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ACTIVIDAD ANTIOXIDANTE DE LA HOJA DE *Tithonia diversifolia* (Hemsl.) A. Gray

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T E S I S

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ANTIOXIDANT ACTIVITY OF *Tithonia diversifolia* (Hemsl.) A. Gray LEAVES

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ABSTRACT

The harmful effects of synthetic antioxidants on human health could be avoided by the use of natural antioxidants. Therefore, two experiments were performed: (1) *Tithonia diversifolia* (Td) dry extracts storage stability: effect of solvent, light and storage time and (2) effect of the methanolic dry extract (MEDEX) of Td on oxidative stability of sunflower crude oil during several days of storage: without MEDEX, with MEDEX and with butylated hydroxytoluene (BHT). The response variables of experiment 1 were total phenolic content (TPC), total flavonoids content (TFC) and antioxidant activity (AA) and those of experiment 2 were TPC, AA, peroxide value (PV) and malondialdehyde (MDA) content. There was no effect of light and time on any variable of the experiment 1, however, MEDEX showed higher values of TFC (5.55 mg/g) than the water dry extract (1.32 mg/g). The sunflower crude oil with MEDEX showed higher values of TPC (1045 mg/kg) and AA (2383 µmol/kg), and lower PV (11.972 meq/kg) values than the oil without MEDEX (975 mg/kg, 1842 µmol/kg and 14.999 meq/kg for TPC, AA and PV, respectively); however, the sunflower oil with BHT showed lower (better) values of MDA (0.445 g/kg) than the oil without MEDEX (0.693 g/kg) and similar values to the oil with MEDEX (0.570 g/kg). In conclusion, methanol is better than water to extract *Tithonia diversifolia* antioxidants, this extract is stable, is not affected by light and it improves most of the variables that indicate oxidative stability of sunflower crude oil.

Keywords: dry extracts, light, solvent, antioxidant activity, lipid oxidation

ACTIVIDAD ANTIOXIDANTE DE LA HOJA DE *TITHONIA DIVERSIFOLIA* (HEMSL.) A. GRAY

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RESUMEN

Los efectos nocivos de los antioxidantes sintéticos sobre la salud humana podrían evitarse por el uso de antioxidantes naturales. Por lo tanto, dos experimentos fueron realizados: (1) estabilidad de almacenamiento de extractos secos de *Tithonia diversifolia* (Td): efecto de solvente, luz y tiempo de almacenamiento y (2) efecto del extracto metanólico (MEDEX) de Td sobre la estabilidad oxidativa de aceite crudo de girasol durante varios días de almacenamiento: sin MEDEX, con MEDEX y con hidroxitolueno butilado (BHT). Las variables respuesta del experimento 1 fueron el contenido fenólico total (TPC), contenido total de flavonoides (TFC) y actividad antioxidante (AA) y las del experimento 2 fueron TPC, AA, índice de peróxido (PV) y contenido de malondialdehído (MDA). No hubo efecto de luz y tiempo en ninguna variable en el experimento 1, sin embargo, MEDEX mostró valores más altos de TFC (5.55 mg/g) que el extracto seco de agua (1.32 mg/g). El aceite crudo de girasol con MEDEX mostró valores más altos de TPC (1045 mg/kg) y AA (2383 µmol/kg) y valores más bajos de PV (11.972 meq/kg) que el aceite sin MEDEX (975 mg/kg, 1842 µmol/kg y 14.999 meq/kg para TPC, AA y PV, respectivamente); sin embargo, el aceite de girasol con BHT mostró valores más bajos (mejores) de MDA (0.445 g/kg) que el aceite sin MEDEX (0.693 g/kg) y valores similares al aceite con MEDEX (0.570 g/kg). En conclusión, el metanol es mejor que el agua para extraer antioxidantes de *Tithonia diversifolia*, este extracto es estable, no se ve afectado por la luz y mejora la mayoría de las variables que indican la estabilidad oxidativa del aceite crudo de girasol.

Palabras clave: extractos secos, luz, solvente, actividad antioxidante, oxidación lipídica.

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1. INTRODUCTION

The oxidative stress, is defined as the imbalance between cellular production of reactive oxygen species and the ability of cells to scavenge them (Khan *et al.,* 2013), in humans, it has been mentioned as a potential contributor to the development of degenerative processes (Pala and Gürkan, 2008), therefore, antioxidants are important to protect human organism against oxidative stress.

