values (26.5327 mg g^{-1} of concentration). In contrast, chavicol and eugenol acetate were present in only five of the 54 accessions analyzed. On the other hand, phenyl ethyl alcohol was the one that showed the lowest percentage (0.0164 mg g^{-1} of leaf) concentration in the collections analyzed. In Table 2, the secondary metabolite information, retention time, Kovats index, concentration (%) and frequency of each of the MS identified in the 54 accessions are presented.

Table 2. Secondary metabolite, retention time, Kovats index, total concentration (%) and frequency of the 47 secondary metabolites identified in 54 accessions of *Persea americana* Miller Drymifolia variety of the germplasm bank of the Faculty of Agrobiology.

Secondary metabolite	TR[1]	IK[2]	[%][3]	F[4]	Secondary metabolite	TR[1]	IK[2]	[%][3]	F[4]
α-pinene[A]	2.79	942	0.982	12	Nerolidol (E)-[E]	7.2	1 577	0.6543	49
Sabineno[A]	3.11	986	0.02	7	Germacrene D-4-ol[E]	7.37	1 606	0.0403	17
β-pinene[A]	3.15	993	4.7539	54	Caryophylene oxide[E]	7.44	1 618	0.5922	46
Myrcene[A]	3.2	1 000	0.5881	49	Tetradecanal[L]	8.1	1 732	1.73	49
Decane[N]	3.26	1 007	0.0933	28	Tetradecanoic acid[F]	8.3	1 766	0.0583	26
Limonene[A]	3.5	1 038	0.0213	8	*Cis*, *cis*, 7, 10- hexadecadienal[L]	9.09	1 903	0.1906	41
Eucalyptus[A]	3.54	1 043	0.7361	53	*Cis*,*cis*,*cis*-7,10,13-hexadecatrienal[L]	9.12	1 909	4.4192	52
β-*cis*-ocimeno[A]	3.63	1 056	0.3887	39	Methyl Hexadecanoate[G]	9.28	1 936	1.7413	40
Undecane[N]	4.01	1 107	0.5654	44	Hexadecanoic acid[F]	9.46	1 967	4.6696	54
Phenyl ethyl alcohol[J]	4.21	1 134	0.0164	9	Fitol[B]	10.37	2 125	4.7608	53
Undecane,4-metil[N]	4.75	1 206	2.7795	43	Oleic acid[F]	10.43	2 137	3.6279	50
Estragole[J]	4.83	1 217	26.5327	54	Ethyl Linoleate[G]	10.72	2 189	0.8929	23
Chavicol[K]	5.14	1 262	0.077	5	Methyl arachidonate[G]	11.16	2 268	3.3274	48
Tridecan[N]	5.45	1 306	0.09388	28	8,11,14-eicosatrienoic acid (*z*,*z*,*z*)-[F]	11.86	2 398	0.6953	21
τ-elemene[E]	5.81	1 359	0.0783	10	4,8,13- Duvatrien-1,3-diol[M]	11.91	2 406	1.1932	28
α-cubebeno[E]	5.89	1 370	0.0389	15	2H-pyrene, 2-(7- heptadecyloxy) tetrahydro-[I]	12.49	2 519	2.1822	46
Copaeno[E]	6.09	1 400	0.398	44	2-methylenenecholestan -3-ol[M]	13.02	2 626	1.5813	34
β-cubebeno[E]	6.17	1 414	0.251	20	Heptacosan[N]	13.4	2 705	3.9402	54
Metileugenol[K]	6.2	1 418	3.5294	49	α- glyceryl linolenate[G]	13.65	2 763	2.9127	45
Caryophylene[E]	6.41	1 451	5.852	54	Cycloartenol acetate[G]	13.84	2 805	0.8669	30
α-humulene[E]	6.62	1 484	0.6148	49	Squalene[C]	14.03	2 848	3.5109	52
Eugenol Acetate[G]	6.98	1 541	0.0518	5	α- tocopherol[D]	15.76	3 170	5.6894	54
Cubenol[E]	6.99	1 543	0.6123	48	Palmitone[H]	16.72	3 301	1.5751	34
Elemicin[K]	7.14	1 568	0.0708	17					

[1]= retention time; [2]= Kovats index; [3]= percent concentration in relation to the total; [4]= frequency number of collections in which each MS was identified; [A]= monoterpene; [B]= diterpene; [C]= triterpene; [D]= polyterpene; [E]= sesquiterpene; [F]= acids; [G]= esters; [H]= acetone; [I]= ether; [J]= aromatic compounds; [K]= phenolic compounds; [L]= aldehydes; [M]= alcohols; [N]= alkanes.

