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Biological efficacy of Phyllanthus amarus Schumn. & Thonn. loaded into a nanoemulsion system on the in vitro culture of pig ovarian follicles

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Outline

Introduction

Phyllanthus (Euphorbiaceae) is a representative genus, native of the American continent, with more than 1000 specimens found in tropical and subtropical countries of the world. In Brazil, this genus is represented by over than 100 plants largely distributed all over the brazilian territories. The specimens of this genus shows medicinal properties such as: diuretic, antidiarrheal, antispasmodic, antiviral, antipyretic, antinociceptive, anti-inflammatory, antidiabetic, antitumoral, antigenotoxic, antimutagenic, hepatoprotection, hypoglycaemic, hypotensive, bactericide, and antioxidant. It is also efficient against jaundice and AIDS [1-5].
Focusing on the genetic diversity of *Phyllanthus* species and its morphological similarities, Sarin et al. (2014), described the importance of the plant genus identification along with pharmacological and phytochemical studies, as well as appropriate protocols for correct identification of species [3].
Specifically, for *Phyllanthus amarus* Schum. & Thorn. and *P. niruri* L. they have been largely published as synonymous because of their morphological similarities. However, in the beginning of this century botanic studies were intensified by using molecular markers for identification of unambiguously *Phyllanthus* species and to indicate the significant differences between the specimens of this genus. Systematic studies at molecular level have been assessed for the *Phyllanthus* genus with high efficient results aiming at acute authentication of plant specimen by using different markers-type linked to a dominant gene. Indeed, there are many differences between *P. amarus* and *P. niruri,* which were found to be in base, seed and stigma type [6]. These herbal have been frequent targets of ethnopharmacological work and are well known since ancient times (about 3000 years). With respect to phytocomponents of *P. amarus*, it is proved that the lignans phyllanthin and hypophyllanthin are the main biomarkers [2, 5, 7]. Phytochemical investigations were conducted with an unambiguous identified *P. amarus* collected from Brazil. Wherein the ethanolic extract, revealed the presence of six bioactive lignans (phyllanthin, hypophyllanthin, isolintetralin, demethylenedioxy-niranthin, 5-demethoxy-niranthin, niranthin) and one triterpene (2*Z*, 6*Z*, 10*Z*, 14*E*, 18*E*, 22*E*-farnesil-farnesol) [2, 7].
Today's current state of *P. amarus* is supported by its scientific findings and its largely applications, of which mainly focusing in the biotechnology field to face the new challenges for this lead medicinal plant. In this sense, the recent progress in the multiple aspects of *Phyllanthus´s* species development have been reviewed based on botanic, phytochemistry and pharmacological aspects as a powerful tool to improve biotechnological researches [2, 4, 5, 7, 8-14]**.**
In view of the medicinal importance of the herbal *P. amarus*, in the present study, a nanoemulsified system was used aiming at to load the hydroalcoholic extract obtained from this plant. The therapeutic effectiveness of the *P. amarus* colloidal-type formulation was confirmed by using *in vitro* experiments applied on antioxidant tests, cell viability and as a medium of ovarian follicular culture for porcine species.

Biological General Comments on Follicular Culture

Concerning to the ovarian culture, it is necessary to know the follicular phases and also the mechanisms involved in folliculogenesis [15]. Specifically, the ovarian follicle is the morphofunctional unit of the mammalian ovary, having two essential functions: endocrine and gametogenic. The endocrine function occurs through the release of estradiol and progesterone and the gametogenic function by the release of the oocyte of the female gamete. The follicle is composed of an oocyte (female germ cell) surrounded by granulosa (a single layer of support cells) and theca cells (additional support cells), having the function of providing an ideal environment for

maturation, viability, growth and release of a mature oocyte in the process of ovulation [16]. In fact, the follicular growth and development can be characterised through proliferation of the granulosa and also by the proliferation of additional support cells and the primordial follicles represent the ovarian pool, being represented by 95% of the preantral follicles, which make up a total of 90% of the total follicular population and antral follicles only 10% [17].

Depending on the stage of development, follicles can be classified as preantral (primordial, primary and secondary) and antral (tertiary and pre-ovulatory). Indeed, the follicular development and growth is a complex mechanism regulated by the interaction of hormones, growth factors and peptides. So, the follicular development from primordial to pre-ovulatory follicle is known as folliculogenesis event, that is characterized by the proliferation and differentiation of granulosa and theca cells and also by the increasing in oocytes. So, ensuring oocyte developmental competence it is necessary a specific medium containing hormones, growth factors, peptides, antioxidants, antibiotics, among others components [17-21].

According to Kidder and Vanderhyden [22] the female fertility process is determined by the quality of the oocyte development, which reflect its ability to undergo meiosis, be fertilized, and give rise to a healthy embryo. The growth of the mammalian oocyte is coordinated with follicle cells and this bi-directional communication has been considered as a critical process for ensuring oocyte developmental competence, which operate within the oocyte-granulosa cell complex to govern oocyte and follicle growth and its differentiation [22,23]. Hence, a fully-grown antral follicle ready for ovulation is called a pre-ovulatory or Graafian follicle. After ovulation, the follicle remnant develops into a highly vascularised endocrine organ, the corpus luteum, and the primary function from which progesterone is produce to support the ensuing pregnancy [22,23]. Briefly, ovarian folliculogenesis, oogenesis, and ovulation are regulated by hormones (gonadotropins) emanating from the pituitary gland [22, 24]. Additionally, it has been shown that the gonadotropin action on the ovary is mediated by intra-ovarian signals provided by multiple families of paracrine factors as well as by direct cell-cell communication via gap junction channels [22, 25-28].

