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Full Length Research Paper

Mesquite (*Prosopis juliflora*) pod extract decreases fertility in female but not male rats

Socorro Retana-Márquez[1]*, Eunice Hernández[2], Floriberta Solano[2], Carlos Romero[1], Gabriela López[1], Lizbeth Juárez-Rojas[1], Fahiel Casillas[1], Philippe Chemineau[3], Matthieu Keller[3] and José Alberto Delgadillo[4]

[1]Departamento de Biología de la Reproducción, Universidad Autónoma Metropolitana-Iztapalapa, San Rafael Atlixco 186, México City C.P. 09340, Mexico.
[2]Maestría en Biología de la Reproducción. Universidad Autónoma Metropolitana, Mexico.
[3]PRC, INRA, CNRS, Université François Rabelais, IFCE, Agreenium, 37380, Nouzilly, France.
[4]Centro de Investigación en Reproducción Caprina, Universidad Autónoma Agraria Antonio Narro. Torreón, Coahuila, México.

The administration of Mesquite pod extract (containing mesquitol, daidzein and genistein) to female and male rats disrupts reproductive variables. However, its effect on fertility is not known. This study evaluated fertility in male and female rats treated with mesquite pod extract, comparing its effects with those of daidzein and estradiol. The following treatments were given for 30 days to groups of female and male rats: vehicle, mesquite pod extract, DAI and E_2. Treatments were administered subcutaneously for 30 days. These extract disrupted both the female and male sexual behavior in a similar way to DAI, but less than E_2. Mesquite pod extract increased the number of days in estrus and decreased lordosis intensity during proestrus. Mesquite pod extract-treated males showed lower testicular and glandular weights, as well as decreased sperm motility, viability and count. In females treated with mesquite pod extract, the number of pups was lower than in control females, and 10 to 20% of pups were dead. These effects were similar to those with DAI-treatment. Despite the lower sperm quality, the fertility of mesquite pod extract- and DAI-treated males seem not to be disrupted, as they could impregnate control females. These results show that mesquite pod extract can disrupt female but not male fertility.

Key words: Mesquite pod extract, daidzein, fertility, offspring, sexual behavior, phytoestrogens.

INTRODUCTION

Discovery of clover disease in ewes (Bennetts et al., 1946) consumption of feed containing large amounts of

plant oestrogens has been suspected to cause temporary or permanent fertility problems in ruminants. It

has been reported that low intake of food with high content of isoflavones causes temporary infertility, and prolonged consumption can cause permanent infertility (Marshall, 1973; Adams, 1990; Adams, 1995).

There are very few studies on the effects of fodder with high phytoestrogen concentration on livestock fertility. A recent study evaluated the effects on conception and early gestation of nulliparous ewes fed for five months before gestation with red clover rich in the phytoestrogen formononetin. Although fecundity was not reduced, lower levels of progesterone were observed as well as increased amount of fetal fluids, which can increase the risk of vaginal prolapse before term (Mustonen et al., 2014). Heifers fed clover had a lesser conception rate, and a greater percentage of heifers returning to estrus than silage-fed heifers. This indicates that isoflavone content in clover disturbs hormonal balance during early pregnancy, leading to a reduction in the fertility of heifers (Hashem et al., 2016).

In women with problems becoming pregnant, a high dietary isoflavone intake has been associated with a higher risk of nulligravidity, that is a higher risk of never becoming pregnant or never giving birth to a live child. Indeed, when isoflavone intake exceeds 40 mg/day, the overall lifetime risk of never becoming pregnant increases 13% (Jacobsen et al., 2014).

In animal models, several plant extracts have been reported to have antifertility activity. The methanolic extract of *Artemisa vulgaris* leaves which is a plant containing flavonoids, possesses estrogenic activity at doses 300 or 600 mg/kg when administered orally from day 1 to 10 of pregnancy causes major implantation failure (100%) in female rats (Shaik et al., 2004). Similarly, the aqueous extract of *Pouzolzia mixta* (a plant native of Africa and used by women as a post-coital contraceptive) administered orally to female rats at a dose of 300 mg/kg for 7 days followed by mating and given an additional treatment for 10 days post-conception, inhibits both implantation and fertility (Sewani-Rusike, 2013). Flower extracts of *Tabernaemontana divaricata*, a plant native of Asia, which also contains flavonoids, is used by women as a traditional medicine for family planning. This possesses estrogenic, anti-implantation and early abortive activity, when administered in doses of 500 mg/kg to female rats (Mukhram et al., 2012). This experimental evidence reinforces the fact that any plant with estrogenic activity can also have potential anti-fertility activity in females.

In men, it has been hypothesized that compounds with endocrine disrupting effects such as phytoestrogens may be associated with decreased semen quality (Xia et al., 2013). Although the evidence is not conclusive, epidemiological studies suggest that phytoestrogen consumption in Chinese and Japanese men can be related to erectile dysfunction (Bai et al., 2004),and lower sperm concentration (Xia et al., 2013; Giwercman, 2011; Iwamoto et al., 2007; Phillips and Tanphaichitr,

2008), causing idiopathic male infertility mainly in Chinese and Japanese men. A similar effect has been observed in American men through the intake of soy based foods (Chavarro et al., 2008).

Mosquite (*Prosopis sp*) is a widespread legume in arid and semi-arid areas in Mexico, Africa and Asia. This leguminous plant is widely used to feed several livestock species due to its high content of protein, carbohydrates, fiber (Kathirvel and Kumudha 2011), minerals and vitamins (Choge et al., 2007; Freyre et al., 2010). In addition, mesquite pod is a source of food for human consumption as bread (Cattaneo et al., 2016; Cruz, 1999), cakes or porridge (Freyre et al., 2010), syrup and beverages (Cruz, 1986), desserts, and as a coffee substitute (Azevedo et al., 1987), which possesses antioxidant activity (Karim and Azlan, 2012). Mesquite contains high amounts of some phytoestrogens such as the flavanol mesquitol (in concentration of 6.4 µg/g) (Sirmah et al., 2009), flavonols such as quercetin, luteolin, and isoharmnetin, the flavone vitexin (Gianinetto et al., 1975), and isoflavones genistein (60.25 µg/g) and daidzein (5.27 µg/g) (González et al., 2015). All these phytoestrogens may contribute to the estrogenic effects of mesquite in livestock and other animals, including human beings. The only reports about the effects of mesquite pod extract on female and male reproduction are from our laboratory. Mesquite pod extract alters estrous cyclicity, decreases lordotic quotient and intensity of lordosis in intact rats. In ovariectomized rats, mesquite pod extract induce vaginal estrus, increased vaginal epithelium height and lordosis; all these effects are similar to those caused by daidzein and genistein (Retana-Márquez et al., 2012). In male rats, mesquite pod extract disrupts sexual behavior, increases testicular germ cell apoptosis, decreases sperm quality and plasma testosterone concentrations in a similar way than genistein and daidzein (Retana-Márquez et al., 2016).

To date, there are no studies about the possible undesirable effects of mesquite pod on fertility when used to feed livestock or human beings. Considering that mesquite pod extract disrupts some reproductive variables in female and male rats, we hypothesize that mesquite pod extract can decrease fertility in female and male rats. Therefore, the aim of this study was to evaluate fertility in rats treated with mesquite pod extract. The percentages of pregnant females observed and the number of offspring per litter were evaluated. The effects were compared with those of daidzein and estradiol used as controls.

MATERIALS AND METHODS

Animals

All experimental procedures used in this study were approved by the Universidad Autónoma Metropolitana's Institutional Animal Care and Use Committee, in accordance with the National Institute

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of Health's Guide for the Care and Use of Laboratory Animals, and Mexican Official Regulation (NOM-062-ZOO-1999).

Adult female (250 to 300 g) and male (300 to 350g) Wistar rats (three months of age) were housed five per cage (50×30×20 cm), under standard vivarium conditions. The colony room was maintained on a 12:12 reversed light cycle (lights off: 10:00) and under controlled temperature (23 ± 1°C). Food and water were available ad libitum throughout the experiments. The rodent diet used was "2018 Teklad global" from Harlan Laboratories. Although this diet contains phytoestrogens DAI and GEN (range from 15.0 to 25.0 µg/g, www.harlan.com), there are no reports confirming any estrogenic effects of this rodent diet (Naciff et al., 2004). This amount is far below the experiment given to the animals in this study. Moreover, all animals in this study were exposed to the same diet ad libitum regardless of their treatment group. The effects of mesquite extract, daidzein (DAI), and estradiol (E_2) were tested in intact rats.

Treatments

The following treatments were given to the experimental groups of females (n=30/group, to have 10 females copulating with males from all the groups) and males (n=10/group. This number was enough to impregnate all females): 1) negative control group: rats received only vehicle injections (corn oil, 0.2 ml); 2) mesquite pod extract group: rats received mesquite pod concentrated extract (3.5 g/kg/day wet pod weight); 3) DAI group: rats received DAI (reference D7802, Sigma, purity >97%; 5 mg/kg/day); 4) positive control group: rats received E_2 (reference E1024-1G, Sigma, purity >98%; 30 µg/kg/day). All the treatments, except mesquite extract were dissolved in corn oil and administered subcutaneously (sc) in the dorsal region of the neck, daily for 30 consecutive days. The volume of injection was 0.2 ml in all cases. Treatment administration was made in the same vivarium room every day.

The dose of mesquite pod extract was selected according to daily consumption in ewes (140 g/day), which is equivalent to 3.5 g/kg/day and corresponds to 1g/0.3Kg/day in the rat. DAI dose was selected according to those used to elicit physiological responses in males, such as reduced reproductive hormone levels and erectile responses in male rats (Retana-Márquez et al., 2016), as well as disrupted estrous cyclicity, reduced body weight, ovarian hypertrophy, and some non-lasting effects on socio-sexual behavior in female rats (Retana-Márquez et al., 2012; Henry and Witt, 2002). The E_2 dose used in this study is known to induce testicular atrophy in males (Ikegawa et al., 1995), and receptive behavior in female rats (Retana-Márquez et al., 2003).

