Chapter 7

Analysis of the Presence of the Betulinic Acid in the Leaves of *Eugenia florida* by Using the Technique GC/MS, GC/FID and HPLC/DAD: A Seasonal and Quantitative Study

Alaíde S. Barreto, Gláucio D. Feliciano,
Cláudia Cristina Hastenreiter da Costa Nascimento,
Carolina S. Luna, Bruno da Motta Lessa,
Carine F. da Silveira, Leandro da S. Barbosa,
Ana C. F. Amaral and Antônio C. Siani

Additional information is available at the end of the chapter

http://dx.doi.org/10.5772/55868

1. Introduction

The betulinic acid (Figure 1) is a known triterpenoid isolated from various organs and species of plants, including flowering *Eugenia* DC [Junges, 1999]. This metabolite shows inhibitory activity on growth of human melanoma cells [Pisha *et al*., 1995], and replication of the AIDS virus [Evers et al. 1996; Soler *et al*., 1996]. In additional betulinic acid derivatives [Chatterjee *et al*., 2000; Galgon *et al*., 2005] induced cell apoptosis of human melanoma. This specificity in melanoma cells makes the substance compared to complex molecules such as taxol, the most promising anticancer drug [Pisha *et al*. 1995]. However, their action is limited only neuroblastomas and melanoma cells and is not active against other cancer cells [Chatterjee, 2000, Pezzuto *et al*., 1999; Pisha, 1995; Mayauxet *al*., 1994]. The betulinic acid also has antibacterial property and inhibits the growth of colonies of *Escherichia coli* and *Staphylococcus aureus*.

Despite all of betulinic acid pharmacological potential, it is obtained by extraction of barks or core of some plant species or by synthetic processes, e.g. using the betulin (alcohol triterpene) as a synthetic intermediate isolated from the bark of *Betula alba* and *Betula pendula* [Galgon., *et al*. 1999]. Therefore, research is necessary to identify new natural sources, which produce large

INTECH
open science | open minds

© 2013 Barreto et al.; licensee InTech. This is an open access article distributed under the terms of the Creative Commons Attribution License (http://creativecommons.org/licenses/by/3.0), which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

quantities of substance easily renewable parts of the plant (leaf) thereby not affecting plant growth, development of chromatographic methods rapid and easy manipulation studies to identify the seasonal best months of collection.

As part of a program conducted in our laboratory involving search for new sources of bioactive metabolites from Brazilian plants, we investigated the leaves of *Eugenia florida*. This species belongs to the family Myrtaceae. Compounds such as flavonoids, triterpenes, tannins and especially essential oils constituted of monoterpenes and sesquiterpenes have already been isolated from the genus Eugenia [Lunardi, et al., 2001]. The species of this family are widely distributed in the Brazilian forests, much of it is popularly known for its edible fruits, wood, essential oils or ornamental purposes [Consolini *et al*., 1999, Costa *et al*., 2005; Siani *et al*., 2000]. The most important genera of this family are: *Melaleuca*, *Eucalyptus*, *Psidium* and *Eugenia* [Siani *et al*., 2000].