Historically, different techniques have been applied to determine the level of apoptosis and recently there arises the question of whether apoptosis is an accurate measure for the interpretation of oocyte quality. Finally, it seems that apoptosis event within the granulosa cells is an integral part of the oocyte development and has limited predictive capability regarding its quality or the ensuing pregnancy rate in the *in vitro* fertilization process [29].

Application of natural resources on *in vitro* follicular culture

In vitro follicular culture, also known as “artificial ovary”, allows to improve the basic knowledge about the mechanisms involved in the ovarian folliculogenesis process. This *in vitro* biotechnology approach allows to test the beneficial or toxic action of drugs on ovarian follicles, before their being used in experiments involving animals and humans. In this sense, pig ovarian tissue has been widely used in order to verify the effect of different substances on follicular development, given the similarity of the ovaries of this species with those of the human species [17].

In order to maintain viability and promote follicle growth, different *in vitro* culture methods have been developed. The effectiveness of a culture system is due to the addition of factors that promote the growth of oocytes that become suitable for the manufacture of *in vitro* embryos. The success of follicular culture may vary according to the species under study along with the size of the follicle and also the culture medium composition [30-35]. In this sense, there are available several commercial means for the culture of ovarian follicles, but the ones that stand out in the literature are α-MEM (Minimum Essential Medium) and TCM-199 (tissue culture medium-199) [35]. It is

worth mentioning that a strongly relevant aspect in the use of an *in vitro* system for the growth of ovarian follicles consist on the cellular medium composition. So, aiming at to ensure the maintenance of viability and *in vitro* follicular growth, different mediums containing electrolytes, antibiotics, hormones, buffers, antioxidant, nutritional, energetic substrates such as lipids, proteins, amino acids, nucleic acids, vitamins, monosaccharides, have been successfully applied [17, 34, 36-40].

Meanwhile, although they are effective as culture medium, the commercial costs are very high, limiting the broad development of scientific research. For this reason, herbal products have been investigated as an alternative *in vitro* cell culture medium, applied for follicular development [19, 41, 42].

Actually, studies with phytotherapeutic components applied as a media of follicular culture are in the initial phase, but it is possible to announce that most results are promising. Highlighting some examples: **i)** coconut water solution (CWS) was evaluated as a medium of culture throughout *in vitro* experiments for the survival and growth of ovarian follicles. Different concentrations (25, 50, 75 or 100%) of CWS were assayed aiming at sequencing the research for animal reproduction. The findings showed that follicular degeneration was kept at the same level after culture in the CWS media, except for CWS in its higher content (100%) that increased the number of degenerated follicles. Meanwhile, addition of supplements to CWS culture media decrease follicular degeneration [19]; **ii)** *Anburana cearensis* extract evaluated in a non-encapsulated approach, as follicular preservation medium, resulted in an excellent viability rates, due to the presence of antioxidant agents of this plant [43]; **iii)** reinforcing this result *Amburana cearensis* extract added by supplements and also FSH (a follicle stimulating hormone gonadotrophic type) improves follicular growth, and can be an alternative culture medium for the development of the goat's preantral follicle [44]; **iv)** different concentrations of saffron (*Crocus sativus L.*) aqueous extract (SAE), were evaluated as an antioxidant agent on IVM of immature mouse oocytes. The findings showed that the maturation rate was significantly higher in all groups treated with different concentrations of SAE compared with the control group. However, when compared with other experimental and control groups, SAE improves oocyte maturation and embryo development at lower concentrations (10 µg/mL and 5 µg/mL) [45]; **v)** *Auxemma oncocalyx* and its major isolated component oncocalyxone A (onco A) were studied on the action of caprine folliculogenesis. Both *A. oncocalyx* and onco A in a concentration-dependent manner, decreased the number of morphologically normal follicles, with no effect on follicular growth. Comparatively, *A. oncocalyx* reduced the percentage of normal follicles more than onco A. So, in a concentration-dependent manner they negatively affect *in vitro* culture of goat ovarian follicles, and are therefore considered toxic for *in vitro* follicular survival and development [46]; **vi)** the effect of hydroalcoholic extract of *Matricaria chamomilla* (25 and 50 µg/mL) applied on preantral follicle culture of mouse ovaries in a three-dimensional culture was analyzed by adding chamomile extract to culture media appeared to decrease follicular function and development. Comparatively, oxygen species levels of metaphase II oocytes were significantly decreased in the lower content of the planta extract and the levels of progesterone and dehydroepiandrosterone hormones significantly increased in the medium added with 50 mg/mL of chamomile extract [47].

Material and Methods

Phytochemical Procedures

Plant material

Phyllanthus amarus was collected in Vila Naval, Alecrim; located at Natal, the capital city of the state Rio Grande do Norte (Brazil). This place is completely preserved from environmental pollution and pesticide contamination. The plant identification was executed by Professor Doctor Maria Iracema Bezerra Loiola (UFRN) and an exsicate (reference no. 1645) is kept in the herbarium of the Botany Department, Ecology and Zoology (UFRN).
The ^{13}C-NMR spectra were recorded in Varian Gemini spectrometer, at 75 MHz. The signal of the solvent, which was employed to solubilize the samples, was used as a reference pattern. Sigma and Merck deuterated solvents were used in NMR spectroscopy. Merck silica gel (230-280 mesh) and, Queel and Vetec, Brazil, solvents (hexane and ethyl acetate) were utilized for the chromatographic column separations. The TLC was realized with Vetec, Brazil, silica gel 60H, chloroform, hexane and ethyl acetate. TCL revelation procedure was performed with sulphuric acid and methanol (1:1), according previously procedure [2,7]. All chemicals utilized in this study were of analytical grade.