Mesquite pod extract preparation

Only mature pods from mesquite (*Prosopis juliflora*) were used. They were collected in summer from the arid Hidalgo region of central Mexico.

The Mesquite pods were dried in an airflow oven at 60°C for 24 h. It was then ground (pod and seeds) in a Thomas-Willey cutting mill with 5 mm diameter sieves and then with 2 and 1 mm diameter sieves. The extract was obtained from 2 kg of powdered fruit by a Shoxhlet extractor to depletion with ethanol-water (90:10 v/v) for 18 h. Ethanol was eliminated by distillation at 78 to 87°C. The final concentration of extract was 1.76 g/ml. The concentrated aqueous extract was administered in a dose of 1g/0.2 ml.

Mesquite pod extract contains: mesquitol in concentration of 6.4 µg/g (Sirmah et al., 1987), isoflavones genistein (60.25 µg/g) and daidzein (5.27 µg/g) (González et al., 2015). Other components of the extract are: crude protein (26.69 to 29.84%), crude lipid (11.89 to 13.75%), total crude fiber (8.78 to 9.89%), ash (3.99 - 4.95%)

and carbohydrates (42.45 to 46.37%). The range of anti-nutritional factors reported are as follows: total free phenolics 4.93 to 8.58%, tannins 6.81 to 9.15%, L-DOPA 2.21 to 4.52%, phytic acid 0.33 to 0.89 g100g-1, and trypsin inhibitor activity 40.4 to 48.2 TIU mg-1 protein (Kathirvel and Kumudha, 2011). The treatments were administered to males and females for 30 days during which, the estrous cycle was monitored in females.

Female measurements

Vaginal cytology and estrous cyclicity

Estrous cycles were monitored by daily evaluation of vaginal smears which were stained with hematoxilin-eosin and evaluated with an optic microscope (Olympus, model CX41RF). Vaginal smears were obtained two hours after the onset of the dark period, under red light (40 Watts). Estrous cycles were classified as follows: (a) 3-day cycle, an irregular shortened cycle usually resulting from a condensed or absent diestrus period; (b) 4-day cycle, a normal length cycle consisting of full estrus, metestrus, diestrus, and proestrus periods; (c) 4 to 5-day cycle, also call normal length cycle that includes an additional 24 h of diestrus, called diestrus II; (d) constant estrus, an irregular estrous cycle defined by the persistence of cornified cells beyond 2 days (Henry and Witt, 2002).

Behavioral testing

Female sexual behavior was assessed in a Plexiglas arena (40x40x50 cm) with males. Testing was done during the first three hours of the dark phase, under red light. Female behavior was assessed through receptivity and proceptivity. Receptivity for each female was determined as a lordosis quotient

$$[LQ = (\text{number of lordosis}/10 \text{ mounts}) \times 100]$$

The intensity of lordosis (extent of dorsiflexion) was quantified according to the lordosis score proposed by Lehman and Erskine (2004). The rating of lordosis intensity (LR) was established based on the degree of spinal dorsiflexion and the extent to which the sagittal ridge of the head lined up in a vertical plane. The rating was based on a scale of no vertebral dorsiflexion (0), slight dorsiflexion coupled with slight movement of the head toward the vertical plane (1), moderate dorsiflexion coupled with vertical movement of the head (2), extreme dorsiflexion coupled with vertical movement of the head (3).

Proceptivity was evaluated by determining the incidence of hopping, darting, and ear-wiggling across the whole receptivity test (Madlafousek and Hlinak, 1977). Females were considered to be proceptive when two of these behaviors are showed during the testing period. Female sexual behavior was assessed in females in Proestrus stage, from day 30 of treatment.

Male measurements

Behavioral testing

Male sexual behavior was assessed three times, one assessment per week. Behavioral testing was performed under dim red lights, 3 h after the onset of the dark phase of the light/dark cycle. Male sexual behavior was assessed by placing the male in a Plexiglas arena (40 x 40 x 50 cm) 5 min before a stimulus receptive female was presented. Female rats were brought into sexual receptivity by

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administering estradiol benzoate (Sigma Chemical, Co. St. Louis MO., Purity 98%, 10 µg/100 µl oil, SC) 44 h before sexual tests.

Progesterone (Sigma Chemical, Co. St. Louis MO., purity 99%, 1 mg/200 µl oil, SC) was administered 4 h prior to testing. After presentation of the female, tests were lasted for 30min. Upon presentation of the female, the following variables of male sexual behavior were recorded: latency to the first mount, latency to the first intromission, and latency to the first ejaculation; number of mounts (mounts with pelvic thrusting) and intromissions (mounts with pelvic thrusting and penile insertion) of the first copulatory series. In addition, ejaculation frequency (number of ejaculations during 30 min of recording), and post-ejaculatory interval (time between ejaculation and subsequent intromission) were recorded. The full description of male sexual behavior variables has been detailed elsewhere (Hull et al., 2006; Meisel and Sachs, 1994).

Sperm analysis

The right cauda epididymis was stored with 1 ml of saline at 37°C. Each cauda epididymis was cut using fine tip dissection scissors to release the sperm stored in the cauda.

Sperm viability

Sperm viability was assessed by using one-step eosin-nigrosin (5% nigrosin, 1% eosin and sodium citrate dissolved in distilled water) staining technique (Lucio et al., 2009). A sample of epididymal sperm suspension (10 µl) was mixed with the colorant solution (10 µl) and analyzed on a pre-warmed slide with a cover slip.

Slides were observed under an optical microscope (Olympus Light Microscope CX 41) 40x objective lens. Unstained spermatozoa were counted as viable whereas stained spermatozoa were counted as dead. Different fields were analyzed randomly until two hundred spermatozoa were counted. Sperm viability was reported as the percentage of viable spermatozoa out of the total count.

Sperm motility

Sperm motility was assessed by counting motile and non-motile sperm from a total of 200 spermatozoa. Sperm motility was expressed as a percentage of motile spermatozoa out of the total spermatozoa counted.

Sperm count

Epididymal sperm count was performed using Neubauer haemocytometer. Epididymal sperm suspension (25 µl) was taken to 500 µl final volume with distilled water. The sperm suspension (10 µl) was placed on the Neubauer haemocytometer and counted in eight different spots. Count discrimination criteria were set using the spermatozoa head position inside the chamber squares that were being analyzed. Formula count was resolved as follows:

Sperm count X # chamber squares (8) X dilution factor (21) X 10, 000 / 2.

Final sperm count was expressed in millions per milliliter.

Fertility evaluation

At the end of 30 days of treatment, females were allowed to copulate with males of all the groups, according to the scheme showed in Figure 1.

Males were allowed to ejaculate twice with each female in order to ensure pregnancy. This was considered at day 0 of pregnancy. Treatments continued for all females during gestation period till day 22. Litter size was evaluated after birth.

Statistical analyses

Lordosis quotient, intensity of lordosis, body and glandular weights, as well as male sexual behavior variables were, analyzed by one-way ANOVA, followed by Newman-Keuls post hoc test. Percentages of females with normal or abnormal cycles, percentages of females presenting lordosis, receptivity and proceptivity, percentages of pregnant females and percentage of dead pups, as well as spermatic variables in males were analyzed by Chi-square test followed by Dunn's multiple comparisons.

The number of offspring was analyzed by a two way ANOVA with treatments of the males in which females are copulated as factors, followed by Newman-Keuls post hoc test. The level of significance was considered with $p < 0.05$. Data are reported as mean ± S.E.M. All analyses were performed with GB-Stat School Pack.

RESULTS

Females vaginal cytology and estrous cyclicity

Vaginal smears showed the treatment-dependent changes in estrous cycles. Normal progression of all stages was observed in the estrous cycle of vehicle females. Mesquite pod extract administration to females caused irregular estrous cycles in 95% of subjects ($p < 0.01$), with an increase in the number of days in estrus (2 to 3) and some 3-day cycles throughout the treatment.

DAI administration also caused an increase in the number of days in estrus (3 to 4) in 87% ($p < 0.01$) of females, and a decrease of days in diestrus. All females treated with E_2 showed shortened cycles (absence of metestrus and diestrus) from the second day of treatment, and constant estrus from day 12 of treatment. Representative estrous cycles of females from the different experimental groups are shown in Figure 2.

Female sexual behavior

The percentage of sexually receptive females exhibiting lordosis behavior throughout the tests in the treated groups (Mesquite, DAI, E_2) was significantly different from the control group. In fact, all females from control and E_2 groups exhibited lordosis behavior. In contrast, mesquite pod extract-treated females (77%) or DAI-treated females (85%) displaying lordosis in proestrus were fewer than control and E_2-females ($p < 0.05$). The lordosis quotient (LQ) was also different among groups.

Indeed, LQ in DAI- and E_2-treated females was similar to control females. In contrast, females treated with

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	♂	♂	♂	♂
♀	CON/CON	MES/CON	DAI/CON	E_2/CON
♀	CON/MES	MES/MES	DAI/MES	E_2/MES
♀	CON/DAI	MES/DAI	DAI/DAI	E_2/DAI
♀	CON/E_2	MES/ E_2	DAI/ E_2	E_2/E_2

Figure 1. Representative estrous cycles of females treated with vehicle (CONTROL), mesquite pod extract (EXT), estradiol (E2) or daidzein (DAI). Control females had normal 4–5 day cycles. Mesquite pod extract, estradiol, and daidzein induced some 3-day cycles, with absence of diestrus periods, as well as an increase in the days in estrus. (E) Estrus; (M) Metestrus; (D) Diestrus; (P) Proestrus.

Table 1. Female sexual behavior in control and experimental females during proestrus. Percentage of females presenting proceptive and receptive behaviors. Lordotic quotient and intensity were evaluated after 30 days of treatment.

