ESCOLA BAHIANA DE MEDICINA E SAÚDE PÚBLICA

CURSO BIOMEDICINA

FERNANDO LUCAS SOARES GUERREIRO

DETERMINAÇÃO DA INIBIÇÃO DA ATIVIDADE DA ENZIMA ACETILCOLINESTERASE POR EXTRATOS DE *Physalis* sp. PARA TRATAMENTO DA DOENÇA DE ALZHEIMER

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Trabalho de Conclusão de Curso apresentado à Escola Bahiana de Medicina e Saúde Pública, como parte dos requisitos para obtenção do título de Bacharel em Biomedicina.

Orientador: Prof. Dr. Ademir Evangelista do Vale

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Este artigo científico foi julgado adequado à obtenção do grau de Bacharel em Biomedicina e aprovada em sua forma final pelo Curso de Biomedicina da Escola Bahiana de Medicina e Saúde Pública.

Salvador – BA, 09 de novembro de 2019.

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Prof. Dr. Ademir Evangelista do Vale

UFBA

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Prof. Dra. Léa Maria dos Santos Lopes Ferreira

Escola Bahiana Medicina de Saúde Pública

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Prof. Msc. Gildeon Silva Marques

UFBA

RESUMO

A Doença de Alzheimer é uma enfermidade neurodegenerativa que atinge principalmente os idosos, o tratamento desta doença tem o princípio da inibição da Acetilcolinesterase, e os principais medicamentos utilizados têm fortes efeitos colaterais que diminuem a qualidade de vida da população idosa. Com essa problemática, o objetivo do estudo foi encontrar alternativas terapêuticas para o tratamento da doença de Alzheimer, com os extratos metanólicos de *Physalis angulata* L., *Physalis peruviana* L. e *Physalis ixocarpa* Brot.. Após a experimentação, os resultados foram muito relevantes, devido ao fato de as três espécies terem apresentado bons resultados, com poucas exceções. Concluindo assim que mais estudos neste campo são essenciais para esclarecer os dados encontrados neste estudo.

Palavras-chave: *Physalis angulata*; *Physalis peruviana*; *Physalis ixocarpa*; Acetilcolinesterase; Inibição.

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SUMÁRIO

DETERMINATION OF INHIBITION OF THE ENZIME ACETYLCHOLINESTERASE BY EXTRACTS OF *Physalis spp*. FOR ALZHEIMER DISEASE TREATMENT

FERNANDO LUCAS SOARES GUERREIRO[1], ADEMIR EVANGELISTA DO VALE[2]

1 Course of Biomedicine, Bahiana - School of Medicine and Public Health, Salvador, Bahia, Brazil
2 Department of Pharmacy, Federal University of Bahia, Salvador, Bahia, Brazil

Abstract

Alzheimer's Disease is a neurodegenerative infirmity that mainly reaches the elder people, the treatment of this disease has the principle of Acetylcholinesterase inhibition and the main medications used have strong side effects that lower the quality of life of the elderly population. With this problematic, the objective of the study was finding therapeutic alternatives for treating Alzheimer's disease, with the *Physalis angulata* L., *Physalis peruviana* L., and *Physalis ixocarpa* Brot. methanolic extracts. After the experimentation the results were highly impressive, owing to the fact that the 3 species exhibit nice results with a few exceptions. Concluding that more studies in this field are essential to clarify the data founded on this study.

Keywords: *Physalis angulata*; *Physalis peruviana*; *Physalis ixocarpa*; Acetylcholinesterase; Inhibition.

Introduction

The Alzheimer Disease (AD) is a neurodegenerative infirmity, which has as main symptoms the loose of memory, the incapacity of learning new information, and psych disorientation [1]. In addition to be the leading cause of Dementia, accounting for 60-70% of cases of this syndrome [2].

Starting from the world scenario, it was estimated in 2017 that there were 50 million cases of Dementia and that each year approximately 10 million new cases appear, indicating an increasingly alarming scenario of the disease, mainly due to aging of the world population, which is a risk factor for AD [1,2].