Chromatography analysis

The vegetal material (roots and the aerial parts of the plant) was chopped and ground after it had been dried in a heater at 40 ^{o}C. The percolation extraction of the crude vegetal (124.5 g of the whole plant) was carried out with a hydroalcoholic solvent (ethanol:watter, 8:2). Several extractions were performed until the greenish colour disappeared. The filtrate was evaporated at reduced pressure, thus obtaining 16.96 g (13.6%) of the hydroalcoholic extract. The chromatographic procedure was realized with 1.00 g of extract, attaining a 43 fraction total, eluted with a mixture of hexane:EtOAc in polarity gradient (99:1 - 0:100). The triterpene 2Z, 6Z, 10Z, 14E, 18E, 22E-farnesil farnesol compound was identified in the F_{11-16} fraction group [eluted with hexane: EtOAc (99:1 to 97:3)]. Lignans were identified from F_{36} to F_{40} fractions [eluted with hexane:EtOAc (70:30 to 50:50)] as well as from the F_{41-43} group (eluted with EtOAc 100%), according previously procedure [2, 7].

Biological Assays

Antioxidant activity

The *in vitro* antioxidant assays applied to the NE-PA formulation were performed by ions ($FeSO_4$ and $CuSO_4$) chelating ability, according to the methodologies previously described [48-51].

Ferrous ion chelating activity

The ferrous ion chelating ability of samples was investigated according to previous studies. Briefly, the reaction mixture, containing samples of $FeCl_2$ (0.05 mL, 2 mM) and ferrozine (0.2 mL, 5 mM), was well shaken and incubated for 10 min at room temperature. The absorbance of the mixture was measured at 562 nm against a blank.

Copper ion chelating activity

The ability to chelate the copper ion was investigated according to previous studies. Pyrocatechol violet, the reagent used in this assay, has the ability to associate with certain cations such as aluminum, copper, bismuth, and thorium. In the presence of chelating agents this combination is not formed, resulting in decreased staining. This reduction thus allows estimating the chelating activity of the copper ion from the *P. amarus* nanoemulsion formulation (NE-PA). The test is performed in 96-well microplates with a reaction mixture containing 2 mg/mL and 5 mg/mL of sample concentration, pyrocatechol violet (4 mM), and copper II sulfate pentahydrate (50 mg/mL). All wells were homogenized with the aid of a micropipette and the solution absorbance was measured at 632 nm. The ability of the samples in chelating the ferrous or copper ion was expressed as the percentage of chelating effect calculating by using the following equation:

$$\% \text{ ion chelating effect} = ([A_{control} - A_{sample}]/A_{control}) \times 100$$

where:
$A_{control}$: absorbance of the control tube (EDTA as the positive control) and
A_{sample}: absorbance of the sample tube.

In vitro culture of pig ovarian follicles

The ovaries (n=10) of adult female pigs crossbreed were used from local slaughterhouses. Immediately after slaughter, the ovaries were collected, washed with alcohol (70%) for 10s and transported to the laboratory into the Minimum Essential Medium (MEM) supplemented with 100 µg/mL penicillin and 100 µg/mL streptomycin plus a commercial buffer (HEPES) at lower temperature (4 ºC), according to the methodologies previously described [17, 20, 21, 28, 34, 37, 38].

In vitro follicular culture

All substances used in the cultivation came from Sigma Chemical Co. (St. Louis, MO, USA). The isolated follicles were grown in a modified α-MEM medium composed of: α-MEM supplemented with 50 ng/mL ascorbic acid, 2 mM glutamine, 2 mM hypoxanthine, 10 ng/mL insulin, 5.5 mg/mL of transferrin, 5 ng/mL of selenium, 1000 ng/mL of FSH and antibiotics (penicillin and streptomycin) (αMEM^{+}), according to the methodologies previously described [17, 20, 21, 28, 34, 37, 38].

Experimental design

Porcine ovarian follicles preantral and initial antral with diameters varying between 150 and 600 µm, were cultured for 4 days, in modified αMEM (αMEM^{+}) or only with the hydroalcoholic extract of *Phyllanthus amarus* Schum. & Thonn loading in the nanoemulsion type system (NE-PA), in low concentrations (2 mg/mL and 5 mg/mL), added with follicle stimulating hormone (FSH) [17, 20, 21, 28, 34, 37, 38].

Isolation and in vitro culture of ovarian follicles

Once collected, the ovaries were carefully dissected, and small sections of the ovarian cortex (1-2 mm) were removed and then transferred to the manipulation medium. For isolation of preantral

(secondary> 150 µm) and initial (tertiary <600 µm) ovarian follicles, fragments of the ovarian cortex were observed under a stereomicroscope (SMZ 645 Nikon, Tokyo, Japan) and once located, the follicles were manually isolated with the aid of 26G needles attached to the syringes. In the experimental sequence, the follicles were transferred to a new manipulation medium for evaluation. Only the follicles with spherical oocyte, surrounded by compact granulosa cells and with no apparent damage to the basement membrane, were selected for the culture. After that, the follicles were randomly distributed in the base culture medium (cultured control) or in the herbal tested medium (NE-PA). The follicles were individually grown in drops of 100 µL under mineral oil for the αMEM treatment (cultured control) and 200 µL in the absence of mineral oil for the treatment follicles by using the NE-PA, in two different concentrations (2 mg / mL and 5 mg / mL). The partial change of the medium was done every 2 days. The cultivation lasted for 4 days and was carried out in a humid atmosphere under CO_2 (5%) and controlled temperature (39 ° C) [17, 20, 21, 28, 34, 37, 38].