Group	Proceptive behavior (%)	Sexual receptivity (%)	Lordotic quotient	Lordosis Intensity
Control	100[a]	100[a]	100 ± 0[a]	2.75 ± 0.04[a]
Extract	43[b]	77[b]	86.5 ± 5.9[b]	1.83 ± 0.10[b]
Daidzein	48[b]	85[b]	98.5 ± 1.5[a]	2.35 ± 0.21[b]
Estradiol	100[a]	100[a]	100 ± 0[a]	3.00 ± 0[a]

[b] Different from control group.

mesquite pod extract group showed lower LQ (86.5; p<0.01; Table 1). The intensity of lordosis (extent of dorsiflexion), was also different depending on the treatment. Maximum lordosis intensity was observed in all control females during proestrus, and this variable did not differ from E_2-treated females. In contrast, females in natural proestrus treated with mesquite extract, besides showing a lower lordosis quotient, had a lower intensity of lordosis (1.83) than control and E_2 females (p<0.01). Low lordosis intensity was also observed in DAI-treated females (2.35) compared to control and E_2 females (p<0.05).

Finally, only 43% of females from the mesquite extract group and 48% of DAI-treated females displayed proceptive behavior (hopping, darting and ear wiggling) during proestrus (p<0.01; Table 1). Since females were copulated with males in order to become pregnant, body and glandular weights were registered in females after weaning. Body weight in females are from mesquite extract, DAI and E_2 groups were lower than in those of

the control group (p<0.05). E_2-treated females showed the lowest body weight (p<0.01). Ovaries and uterus weights in females are from mesquite pod extract, DAI and E_2 were not different from controls, but vagina weight increased in all experimental groups compared to the control group (p<0.05). No data on females treated with E_2 are shown, since all females were in estrus due to the hormonal treatment (Table 2).

Males

Body and glandular weights

At the end of the 30 days of treatments, body weight of males treated with mesquite pod extract and E_2 was lower than that of control males (p<0.01; Table 3). No difference was observed in males from DAI group compared to control males. Testicular weight in males from all the experimental groups was lower than that of

Table 2. Body and glandular weights in control and treated females after weaning.

Groups	Body Weight (g)	Ovaries weight (mg)	Uterus weigth (mg)	Vagina weigth (mg)
Control-Proestrus	301.8 ± 11.5[a]	71.2 ± 5.1[a]	630.0 ± 97.7[a]	97.5 ± 12.5[a]
Mesquite pod extract – Proestrus	265.9 ± 6.9[b]	75.6 ± 2.4[a]	778.7 ± 113.5[a]	155.7 ± 7.1[b]
Daidzein – Proestrus	255.6 ± 6.7[b]	78.0 ± 2.4[a]	792.0 ± 60.4[a]	168.0 ± 22.4[b]
Estradiol – Estrus	225.5 ± 5.7[c]	31.4 ± 1.4[c]	720.0 ± 41.7[a]	143.0 ± 9.2[b]
Control – Diestrus	284.6 ± 3.1[a]	70.0 ± 9.1[a]	647.5 ± 177.2[a]	122.5 ± 13.1[a]
Mesquite pod extract - Diestrus	251.0 ± 16.8[b]	63.7 ± 2.6[a]	860.0 ± 110.0[a]	160.0 ± 20.0[a]
Daidzein - Diestrus	238.3 ± 10.2[b]	83.0 ± 6.3[a]	744.0 ± 60.7[a]	142.0 ± 15.6[a]

[a] Not different from control group; [b] different from control group, p<0.05; [c] different from all the groups, p<0.01

Table 3. Body and glandular weights in control and treated males after 30 days of treatment.

Group	Body weigth (g)	Testes weight (g)	Epidydmis (mg)	Seminal gland (g)	Prostate (mg)
Control	404.94 ± 8.26[a]	1.88 ± 0.03[a]	758 ± 8.13[a]	2.05 ± 0.09[a]	685 ± 31.13[a]
Extract	352.45 ± 14.11[b]	1.68 ± 0.03[b]	692 ± 20.91[b]	1.55 ± 1.13[b]	553 ± 31.02[b]
Daidzein	393.58 ± 14.31[a]	1.64 ± 0.03[b]	611.11 ± 30.75[b]	1.74 ± 1.09[b]	603 ± 51.98[a]
Estradiol	312.13 ± 7.44[b]	0.36 ± 0.01[c]	0.24 ± 0.02[c]	0.17 ± 0.008[c]	0.13 ± 0.01[c]

[b] different from the control group, p<0.01; [c] different from all the groups, p<0.01.

Table 4. Sperm total motility, viability, sperm concentration, and percentage of abnormalities observed in control and treated males with 30 days of treatment.

Group	Motility (%)	Viability (%)	Count x 10^6 (Mean ± SEM)	Abnormalities (%)
Control	89.25 ± 0.85[a]	92.2 ± 1.11[a]	173.64 ± 3.33[a]	0.9 ± 0.14[a]
Extract	55.75 ± 5.89[b]	68.5 ± 3.77[b]	105.98 ± 14.27[b]	2.75 ± 0.78[b]
Daidzein	70.33 ± 2.45[b]	72.72 ± 2.48[b]	95.65 ± 9.71[b]	3.72 ± 0.78[b]
Estradiol	25.5 ± 7.31[c]	29.33 ± 3.01[c]	10.64 ± 5.61[c]	5.17 ± 0.18[c]

[b] different from the control group, p<0.01; [c] different from all the groups, p<0.01.

Prostate, seminal glands and epididymis weights from males of the mesquite extract, DAI and E2 groups were lower than those of control males (p<0.05). Atrophic glands and epididymis were observed only in males from E2 group (p<0.01; Table 3).

Sperm parameters

In males treated with mesquite pod extract, DAI or E2, the values of total sperm motility, sperm viability and sperm count were lower than in control males (p<001; Table 3). E$_2$-treatment caused the most disruptive effects, and only few spermatozoa were obtained from atrophic epididymi of males after 30 days of treatment. In males from mesquite pod extract and DAI groups, these values were lower compared with those of control males;

values registered in mesquite pod extract- and DAI-treated males did not differ significantly between them.

Finally, the percentage of sperm abnormalities increased in males from all experimental groups, with the highest percentage in the E$_2$ group (p<0.01). Abnormalities consist of tailless heads, head and flagellum errors, as well as double head spermatozoa. The percentage of sperm abnormalities observed in mesquite extract and DAI groups did not differ between them (Table 4).

Male sexual behavior

All males from experimental groups copulated, and all males from the control and mesquite extract groups ejaculated; 93% of DAI-treated males and 20% of E$_2$-

Table 5. Male sexual behavior variables (X ± S.E.M.). Males were treated during 30 consecutive days.

Variables	Control	Extract	Daidzein	Estradiol
Mount Latency (s)	7.2 ± 1.04[a]	54.5 ± 21.04[b]	14.5 ± 2.72[b]	145.5 ± 45.99[b]
Intromission Latency (s)	9.9 ± 1.03[a]	102.9 ± 30.49[b]	49.25 ± 15.34[b]	211.11 ± 74.75[b]
Ejaculation latency (s)	283.1 ± 25.58[a]	519.2 ± 84.35[b]	422.33 ± 18.30[b]	992.0 ± 162.0[b]
Number of mounts	3.6 ± 0.49[a]	12.5 ± 3.74[b]	8.66 ± 0.91[b]	16.0 ± 6.0[b]
Number of ejaculations	3.0 ± 0[a]	2.0 ± 0[b]	1.7 ± 0.21[b]	0.2 ± 0.13[b]
Postejaculatory interval	286.5 ± 26.40[a]	441.8 ± 62.14[b]	409.0 ± 75.32[b]	684.5 ± 278.5[b]

[b] Different from control group.

Table 6. Percentages of females pregnant after copulating with males from control and experimental groups after 30 days of treatment.

Group	Pregnant females (%)	Dead pups (%)
C-C	100	0
C-M	100	0
C-D	100	0
C-E$_2$	0	-
M-C	90*	20*
M-M	100	10
M-D	100	10
M-E$_2$	0	-
D-C	90*	10
D-M	70*	0
D-D	90*	20*
D-E$_2$	0	-
E$_2$-C	0	-
E$_2$-M	0	-
E$_2$-D	0	-
E$_2$-E$_2$	0	-

*p<0.05 compared with C-C group. C-C: control female-control male; C-M: control female-mesquite male; C-D: control female daidzein male; C-E$_2$: control female-E$_2$ male; M-C: mesquite female-control male; M-M: mesquite female-mesquite male; M-D: mesquite female-daidzein male; M-E$_2$: Mesquite female-E$_2$ male; D-C: daidzein female-control male; D-M: daidzein female-mesquite male; D-D: daidzein female-daidzein male; D-E$_2$: Daidzein female-E$_2$ male; E$_2$-C: E$_2$ female-control male; E$_2$-M: E$_2$ female-mesquite male; E$_2$-D: E$_2$ female-daidzein male; E$_2$-E$_2$: E$_2$ female-E$_2$ male.

treated males ejaculated. Male sexual behavior was disrupted in all experimental subjects: males treated with mesquite pod extract, DAI or E$_2$ displayed longer mount, intromission and ejaculation latencies than control males (p<0.05). The number of mounts in males treated with mesquite extract, DAI or E$_2$ was higher than in the control group (p<0.05). The number of ejaculations was lower in males treated with mosquite, DAI and E$_2$

(p<0.05).

The post ejaculatory interval was also higher in experiment than in control males (Table 5).

Fertility: number of pups per litter

Regarding fertility, all control females became pregnant when copulating with males from vehicle, mesquite extract or DAI groups (Table 6). No dead pups were observed after birth.

Most females treated with mesquite pod extract (90%) became pregnant when copulating with control males. All of the mesquite extract-treated females became pregnant after copulating with mesquite extract- or DAI-treated males. In the case of DAI-treated females, only 70% of those copulating with males treated with mesquite pod extract got pregnant (p<0.05). None of the E$_2$-treated females became pregnant and none of the females copulating with E$_2$-treated males got pregnant (Table 6).