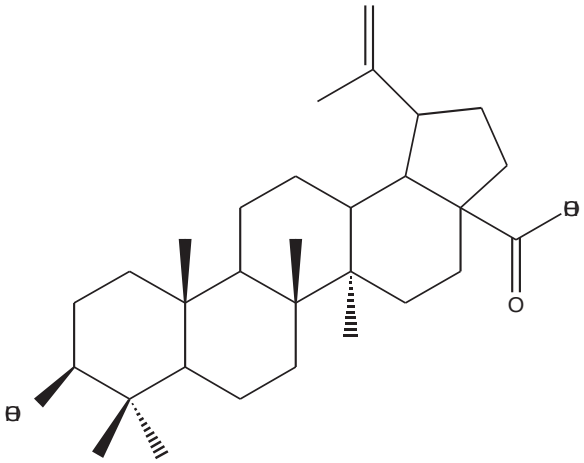


Figure 1. Betulinic acid

1.1. Seasonal variation

Since the fourth century B.C. there are reports of procedures for the collection of medicinal plants. The executioners Greeks, e.g., they collected their samples of poison hemlock (*Conium maculatum*) morning when levels are higher alkaloid coniina [Robinson, 1974]. Temporal and spatial variations in the total content, as well as the relative proportions of secondary metabolites in plants occur at different levels and, despite the existence of a genetic control, the expression may undergo changes resulting from the interaction of biochemical processes, physiological, ecological and evolutionary [Gobbo, Lopes, 2007]. In fact, the secondary metabolites represent a chemical interface between plants and the surrounding environment. Therefore, their synthesis is often affected by environmental conditions [Gobbo, Lopes, 2007].

http://dx.doi.org/10.5772/55868

Several factors that can coordinate or alter the rate of production of secondary metabolites, genetic factors, physical environment, collection method (date, time, etc.), drying conditions and transport, storage, pH of the soil, growing conditions, nutrient soil, plant part used, interactions between plants, the presence of microorganisms, can directly affect the concentration of the chemical components of each species [Silva, 1996]. Some factors have correlations with each other and not act alone, and may jointly affect the secondary metabolism, e.g. development and seasonality, rainfall and seasonality, temperature and altitude, among others [Gobbo, Lopes, 2007]. It should also be noted that, often, the changes may result from leaf development and / or appearance of new organs concomitant with constancy in the total content of secondary metabolites. This may cause decrease in the concentration of these metabolites by dilution may, however, result in a higher total amount due to the increase of biomass. [Hendriks *et al*., 1997; Spring, Bienert, Klemt 1987]

The seasonal variation (collection of plants at different times or seasons), is a very significant factor in the percentage and production of secondary metabolites [Mitscher, Pillai, Shankel, 2000; Navarro *et al*., 2002]. Due to its importance, it is necessary a study of seasonality, when working with medicinal plants [Mitscher, Pillai, Shankel, 2000; Navarro *et al*., 2002].

This work had as main objectives to develop a protocol for the quantification of betulinic acid present in the leaves of *Eugenia florida* by using the technique GC/MS, GC/FID and HPLC/ DAD and through studies demonstrates the potential of this seasonal vegetable production as a source of natural metabolite.

2. Material and methods

2.1. General experimental procedures

1H and 13C NMR spectra were recorded on a Brucker AM-200 and 500MHz) chemical shifts are given in d values referred to internal tetramethlysilane (TMS), EIMS (MS Agilent 5973; 70eV) and Infrared (IV) spectra were recorded on a Nicolet spectrophotometer with Fourier transform Model Magna–IR 760 wavelengths are expressed in reciprocal centimeter (cm^{-1}).

HPLC analysis were performed at room temperature using the following system: second pump system (Shimadzu LC10AD model, Japan) a photodiode array detector (Shimadzu, SPD10ADVP model), an auto injector (Shimadzu SIL10ADVP model) an oven (Shimadzu, CTO 8-A model) and under the following conditions: Shimpack C-8 (1cm x 4.6mm i.d.) guard column and C-18 column (25cmx4.6mm i.d.; 5μm particle size) was from Zorba Zx provided by Agilent Technologies (USA). The system was controlled by Class Vp (Shimadzu, Japan) software 5.16. The gradient mobile phase was carried acetonitrile (Tedia, Brazil) and water HPLC grade. The mobile phase was degassed with helium and the flow rate adjusted to 1mL. min^{-1}. The water was acidified with TFA (0.05% v/v). All samples were injected automatically (10μ L) in triplicate.

In GC/FID experiments, 1mg each extracts [August 2009 to July 2010] and standard (Carl Roth – Karlsruhe, Germany) were transferred into glass vial and submitted methlylation with

CH_2N_2 with 100% yield. Those samples were dissolved in CH_2Cl_2 in concentrations 1.4mg/mL and injected for GC-FID and GC-MS analysis. The identification of methylated betulinic acid in extracts was done with use Wiley and NBS peak matching library search system. Authentic standard of the betulinic acid and data reported in the literature were also used for further identification as described.