Regarding the pathophysiology of AD, the disease is initiated by the formation of extracellular plaques in the synaptic clefts, which are constituted by a cluster of peptide beta-amyloid (Aβ) and hyperphosphorylated tangles of tau protein in the intracellular environment [3]. These factors lead to chronic inflammation of the nervous tissue and thus trigger the impairment of synapses, senescence of cholinergic neurons, and involvement of brain tissue. For being a chronic inflammation, this damage leads to the replacement of brain tissue by fibrotic tissue leading to loss of tissue function [4].

Regarding the pharmacological treatment of AD in Brazil, according to the Brazilian Alzheimer's Association (ABRAz), primarily three drugs (rivastigmine, donepezil, and galantamine) are used. These have an inhibitory action on the enzyme acetylcholinesterase. All these drugs bring an initial attenuation of symptoms, however, with the evolution of the disease, this improvement becomes increasingly smaller, besides these medications bring numerous side effects [5,6]. From this scenario, there is a large number of research investigating new ways of treating AD, among which there are some that seek the answer in herbal medicine, precisely to try to solve the problem of current medications that have a large number of adverse effects.[6].

With this problem in the treatment of AD, much research has been conducted to investigate alternative treatments using medicinal plants, including some with one or more of the 120 species of *Physalis spp.* which is part of the Solanaceae family, which is widely distributed in most tropical and subtropical areas. Several discoveries have already been made about the action of the groups of substances extracted from this vegetable, known as physalins, withanolides, and carotenoids. These groups of substances have great diversity in their actions, such as anti-inflammatory, antioxidant, and anti-proliferative actions on tumor cells.[7].

In addition to these beneficial effects of *Physalis* spp. substance groups, we have many other actions that have been shown to be effective and others that need further study, and we need to discuss the substances separately. Beginning with withanolides, these are steroids, they have liposolubility and are formed by a set of non-benzene carbon rings. This group gives rise to two types of substances, primarily physagulins and acnistinas. These elements have two actions that have been thoroughly studied, their trypanocidal action (*T. cruzi*) and their antitumor effect. [8] [9]. Starting with carotenoids, substances that pigment some fruits and vegetables, are formed by a long chain of carbons and hydrogens by two non-benzene rings at their ends. Regarding the action of this compound, there are studies that correlate the intake of carotenoids with the prevention of several chronic diseases, from its antioxidant effect [10].

Finally, we mention the physalins, it has many subtypes, but the most studied are the types B, D, E, F, G, and H. These subtypes have been widely studied and their functions are described, however, this does not mean that the others have not been evaluated, it just shows that there are many other subtypes that need to be determined from more in-depth studies, as well as physagulins and "acnistins" [9].

From this scenario presented, the aim of this study is to determine the inhibition of the enzyme acetylcholinesterase by extracts from *Physalis* spp. for prospecting new drugs to treat Alzheimer's disease. And thus, contribute to the knowledge of the biological potential of the species of *Physalis*.

Materials and Methods

Preparation of vegetable material

Physalis angulata L., *Physalis ixocarpa* Brot. and *Physalis peruviana* L. extracts were provided by the adult individuals that were collected in summer (November 2017) at the Horto Florestal Experimental Unit of the State University of Feira de Santana (UEFS), Bahia, Brazil. Specimens were harvested at physiological maturity. A voucher specimen was deposited in the Herbarium of UEFS (Voucher number 110448). The plant material was oven dried under air circulation (FANEM, model 320-SE) under an average temperature of 40° C for five days. They were then grounded in a Wyllie mill (TE-650 / Tecnal) and placed to macerate in glass recipient separately. 0.5 L MeOH was added to each well and after 96 hours the methanolic extract was filtered. The extract was then concentrated in the rotary evaporator apparatus (BUCHI-RII, SWITZERLAND) and stored in properly weighed glass vials.