Synthetic antioxidants such as butylated hydroxytoluene (BHT), butylated hydroxyanisole (BHA) and tertiary butyl hydroquinone (TBHQ) are extensively used as food preservatives (Kamkar *et al.,* 2010); however, there are reports of the harmful effects of these synthetic antioxidants (Altmann *et al.,* 1985; Lindenschmidt *et al.,* 1986; Blaszczyk and Skolimowski, 2007).

There is an increasing interest in the search for naturally occurring antioxidants (Kamkar *et al.,* 2010), such as polyphenols and carotenoids (Trebušak *et al.,* 2014), flavonoids are the most abundant polyphenols within the plant kingdom (Quiñones *et al.,* 2012). The yield and antioxidant activity of the extracts depend on the solvent used for extraction (Spigno *et al.,* 2007); polar solvents such as methanol, ethanol, either and their aqueous mixtures, are mostly recommended for the extraction of phenols from a plant matrix (Anwar and Przybylski, 2012). During the extraction and storage, the extracts can be degraded. It has been reported that light is one of the most important factors that facilitate degradation reactions (Liazid *et al.,* 2007). Although, evidence has been reported about the phenolic content, flavonoid content and the antioxidant activity of dry extracts of leaves from *Tithonia diversifolia* Hemsl. A. Gray (Kuroda *et al.,* 2007; Di Giacomo *et al.,* 2015; Kolawole *et al.,* 2011; Tania *et al.,* 2016), the combined

effect of solvent and light during storage time on the stability of *Tithonia diversifolia* phenolic compounds has not been reported.

Fats and oils get easily oxidized during processing, transportation and preservation (Yang *et al.,* 2014), therefore, the oxidative stability is one of the most important indicators for maintaining the quality of edible oils in the oils and fats industry (Tan *et al.,* 2002). Sunflower oil, one of the three important edible oils, due to its higher content of polyunsaturated fatty acids, is more susceptible to oxidation (Chen *et al.,* 2014). Oxidation of oils, not only produce undesirable flavours, rancid odours, discoloration and other forms of spoilage (Hraš *et al.,* 2010), also, it could have harmful effects on human health (Laguerre, 2007). The antioxidant activity of natural antioxidants in oil has been reported (Zhang *et al.,* 2010; Chen *et al.,* 2014; Yang *et al.,* 2016). However, the effect of methanolic dry extracts of leaves from *Tithonia diversifolia* on the oxidative stability of oils has not been evaluated.

2. OBJECTIVES

Determine the effects of solvent, light and storage time on antioxidant activity, and total phenolic and flavonoids content in dry extracts of *Tithonia diversifolia* (Hemsl.) A. Gray leaves.

Evaluate the effects of the methanolic dry extract of *Tithonia diversifolia* A. Gray leaves on the oxidative stability of sunflower crude oil (*Helianthus annuus* L.).

3. HYPOTHESIS

The antioxidant activity, and total phenolic and flavonoids content of dry extracts of *Tithonia diversifolia* (Hemsl.) A. Gray leaves are not affected by type of solvent, light and storage time.

The methanolic dry extract of *Tithonia diversifolia* A. Gray leaves improve the oxidative stability of sunflower crude oil (*Helianthus annuus* L.).

4. LITERATURE REVIEW

4.1 Effect of light on phenolic compounds and lipid oxidation

Unsaturated fatty acids and phenolic compounds by themselves do not absorb visible light and normally are not subject to direct oxidation by ultraviolet light; however, oils, fatty foods and phenols do undergo accelerated oxidation when exposed to daylight or artificial lights (Faria and Mukai, 1983; Stylidi *et al.,* 2004). This is due to impurities present in vegetable oils and to colored substances in fatty foods (Rawls and Van Santen, 1970), and other phytochemicals obtained in plants extracts such as chlorophylls (Hojnik *et al.,* 2007), in which absorb light and thus may contribute to photosensitization. On the one hand chlorophylls are needed for the use of light energy in photosynthesis, on the other hand, the same molecules carry the potential danger of being a singlet oxygen producer (Krieger-Liszkay, 2005). Production of singlet oxygen ($^1O_2$) in plants occurs mainly in the chloroplasts from chlorophyll triplet state (^{3}Chl*) (Krieger-Liszkay, 2005; Triantaphylide`s and Havaux, 2009). The photosensitizer (chlorophyll) absorbs the ultraviolet or visible radiation energy rapidly and becomes an unstable, excited, singlet state molecule (chlorophyll singlet state, ^{1}Chl*) (Min and Boff, 2002; Krieger-Liszkay, 2005). If the energy is not efficiently used, the spins of the electrons in the excited state can rephase and give rise to a lower energy excited state: the chlorophyll triplet state (^{3}Chl*) (Krieger-Liszkay, 2005). The ^{3}Chl* has an even longer lifetime and can react with triplet oxygen ($^3O_2$) to produce the very reactive $^1O_2$ (Min and Boff, 2002; Krieger-Liszkay, 2005). The $^1O_2$ reacts directly with the unsaturated fatty acids of vegetable oils to produce a mixture of conjugated and nonconjugated hydroperoxides (Terao and Matsushita, 1977; Frankel *et al.,* 1985). Also, it has been demonstrated that phenolic compounds can be oxidized by $^1O_2$ that result in the formation of phenoxyl radical and Quinone structure (Li and Hoffman, 2000, Al-Nu'airat *et al.*, 2018).