2.2. Plant material

Healthy leaves of *Eugenia florida* and adults were collected during 12 months [August 2009 to July 2010] on the campus Oswaldo Cruz Foundation, state of Rio de Janeiro. The specie identification was carried out by biologist Sergio Monteiro of Oswaldo Cruz Foudantion [Laboratory of Production and Processing of Raw Plant (LPBMPV)], and a voucher specimen was deposited in the Herbarium of the Botanical Garden of Rio de Janeiro with the number RB 328061.

2.3. Methylation

A solution of diazomethane (CH_2N_2) in ether was prepared and added (excess) in drops of the solutions of extracts, EF-1 and standard (1mg) $CHCl_3$ or MeOH. The resulting solutions were allowed to stand for 12 hours and the ether removed by passing a stream of N_2 [Leonard, Lygo, Procter, 1995].

2.4. Betulinic acid quantification

GC analysis was performed on 6890N (Agilents Technologies, Network series) equipped whit a HP-5 column (30 x 0,25mm; 0,25μm liquid phase). Oven temperature program of 70°C - 300°C at 5°C/min; carrier gas: helium 11,3L/min; split mode of (20:1) and finally held for 30min. The mass spectrometer unit was performed with the same conditions the GC analysis. The calibration curve of the GC / FID was made in triplicate from different concentrations of esterified betulinic acid standard (0.1 to 1$\mu g.mL^{-1}$) and the curve was constructed using the average values of the detector response. The detector response was linear to the concentration internal of 0.1 to 1$\mu g.mL^{-1}$ ($r^2 = 0.999$, Figure 2a).

HPLC grade acetonitrile was purchased from TEDIA (Brazil); 0,05% TFA (Trifluoroacetic acid) from Vetec (Brazil). Water was purified by Milli-Q_{plus} system from Milipore (Milford, MA, USA). Betulinic acid was purchased from Carl Roth (Karlsruhe, Germany with 99%). The 30mg ethanol extracts were then dissolved in 5mL of mobile phase. The mobile phase consisted of a gradient of 0,05% aqueous trifluoroacetic acid: acetonitrile delivered at a 1.0$mL.min^{-1}$ as follows initial (t= 0 min) 30:70, linear gradient over 20min to 15:85, linear gradient over 10min to 100:0 and a new linear gradient over 20min (30:70); 40min as total time of analysis. Flow rate was 1$mL.min^{-1}$. Quantification was performed using the detector set at a wavelength of 210nm. Injection volume was 30μl. The peak of betulinic acid was identified in each chromatogram from of the ethanol extracts monthly (twelve months) with the help of injection of the standard solution of betulinic acid or comparison of the UV spectrum. The calibration curve of the HPLV-UV was made in triplicate from different concentrations of betulinic acid standard (0.1 to 1$\mu g.mL^{-1}$) and the curve

http://dx.doi.org/10.5772/55868

was constructed using the average values of the detector response. The detector response was linear to the concentration internal of 0.1 to 0,5μg.mL^{-1} (r^2 = 0.9994, Figure 2b).