The methanolic extracts, totaling 13 extracts, were obtained from different parts of the plant such as stem, root, leaf, fruit, and flower. One week after this process, the flasks were weighed again to determine the weight of the extract so that way the concentration of the extracts could be defined when diluted in 70% aqueous alcohol solution

Triage of the extracts

The methodology applied is an adaptation of Ellman's method (1961)[11]. Based on that the 13 extracts were diluted to a concentration of 1 mg/mL and tested in triplicate. Also, in triplicate were tested blank and positive control (Physostigmine 500µM/mL) to ensure the safety of the results. Thus, this step of the experiment used a 96-well plate. Each well contains 140µL phosphate buffer (PO4), 10µL Acetylcholinesterase (AChE) enzyme (0.5 U/mL), 20µL Ellman reagent (DNTB) (10 mM) and 20µL sample. At this time 0, the absorbance of the wells was measured with the aid of an ELISA reader with 405nm wavelength, after adding 10µL of acetylthiocholine iodide (ATCI) (15 mM) the read was made again. After these two measurements, the plate was incubated at room temperature for 10 minutes, and its absorbance was measured again on the ELISA reader at this new time. Thus, the plate was placed in these 10-minute cycles and had its absorbance measured at all these moments of the cycle until reaching the time of 60 minutes.

This experiment is based on the principle of DNTB reaction with thiocoline, which is the product of AChE with ATCI, which when in contact with thiocoline forms a yellow anion, this staining is measured by the ELISA reader in the form of absorbance.

To calculate the percentage of inhibition of extracts on AChE, the following formula was used:

$$\text{\% of inhibition} = \frac{\text{Blank} - (\text{Test Absorbance Average} - (\text{Test Absorbance without ATCI} - \text{Test Absorbance with ATCI})}{\text{Blank}} \text{ X } 100$$

At this stage, extracts that had inhibition percentage > 2% were selected to continue the study, thus, 5 extracts were excluded from the study. As described in Table 1.

Table 1: Extracts that was included and excluded of the study

Extract	*Physalis angulata* L.	*Physalis ixocarpa* Brot.	*Physalis peruviana* L.
Stem	Excluded	Included	Included
Root	Included	Included	Included
Leaf	Included	Excluded	Excluded
Fruit	Included	Excluded	Included
Flower	*	Excluded	*

Notes: *Those extracts were not available.

Acetylcholinesterase inhibition assay

To perform this step a new preparation of the vegetable material was made, just as it was used in the first topic of this methodology. The choice was taken based on their inhibition percentage on the "Triage of the extracts". Those with <2% inhibition percentage were excluded. However, in this step, only the 8 extracts approved on the triage were used. These 8 extracts were diluted in the 8 mg/mL, 4 mg/mL, and 2 mg/mL concentrations, and the methodology explained on the "Triage of the extracts" were performed again.

Statistical analysis

The graphics and linear regression were made using the GraphPad Prism software 8.2.1 (GraphPad Software). The values of R^2 varying between 0.75 and 1.0 and those with p value ≤ 0,05 were considered statistically significant. IC50 was estimated by means of a linear regression equation.

Results and Discussion

Evaluation of Acetylcholinesterase Inhibition

The data collected to plot the 3 graphics below were obtained in the mark of 50 minutes of the "Acetylcholinesterase inhibition assay" for all the 8 extracts selected. This time was chosen based on the peak of inhibition of the samples. Starting with the results presented in the extracts of the *Physalis angulata* L. (Figure 1). The leaf extract was the best sample, not only for the *Physalis angulata* L., but for all of the 8 tested in this study presenting 39,25% of inhibition and an IC50 of approximately 11,33 mg/ml, followed by the root extract which presented

27,05% of inhibition and an IC50 of 13,64 mg/ml. Lastly the fruit extract which presented 24,00% of inhibition and an IC50 of 20,62 mg/ml.

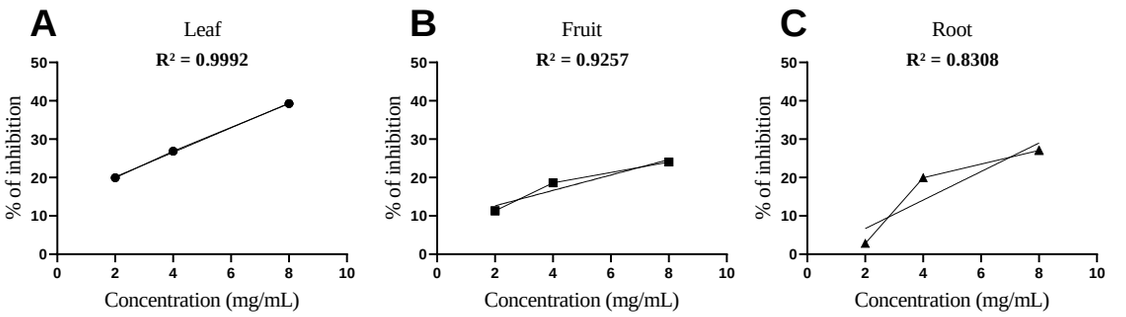

Figure 1: Inhibition percentage of AChE by *Physalis angulata* L. extracts in the concentrations 2 mg/mL, 4 mg/mL, and 8 mg/mL.