The number of litters was different depending on the treatment. Significantly reduced litters were observed in mesquite extract-treated females copulating with control, mesquite extract- or DAI-treated males (p<0.05). The same was observed in DAI-treated females copulating with mesquite pod extract- and DAI-treated males (Table 7). The mortality in pups born from females treated with mesquite pod extract or DAI increased in 10 to 20% compared with control females (p<0.05). No litter was obtained from E$_2$-treated females and females copulating with E$_2$-treated males (Table 6).

DISCUSSION

Results of this study show that mesquite pod extract disrupts sexual behavior and reproduction in female and male rats. In females, mesquite pod extract induces an increase in the number of days in estrus and a decrease in the intensity of lordosis during proestrus, as well as proceptivity and receptivity. Body weight decreased while vaginal weight increased in all experimental

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Table 7. Average number of litters born from control and experimental females copulating with control or experimental males. Females and males received treatments during 30 consecutive days.

Groups	C-C	C-M	C-D	M-C	M-M	M-D	D-C	D-M	D-D
Litters	9.25 ± 0.45^a	9.55 ± 0.74^a	8.33 ± 0.64^a	5.12 ± 0.91^b	6.62 ± 0.70^b	5.00 ± 0.75^b	6.57 ± 1.35^a	6.42 ± 1.08^b	5.28 ± 1.20^b

[b] Different from control group. C-C: control female-control male; C-M: control female-mesquite male; C-D: control female-daidzein male; M-C: mesquite female-control male; M-M: mesquite female-mesquite male; M-D: mesquite female-daidzein male; D-C: daidzein female-control male; D-M: daidzein female-mesquite male; D-D; daidzein female-daidzein male.

females compared with controls.

In addition, the number of pups born from females treated with mesquite pod extract was lower than in control females, and the mortality of pups increased between 10 and 20%. In males, mesquite pod extract causes a decrease of body, testes and glandular weights, as well as decreased sperm motility, viability and count after 30 days of treatment. The effects of mesquite pod extract on male sexual and reproductive variables were similar to those observed in animals treated with DAI, but less pronounced than in animals treated with E_2. Altogether, these findings support our initial hypothesis which states that, mesquite pod extract decreases fertility in female and male rats.

The decrease in body weight observed in experimental females and males can be attributed to the estrogenic effects of mesquite pod extract and DAI, although the most pronounced effects were observed in subjects treated with estradiol. Estradiol has the ability to control energy balance, food intake, and body fat distribution which may be mediated through its interaction with orexigenic and anorexigenic hormones.

In rats and mice, estrogen exerts a tonic inhibitory effect on meal size and daily food intake throughout the ovarian cycle and a cyclic inhibitory effect during the peri-ovulatory phase. Estradiol acts via the estrogen receptors (ERs) in the hypothalamus to reduce feeding which may mediate its anorectic effects by decreasing the expression or releasing orexigenic neuropeptides such as neuropeptide Y (NPY) and Ghrelin, at the same time can increase the central sensitivity to anorexigenic peptides such as Leptine and Cholecystokinin, and increasing the neurotransmission of Serotonin (Brown and Clegg, 2010).

Considering these effects of estradiol and to a lesser extent those of DAI and phytoestrogen content (mesquitol (6.4 µg/g), Genistein (60.25 µg/g) and Daidzein (5.27 µg/g) in mesquite pod extract, it is possible that the reduced overall condition of both females and males prior to mating could decrease their fertility, with a negative effect on reproductive performance. Although reproductive organs in females did not decrease due to the treatments, it is possible that the changes in the female reproductive system caused by low body weight can lead to reduction or even suppression of ovulation and other disorders generated

by a complex hormonal balance of the hypothalamic pituitary-ovarian system, with consequent reduction in the levels of hormones involved in fertility, such as GnRH, LH, FSH, and estrogen. This can be evaluated in other studies. All these changes are able to modify ovarian function, preventing the development of appropriate conditions for ovulation to occur. Furthermore, the nutritional status of the mother is of great importance for the survival of embryos and the health of offspring. Considering the decrease in body weight data in Table 2 (females) as well as body and glandular data in Table 3 (males), it seems necessary to evaluate the nutritional status of animals treated with mesquite pod extract, DAI and E_2.

The alterations in estrous cycle and female sexual behavior due to mesquite pod extract could be explained by its content of phytoestrogens and their disruptive effects on the gonadal axis. In the hypothalamus, phytoestrogens can disrupt the control of GnRH neurons by Kisspeptin (Patisaul and Adewale, 2009; Patisaul and Jefferson, 2010; Jefferson et al., 2005), leading to a decrease in GnRH, in the same way that estradiol does (Ördög and Knobil, 1995). Phytoestrogens can also attenuate the preovulatory surge of LH and FSH by suppressing circulating estrogen (Hooper et al., 2009; Trock et al., 2006), decreasing ovarian steroidogenesis and foliculogenesis stimulation.

In ovary, phytoestrogens can cause an absence of corpora lutea, large antral-like follicles with degenerating or no oocytes and ovarian cysts (Kouki et al., 2003). All these effects contribute to female infertility.

Regarding female sexual behavior, this is mediated by ERα subtype (Krege et al., 1998), located in the hypothalamic ventromedial nucleus (VMN) (Chang et al., 2008; Kuiper et al., 1997), which is an important structure for the control of that behavior (Rubin and Barfield, 1980). The fact that neither mesquite pod extract nor DAI stimulate female sexual behavior, decreasing lordosis quotient and intensity might be explained by their higher affinity for ERβ (30 times higher) than ERα (Goldberg et al., 1996). Estradiol induces a stimulatory effect on female sexual behavior through its binding to both estrogen receptor subtypes α and β (Pfaff et al., 2006). Therefore, no inhibitory effect was observed in this respect.

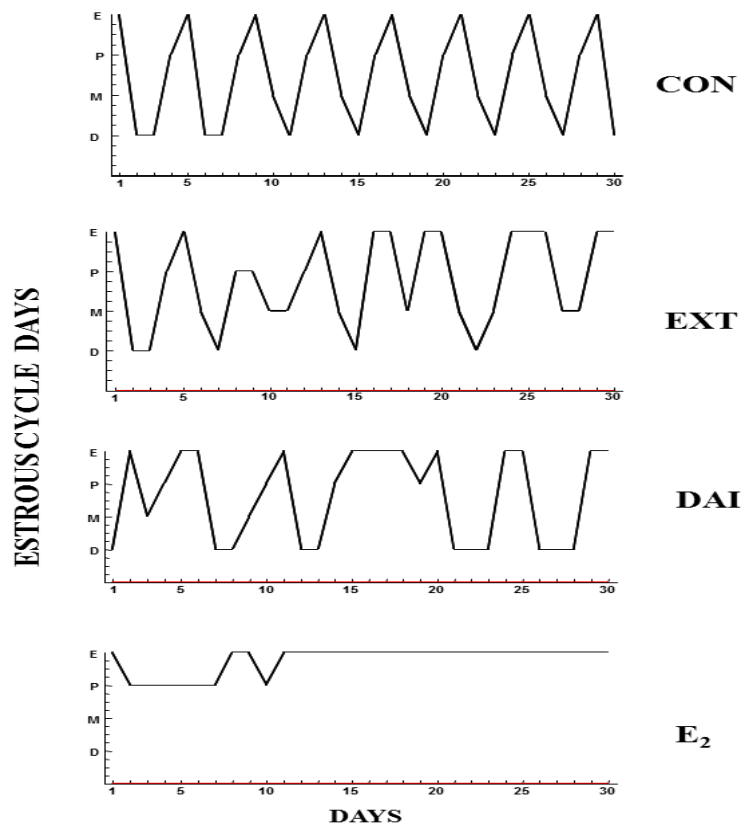The current study also shows that mesquite pod

Figure 2. Representative estrous cycles of females treated with vehicle (CONTROL), mesquite pod extract (EXT), estradiol (E2) or daidzein (DAI). Control females had normal 4–5 day cycles. Mesquite pod extract, estradiol, and daidzein induced some 3-day cycles, with absence of diestrus periods, as well as an increase in the days in estrus. (E) Estrus; (M) Metestrus; (D) Diestrus; (P) Proestrus.

extract adversely affects pregnancy outcome, although the extract did not impair pregnancy. Females exposed to the extract, delivered fewer pups than control and DAI treated females, with 10 to 20% of mortality in born pups.

In regard to DAI-treated females, disruption of female sexual behavior and a reduction in the percentage of pregnant females, decreased numbers of pups where higher number of dead pups was also observed. Isoflavone intake can attenuate lordosis and decrease proceptive behavior in female rats (Patisaul and Jefferson, 2010). Additionally, it has been reported that neonatal treatment with genistein in female mice causes a significant decrease in the number of live pups (Jefferson et al., 2005). Taken together, the results obtained in this study suggest that both mesquite extract and DAI could have anti-zygotic, blasto-cytotoxic, or anti-implantation activity, causing a decrease in the number of implantation sites in the uterus, as has been observed in females treated with genistein (Jefferson, et al., 2005) and with extracts of other plants. The fact that E_2

treatment suppresses estrous cyclicity in females due to its overstimulation of vaginal epithelia, causes persistent vaginal cornification, as has been demonstrated earlier (Retana-Márquez et al., 2012). The fact that estradiol-treated females did not become pregnant is due to, the inhibition of the gonadotropin peak caused by suppressing the Gonadotropin releasing hormone (GnRH) pulse due to estradiol overdoses (Ördog and Knobil, 1995), thus suppressing ovulation.

Concerning male's sexual behavior, glandular weights and sperm quality, they decreased mesquite pod extract in a similar way as DAI treatment. These results confirm that mesquite pod extract disrupts reproduction in male rats. The effects of mesquite pod extract and DAI in male reproductive variables should be considered to explain the lower number of pups and/or dead pups, since the quality of sperms is also important for the progression of the zygote to blastocyst (Casillas et al., 2016). The results of this work show that 100% of control females copulating with control, mesquite pod extract- or DAI-treated males became pregnant, the number of

pups was the highest and all pups were alive. This indicates that despite the reduction in sperm viability, motility and count, the treatments did not disrupt the reproductive ability of males. However, when the females receiving mesquite pod extract or DAI copulated with control, mesquite extract or DAI-treated males, the number of pregnant females and the number of pups decreased. These results indicate that female reproduction seems more vulnerable to the effect of mesquite pod extract and DAI than in males.