Evaluation of follicular viability with fluorescent markers

The isolated ovarian follicles were analyzed using a viability test based on the simultaneous determination of living and dead cells by the fluorescent markers calcein-AM and ethidium homodimer-1, respectively. While the first compound detects the activity of intracellular viable cell esterases, the last one marks nucleic acids from non-viable cells with plasma membrane damage. The test was performed by adding 4 µM calcein-AM, 2 µM homodimer ethidium-1 (Molecular Probes, Invitrogen, Karlsruhe, Germany) and glutaraldehyde to the follicles suspension, followed by incubation at 39 ° C for 30 minutes. The analysis was then carried out using a fluorescence microscope (Nikon, Japan) equipped with an image analysis system composed with: DS-U2 module, DS-Ri1 digital camera and NIS-elements software. Oocytes and granulosa cells were considered alive if the cytoplasm was marked by calcein-AM (green) and the chromatin was not marked by homodimer-ethidium-1 (red) [17, 20, 21, 28, 34, 37, 38].

Evaluation of follicular diameter

The follicular diameter was evaluated at both beginning and the end of the culture, with the aid of a micrometric eyepiece inserted in a stereoscope (Nikon SMZ 645, in Tokyo, Japan) (100x). The diameter of the oocytes was measured at the end of the culture period. Both diameters were measured and calculated from the basement membrane, at a right angle (90º) in the largest cross section of each oocyte and growing follicle. Two perpendicular diameters were recorded for each structure to be measured and the average of these two values constituted the diameters of the follicle or oocyte. The formation of the antral cavity was characterized by the appearance of a translucent cavity visible between the granulosa cells and oocyte extrusion rates were also recorded. Regarding follicular growth, the average increase in follicular diameter was calculated as follows: diameter of viable follicles after 4 days of culture minus the diameter of viable follicles on the fourth day [17, 20, 21, 28, 34, 37, 38].

Statistical analysis

Data on continuous variables (follicular diameter) were evaluated for homoscedasticity and homogeneity (Bartlett and Shapiro - Wilk tests, respectively) to confirm ANOVA requirements. Then, the Student's - Newman – Keuls test (SNK) was used with the results expressed as mean ±

standard error (SE). Discrete variables (follicular integrity and viability) were analyzed by using the chi-square test and the results expressed as a percentage, according to the methodologies previously described [17, 20, 21, 28, 34, 37, 38].

Results and Discussion

Herbal Approach

The plants used by different ethnic groups to treat diseases are correlated with specific factors, such as the ease of obtaining the drug, low cost and proven therapeutic efficiency. In Brazil, this set of benefits, linked to scientific validations, made it possible to include some herbal medicines in the public health program of the Unified Health System (SUS). Among which are species of the genus *Phyllanthus* (*P. amarus, P.niruri, P. tenellus and P. urinaria*). However, most medicinal plants are still used with an empirical basis for their medicinal properties. In this context, it is known that 80% of people worldwide relying on herbal uses for primary healthcare. Meanwhile, the improper use of medicinal plants can cause harmful effects to human health, which includes serious cases of hepatitis due to the abusive use of plants. So, scientific concerns surrounding their safety have been strongly recognized. Although many of them remain untested and their use are either poorly monitored or not even monitored at all [52-57].

This is not the case of *Phyllanthus* genera which specimens have been frequent targets of ethnopharmacological studies over the world, mainly in Brazil, the U.S.A, Malaysia, Cuba, Peru, Caribbean, China, Nigeria, Africa and India [1-5, 58-63]. Relying on scientific results for the herbal *P. amarus,* wherein highlighting its safety uses and some therapeutic properties such as bactericide, antiviral, antioxidant, immunosuppressive, hepatoprotector, anti-inflammatory, antigenotoxic, and antimutagenic [1-5, 58-85] in the present study, a colloidal nanoemulsified system was assessed to load the hydroalcoholic extract of P. amarus aiming at validate its therapeutic effectiveness as a medium of ovarian follicular culture.

Hence, the phytochemical study realized for the *Phyllanthus amarus* Schum. & Thorn. hydroalcoholic extract enabled through NMR analysis, the identification of the terpene 2Z, 6Z, 10Z, 14E, 18E, 22E-farnesil farnesol and bioactive lignans such as: isolintetralin (2,3-demethoxy-*seco*-isolintetralin diacetate), demethylenedioxy-niranthin, 5-demethoxy-niranthin, niranthin, phyllanthin and hypophyllanthin (Figure 2.1), which are reported as antioxidant, anti-inflammatory and hepatoprotector agents [4, 12, 85-90]. The spectroscopic data of the identified *P. amarus* biomarkers terpenoid and lignans (Figure 2.1) described below, are in accordance with the literature [2, 7, 89,90].

Terpenoid compound ^{13}C-NMR ($CDCl_3$) characterization

2*Z*, 6*Z*, 10*Z*, 14*E*, 18*E*, 22*E*-farnesil farnesol (shortly named farnesil farnesol): δ: 4.06 (H-1), 5.40 (H-2), 2.02 (H-4), 2.05 (H-5), 5.07 (H-6), 2.02 (H-8), 2.05 (H-9), 5.07 (H-10), 2.02 (H-12), 2.05 (H-13), 5.07 (H-14), 2.05 (H-16), 2.02 (H-17), 5.07 (H-18), 2.05 (H-20), 2.02 (H-21), 5.07 (H-22), 1.66 (H-24), 1.57 (H-25), 1.57 (H-26), 1.57 (H-27), 1.66 (H-28), 1.66 (H-29), 1.73 (H-30). ^{13}C-NMR ($CDCl_3$) δ: 59.18 (C-1), 125.19 (C-2), 139.98 (C-3), 32.11 (C-4), 26.57 (C-5), 125.10 (C-6), 135.54 (C-7), 26.57 (C-9), 125.10 (C-10), 135.54 (C-11), 32.11 (C-12), 26.57 (C-13), 124.57 (C-14), 135.41 (C-15), 39.54 (C-16), 29.84 (C-17), 124.42 (C-18), 135.06 (C-19), 39.54 (C-20), 26.93 (C-21), 124.30 (C-22), 135.06 (C-23), 25.70 (C-24), 16.18 (C-25), 14.30 (C-26), 14.47 (C-27), 22.88 (C-28), 22.88 (C-29), 22.88 (C-30).