The other species analyzed in this study was the *Physalis peruviana* L., which presented great results in the experiments (Figure 2). Starting with the fruit extract that exhibited 25,27% of inhibition and an IC50 of 16,70 mg/mL, the root extract presented 21,15% of inhibition and an IC50 of 23,40 mg/mL, and the stem extract which presented 19,06% of inhibition and an IC50 of 20,18 mg/mL.

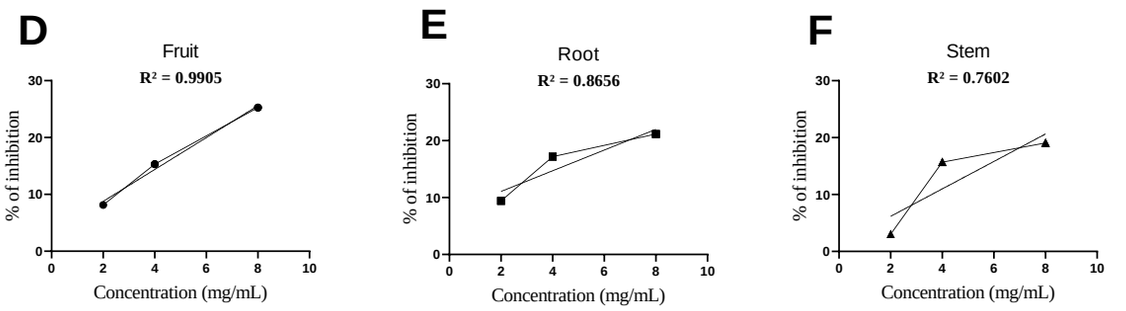

Figure 2: Inhibition percentage of AChE by *Physalis peruviana* L. extracts in the concentrations 2 mg/mL, 4 mg/mL, and 8 mg/mL.

The last plant tested were *Physalis ixocarpa* Brot. which unlike the other 2 species tested, this plant had only 2 extracts experimented (Figure 3). The stem extract presented 19,10% of inhibition and an IC50 of 26,32 mg/mL, and the root extract presented an inhibition of 17,10% with the minor concentration and 12,70% of inhibition with the major concentration, and the IC50 were not calculated owing to the fact that the linear regression equation of this extract was not valid, resulting in negative numbers. This event may occur due an operator error or due the state of the materials used.

Similar to many diseases, ancient writings describing the symptoms of AD also suggest herbal extracts, such as *Withania somnifera*¸ described in ancient Sanskrit from India as a promoter of

learning and memory recovery [12]. That is, herbal medicine of AD is not a current issue, especially for some of the Asian countries that have a legacy in the use of traditional medicine.