4.2 Effect of solvent on phenolic compounds content

Solutions, can be defined as homogeneous liquid phases consisting of more than one substance in variable ratios, where, usually, the solute(s) is/are the minor component(s) and the solvent is the component in excess (Katritzky *et al.*, 2004). The choice of a good solvent is important in chemistry because of the crucial role of solvent in many chemical processes (Gramatica *et al.*, 1999). Since the antioxidant compounds found in plants have different polarities, different solvents are used to isolate antioxidants (Askoy *et al.*, 2013). The term "polarity" is usually related to the capacity of a solvent for solvating dissolved charged or dipolar species (Katritzky *et al.*, 2004). Trying to understand the properties of solvents and to facilitate solvent choice has led to the development of many solvent scales. These scales are based on diverse physicochemical phenomena including reaction rates, solvatochromic effects, and reaction enthalpies, among others (Katritzky *et al.*, 2005). Solvatochromism is defined as the pronounced change in position and sometimes intensity of an electronic absorption or emission band accompanying a change in the polarity of the medium. This can be even detected visually by the change of solution colour when going from one solvent to another (El-Ayaan *et al.*, 2001). Table 1, shows a solvents classification based on solvatochromic method. The extract yields and resulting antioxidant activities of the plant materials are strongly dependent on the nature of extracting solvent, due to the presence of different antioxidant compounds of varied chemical characteristics and polarities that may or may not be soluble in a particular solvent (Sultana *et al.*, 2009). Polar solvents such as methanol, ethanol, either and their aqueous mixtures, are mostly recommended for the extraction of phenolics from a plant matrix (Sultana *et al.*, 2009; Anwar and Przybylski, 2012). Also, water and acetone are solvents commonly used in extraction processes (Askoy *et al.*, 2013).

Table 1. Solvents classification. Solvatochromic parameters for some selected solvents.

Solvent	α	β	π*
Diisopropil ether	0.00	0.49	0.27
Diethyl ether	0.00	0.47	0.27
Diphenyl ether	0.00	0.13	0.66
Acetone	0.08	0.48	0.71
Nitrobenzene	0.00	0.39	1.01
Acetonitrile	0.19	0.31	0.75
Toluene	0.00	0.11	0.54
Benzene	0.00	0.10	0.59
Chloroform	0.44	0.00	0.58
Isopropanol	0.76	0.95	0.48
n-butanol	0.79	0.88	0.47
Ethanol	0.83	0.77	0.54
Methanol	0.93	0.62	0.60
Ethylene glycol	0.90	0.52	0.982
Water	1.17	0.18	1.09

Adapted from De Juan *et al.* (1997). α = hydrogen bond acidity. β = basicity. π* = dipolarity/polarizability.

4.3 Reactive oxygen species and lipid oxidation

Reactive oxygen species (ROS) is a phrase used to describe a variety of molecules derived from molecular oxygen (Turrens, 2003); and includes free radicals (atoms or molecules containing one

or more unpaired electrons; Fotina *et al.* 2013) and non-radical molecules (Kregel and Zhang, 2007). "Oxidative stress" is an expression used to describe various deleterious processes resulting from an imbalance between the excessive formation of ROS and/or reactive nitrogen species and limited antioxidant defenses (Turrens, 2003). Increased oxidative stress is associated with increased lipid oxidation (Tsaluchidu *et al.,* 2008). Lipid oxidation can be described generally as a process under which oxidants such as free radicals attack lipids containing carbon-carbon double bond(s), especially polyunsaturated fatty acids (PUFA) (Ayala *et al.,* 2014). Lipid oxidation may contribute to the deterioration of product quality due to the formation of hydroperoxides. Hydroperoxides are susceptible to further oxidation or decomposition, forming secondary reaction products such as aldehydes (Repetto *et al.,* 2012), ketones, acids, and alcohols, these compounds produce undesirable aromas as well as flavors and can decrease the quality of the food product (Fratiani *et al.*, 2010).

