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In vitro mutagenicity assay (Ames test) and phytochemical characterization of seeds oil of *Helianthus annuus* Linné (sunflower)

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ABSTRACT

The objective of this research was to investigate the genotoxic potential of the oil of *H. annuus* L. (sunflower) seeds via the Ames test as well as its oxidative properties and lipid composition. The pre-incubation method, system metabolic activation (S9 fraction) and five *S. typhimurium* strains (TA97, TA98, TA100, TA1535 and TA102) were employed for the Ames test. The oxidative stability and fatty acid composition were analyzed by standard methods and gas chromatography. A revertant analysis showed no significant differences between the treatment doses (10–200 μl/plate) and the negative controls, regardless of $S9^+$ and $S9^-$, and included all of the *S. typhimurium* strains. Chromatographic analysis showed high levels of polyunsaturated fatty acids, followed by monounsaturated, saturated and total trans-isomers. Among the polyunsaturated, monounsaturated and saturated fatty acids, linoleic, oleic and palmitic acids predominated. The results suggest that the sunflower oil is not genotoxic as indicated by frameshift mutations and base pair substitutions regardless of the treatment dose, but shows dose-dependent toxicity. The oxidative properties of the sunflower oil were consistent with the requirements of national and international standards. However, its composition could also indicate phytotherapeutic properties.

1. Introduction

The cultivated sunflower (*Helianthus annuus* L.) is one of the 67 species of the genus Helianthus and is a dicotyledonous plant and member of the Compositae (Asteraceae) family, having a typical composite flower [1]. Sunflower seed oils are high in saturated fatty acids (lauric acid C12:0, maristic C14:0 palmitic C16:0 and stearic C18:0), monounsaturated fatty acids (oleic acid C18:1, n-9), and poly-unsaturated fatty acids (linoleic acid C18:2, n-6, and α-linolenic C18:3, n-3) [2]. The unsaturated fatty acids are the most abundant, especially linoleic acid. In turn, linoleic acid makes vegetables oil more susceptible to lipid oxidation [3] and therefore favors the formation of substances (e.g., peroxides, hydroperoxides and free radicals) that cause spoilage [4] in addition to the genotoxicity caused by the reaction between these substances and DNA molecules [5]. In addition, linoleic acid is a precursor of arachidonic acid [6], which participates in the synthesis of biologically active mediators, such as prostaglandins, thromboxanes and leukotrienes. These substances act as inflammatory mediators [7,8], stimulating

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local neovascularization, cell migration, proliferation and the differentiation of fibroblasts, along with extracellular matrix synthesis that acts directly on healing [9,10].

Sunflower seed oil has potential phytotherapeutic properties [10], and some research also supports the phytotherapeutic effectiveness of the oil and an aqueous or alcoholic extract of sunflower seeds for the relief of asthmatic symptoms and other diseases [11], gastric protection [12,13], healing properties [14], anti-inflammatory action [15–17] and antimicrobial properties [12,14,18,19]. In addition, a limited number of investigations that investigated the genotoxic action of various oils, including sunflower seed oil, have gone unnoticed. For example, vegetable oils for human consumption showed high (linseed oil) and weak (sesame oils, wheat germ and soybean) genotoxic responses or even the absence of genotoxicity (sunflower oil, olive oil and refined olive oil extra-virgin) according to a mutation and somatic recombination test (SMART) in *Drosophila melanogaster* [20]. In another study, lymphocytes incubated with an aqueous extract of sunflower oil submitted to thermal stress exhibited high rates of chromosomal breakage and were significantly different from those of lymphocytes incubated with the same concentrations of the aqueous extract of sunflower oil in the absence of heat. Furthermore, in tests with HepG2 or HUVEC cells, sunflower oil subjected to heat stress was clastogenic and showed dose-dependent cytotoxicity [21]. The absence of clastogenicity and/or aneugenicity in two sources of oil and a tincture of *H. annuus* L. (sunflower) seeds was also confirmed by in vivo micronucleus assays in mouse bone marrow and was dose-independent, time-independent and sex-independent, except for the oil. However, systemic toxicity of sunflower oil might be dependent on its origin and dose [22].

Thus, further studies on the genotoxicity of sunflower extracts and oils (seeds, flowers and leaves) must be conducted to determine their effects and potential genotoxic mechanisms, especially for setting limits for human use. The Ames test (Salmonella/Microsome test) has been employed as an indicator of the carcinogenic potential in mammals and uses bacterial strains of *S. typhimurium* that are auxotrophic for histidine (*his*$^-$) (i.e., are unable to grow in a minimal culture medium without histidine) because of the presence of mutations in the histidine operon. These strains are used to detect gene mutations, base pair substitutions and frameshift types. However, revertant colonies (i.e., histidine prototrophs) can be quantified after exposure to test substances in the presence or absence of an exogenous metabolic activation system, which indicates the occurrence of gene mutations by the restoration of bacterial metabolism and growth in minimal culture medium [23–25]. Thus, this *in vitro* assay can be used in the screening of new chemicals and drugs as well as to provide a high predictivity of carcinogenicity due to mutagenesis [24]. Some vegetable oils (*Ocimum selloi* [26], *Melaleuca alternifolia* and *Lavandula angustifólia* [27], *Azadirachta indica* [28], *Curcuma longa* L. [29]) have been previously evaluated by the Ames test.