Lignan compounds ^{13}C-NMR ($CDCl_3$) characterization

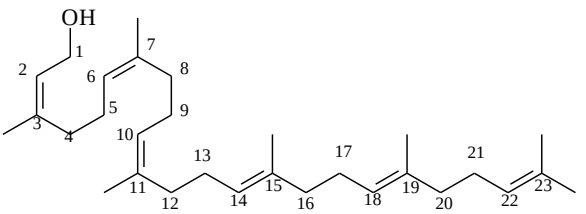
2,3-Demethoxy-*seco*-isolintetralin diacetate (shortly named isolintetralin): δ: 133.69, 132.14 (C-1', C-1), 112.23, 110.87 (C-2', C-2), 147.18, 147.01 (C-3', C-3), 148.90, 148.78 (C-4', C-4), 112.23, 111.03 (C-5', C-5), 121.91, 121.18 (C-6', C-6), 35.03, 35.54 (C-7', C-7), 40.79 (C-8', C-8), 55.84, 55.93 (O$\underline{C}H_3$-Ar), 101.31 (O-$\underline{C}H_2$-O).
5-Demethoxy-niranthin: δ: 133.69 (C-1'), 135.77 (C-1), 112.23 (C-2'), 110.87 (C-2), 148.90 (C-3'), 147.18 (C-3), 147.01 (C-4'), 111.03 (C-5'), 108.12 (C-5), 121.18 (C-6'), 121.91 (C-6), 35.03 (C-7'), 35.54 (C-7), 42.00 (C-8'), 40.79 (C-8), 59.05, 58.89 (O$\underline{C}H_3$-alquil), 56.56, 56.48 (O$\underline{C}H_3$-Ar), 101.23 (O-$\underline{C}H_2$-O).

Niranthin: δ: 135.77 (C-1), 101.31 (C-2), 133.69 (C-4), 148.78 (C-5), 108.16 (C-6), 35.03 (C-7), 40.79 (C-8), 133.69 (C-1'), 112.23 (C-2'), 147.18 (C-3'), 148.90 (C-4'), 111.03 (C-5'), 121.18 (C-6'), 35.03 (C-7'), 40.79 (C-8'), 72.72 ($\underline{C}H_2$-OMe), 101.23 (O$\underline{C}H_2$O), 58.89 (O$\underline{C}H_3$-alquil), 55.99, 55.93, 55.84 (O$\underline{C}H_3$-Ar).

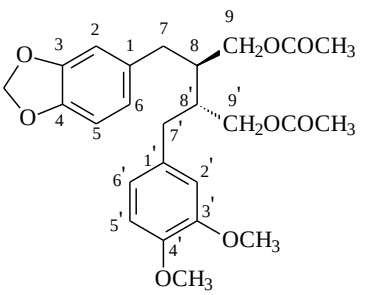
Demethylenedioxy-niranthin: δ: 135.77 (C-1), 108.12 (C-2), 148.78 (C-3), 148.90 (C-4), 148.78 (C-5), 106.55 (C-6), 37.75 (C-7), 40.79 (C-8), 133.69 (C-1'), 112.23 (C-2'), 147.18 (C-3'), 147.01 (C-4'), 111.03 (C-5'), 121.91 (C-6'), 35.03(C-7'), 40.79 (C-8'), 71.36, 72.72 (C-9 and C-9'-$\underline{C}H_2$-OCH$_3$), 58.89, 55.99 (O$\underline{C}H_3$-alquil), 55.93, 55.84 (O$\underline{C}H_3$-Ar).

Phyllanthin: δ: 133.75 (C-1, C-1'), 112.27 (C-2, C-2'), 148.85 (C-3, C-3'), 147.08 (C-4, C-4'), 111.07 (C-5, C-5'), 121.25 (C-6, C-6'), 35.11 (C-7, C-7'), 40.88 (C-8, C-8'), 72.78 (C-9), 72.66 (C-9'), 55.92, 56.00 (O$\underline{C}H_3$-Ar), 58.98 (O$\underline{C}H_3$-9, 9').

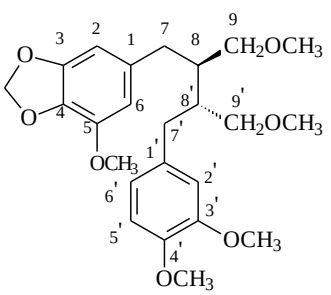
Hypophyllanthin: δ: 131.96 (C-1); 138.15 (C-1'); 106.55 (C-2); 111.84 (C-2'); 143.52 (C-3); 148.85 (C-3'); 133.75 (C-4); 147.08 (C-4'); 147.08 (C-5); 110.75 (C-5'); 115.25 (C-6); 120.60 (C-6'); 33.30 (C-7); 42.08 (C-7'); 35.62 (C-8); 45.59 (C-8'); 75.47 (C-9, $\underline{C}H_2$-OCH$_3$); 71.87 (C-9'); 56.63; 55.92 (O$\underline{C}H_3$-Ar); 58.98; 59.12 (O$\underline{C}H_3$-9; 9'); 101.33 (O-$\underline{C}H_2$-O).