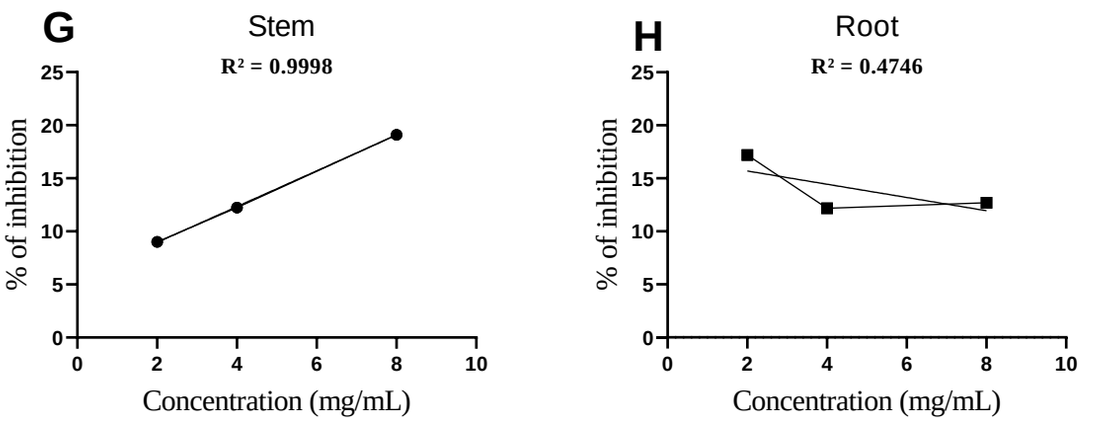

Figure 3: Inhibition percentage of AChE by *Physalis ixocarpa* Brot. extracts in the concentrations 2 mg/mL, 4 mg/mL, and 8 mg/mL.

However, few studies have demonstrated the anticholinesterase effect of these three species studied in the present study, however, one study found a substance called N-trans-p-Coumaroyltyramine in *Physalis minima* species that has several pharmacological actions, one of them being the inhibitory action on acetylcholinesterase [13]. In addition to the genus *Physalis*, studies have shown that ethanolic extracts of *Pimpinella anisoides* fruit have inhibitory action against AChE and presented an IC50 of 227.5 μg / ml [14].

The results presented by the 3 studied *Physalis* species (Table 2) were excellent for the most part. Starting with *Physalis angulata* L., this was the species that presented the most potent extract, the leaf extract. It presented the highest inhibition percentage, the smallest IC50 and had a value of R^2 very close to 1, which represents a high linearity. ($p < 0{,}0001$), could be a potent candidate for future studies. So far the main pharmacological actions of *Physalis angulata* L. are: antihistamine action, anti-asthmatic action, malaria treatment, hepatitis treatment, dermatitis treatment, rheumatism treatment, anti-mutagen, anticoagulant[15,16].

With this wide variety of pharmacological actions, we infer that the medicinal potential of this species is very promising, as presented in this study where all 3 extracts of the species showed statistically significant results, with $p \leq 0.05$.

The second species tested was *Physalis peruviana* L., which was shown to have potent extracts, such as its fruit extract, which is in agreement with the findings of Das; De, (2015). And like *Physalis angulata* L., this species also showed statistically significant findings in all its tested extracts.

The last species that were experimented, *Physalis ixocarpa* Brot., had only 2 extracts one of them presented to be promising, the stem extract, that presented an excellent R^2, IC50, and

inhibition percentage, further the *p* value <0,0001, representing a statically significant result. In counterpart the other extract, root extract, presented an inverted result owing to the fact that the minor concentration of 2 mg/mL had a higher percentage of inhibition than the major concentration of 8 mg/mL. To explain that event as discussed previously the most probable theory is an error linked to the execution of the experiment. This species has a lack of information in the literature, making this present study a pioneer testing this plant on an acetylcholinesterase inhibition assay. In other words, further studies are needed to enrich the discussion of this species.

Table 2: Main results presented by the extracts

Extract	I%(8mg/mL)	IC50(mg/mL)	R^2	*p* value
Physalis angulata L. Leaf	39,25	11,33	0,9992	< 0,0001*
Physalis angulata L. Fruit	24,00	20,62	0,9257	< 0,0001*
Physalis angulata L. Root	27,05	13,64	0,8308	0,0006*
Physalis peruviana L. Fruit	25,27	16,70	0,9905	< 0,0001*
Physalis peruviana L. Root	21,15	23,40	0,8656	0,0003*
Physalis peruviana L. Stem	19,06	20,18	0,7602	0,0022*
Physalis ixocarpa Brot. Stem	19,10	26,32	0,9998	< 0,0001*
Physalis ixocarpa Brot. Root	12,70	N.O.	0,4746	0,1301

Notes: 1. Relevant results presented by Prism software linear regression.

* $p \leq 0,05$/ N.O. Not Obtained

Conclusion

Based on all the data presented on this study is possible to infer that the 3 species demonstrated a high potential inhibiting the acetylcholinesterase with low concentrations. Then is required further studies in vivo and in vitro to evaluate toxicity, biodisponibility, and other properties of the herbal medicine presented in this paper.