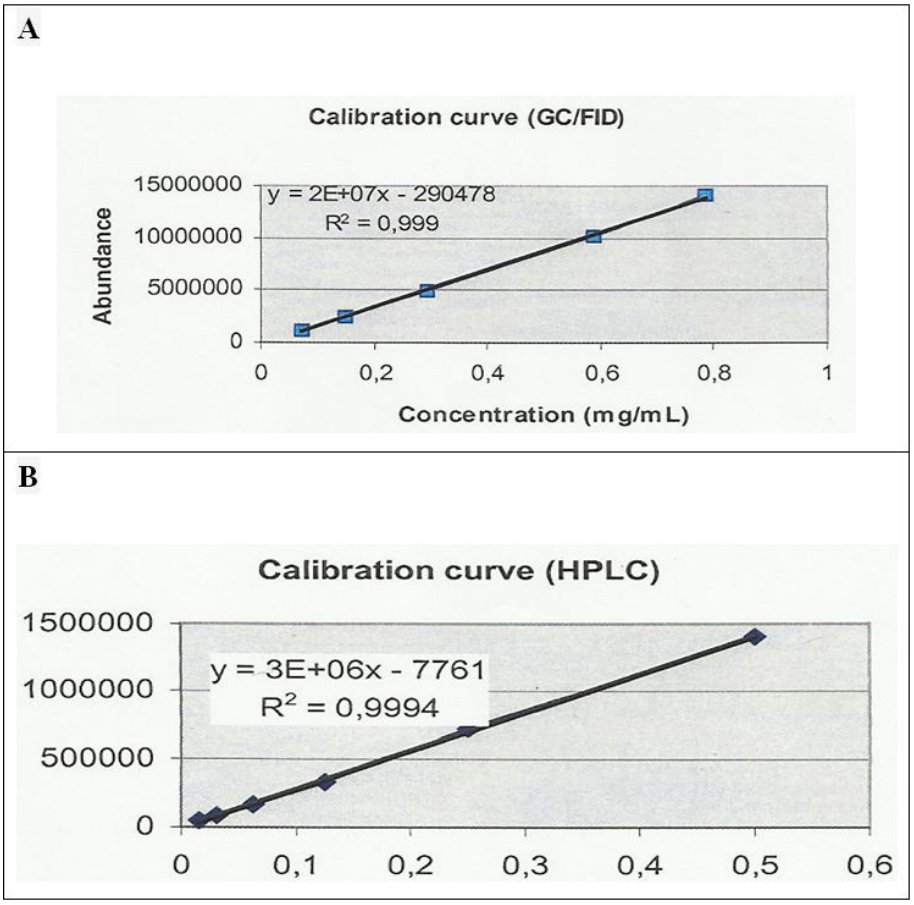


Figure 2. Calibration curve of Betulinic acid, A: GC-FID and B: HPLC-UV

3. Results and discussion

3.1. Extraction of betulinic acid

The leaves of *Eugenia florida* (17.1 kg) were dried at 400C, ground and subjected to soxhlet extraction with ethanol. The diluted extract was removed under reduced pressure (4.7 g). An aliquot of the methanol extract (200 mg) was dissolved in methanol (20ml) and recrystallized using mixtures of $CHCl_3$ and MeOH. Recrystallization was obtained a white crystal (EF – 1; 50mg).

EF-1 was analyzed by spectrophotometer 1H and 13C NMR (Bruker AC 200, 200MHz) using as solvent chloroform ($CDCl_3$) and methanol (CD_3OD) deuterated at a ratio of 9:1 to tetramethylsilane (TMS) as internal reference standard. An aliquot of EF-1 (5 mg) was methylated with diazomethane and subjected to mass spectrometry (MS; Agilent Technologies). The spectral data obtained were compared with the literature [Oliveira *et al*., 2006].

The substance showed an EF-1 in the form of white crystals and the IR spectrum showed a broad band at 3450cm^{-1} by a characteristic of hydroxyl groups and acid, a broad band at 2942cm^{-1} one of alkyl groups and bands at 1686cm^{-1} and 1639cm^{-1} corresponding respectively to the axial deformation of carbonyl acid and alkene.

The information that led to elucidation of the structure was obtained from experiments nuclear magnetic resonance spectra [DEPT, HMQC, ^1H-^1H COSY (homonuclear correlation spectroscopy) and HMBC experiment] which indicate a known pattern of the terpenes series lupanos (Nick *et al*., 1994, Mahato, Kundu 1994; Budzikiewicz *et al*., 1964). The ^1H NMR spectrum showed two signals of multiplet in δ_H 4.69 and 4.58, referring to vinyl hydrogen (H-20), δ_H1.66 a signal corresponding to the methyl group bonded to carbon and fifth signals sp^2 corresponding to the methyl tertiary (δ_H 0.74; 0.85, 0.94, 0.96 and 1.00). The 1^3C NMR spectrum confirmed the presence of signals in vinyl 152.02 and 110.15 ppm (double bond), carbonyl acid in 180.03ppm and secondary alcohol in 79.69 ppm [Nick *et al*., 1994; Mahato, Kundu, 1994].