Nonetheless, males treated with mesquite pod extract impregnated only 70% of females treated with DAI, although no pups were dead. This result could suggest that mesquite extract might contribute to decrease in male fertility. Concerning the effects of estradiol in male reproduction, large doses of estradiol induce testicular germ and Leydig cell apoptosis, causing a loss of almost all cellular types, which induces severe testicular atrophy and undetectable plasma levels of testosterone (Retana-Márquez et al., 2016), as was corroborated in this study. The consequent absence of testosterone and its metabolite, 5-Hydroxytestosterone, necessary for penile erection (Manzo et al., 1999), causes erectile dysfunction, thus increasing mount latency and the number of mounts, which leads to lack of intromission and ejaculation (Meisel and Sachs, 1994). On the other hand, the absence of sperm caused by great doses of estradiol prevents the possibility of oocyte fertilization and therefore no pregnancy occurs.

Conclusions

Mesquite pod extract can disturb the estrous cycle, sexual behavior and fertility of female rats, decreasing the rate of pregnancy and the number of pups, as well as increasing the number of dead pups at birth. In males, despite mesquite pod extract and DAI decreasing sexual behavior and sperm quality, these effects seems not to disturb fertility, since they can fertilize control females. However, mesquite extract or DAI treatments in males could also contribute to decreased pregnancy rates and the number of live pups. Considering that mesquite is used to feed livestock and also for human consumption, the findings of this study could be taken into account for the possible side effects on reproduction and fertility in males and females.

It is important to evaluate the nutritional status of animals fed mesquite because its content of mesquitol, daidzein and genistein can account for a decrease in body weight, thus altering their reproductive performance.

Conflict of Interests

The authors have not declared any conflict of interests.

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11

Full Length Research Paper

Euterpe oleracea Mart. (açai): an old known plant with a new perspective

Heitor Ribeiro da Silva[1,2], Daniele da Cruz de Assis[2], Ariadna Lafourcade Prada[1,2], Hady Keita[2], Jesus Rafael Rodríguez Amado[2] and José Carlos Tavares Carvalho[2]*

[1]Programa de Pós-graduação em Inovação Farmacêutica, Departamento de Ciências Biológicas e da Saúde, Universidade Federal do Amapá, Brazil.
[2]Laboratório de Pesquisa em Fármacos, Curso de Farmácia, Departamento de Ciências Biológicas e da Saúde, Universidade Federal do Amapá, Brazil.

The açai (*Euterpe oleracea* Mart) fruit pulp is extensively used in Brazil as food among other uses. The health benefits of açai are largely reported by the Amazon inhabitants. Nonetheless, just a few pharmacological and toxicological studies were made to probe the innocuousness and the safety of the use of this product. The aims of this work were to update knowledge about the chemical composition, pharmacological and toxicological studies of the fruits and to identify possible vacuum of knowledge in the use, evaluation, and characterization of *E. oleracea* Mart (Açai) as a promising Amazon superfruit. It was made a draw out internet revision, especially in databases as NCBI, SCOPUS, PUBMED, SCIELO, and ELSEVIER by using the keywords *E. oleracea*, açai, nutraceuticals and food supplementations. Also, it was looked for each one of the ethnobotanical uses reported for this plant species combined with the first keywords. A complete record of the chemical composition of this species was achieved. Just two studies in humans were found in the literature using the açai fruit pulp. There is no sufficient systematic evidence to assure that all of the ethnobotanical uses of this species are true. A great emptiness of scientific knowledge related to the real benefits of this plant species exist. There exist neither pharmaceutical forms nor standardized product derived from the açai fruit. Until now, the number of scientific studies that allow the validaton of the ethnopharmacological practices, the innocuousness and the safety of the use of this plant fruit is insufficient.

Key words: *Euterpe oleracea*, açai, nutraceutical, food supplementation.

INTRODUCTION

Euterpe oleracea Mart (EOM), commonly known as acai, has long been used by the inhabitants of the Amazon. This is a plant with many beneficial health effects, which has been used in other countries of Europe, North America and Middle-Eastern (Menezes et al., 2011). The increase of the interest in the international community for the açai was clearly related by Heinrich et al. (2010). They demonstrated by using an overall search for açai
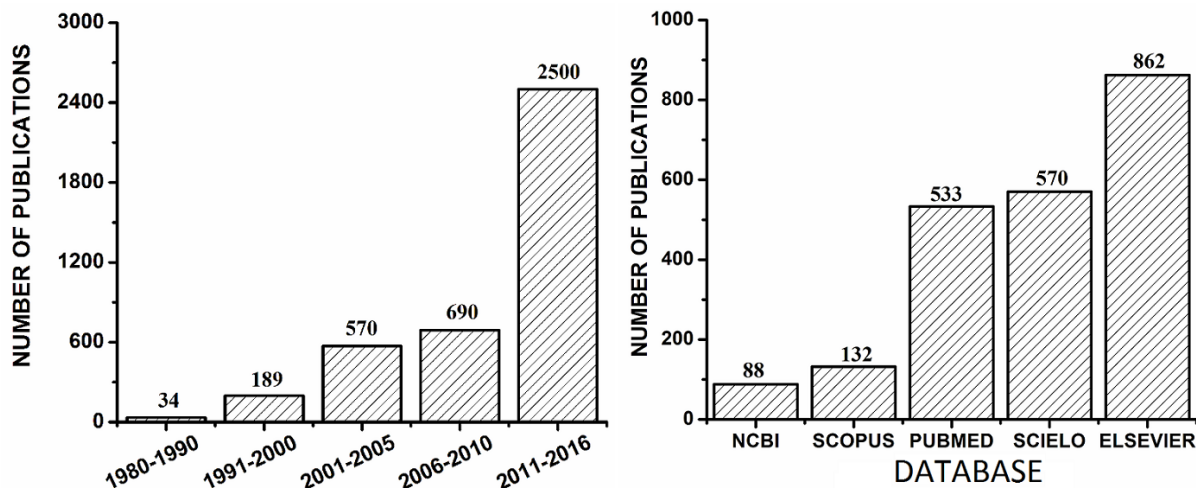
Figure 1. Publications including the term *Euterpe oleracea* Mart. (açai). (A) In international scientific database; (B) Represent reports for this species in Google (Period: 1980-2016).

from 2004 to 2010, that it was an increase in the searches about this plant all over the world and especially in USA, UK, Australia, New Zealand and Canada.The harvest period of this fruit is between August and December and approximately 1000 jobs are created every year for the local populations in the Northern Brazil. The northern states of Brazil produce 95% of all the country's açai (Heinrich et al., 2010). Just in 2015, 198.9 thousand ton of the acai were produced in Brasil; of it, 54% were produced in Para, 33.6% in Amazonas, 7% in Maranhão, 2% in Acre, and 1.1% in Amapá, Rondônia, and Roraima (0.9%).

The intake of açai fruit pulp with chicken meat, fish, and vegetals as tapioca or maize flour is a traditional practice of the Brazilian population. The açai fruit pulp is also used for the preparation of pies, jellies, creams, ice creams and liqueurs as run and wines (Rogez, 2000).

The interest for the use of this plant is continually increasing over the years (Schauss, 2016). To this regard, it was observed that there are a lot of publications (On the Internet) about the use of this species as nutraceutical and as a cosmetic ingredient. The majority of these publications lack the scientific perspectives with no serious data to support their characteristics.

Several ethnobotanical uses have been linked to the chemical composition of the açai fruits pulp (Portinho et al., 2012). Different parts of this plant have been used by Amazonian populations (Bourdy et al., 2000). Over the years, studies have been made to identify chemical characteristics of the fruits of EOM. The main secondary metabolites present in the fruits of açai are phenolics, principally flavonoids and anthocyanins (Costa et al., 2013). The dissimilar chemical composition of the açai fruit pulp allows their use in nutraceuticals, cosmetic and food industries (Schauss, 2015; Schauss, 2016). However, there are few scientific studies supporting the

pharmacological and toxicological properties of the açai fruit pulp.

This paper aims to update knowledge about the chemical composition, pharmacological and toxicological studies related to the açai fruits and to identify possible vacuum of knowledge in the use, evaluation and characterization of *E. oleracea* Mart (Açai) as a promising Amazon superfruit.

MATERIAL

A draw out revision was made in databases as NCBI, SCOPUS, PUBMED, SCIELO, and ELSEVIER by using the keywords *E. oleracea*, açai, nutraceuticals and food supplements. Also, it each one of the ethnobotanical uses reported for this plant species (anti-inflammatory, anticancer, antioxidant, cardiovascular, dyslipidemic, neuroprotective, renal diseases, cosmetic, food, toxicity test, and pharmacological test) was looked for combined with the first keywords. The review was made from 1980 until 2016.

RESULTS AND DISCUSSION

The increase on the interest in this Amazonian fruit is noticeable. Figure 1 shows the increase of the research to prove the biological, nutraceutical and pharmaceutical activity of this species from 1980 to 2016. From 1980 up to 2016, 3983 publicatons were made about *E. oleracea* Mart. Nonetheless, just 2181 (54.75%) of these publications were found in the scientific database. In the last five years, it was an augment of 173% on the publications about this plant. Nonetheless, just 33% of these publications were publicized in scientific database. These are evidence of the increasing interest on this plant but just a few amounts of these are scientifically founded.

Figure 2. A plantation of *Euterpe oleracea* Mart (açai) palm tree in the Recanto Santa Clara, located at -0.96039 N; -51.268450 O, Mazagão, Amapá, Brazil.
Source: Authors.