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References

1. Syad AN, Shunmugiah KP, Kasi PD. Assessment of anticholinesterase activity of gelidiella acerosa: Implications for its therapeutic potential against alzheimer's disease. Evidence-based Complement Altern Med. 2012;2012.
2. World Health Organization. Dementia [Internet]. 2017 [cited 2019 Mar 23]. Available from: https://www.who.int/news-room/fact-sheets/detail/dementia
3. Jiang F, Gao P, Zhang Z, Yang D, Jin M, Liu X, et al. Optimization of Extraction Conditions for Flavonoids of Physalis alkekengi var. franchetii Stems by Response Surface Methodology and Inhibition of Acetylcholinesterase Activity. J Mex Chem Soc. 2018;59(1):59–66.
4. Martinez B, Peplow P V. Amelioration of Alzheimer ' s disease pathology and cognitive deficits by immunomodulatory agents in animal models of Alzheimer ' s disease. Neural Regenaration Res. 2014;
5. Associação Brasileira de Alzheimer. Tratamento [Internet]. 2019 [cited 2019 Apr 28]. Available from: http://abraz.org.br/web/sobre-alzheimer/tratamento/
6. Perry EK, Pickering AT, Wang WEIWEI, Houghton PJ. Medicinal Plants and Alzheimer ' s Disease : from Ethnobotany to Phytotherapy **. J Pharm Pharmacol. 1999;(Mann 1992):527–34.
7. Sun CP, Qiu CY, Yuan T, Nie XF, Sun HX, Zhang Q, et al. Antiproliferative and Anti-inflammatory Withanolides from Physalis angulata. J Nat Prod. 2016;79(6):1586–97.
8. Tomassini TCB, Barbi NS, Ribeiro IM, Xavier DCD. Gênero Physalis - uma revisão sobre vitaesteróides. Quim Nova. 2000;23(1):47–57.
9. Salgado RE, Arana G. Physalis angulata L. (Bolsa mullaca): A Review of Its Traditional Uses, Chemistry and Pharmacology. Bol Latinoam y del Caribe Plantas Med y Aromat. 2013;12(5):431–45.
10. Severo J, Lima CSM, Coelho MT, Rufatto ADR, Rombaldi CV, Silva JA. Atividade antioxidante e fitoquímicos em frutos de Physalis (Physalis peruviana , L .) durante o amadurecimento e o armazenamento. Rev Bras Agrociências. 2010;16(1–4):77–82.
11. Ellman GL, Courtney KD, Andres V, Featherstone RM. A new and rapid colorimetric determination of acetylcholinesterase activity. Biochem Pharmacol. 1961;7(2):88–95.
12. Houghton PJ, Howes MJ. Natural products and derivatives affecting neurotransmission relevant to Alzheimer's and Parkinson's disease. NeuroSignals. 2005;14(1–2):6–22.
13. Sudhamalla B, Gokara M, Ahalawat N, Amooru DG, Subramanyam R. Molecular dynamics simulation and binding studies of β-sitosterol with human serum albumin and its biological relevance. J Phys Chem B. 2010;114(27):9054–62.
14. Menichini F, Tundis R, Loizzo MR, Bonesi M, Marrelli M, Statti GA, et al. Acetylcholinesterase and butyrylcholinesterase inhibition of ethanolic extract and monoterpenes from Pimpinella anisoides V Brig. (Apiaceae). Fitoterapia [Internet]. 2009;80(5):297–300. Available from: http://dx.doi.org/10.1016/j.fitote.2009.03.008

15. Rathore C, Dutt KR, Sahu S, Deb L. Antiasthmatic activity of the methanolic extract of Physalis angulata linn. J Med Plant Res. 2011;5(22):5351–5.

16. T.K. Lim. Edible Medicinal and Non-Medicinal Plants. Vol. 6. 2013. 326–332 p.

17. Das S, De B. Acetylcholinesterase inhibitory potentiality of some minor fruits of West Bengal, India. J Pharm Bioallied Sci. 2015;0(0):0.

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For Patent Reference: Aviv H, Friedman K and Vered V. Submicron emulsions as ocular drug delivery vehicles. U.S. Patent US 5496811; 1996.