The methylation EF-1 with diazomethane promoted the removal of hydrogen from the carboxyl acid and incorporation of a methyl group from the diazomethane, leading to formation of an ester, molecular weight 470. The derivatization and the formation of the ester are ideal possible to decrease the molecular interactions between the sample and a chromatographic column and thus decrease the retention time. An aliquot of esterified EF-1 (1.4mg) was subjected to MS electron impact (70 eV). The MS spectrum of esterified EF-1 confirmed the presence of a terpene class of lupanos due to the absence of peaks m/z 218 and m/z 203 characteristic of the series oleanane and ursane (rearrangement retro Diels-Alder ring C). The presence of the methyl ester group at C-28 is confirmed by the ion m/z 262 (10%). Other peaks were obtained m/z 208 (5%), m/z 190 (10%) and m/z 189 (100%) from the break ring C and the molecular ion m/z 470 5% [Budzikiewicz *et al*., 1964]. The spectral data obtained from the EF-1 and the ester data were similar to those observed in the literature to betulinic acid [Nick *et al*., 1994, Mahato, Kundie 1994; Budzikiewicz *et al*., 1964].

After calibration with standard of betulinic acid, the monthly extracts from leaves of *Eugenia florida* were analyzed. Those extracts were analyzed in triplicate and the average areas

http://dx.doi.org/10.5772/55868

corresponding to betulinic acid was calculated. From these average areas, percentage composition of the betulinic acid in the extract were calculated using the linear equation generated during calibration of betulinic acid (Figure 2) carried out in HPLC-UV and GC-FID (Table 1).

Month (year)	CG-FID (%)	HPLC (%)
August (2009)	7.43	8.01
September (2009)	16.83	8.57
October (2009)	26.27	11.18
November (2009)	17.97	8.36
December (2009)	8.24	4.83
January (2010)	6.90	2.79
February (2010)	7.92	5.12
March (2010)	23.03	6.12
April (2010)	9.68	6.31
May (2010)	14.85	9.16
June (2010)	19.33	9.99
Jully (2010)	15.23	5.62

Table 1. Quantification (w/w) of betulinic acid present in ethanol extracts from leaves *of Eugenia florida* determined by GC-FID and HPLC-UV at 210nm

4. Conclusions

Several activities are being attributed to betulinic acid, however, despite all of their potential pharmacological, it is still obtained by extraction of the bark and heartwood of some [Soler, 1996], synthetic processes [Evers et al., 1996] and by biotransformation [Galgon, 2005]. Unlike these traditional species whose income was less than 3%, we found that betulinic acid was present in all extracts analyzed (table 1), with yields well above those found in the literature.

The betulinic acid level in the *E. florida* leaves increased significantly in the May, June, Jully (autumn - winter) and, September, October and November (winter) which was mainly due to the accumulation of this compound in vegetal tissue. Some authors related with the pentacyclic triterpenes, just as betulinic, acid ursolic, acid, β-amyrine and lupeol, are supposed to be toxic to insects, due to their ability to inhibit acyl chain packing in the lipid bilayers of the insect membranes [Rodriguez *et al*., 1997; Prades et al., 2011].

These fluctuations observed in the months described in Table 1 may be related to the chemical ecology of *Eugenia florida* as, for example, the attraction of pollinators or the reproductive phenology of the specimens

It is possible that the increased concentration of betulinic acid in the month of March is due to the large amount of rainfall characteristic of the Rio de Janeiro, state. However, more research is needed to determine whether other factors may be influencing the concentration of this metabolite, verify that specimens from other regions have the same or different behavior and examine whether the effect of the solvent can affect the increase in the concentration of this metabolite

Acknowledgements

The authors thank the (FAPERJ) for the financial support. We also acknowledge the Fundação Oswaldo Cruz, Farmanguinhos – PMA (Plataforma de Métodos Analíticos) for GC/FID, GC/MS analysis.