Botanical aspects

The açai palm belongs to the Arecaceae family. This family has about 200 genera and about 2600 species distributed in tropical and subtropical areas (Jones, 1995). Of the native species from Brazil, the most important are *E. oleracea, Euterpe edulis,* and *Euterpe precatoria*. The first is popularly known as *Palmiteira*, *açai de Pará* and açai real. This species was the main source of raw material in the Palmito industry (Palm's heart pickled) (Choi et al., 1998). The botanical classification of this species, according to Cronquist is Kingdom Plantae; Division: Magnoliophyta; Class: Liliopsida; Order: Arecales, Family: Arecaceae; Genus: Euterpe; Species: *E. oleracea*. The binomial name of this species is *E. oleracea* Martius 1824 (Schauss, 2015, 2016). Figure 2 show the açai palm tree and the collected fruit ready to be commercialized.

Chemical composition

Species *E. oleracea* Mart has been extensively investigated for their chemical composition. Table 1 present all chemical compounds reported, until now, for the fruit of *E. oleracea* Mart.

The açai fruit pulp is rich in polyphenols like flavonoids and anthocyanins and content a diversity of fatty acids (Silva and Rogez, 2013). Anthocyanins are glycosidic derived from anthocyanidins. At low pH, they are predominantly present in the form of flavylium cation, giving a reddish color in aqueous solutions. At higher pH, the flavylium cation is converted into other species, some of them being uncolored (Cheminat and Brouillard, 1986).

Açai fruit pulp contains between 88.0 and 211.0 mg/L of total anthocyanins (Lichtenthaler et al., 2005) as cyanidin-3-glucoside, cyanidin-3-rutinosídeo, cyanidin-3-arabinoside (Bobbio et al., 2000) and cyanidin 3-acetyl

Table 1. Chemical compounds reported for the fruit of *Euterpe oleracea* Mart.

Groups	Compound	Reference
Anthocyanins	cyanidin-3-O-glucoside, cyanidin-3-O-rutinosídeo, cyanidin-3-arabinoside, cyanidin-3-arabinoside, cyanidin 3-acetyl hexose, peonidin 3-rutinoside, peonidin 3-glucoside, cyanidin 3-sambubioside	Del Pozo-Insfran et al. (2004), Gouvea et al. (2012), Muñiz-Miret et al. (1996), Schauss et al. (2006a)
Flavonoids	Quercetin, quercetin arabinopyranoside, orientin, isoorientina, isovitexin, rutin, epicatechin, catechin, taxifolin desoxihexose, apigenin, crisoeirol, 5,4'-dihydroxy-7, 3 ', 5'-trimethoxy flavone, luteoline diglicoside, astilbin, quercetin rhamnoside, protoanthocyanidin, procyanidin dimeric, quercetin rutinoside, scoparin, kaempferol rhamnoside, kaempferol rutinoside	Bobbio et al. (2000), Lichtenthaler et al. (2005), Del Pozo-Insfran et al. (2004), Del Pozo-Insfran et al. (2006), Dias et al. (2012), Gallori et al. (2004), Pacheco-Palencia et al. (2009), Schauss et al. (2006ª), Vera de Rosso et al. (2008)
Phenolic	Ferulic acid, benzoic acid, p-hydroxybenzoic acid, gallic acid, pirocatéquic acid, ellagic acid, vanillic acid, p-coumarinic acid, glycoside ellagic acid, chlorogenic acid, escoparine, dihydrokaempferol, velutine, pinoresinol, syringaresinol, 3-hydroxy-1-(4-hydroxy-3,5-dimetoxyphenil)-1-propanona, dihydroconiferyl alcohol, lariciresinol	Gordon et al. (2012), Kang et al. (2010, 2011), Lichtenthaler et al. (2005), Pacheco-Palencia et al. (2009), Ribeiro et al. (2010), Rojano et al. (2011), Schauss et al. (2006b)
Fatty acids	Saturated: butyric, caproic, caprylic, capric, undecanoic, lauric, tridecanoic, myristic, pentadecanoic, margaric, stearic, nonadecanoic, eicosanoic, behenic, tricosanoic, lignoceric; Monounsaturated: tridecenoic, myristoleic, pentadecenoic, palmitoleic, margaroleic, oleic, elaidic, gadoleic, erucic, nervonic; Polyunsaturated: linoleic, linolenic, gamma linolenic, eicosadienoic, eicosatrienoic, homogamma linolenic, arachidonic, eicosapentaenoic, docosadienoic, docosahexaenoic	Nascimento et al. (2008), Schauss et al. (2006a)
Sterols	Campesterol, stigmasterol, b-sitosterol	Schauss et al. (2006a)
Aminoacids	Aspartic acid, threonine, serine, glutamic acid, glycine, alanine, valine, methionine, isoleucine, leucine, tyrosine, phenylalanine, lysine, histidine, arginine, proline, hydroxyproline, cysteine, tryptophan	Schauss et al. (2006a)
Sugars	Fructose, lactose, sucrose, glucose, maltose	(Schauss et al., 2006a)
Lignans	(+)-isolariciresinol, (+)-5-methoxy-isolariciresinol, (+)-lariciresinol (8), (+)-pinoresinol), (+)-syringaresinol	Chin et al. (2008), Da Costa et al. (2010), Ribeiro et al. (2010)
Carotenoids	α-carotene, β-caroten, lutein, tocopherols A, B, C, D, chlorophyll	Da Costa et al. (2010), Darnet et al. (2011), Schaus et al. (2006a)
Vitamins	Vitamin E, vitamin A, vitamin B1, vitamin B2, vitamin B3, vitamin B5, vitamin C, vitamin K	Rogez (2000)
Trace elements	Lead, cadmium,mercury, arsenic, potassium, magnesium, phosphorus, calcium, sodium, zinc, iron, copper	Pesce (2009); Schauss et al. (2006a)

Table 2. Pharmacological and toxicological studies using Euterpe oleracea Mart. and some extracts derived from.

Activity	Refference	*In vivo/ In vitro*	Biomodel/Assay
Antioxidant	Chin et al. (2008)	in vitro	DPPH
	Choi et al. (1998)	in vitro	DPPH and assay of superoxide anion
	De Bem et al. (2014)	in vivo	Rat model: first antioxidant defense system
	De Souza et al. (2010)	in vivo	Rat model: protein oxidation and first defense antioxidant system
	Gordon et al. (2012)	in vitro	TEAC AND TOSC
	Hogan et al. (2010)	in vitro	TEAC AND TOSC
	Kang et al. (2011)	in vitro	ORAC
	Lichtenthaler et al. (2005)	in vitro	TOSC
	Pacheco-Palencia et al. (2008)	in vitro	TEAC
	Rojano et al. (2011)	in vitro	ABTS, DPPH, FRAP AND ORAC
	Rufino et al. (2010)	in vitro	ABTS, DPPH, FRAP
	Santos et al. (2008)	in vitro	ABTS
	Schauss et al. (2006b)	in vitro	SOD, ORAC, NORAC, HORAC AND TAO
	Spada et al. (2009)	in vitro	SOD-TBARS
	Matheus et al. (2006)	in vitro	SNAP with cell culture. Nitric oxide-trapping capacity
	Rocha et al. (2007)	in vivo	Rat model: Determination of NO formation
Antineoplasic	Del Pozo-Insfran et al. (2006)	in vitro	Cellular proliferation and apoptosis
	Hogan et al. (2010)	In vitro	Rat, induction of apoptosis of C-6 brain glioma cells
	Silva et al. (2014)	In vitro	Human cell line, antitumorigenic potential in the MCF-7 cell line
Anti-inflammatory	Favacho et al. (2010)	in vivo	Rat model: edema
	Kang et al. (2011)	in vivo	Rat, SEAP
	Schauss et al. (2006b)	in vivo	Rat, cyclooxygenase (COX)-1 and COX-2 inhibition
	Matheus et al. (2006)	In vitro	Rat, production of NO in macrophage cell line
Genotoxicity	Ribeiro et al. (2010)	in vivo	Rat model: micronucleus test and comet assay
Cytoprotective	Chin et al. (2008)	in vitro	Cultured MCF-7 cells stressed by H_2O_2
Dislipidemic	De Souza et al. (2010)	in vivo	Rat model: Hypocholesterolemic
	De Souza et al. (2012)	in vivo	Rat model: Mediation of the Hypocholesterolemic activity
	Udani et al. (2011)	in vivo	Humans overwight, evaluation of lipid profile and metabolic parameter
	Xie et al. (2011)	in vivo	Rat model: Atherosclerosis

DPPH: 2,2-diphenyl-1-picrylhydrazyl; TEAC: trolox equivalent antioxidant capacity; TOSC: total oxidant scavenging capacity; ORAC: oxygen radical absorbance capacity; ABTS: 2,2′-azino-bis-3-ethylbenzthiazoline-6-sulphonic acid; FRAP: ferric reducing antioxidant power; SOD: superoxide dismutase; HORAC: hydroxyl radical antioxidan capacity; NORAC: peroxynitrite radical averting capacity; TAO: total antioxidant; TBARS: thiobarbituric acid reactive substances.

hexose (Dias et al., 2012). Cyanidin-3-glucoside and cyanidin-3-rutinoside are the anthocyanins with a higher presence in açai fruit pulp (Pacheco-Palencia et al., 2009; Vera de Rosso et al., 2008). To anthocyanins content, the antioxidant properties of the açai fruit pulp has been attributed (Del Pozo-Insfran et al., 2004; Muñiz-Miret et al., 1996).

The main phenolic and flavonoids reported for the acai fruit pulp were quercetin, orientin and its derivatives (Pacheco-Palencia et al., 2009; Schauss et al., 2006b). The phenolic profile of the açai fruit pulp was reported (Del Pozo-Insfran et al., 2004). Ferulic acid, p-hydroxybenzoic acid, gallic acid, pyrocatecolic acid, ellagic acid, vanillic acid, p-coumaric acid and glycoside

ellagic acid were the majorities. Other authors reported the presence of other phenolic compounds as epi-catechin, catechin, rutin, orientin, isoorientin, isovitexin, scoparone, taxifolin deoxihexose, apigenin, crisoeirol, dihydrokaempferol, velutine; 5,4'-dihydroxy-7, 3', 5'-trimethoxy flavone, luteolin diglycoside and procyanidin dimers (Gordon et al., 2012; Rojano et al., 2011).