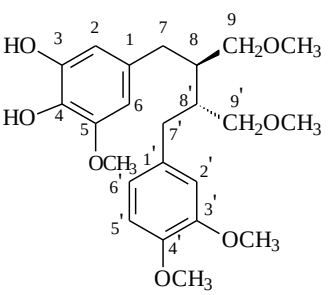
Farnesil farnesol



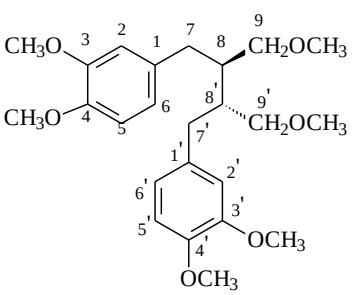
Isolintetralin



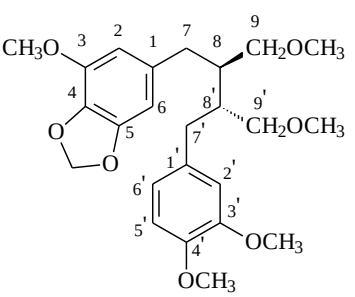
Niranthin



Demethylenedioxy-niranthin



Phyllanthin



Hypophyllanthin

FIGURE 2.1
Chemical structures of some *Phyllanthus amarus* bioactive constituents.

Colloidal System Approach

The nanoemulsion system (NE) was prepared according to the previously described methodology, by using as sufactant Tween 80 (preferential content ranging from 9% to 15%), and as organic phase a vegetal oil, widely used in human feed (ranging from 0,5% to 2%), under a neutral aqueous medium (bidistilled water, in a preferential content ranging from 90,5% to 83,0%). The applied procedure to obtain the NE-type system required a biocompatible mixture of surfactant/phase oil affording a o/w colloidal systems without co-surfactant need. Specifically, to prepare the NE-carrier precise amounts of oil phase and surfactant were mixed together using a magnetic bar at moderate temperature and speed (on a magnetic stirring plate), followed by addition of water. Both phases were gradually heated (55 °C to 65 °C) and during the water addition (20 minutes for each 10 mL of solution) the highest temperature was maintained [91]. The phase diagrams (Figure 2.2) were obtained from the titration with bidistilled water and the oil phase of predetermined mixtures.

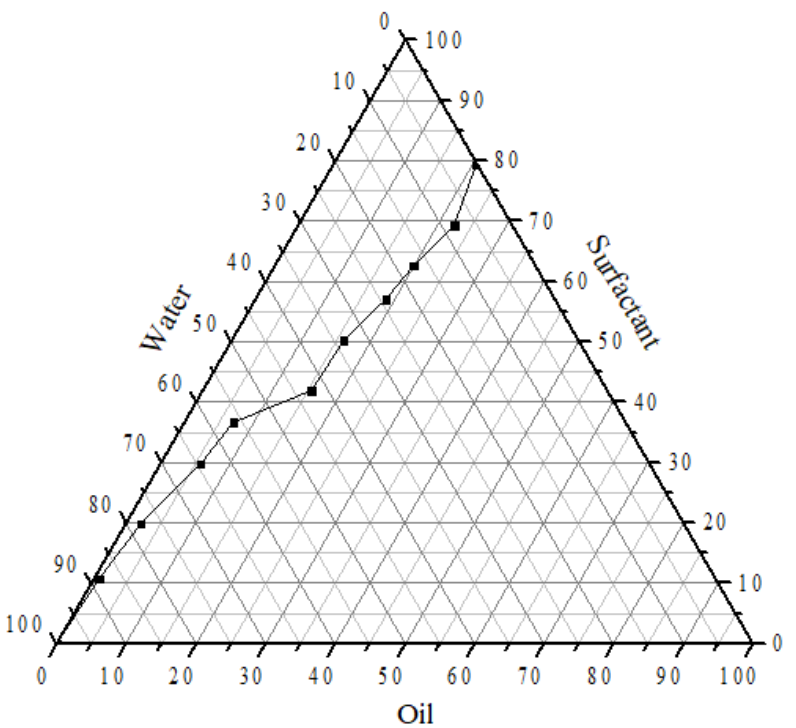


FIGURE 2.2
Ternary phases diagram of the NE-PA nanoemulsion system.

Sequentially, the *P. amarus* hydroalcoholic extract in two different content (2 mg/mL and 5 mg/mL) were solubilized in this carrier colloidal system and after its satisfactorily loading, and then was so called NE-PA formulation (or NE-PA colloidal system among other similar options). The obtainable NE-PA single-phase o/w-type nanoemulsion system remained isotopically stable after centrifuge procedure (3500 rpm) as well as by water dilution (upon thirty dilutions) ensuring no phase change. Indeed, the biotechnological *P. amarus* colloidal based formulation (NE-PA) is already protected under a patent request (BR102017012286-7B1).

Shortly, the physicochemical characterization of the NE-carrier without incorporation of the *P. amarus* hydroalcoholic extract, showed: **i)** c.m.c. (critical micellar concentration, with experimental value ranging from 6,0 x 10^{-3} g/mL to 9,0 x 10^{-3} g/mL); **ii)** when the interface is saturated with the surfactant molecules self-assembling are formed and the surface tension (ranging from 4 x 10^{-2} N/m to 6 x 10^{-2} N/m) reached its minimum value at the c.m.c. and remains constant above it; **iii)** viscosity with experimental value ranging from 2 x 10^{-3} N·s/m² to 4 x 10^{-3} N·s/m²); **iv)** reached strongly small droplet size (ranging from 5 nm - 18 nm). So, the biotechnological *P. amarus* colloidal based formulation (NE-PA) is already protected under a patent request [92].
Generally, nanoemulsion systems shows high kinetic stability, large specific surface area and become inherently unstable at the oil-water interface such as lipid oxidation, but do not scatter light strongly, hence are highly stable to gravitational separation and droplet aggregation and can be designed as metastable dispersions systems and may be used to extend the shelf life of commercial products [91-95]. However, this rule cannot strictly be applied for systems containing antioxidant stabilizers such as the antioxidant compounds (lignans) presented in the polar extract (hydroalcoholic extraction) of the herbal *Phyllanthus amarus*. So, the NE-PA nanoemulsion systems is a metastable dispersion of nanodroplets of one liquid (Tween 80® and the oil phase) into a large water medium.