Author details

Alaíde S. Barreto[1,3], Gláucio D. Feliciano[1,4],
Cláudia Cristina Hastenreiter da Costa Nascimento[1], Carolina S. Luna[2], Bruno da Motta Lessa[2], Carine F. da Silveira[2], Leandro da S. Barbosa[2], Ana C. F. Amaral[2] and Antônio C. Siani[2]

1 Laboratory of Analysis Chemical - Biological (LAQB), Foundation of State University Center West Zone (UEZO), Brazil

2 Natural Products Laboratory, Oswaldo Cruz Foundation Farmanguinhos - Oswaldo Cruz Foundation (Fiocruz), Rio de Janeiro, Brazil

3 Department of Chemistry, University Severino Sombra (USS) Vassouras, Rio de Janeiro, Brazil

4 Universidade Estácio de Sá (UNESA), Área De Ciências Biológicas e da Saúde, Rio de Janeiro, Brazil

References

[1] Budzikiwicz, H, Djerassi, C, & Williams, D. H. (1964). Structure Elucidation of Natural Products by Mass Spectrometry. Volume II: Steroids, terpenoids, sugars and miscllaneous classes Holdey-day, INC, São Francisco, London, Amsterdam, 306p.

[2] Chatterjee P; Kouzi SA; Pezzuto JM; Hamann MT; ((2000). Biotransformation of the antimelanoma agent betulinic acid by Bacillus megaterium ATCC 13368. Applied and environmental microbiology. , 66(9), 3850-3855.

http://dx.doi.org/10.5772/55868

[3] Consolini, A. E, Baldini, O. A. N, & Amat, A. G. (1999). Pharmacological basis for the empirical use of *Eugenia uniflora L.* (Myrtaceae) as anthypertensive. Journal of Ethnopharmacology, , 66, 33-39.

[4] Evers, M, Poujade, C, Soler, F, Ribeil, Y, James, C, Lelièvre, Y, Geguen, J. C, Reisdorf, D, Morize, I, Pauwels, E, De Clercq, E, Hènin, Y, Bousseau, A, & Mayaux, J. F. Le Pecq, J.B.; Dereu N. ((1996). *Betulinic Acid Derivatives: A new class of HIV type 1 specific inhibitors with a new mode of action*. Journal of Medicinal of Chemistry , 39, 1056-68.

[5] Galgon, T, Wohlrab, W, & Drager, B. (2005). Betulinic acid induces apoptosis in skin cancer cells and differentiation in normal human keratinocytes. Experimental Dermatology. October, , 14(10), 736-743.

[6] Galgon, T, Höke, D, & Dräger, B. (1999). *Identification and Quantification of Betulinic Acid. Phytochem* Anal. 10. , 187-190.

[7] Gobbo-neto, L, & Lopes, N. P. (2007). Medicinal plants: factors of influence on the content of secondary metabolites. Química Nova. Mar./Apr. São Paulo., 30(2)

[8] Hendriks, H, Wildeboer, Y. A, Engels, G, Bos, R, & Woerdenbag, H. J. (1997). The content of parthenolide and its yield per plant during the growth of *Tanacetum parthenium.* Planta Medica , 63, 356-359.

[9] Junges, M. J, Fernandes, J. B, Vieira, P. C, Silva, M. F. G, & Filho, E. R. (1999). *The use of ^{13}C and ^{1}H-NMR in the structure elucidation of a new nor-lupane triterpene*. Journal Brazilian Chemical Society , 10(4), 317-320.

[10] Leonard, J, Lygo, B, & Procter, G. Advanced Pratical Organic Chemistry, 2 nd ed. Chapman & Hall, (1995).