The lipid fraction of the pulp contains between 68.0 and 71.0% of mono-unsaturated fatty acids and 7.8 and 10.6% of poly-unsaturated fatty acids, including linoleic acid, oleic acid and palmitic acid (Nascimento et al.,2008) in high concentrations. A large number of other minor fatty acids have been reported. Schauss et al. (2006a) reported the presence of nineteen amino acids in the lyophilized powder of açai fruit pulp, corresponding to 7.59% of the lyophilized fruit pulp. Furthermore, three sterols campesterol and stigmasterol were also reported (0.48 mg/g of dry weight) with 0.44 mg/g of dry weight of b-sitosterol.

A range of lignans has also been reported. Of the nine lignans isolated [including (+)-isolariciresinol, (+)-5-methoxy-isolariciresinol, (+)-lariciresinol (8), (+)-pinoresinol), (+)-syringaresinol], pinoresinol is well known as an antioxidant and monoterpenoids and norisoprenoids (Chin et al., 2008), α-carotene, β-carotene and lutein (Da Costa et al., 2010; Ribeiro et al., 2010), tocopherols A, B, C and D and vitamin E and chlorophyll 394 and 20.8 mg/100 g of dry pulp, respectively (Darnet et al., 2011). In the same way, Rogez (2000), reported (for each 100 g of the fruit pulp) the presence of vitamin A 146 IU, vitamin B1: 11.8 ug, vitamin B2: 0.32 ug, vitamin B3: 1738 µg, vitamin B5: 1389 µg, vitamin B6: 257 µg, vitamin C: 0.01 mg, vitamin E: 20 µg and vitamin K 2.07 µg. Pesce (2009) reported (in 100 g of dry pulp) the presence of trace elements as potassium 932 mg, magnesium 174 mg, phosphorus 124 mg, calcium 286 mg, sodium 56.4 mg, zinc 7 µg, iron 1.5 µg and copper 1.7 µg.

The açai fruit pulp content is a complete chemical composition that made them an excellent nutraceutical complement of vitamins, mineral, fatty acids and antioxidants compound like anthocyanins, polyphenols, and flavonoids.

These compounds can help to prevent several degenerative diseases. Besides this, the chemical composition of the açai fruit pulp can justify the fact that peoples living on the banks of the rivers having the açai fruit pulp as the basis of their diet as they lack other foods that will enable them to balance their nutrition. They are strong people and healthy from childhood. On the other hand a lot of ancient peoples in this region were observed just for intake of açai fruit pulp as a basis diet, just accomplished by some grains and cereals like maize, wheat, and oats.

Ethnobotanical and pharmacological uses of açai

Açai fruit pulp, the whole fruit, and the root of the açai palm tree have been used by Amazonian tribes as the remedy for treating diarrhea, parasitic infections, bleeding, and ulcer (Schauss, 2015, 2016). The decoction of the açai crushed seed has been used for the treatment of fever, menstrual pain, liver diseases, and malaria. The root mixed with other medicinal plants was used as antimalarial (Vigneron et al., 2005), for the treatment of the prostate cancer (Homma et al., 2006) and for the treatment of leishmaniasis (Odonne et al., 2011). Some of these ethnobotanical uses can be attributed to the presence of metabolites like phenolics, flavonoids, and anthocyanins. Nonetheless, there is a not reported study about these conditions.

Despite the great number of publications on the internet about the uses of EOM, just a few number of them are significantly and scientifically founded. Table 2 presents the most significant studies reported for this species in the scientific database. The fact that just one study in humans was made is interesting (Udani et al., 2011).

Is contradictory that the fact that the açai fruit pulp, probably the most consumed vegetal in the North and Northeast of Brazil, lack off the studies to probe the innocuousness, security and efficacy of their use by the population as food and as an ethnobotanical remedy. Still, more is curious that no work in humans was conducted to evaluate the real benefits of the majority of the popular uses of this product. Consequently, if this plant and especially the fruits have been used for centuries with certain "innocuousness", is mandatory to confirm the ethnopharmacological use in order to validate, scientifically, the efficacy and safety of their use.

Antioxidant activity

Antioxidant activity is the most studied property of the *E. oleracea* Mart. Data about the açai fruit antioxidant potential are disagreeing. Del Pozo-Insfran et al. (2004) reported that anthocyanins are the predominant factor of the antioxidant capacity of açai pulp. Kang et al. (2010) concluded that the predominant factor in the açai antioxidant activity is the presence of seven flavonoids present on it (orientin, isoorientin, vitexin, luteolin, criseriol, quercetin, and dihydrokaempferol). Our judgment, both authors are on the right because the principal antioxidant effect of the natural extracts is the synergy among all of the compound present in the extracts, which are able to efficiently inactivate reactive nitrogen and oxygen species.

Spada et al. (2009) have shown an antioxidant activity of açai frozen fruit pulp in the cerebral cortex,

hippocampus, and cerebellum of rats treated with hydrogen peroxide (H_2O_2). Pretreatment of tissues with açai extract decreased the H_2O_2-induced damage of both lipids and proteins. The extract of the fruit was also able to reduce the activities of the antioxidant enzymes superoxide dismutase and catalase to basal levels. They observed a negative correlation between the polyphenol content of açai and the levels of lipid (r=0.689; P<0.05) and protein damage (r=0.569; P<0.05), suggesting the participation of polyphenols in the observed antioxidant activity.

Practically, all the compounds presents in açai fruits are recognized antioxidants. The synergistic antioxidant action of fatty acids, vitamins, sterols, flavonoids, anthocyanins and phenolics makes the pulp of this fruit a powerful antioxidant. The antioxidant activity of the majority of this compounds in others vegetal species were reported (Ismail et al., 2010; Lee et al., 2002; Liolios et al., 2009).

Some sickness like diabetes, hepatitis, and some degenerative diseases promote an imbalance in the body antioxidant defense. It could be interesting to test the preventive or regenerative activity of the differents açai fruit extracts as a way for the evaluation of the real benefit of these extracts in differents related pathologies. On the other side, there was no publishing article evaluating the benefits of the açai fruit pulp (or in derived extracts) in humans' model. Thus, a lack of these studies is a real necessity.

The use of this product must be cautiously in patients with diabetes or those using antidiabetic agents as, according to human research, açai may lower glucose and insulin (Udani et al., 2011).

Assays in cardiovascular diseases

The açai fruit pulp contains a large number of fatty acids, including linoleic acid, oleic acid, palmitic acid and other fatty acids (Schauss et al., 2006a). These substances showed a cardioprotective effect in rats improving the lipid profile (Bhattacharya et al., 2006). Another study reported a vasodilator effect on mesenteric vessels of rats related to a lipid profile improved by the açai fruit extract (Mantovani et al., 2003; Xie et al., 2011) reported the atheroprotective effects of açai fruit pulp in apolipoprotein E-deficient in mice, mediated by a reducing lipid peroxidation through boosting antioxidant enzymes and inhibiting pro-inflammatory cytokine production.

One study was conducted in overweight patients who consumed 100 g açai pulp twice daily for 1 month. There was a reduction in glucose levels from 98.0 ± 10.1 to 92.8 ± 10.9 mg/dl. There were also reductions in total

cholesterol and triglycerides (Udani et al., 2011). Animals' feed with hypercholesterolemic diet, treated with açai pulp extract, showed an improvement in the lipid profile. These results suggest that açai pulp promotes a hypocholesterolemic effect in a rat model of dietary-induced hypercholesterolemia (De Souza et al., 2010). A diet rich in antioxidants can improve both the lipid metabolism and glucose homeostasis reducing complications in the two types of diabetes and in metabolic syndrome (Dembinska-Kiec et al., 2008).

In this sense, until we know, there is only one study in humans and the report is not enough to confirm the potential utility of the açai fruit to control dyslipidemic disorders and other imbalances of the lipidic profiles. Thus, the richness in chemical compounds of these products could be taken advantage for this kind of treatment, but other studies are needed.

Anti-inflammatory activity

The oily extract of the açai fruit reduced the number of neutrophils migrating in a carrageenan-induced peritonitis model in rats. These results suggested that the oil of açai fruit has anti-inflammatory and antinociceptive activity. It was attributed to the presence of flavonoids and a lot of unsaturated lipid present in the extract (Favacho et al., 2011). On the other side, açai fruit pulp showed potential cyclooxygenases COX-1 and COX-2 inhibitor activity (Schauss et al., 2006b).

The anti-inflammatory effects of the açai extract were screened by the secretion embryonic alkaline phosphatase (SEAP) assay. This assay is designed to measure NF-jB activation. It studied the capacity of activation of NF-jB of the açai extract by the secretion embryonic alkaline phosphatase (SEAP) assay in rats. A dose-dependent SEAP inhibitory activity in RAW-blue cells induced by lipopolysaccharides was observed. It was also observed an inhibition of SEAP induced by oxidized LDL, indicating a potential atheroprotective effect in rats (Kang et al., 2011).

Açai extracts inhibited lipopolysaccharide and interferon-gamma-induced nitric oxide (NO) production in a macrophage cell line. Overproduction of NO may lead to activation of NO synthase, leading to the generation of cells mediating inflammatory processes. The mechanism of action was associated with inhibition of NO synthase expression (Matheus et al., 2006).

The anti-inflammatory activity of açai fruit is still no conclusive, the studies made until now are none conclusive, in some of them was used the oily fraction of the açai fruit pulp, in others, they use the açai fruit pulp mixture with other species (Jensen et al., 2008; Schauss, 2016). There is not any report of the evaluation of any

formulation made by using and extract or the lyophilized fruit pulp. We do not find ethnopharmacological reports for the use as anti-inflammatory.