Estragole was the secondary metabolite highlighted by being present in higher concentration and in the 54 collections analyzed. These results are similar to those reported in similar works in germplasm different *Persea* species from different regions of Mexico (Rincón-Hernández *et al*.,

2011). The evaluation of the content of this MS in each of the collections is interesting, for example, the interaction avocado creole-*Trioza anceps* has been evaluated, the trees with the highest concentration of estragole showed less presence of leaf galls (Torres-Gurrola *et al*., 2009).

In addition, estragole has been identified as the main component in other species and, with important biological defense activities, as an insecticide (López *et al*., 2008) in *Ocimum basilicum* induced contact mortality to the rice weevil (*Sitophilus oryzae*) (Pascual-Villalobos *et al*., 2004), it has also been used against the red flour beetle (*Tribolium castaneum* Herbst) and the grain borer (*Rhyzopertha dominica*) in *Pimpinella anisum*, it was reported with bactericidal effect, it has been related to antifungal activity (Fontenelle *et al*., 2008), larvicide (Senthilkumar *et al*., 2008) and genotoxic (Zani *et al*., 1991).

In the present study, three previous collections of the municipality of Tingambato, Michoacan, Mexico, were those that presented the highest concentration of estragole, so it would be expected that these collections could present better defense attributes to be used as selected rootstocks.

In the Figure 1 shows the variation determined in the content of estragole in the 54 collections analyzed.

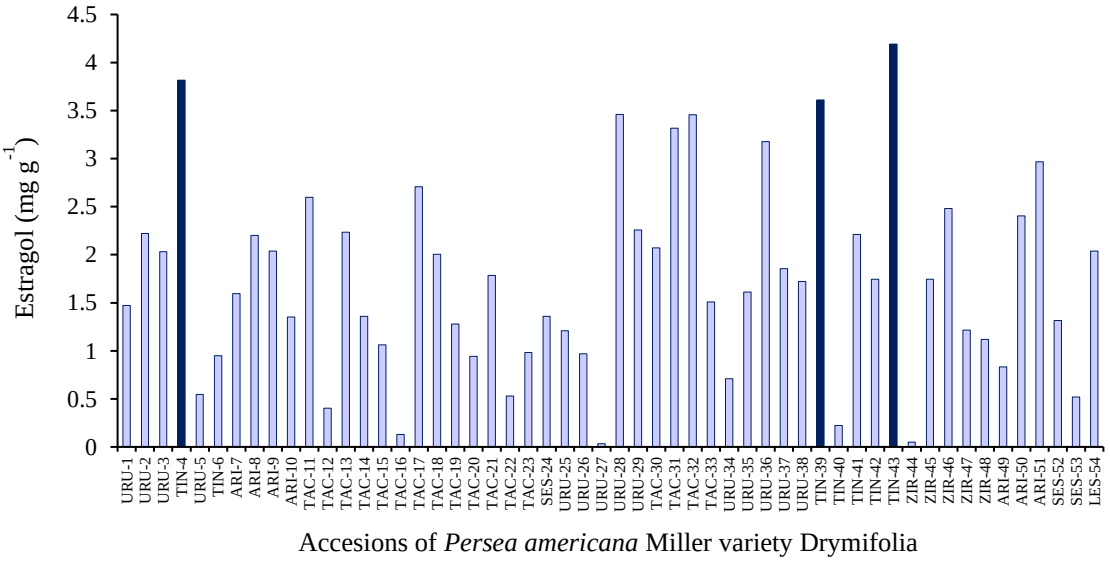


Figure 1. Histogram for concentration of estragole in 54 accessions of *Persea americana* Miller variety Drymifolia collected in the municipalities of Ario (ARI), Los Reyes (LES), Salvador Escalante (SES), Tacámbaro (TAC), Tingambato (TIN), Uruapan (URU) and Ziracuaretiro (ZIR) established in the germplasm bank of the Faculty of Agrobiology.

The values of the coefficient of determination (R^2) oscillated between 0.33 and 0.46, in relation to the coefficients of variation (CV), the values fluctuated between 98.13 and 896.43. The highest CV value was obtained for the eugenol acetate in contrast to the *Cis*, *cis*, *cis* 7, 10, 13-hexadecatrienal. Likewise, the average values ranged from 0.00108 to 0.28877 in percentage of total concentration.

Later, in the Stepwise discriminant analysis, the tridecane was identified as one of the MS that did not provide information to explain the variation in MS concentration and when reviewing the Pearson correlation values, the dean was highly correlated (<0.0001) with the tridecane.

These results are explained by the fact that the collections present differences in their gene expression and the chemical profile is subject to strong genetic control, as it is in other tree species (Langenheim and Stubblebine 1983; Gershenzon *et al*., 2000; McConkey *et al*., 2000).