[11] Lunardi, I, Peixoto, J. L. B, Silva, C. C, & Shuquel, I. T. A. Basso E.A.E; Vidotti G.J ((2001). Triterpenic Acids from Eugenia moraviana. Journal Brazilian Chemical Society, , 12, 180-183.

[12] Mahato, S. B, & Kundu, A. P. (1994). C NMR spectra of pentacyclic triterpenoids- A compilation and some salient features. Phytochemistry , 37, 1517-1575.

[13] Mayaux, J. F, Bousseaux, A, Panwels, R, De Clerq, E, & Pecq, J. B. (1994). *Triterpenes derivatives that block the entry of human immunodeficiency virus type I into cells.* Proceedings National Academy Sciences, , 91, 3564-3568.

[14] Mitscher, L. A, Pillai, S, & Shankel, D. M. (2000). Some transpacific thoughts on the regulatory need for standardization of herbal medical products. Journal Food and Drug Anal, n. 4, , 8, 229-234.

[15] Navarro, F. N, Souza, M. M, Neto, R. A, Golin, V, Niero, R, & Yunes, R. A. Delle Monache, F.; Cechinel Filho, Phytochemical analysis and analgesic properties of Curcuma Zedoaria grwn in Brasil. Phytomedicine, n.9, , 2002, 427-432.

[16] Oliveira, B. H, Santos, C. D. A, & Espíndola, A. P. D. M. (2002). *Determination of the triterpenoids, Betulinic Acid in Doliocarpus schottianus by HPLC*. Phytochemistry. Anal. , 13, 95-98.

[17] Oliveira, C. M, Moos, H. W, Chayer, P, & Kruk, J. W. *Variations in D/H and D/O from New Far Ultraviolet Spectroscopic Explorer Observations.* The Astrophysical Journal, (2006). , 642, 283-306.

[18] Pezzuto, J. M, Dasgupta, T. K, & Kim, D. S. H. L. United States Patent nº 5,869,535; February 9, (1999).

[19] Pisha, E, Chai, H, Lee, I. S, Chagwedera, T. E, Farnsworth, N. R, Cordell, G. A, Beecher, C. W. W, Fong, H. H. S, Kinghorn, A. D, Brown, D. M, Wani, M. C, Wall, M. E, Hieken, T. J, Dasgupta, T. K, & Pezzuto, J. M. (1995). *Discovery of betulinic acid as a selective inhibitor of human-melanoma that functions by induction of apoptosis.* Nature Medicine , 1, 1046-1051.

[20] Prades, J, Vögler, O, Alemany, R, & Gomez-florit, M. Funari, S.S; Ruiz-Gutiérrez, V.; Barceló, F. ((2011). Plant pentacyclic triterpenic acids as modulators of lipid membrane physical properties Biochimica et Biophysica Acta (BBA)- Biomenbranes. , 1808

[21] Robinson, T. Metabolism and function of alkaloids in plants. Science, (1974). , 184, 430-435.

[22] Rodriguez, S, Garda, H, Heinzen, H, & Moyna, P. (1997). Effect of plant monofunctional pentacyclic triterpenes on the dynamic and structural properties of dipalmitoylphosphatidylcholine bilayers Chemical Physical Lipids , 89-119.

[23] Siani, A. C, Sampaio, A. L. F, Sousa, M. C, Henriques, M. G. M. O, & Ramos, M. F. S. (2000). Óleos essenciais, potencial antiinflamatório. Revista Biotecnologia Ciência & Desenvolvimento, n 16, , 3, 38-43.

[24] Soler, F, Poujade, C, Evers, M, Carry, J. C, Hènin, Y, & Mayaux, J. F. Le Pecq J. B.; Dereu, N. ((1996). *Betulinic acid derivates: a new class of HIV type 1 entry*. J. Med. Chem. , 39, 1069-83.

[25] Spring, O, Bienert, U, & Klemt, V. (1987). Sesquiterpene lactones in glandular trichomes of sunflower leaves. Plant Physiol. , 130, 433-439.