Biological Assays

Antioxidant analysis

The higher NE-PA nanoemulsion antioxidant effect (Table 2.1) is correlated with the biocompounds present in the *P. amarus* hydroalcoholic extract as well as with the nutrients of the oil phase. In fact, the drug action was facilitated which the low dielectric constant of the nanoemulsion system, as well as its interfacial region (intermediate dielectric constant between oil and water), and the aqueous phase (high dielectric constant), which justify the highest efficiency of the NE-PA formulation.

TABLE 2.1
Antioxidant activity percentage of *Phyllanthus amarus* NE-PA formulation.

Nanoemulsion Formulation	Ferrous ion chelating activity (%)	Copper ion chelating activity (%)
NE-PA	23.43 ± 2.36	55.59 ± 0.02

The *Phyllanthus amarus* Schumn. & Thonn. hydroalcoholic extract carried by the NE-nanoemulsion system showed efficacy on its antioxidant activity, that indicated the higher availability of its antioxidant active constituents, and the foremost importance of the NE-PA formulation, particularly consist in a colloidal oil/water type system without the presence of co-surfactant

component. Corroborating with this finding different extract type obtained from *P. amarus* showed to be antioxidant and reinforce this herbal importance in the prevention of different diseases [96-100].

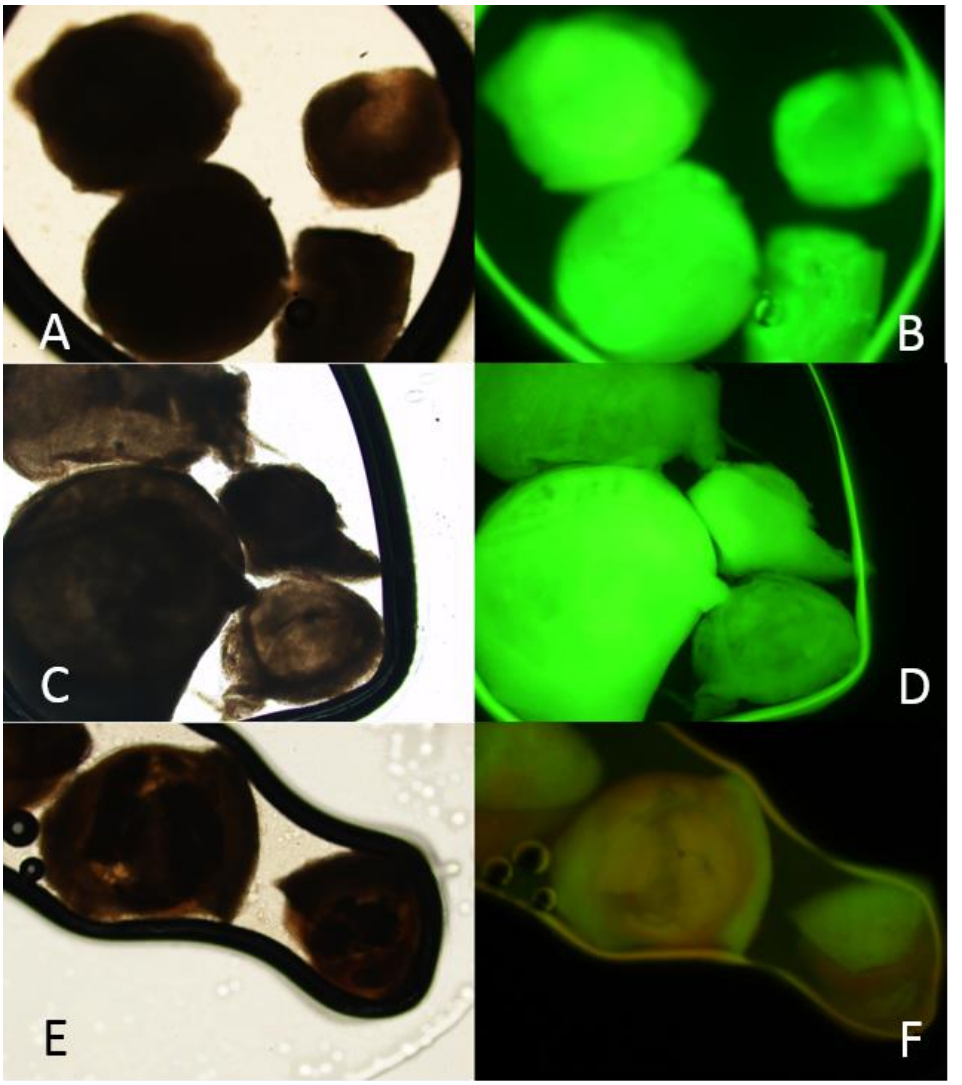
Follicular Viability

TABLE 2.2
Percentage of follicular integrity, viability and follicular diameter of porcine ovarian follicles, after 4 days of culture.