The results obtained from the one-way analysis of variance, performed for 47 MS content identified by analyzing 216 individuals from 54 collections of *P. americana* Miller variety Drymifolia, indicated significant statistical differences ($p< 0.05$) for concentration for ten of them and differences highly significant ($p< 0.01$) for phenyl ethyl alcohol and 8,11,14 -eicosatrienoic acid (z,z,z)-. Table 3 shows the simple statistics obtained for the 12 secondary metabolites that presented significant statistical differences.

Table 3. Simple statistics mean squares of error and probability of 12 MS with significant statistical differences obtained from the analysis of variance practiced for concentration of 47 MS identified in 54 collections of *Persea americana* Miller Drymifolia variety of the germplasm bank of the Faculty of Agrobiology.

Secondary metabolites	R^{2*}	CV^{1}	DE^{2}	Mean	CME^{3}	Pr>F
Decane	0.34	196.54	0.01199	0.0061	0.00014	0.0131
Phenyl ethyl alcohol	0.46	473.28	0.00509	0.00108	0.00003	<0.0001
Undecane 4 methyl	0.41	128.08	0.23263	0.18162	0.05411	0.0002
Tridecan	0.38	198.86	0.0122	0.00613	0.00015	0.0019
β-cubebeno	0.41	341.69	0.05605	0.0164	0.00314	0.0002
Eugenol Acetate	0.4	896.43	0.03039	0.00339	0.00092	0.0004
Caryophylene Oxide	0.33	170.22	0.06588	0.0387	0.00434	0.028
Cis,cis 7,10 hexadecadienal	0.33	271.52	0.03382	0.01246	0.00114	0.0341
Cis,cis,cis-7,10,13 hexadecatrienal	0.34	98.13	0.28338	0.28877	0.0803	0.019
8,11,14-eicosatrienoic acid (*z,z,z*)-	0.43	299.19	0.13595	0.04544	0.01848	<0.0001
4,8,13-duvatrien 1,3-diol	0.34	222.07	0.17315	0.07797	0.02998	0.0184
2-methylenecholestan-3-ol	0.39	175.88	0.18174	0.10333	0.03303	0.0009

*= coefficient of determination; 1= coefficient of variation; 2= standard deviation; 3= Mean square of the error.

To reduce the number of variables to be considered for the explanation of the variability in relation to the concentration of secondary metabolites, a principal component analysis (PCA) was carried out. The results indicated that the first three main components (CP), explain together 29.64% of the variance that exists between the accessions, while with 17 it is explained 80%.

Similar results have been obtained in works of *in situ* morphological characterization of *Persea* and other fruit species, since it has been observed that in this type of work is not achieved more than 80% of the variance explained with the first three components (Tofiño *et al*., 2012; López-Gúzman *et al*., 2012; Guillén-Andrade *et al*., 2013; Medina-Torres *et al.*, 2015; Montes-Hernández *et al*., 2017).

In Figure 2, the distribution of the 54 collections is shown according to three main components. When analyzing the eigenvectors with values above 0.3, it was determined that the CP1 is a function of the presence of the eucalyptus, caryophyllene and α-humulene, the CP2 was determined in terms of the decane, undecane, 4-methyl and tridecan. finally, CP3 is a function of chavicol and τ-elemeno. It is important to note that the tridecane and the undecane, 4-methyl with the highest weight in CP2 are reported for the first time in *Persea americana* Miller variety Drymifolia.

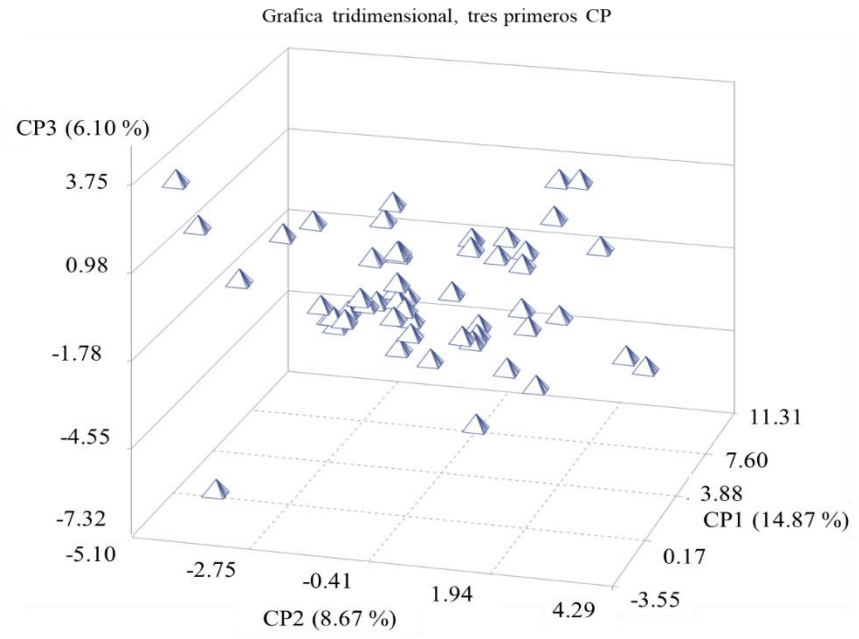


Figure 2. Dispersion of 54 collections of *P. americana* Miller variety Drymifolia from the germplasm bank of the Faculty of Agrobiology, based on three main components.