Neuroprotective activity

One study examined whether açai fruit extract afforded protection against β-amyloid (Aβ)-mediated loss of cell viability and oxidative stress associated with anti-fibrillar effects. PC12 cells were exposed to either Aβ1–42, Aβ25–35 or tertbutyl hydroperoxide (t-BHP), alone or in the presence of açai extract (0.5 to 50 μg/ml). The study shows that exposure to Aβ1–42, Aβ25–35 or t-BHP decreased PC12 cell viability. Pretreatment with açai extract significantly improved cell viability following Aβ1–42 exposure. Açai extract inhibited the thioflavin T fluorescence and disrupted Aβ1–42 fibril and aggregate morphology. In comparison with other phenolics, açai was most effective at inhibiting Aβ1–42 aggregations. Inhibition of β-amyloid aggregation may underlie a neuroprotective effect of açai (Wong et al., 2013).

The β-amyloid proteins are strongly implicated in Alzheimer's disease (Murphy and Levine, 2010; Schauss, 2016). A negative correlation was reported between the polyphenol content of açai and the levels of lipids and proteins damage. These data suggested that açai has a positive contribution to the prevention of the development of age-related neurodegenerative diseases. Nonetheless, further investigation is needed to evaluate the role of chemical compounds present in açai in these findings.

Anticancer effect

An anthocyanin-rich extract from açai fruit was used (AEA) to investigate the antioxidant properties and antiproliferative activity against C-6 rat brain glioma cells and MDA-468 human breast cancer cells. AEA remarkably suppresses proliferation of C-6 rat brain glioma cells, but has no effect on the growth of MDA-468 human breast cancer cells. Further experiments demonstrated that the AEA treatment dose-dependently inhibited the growth of C-6 rat glioma cells with an IC_{50} of 121 μg/ml. The DNA ladder fragmentation results indicated that AEA-induced apoptosis of C-6 rat brain glioma cells (Hogan et al., 2009).

Açai fractions containing polyphenolic compounds reduced the proliferation of HL-60 leukemia cells through caspase-3 activation in a dose- and time-dependent manner. The mechanism of action is associated with polyphenolic phytochemicals activating caspase-3, leading to cell death or apoptosis (Del Pozo-Insfran et al., 2006).

In another work, the anticancer activity in different human malignant cell lines derived from breast and colorectal adenocarcinomas was evaluated. Cell lines were treated with 10, 20, and 40 μg/mL of bark, seed, and total açai fruit hydroalcoholic extracts for 24 and 48 h. After treatment, cell viability was measured and cell morphological features were observed. The study demonstrated that açai possesses antitumorigenic potential in the MCF-7 cell line. This fact demonstrated the need to identify the compounds responsible for this activity and the molecular target in the cell (Silva et al., 2014). As observes, the real anticancer potential of the açai fruit pulp and the açai fruit extracts are still unexplored and just a few studies have been made to this regard, despite that the ethnopharmacological use in cancer is well reported for the population.

Use of açai extract in renal diseases

The use of açai extract in reduced acute renal failure (ARF) was reported. The study investigated the effect of açai fruit extract on glycerol-induced ARF in rats. Results showed for a different dose a significant decrease in serum urea, serum creatinine, and blood urea nitrogen. Moreover, there was significant amelioration in renal oxidative stress markers and renal histopathological changes. These results suggest that açai fruit extract has a potential effect in ameliorating renal damage involved in ARF (Unis, 2015).

Other study examined the effect of açai seed extract (ASE) on cardiovascular and renal alterations in adult offspring rats, whose mothers were fed with low-protein diet during pregnancy. It was observed that hypertension and the reduced acetylcholine-induced vasodilation in the low-protein group were prevented by ASE. This product improved nitrite levels and the superoxide dismutase and glutathione peroxidase activity in low-protein, with a decrease of malondialdehyde and in the protein carbonyl levels. Kidney volume and glomeruli number were reduced and glomerular volume was increased in low-protein group. These renal alterations were prevented by ASE (De bem et al., 2014).

A study to investigate the possible mechanisms of renal injury attenuation caused by açai extract in a rat renal (I/R) model was reported. Rats were administered with açai extract at 500 and 1000 mg/kg for 15 days, before bilateral renal I/R induction. Serum and kidneys were isolated and used for subsequent biochemical analysis. The açai extract significantly and dose-dependently attenuated I/R-induced renal damage. It suppressed the levels of blood urea nitrogen (BUN), serum creatinine, and renal tissue content of kidney injury molecule-1 (KIM-1). In addition, the serum lactate dehydrogenase (LDH) activity was inhibited. Moreover, renal contents of malondialdehyde (MDA), myeloperoxidase (MPO),

interferon-gamma (IFN-γ), caspase-3, collagen IV, and endothelin-1 were reduced, while renal interleukin-10 (IL-10) content was increased by açai extract administration (El Morsy et al., 2014).

The reported studies assuring the renal protective activity function did not specify the nature of the extract used. Finally, there is no study in humans, for the evaluation of the renal function or renal failure. Thus, this kind of studies is imperative.

Uses of açai in cosmetics

The high content of anthocyanins and phenolic compounds with important antioxidant activity was used in cosmetic preparation for the treatment and prevention of skin damages (Herculano, 2013). Among these products, both the extract and the pulp of açai fruits are used as moisture agents in creams, hair conditioner, and shampoo. The açai fruit pulp has properties of nutrition and capillary brightness. The oil extracted from the pulp is used in shampoos and body lotions (Hogan et al., 2009). The glycolic extract of açai was used to prepare a sunscreen emulsions (o/w). The resulted cream showed a good protection UV-A and UV-B factor (Daher et al., 2014).

In the face of all reports that can be found on the Internet promoting the use of cosmetics with açai fruit pulp or açai extracts as active principle, there are no reports of the studies evaluating the effectiveness and the security of all of these products. Thus, there are a lot of cosmetics and nutraceutical products using some product derived from açai as an active principle without scientific foundation. Nonetheless, this represents an excellent opportunity to do research in this area to justify scientifically the use of these products.

Pharmaceutical forms and foods based on açai

Due to the richness in phytochemical substances present in the açai fruit pulp, it is used in a variety of formulations. The freeze dried açai fruit pulp was used in a formulation for the erectile dysfunction. An increase in the time of shelf life of this product was observed (Clewell et al., 2010). Tablets and açai capsules can be found in the market. Everything is marketed as the nutritional supplement. In these formulations, the lyophilized açai fruit pulp was used (Empresa Saúdeja, 2015). In a general way, a lot of cosmetic and nutritional preparations containing açai fruit pulp including juices, powders, capsules, liquids, creams, and lotions can be found in the market. However, there is no product registered as a medicament (Medicament: A preparation containing a tested active drug used to diagnose, cure, treat, or prevent disease) (United States Pharmacopoeia, 2012). In all cases, there is no scientific evidence supporting the biological activities attributed to these preparations.

Toxicological studies involving açai fruit pulp

The toxicity of a mixture of the açai fruit pulp with a berry functional juice was studied. The mixture was neither cytotoxic nor genotoxic. The LD_{50} based on a 14-day acute oral toxicity study was greater than 2000 mg/kg body weight (Schauss et al., 2010). In another study, the genotoxicity of açai fruit pulp was investigated in Swiss albino mice by using a doxorubicin (DXR)-induced DNA damage model. The protective effect of açai fruit pulp was observed in both acute and subacute treatments when administered prior to DXR. The protective effects were associated with the phytocompound presents in the açai fruit pulp. Despite that the pulp of this fruit, it is used as food in Brazil and other parts of the world, and besides that, it is widely available in a variety of forms, including juices, powders, and capsules, etc., açai is not listed on the U.S. Food and Drug Administration (FDA) Generally Recognized As Safe (GRAS) list (Schauss, 2016).

Açai has been very well accepted by the population of big cities, attracted by the nutritional and medicinal properties of the fruit (Rogez, 2000). With the constant increase of açai consumption, a standardization of the quality of this product is required (Bhattacharya et al., 2006; Boghani et al., 2012; Nogueira et al., 2005).

Nonetheless, the evaluation of the safety of any pharmaceutical and food product is an imperious necessity. Thus, there is a lack of studies to demonstrate the safety in the use of the açai. Taking into account that the açai fruit is one of the most consumed vegetal product in the North and Northeast of Brazil, in some Asian country and in North America toxicological studies by means of the international standards are imperative to assure the innocuity of this product. On the other side, there is no published results talking about the safety and efficacy of any of the nutraceutical products that are sold in the market.

Nutritional composition

Each 100 g of the dry açai fruit pulp content has 533.9 calories, 32.5 g of total fats, 8.1 g of saturated fats, 13.5 mg of cholesterol, 52.2 g of carbohydrates, 44.2 g of dietary fiber, 1.3 g of sugar, and 8.1 g of proteins. Also, it contains vitamin A 1002 UI, vitamin C 0.1 mg, calcium260 mg, and sodium 30.4 mg (Costa et al., 2013; Schauss et al., 2006a).

For the diet, the great contribution of açai is their energetic value because of the high content of lipids (70

to 90% of the calories) (Crozier et al., 2011; Rufino et al., 2010). The high fiber content and a considerable amount of anthocyanins present in açai make this fruit a great help to prevent chronic degenerative diseases (Rufino et al., 2010). Açai is also an important source of trace elements such as calcium, phosphorus, sodium, zinc, iron, manganese, copper, boron, chromium, magnesium, potassium, and nickel (Crozier et al., 2011).

Conclusions

Despite the widespread use of açai by populations of Brazil and other countries, there are few scientifically based studies on nutrition and medicinal properties of the fruit pulp of this plant. The antioxidant activity has been the most investigated. No dosage forms using açai as the active ingredient were registered; until now, as a medicine to the health authorities of any country. Just two studies, in humans were found in the literature using the açai fruit pulp. There are no sufficient systematic evidence to assure that all of the ethnobotanical uses of this species could be scientifically founded. A great emptiness of scientific knowledge related to the real benefits of this plant species exists. There exist neither pharmaceutical forms nor standardized product derived from the açai fruit pulp. Until now, the number of scientific studies that allow the validation the ethnopharmacological practices, the innocuousness and the safety of the use of this plant fruit is insufficient.

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Conflict of interests

The authors have not declared any conflict of interests

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