To contribute to the information on the genotoxic potential of vegetable oils, this study evaluated the mutagenic effects of the pharmaceutical oil of *H. annuus* L. seeds (sunflower) in the Ames test using *S. typhimurium* strains TA97a, TA98, TA100, TA102 and TA1535. The oxidative properties and lipid composition of this oil were also assayed in oxidative stability tests (iodine, peroxide and acidity index) and by gas chromatography (GC).

2. Material and methods

2.1. Phytotherapeutic sunflower oil

The pharmaceutical oil of *H. annuus* L. (sunflower) seeds (CAS # 8001-21-6) was purchased commercially and stored according to the manufacturer's recommendations (Fagron Farmacêutica do Brasil, São Paulo, SP, Brazil, lot 14010155B: relative density equal to 0.923 g/cm^3, iodine index equal to 126 g/100 g, acidity index equal to 0.03% and peroxide index equal to 0.03 mequiv. O_2/kg).

2.2. Ames test (Salmonella/Microsome test)

Bacterial strains of *S. typhimurium* TA97a, TA98, TA100, TA102 and TA1535 were kindly provided by Companhia Ambiental do Estado de São Paulo (CETESB, SP, Brazil) to Laboratório de Ecotoxicologia e Microbiologia Ambiental of Prof. Dr. Abílio Lopes – LEAL, stored and maintained as provided in the standard protocol [25]. The test was performed with the pre-incubation method according Mortelmans and Zeiger (2000) [24] and Guideline for testing of chemicals [25]. Initially, each bacterial strain was grown in 20 ml of nutrient broth (Nutrient Broth Oxoide no. 2, code # CM0067, Thermo Fisher Scientific Inc.) at 37 °C for 16 h (overnight) under constant shaking at 160–170 r.p.m (Incubator with Orbital Agitation Platform – Shaker model 430, Nova Ética, Vargem Grande Paulista, SP, Brazil). Then, 100-μl aliquots of each freshly grown bacterial culture (1–2×10^9 CFU/ml) were added to assay tubes containing (*i*) a known volume of sunflower oil (10, 20, 50, 100 and 200 μl/plate) and 500 μl of the S9 mixture [phosphate buffer, NADPH glucose-6-phosphate, solution of salts ($MgCl_2$ and KCl), and the S9 fraction (S9 microsomal fraction of homogenized rat liver: post-mitochondrial fraction supplemented with a cofactor, prepared from the liver of rodents treated with an agent enzyme inducer, arocloror 1254, MOLTOX®, Molecular Toxicology, USA)] – system with metabolic activation – or (*ii*) known volumes of sunflower oil (10, 20, 50, 100 e 200 μl/plate) and 500 μl of phosphate buffer (Na_2HPO_4 and $NaH_2PO_4{\cdot}H_2O$) – system without metabolic activation –, and pre-incubated at 37 °C for 30 min.

Prior to testing, aliquots of the S9 fraction were prepared according to the manufacturer's specifications and stored in 2-ml sterile Eppendorf-type tubes at −20 °C. The reagent 2-Aminoanthracene (2.5 μg/plate; CAS Number 613-13-8, Cat. #A38800 Aldrich, Sigma-Aldrich Chemical Co.) was used as a positive control in analysis systems with metabolic activation of all of the *S. typhimurium* strains. For analysis systems without metabolic activation and as a positive control, 4-Nitroquinoline *N*-oxide (0.5 μg/plate; CAS Number 56-57-5, Cat. #N8141 Aldrich, Sigma-Aldrich Chemical Co.) was used in the assays with the TA97a, TA98 and TA100 strains and sodium azide (5 μg/plate; CAS Number 26628-22-8, Cat. #V000494 Vetec, Sigma-Aldrich Chemical Co.) and hydrogen peroxide (50 μg/plate; CAS Number 7722-84-1, Cat. #H1009 Sigma, Sigma-Aldrich Chemical Co.) were employed in the assays with strains TA1535 and TA102, respectively. Phosphate buffer was used as a negative control of analysis systems with and without metabolic activation with all *S. typhimurium* strains [24,25].

After the pre-incubation period, 2 ml of surface agar (top agar) adjusted to 45 °C [10.3 mM NaCl, 0.5 mM biotidine solution (histidine and biotin) and bacteriological agar 0.6% (w/vol)] were added to each test tube, vortexed for 30 s and dispensed on Petri dishes (90 mm × 150 mm) containing 20 ml of minimal Vogel Bonner medium [20 ml of 50 × (10 g of $MgSO_4{\cdot}7H_2O$, 100 g of $C_6H_8O_7{\cdot}H_2O$, 500 g of K_2HPO_4, 175 g of $NaNH_4HPO_4{\cdot}4H_2O$, 1000 ml of H_2O type 1 q.s.p.); 200 ml of glucose solution 10% (w/vol); 780 ml of bacteriological agar 1.92% (w/vol)] for the TA98, TA100, TA102 and TA1535 strains, and minimal agar with added glucose at 0.4% (w/vol) for strain TA97a. These plates were kept at room temperature until the complete solidification of the top agar. Revertants (*his*$^+$) were counted after incubation at 37 °C for 66 h [24] and Guideline for testing of chemicals [25].