On the other hand, the dendrogram presented in Figure 3, generated from the cluster analysis based on Ward's grouping method, showed that accessions are not associated in a logical way in relation to their geographical origin. These results were corroborated when performing the Mantel test, the correlation value obtained was r= -0.064 and a significance of 0.069, which confirmed the non-relationship between the geographic origin of the collections and the MS content identified in 54 collections.

These results may be masked because the leaf tissue samples were isolated from the collections established in the germplasm bank; that is, they are in a single environment.

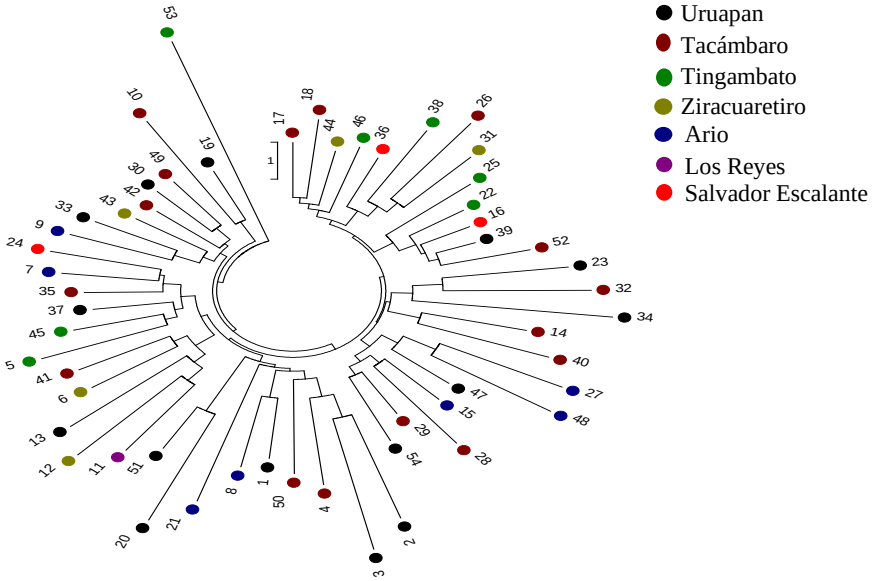


Figure 3. Complete clustering dendrogram of 54 collections of *Persea americana* Miller variety Drymifolia, based on data on the concentration of 47 secondary metabolites. The colored circles indicate the place of origin of the germplasm.

The results of the aforementioned test are presented in Figure 4.

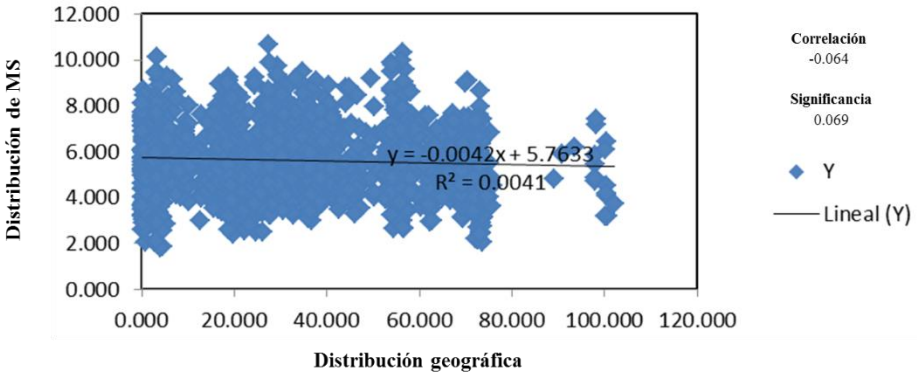


Figure 4. Linear correlation between the content of secondary metabolites and geographic distribution of 54 collections of collections of *Persea americana* Miller variety Drymifolia.

Conclusions

The 54 collections established in the germplasm bank of the Faculty of Agrobiology of *Persea americana* Miller variety Drymifolia, are contrasting in the type and concentration of 47 identified secondary metabolites. Estragole was a constant in terms of content and greater concentration in all the germplasm analyzed. The identification of 16 new secondary metabolites in the evaluated germplasm is an important contribution of the present work.

Finally, no relationship was determined between the chemical profile and the geographical origin of origin of the collections analyzed.

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