Treatments	Integrity (%)		Viability (%)	Follicular diameter (μm)	
	Day 0	Day 4	Day 4	Day 0	Day 4
α-MEM^{+}- Control+	100.00 ^{Aa}	86.6 ^{Ab}	83.3 ^{A}	243.3 ± 16.2 ^{Aa}	308.3 ± 42.1 ^{Ab}
NE	100.00 ^{Aa}	83.3 ^{Ab}	76.6 ^{A}	267.6 ± 17.8 ^{Aa}	269.4 ± 34.2 ^{Ba}
NE- PA2	100.00 ^{Aa}	83.3 ^{Ab}	73.3 ^{A}	259.7 ± 20.3 ^{Aa}	298.3 ± 32.6 ^{Ab}
NE- PA5	100.00 ^{Aa}	63.3 ^{Bb}	56.6 ^{B}	260.5 ± 18.9 ^{Aa}	303.8 ± 37.9 ^{Ab}

^{a,b}Different letters denote statistical differences between culture days ($p<0.05$).
^{A,B}Different letters denote statistical differences between treatments within the same day of culture ($p<0.05$).

The cell viability findings for the NE-PA nanoscale formulation showed that there was no statistical difference in follicular viability for the cultured control group and the assessed herbal group, which was treated with two different concentrations (2 mg/mL and 5 mg/mL) of the *P. amarus* hydroalcoholic extract loaded into the nanoemulsion carrier, so called NE-PA. Hence, the observed result 83.3% for NE-PA2 and 63.3% for NE-PA5 (Table 2.1), makes the NE-PA colloidal system an effective commercial product to be largely applied in the *in vitro* follicle growth for mammalian specimens.
Statistically, there was no significant difference between the modified αMEM (cultured control) and NE-PA groups, with regard to follicular diameters throughout the culture period. It was also proved that the nanoemulsion carrier system (NE) without the herbal extract, does not present toxicity. In addition, there was a significant increase in the follicular diameter of the herbal NE-PA formulated system (tested in two concentrations) over the treatment period, in which at least 30 follicles were evaluated per group. However, for NE-PA at the highest concentration (5 mg/mL) there was a lower percentage of follicular viability when compared to the other groups (Table 2.2).
Figures 2.3B, 2.3D and 2.3F represent the fluorescence analysis, with 2.3B and 2.3D follicles stained in green with calcein (viable) and 2.3F follicles stained in red with ethidiohomodimer (Degenarated).



A, B = NE-carrier system (nanoemulsion without herbal)

C, D = NE-PA2 (extract of *P. amarus* load at the lower concentration, 2 mg/mL)

E, F = NE-PA5 (extract of *P. amarus* load at the higher concentration, 5 mg/mL)

FIGURE 2.3
Porcine ovarian follicles after 4 days of *in vitro* culture in the NE carrier formulation and NE-PA system as a new green medium of culture.

Conclusion

Nowadays, it is notorious that the limitations of chemical and morphological approaches for plant authentication, had generated the need for newer methods in quality control of botanicals. In that, the assess of genetic variation protocols are the most relevant point. In this sense, the importance of the *Phyllanthus* unambiguous species identification became mandatory, because of their morphological similarities. The present work focusing on the genetic diversity question of *Phyllanthus amarus* Schum. & Thorn. along with phytochemical, nanobiotechnological and also pharmacological studies, enable a newer therapeutic application to the herbal *P. amarus*.

Taking in account the medicinal importance of this species, in the present study, a nanoemulsified system (NE) based on the loading of *P. amarus* hydroalcoholic extract, so called NE-PA system, was evaluated in the *in vitro* experiment applied on cell viability of porcine ovarian follicles. Specifically, NE-PA was assayed as a medium of culture for pig follicular development, as well as tested as an antioxidant therapeutic product.

The NE-PA formulation, co-surfactant free, emulsifier two phases mixtures composed by oil/Tween 80® (organic phase) and water (inorganic phase), and was developed with the objective to define a low-energy approache by using water titration method. Indeed, the applied relatively simple manufacturing process allowed to construct a nontoxic nanoemulsion-type system that after loading *P. amarus* hydroalcoholic extract, become appropriate for several biological applications.

In order to investigate the cellular viability and the follicular development of pig ovarian follicles, the nanoemulsion NE-PA was evaluated on the *in vitro* culture of pig ovarian follicles. The diameters of the analyzed follicles ranged from 150 µm to 600 µm which were cultured for 4 days into a modified α-MEM (α-MEM^{+}) as cultured control as well as in the presence of the NE-PA loading lower concentration (2,0 mg and 5 mg) of the *P. amarus* hydroalcoholic extract.

The modified α-MEM culture of follicular treatment was carried out with the individually follicles incubated in 100 µL drops under mineral oil. In the NE-PA treatment of follicular culture, the tested follicles were incubated in 200 µL drops in the absence of mineral oil. The parameters evaluated in this experiment were the follicular viability rate through vital dyes (ethidium homodimer and calcein) and follicular diameter. In this protocol, at least 30 follicles per group were evaluated and the findings showed that there was no statistical difference in between the control group not cultivated, cultured control and treatment by using the NE-PA colloidal nanosystem.

Viability data was compared using Chi-square test, data for follicular diameters was submitted to ANOVA, and treatments was compared using Student-Newman-Keuls (SNK) test. Additionally, the antioxidant activity of the NE-PA formulation was evidenced by using ion chelation testes (iron and copper). So, in the teste protocol NE-PA act doing the expected functional therapy as a nutritional and antioxidant basic needs for the pig oocyte development and for a short time, does not require feasibility damage these follicles. Indeed, the *P. amarus* therapy correlated to its phytochemical compounds plays an important role in the development of green medicines, which based in the general literature and also in this present finding, is biologically safe. From now on the NE-PA (2 mg/mL) system advances, limitations and prospects are the remnant development which may consist on a new nontoxic and nutritive medium to be largely applied in the *in vitro* follicular culture for several different species as well as in different pharmacological assays, including human oral uses.

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Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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