4.4 Oxidative stability of oils: primary and secondary products of lipid oxidation

Monitoring the concentration of primary or secondary oxidation products is generally used to determine the rate at which the oxidation process proceeds (Mohammadi *et al.,* 2013). Resistance to oxidation of an oil or fat is known as oxidative stability (OS) and can be expressed as the period of time necessary for the secondary products of the reaction to be formed and detected under different conditions (Pardauil *et al.*, 2011). The OS of oils and fats with added antioxidants can be determined during storage under normal ambient conditions and packing. However, in general, oxidation can take a long time to occur, e.g. a few days to a few months, which is impractical for routine analysis. For this reason, accelerated oxidation tests are conducted (Abdelazim *et al.,* 2013).The process of lipid peroxidation is initiated by the abstraction of a

hydrogen atom in an unsaturated fatty acyl chain and propagated as a chain reaction (Shyamala *et al.,* 2005). Peroxide value (PV) indicates the state of primary oil oxidation (Yang *et al.,* 2016). This index is related to the hydroperoxides, which are unstable and readily decompose to form mainly mixtures of volatile aldehyde compounds (Mohdaly *et al.,* 2011). The PV indicates the quantity of the hydroperoxides reported as milliequivalents of active oxygen in 1000 g of an oil sample (Yang *et al.,* 2014). The peroxides present are determined by titration against thiosulphate in the presence of potassium iodide (KI), and starch is used as indicator (Chen *et al.,* 2014). For secondary reaction products, the most prominent and currently used assay as an index for lipid peroxidation products is the thiobarbituric acid assay (Garcia *et al.,* 2005). The method is based on the spectrophotometer quantization of the pink complex formed with peak absorbance at 532 nm after reaction of one molecule of malondialdehyde (MDA) with two molecules of 2-thiobarbituric acid (TBA) (Figure 1) (Chen *et al.,* 2014). TBA is defined as the quantity of MDA (in mg) present in 1 kg of sample (Zhang *et al.,* 2010). Antioxidants can be added to the product to retard or minimize the deteriorative events; these additives prolong shelf life and protect against microbial contamination (Fratiani *et al.,* 2010)

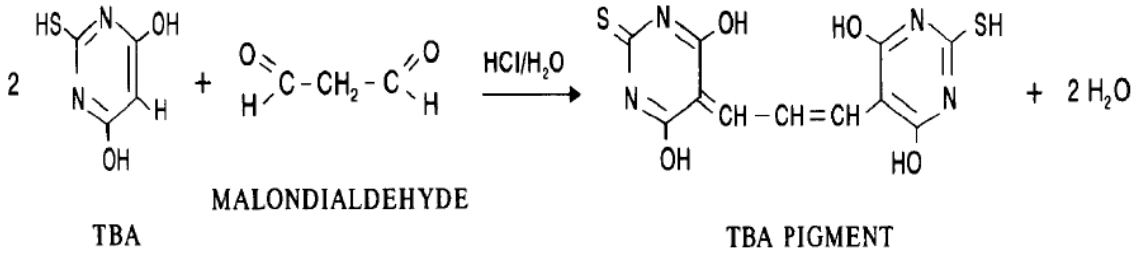


Figure 1. Reaction between TBA and MDA to form the TBA pigment (Fernández *et al.,* 1997).

4.5 Antioxidants and antioxidant activity: 1,1-diphenyl-2-picrylhydrazyl method

Antioxidants are used by the food industry to delay the oxidation process (Brand-Williams, 1995). An antioxidant is a molecule that inhibits the oxidation of another molecule (Mehta and Gowder, 2015). In the food industry, synthetic antioxidants have been used, butylated hydroxytoluene (BHT), butylated hydroxyanisole (BHA) (Hocman, 1988) and ethoxyquin (EQ) are the main synthetic antioxidants (Hilton, 1989). The safety concerns regarding the use of synthetic antioxidants have increased globally (Yang *et al.,* 2016).

There are reports of the harmful effects these synthetic antioxidants (Altmann *et al.,* 1985; Lindenschmidt *et al.,* 1986; Blaszczyk and Skolimowski, 2007). And the benefits of adding natural, plant-based antioxidants to lipids have led to an increasing interest in the search for naturally occurring antioxidants (Kamkar *et al.,* 2010, Yang *et al.,* 2016). The natural antioxidants can improve the oxidative stability of vegetable oils (Chen *et al.,* 2014: Yang *et al.,* 2016). The "antioxidant activity" represents the ability to inhibit the process of oxidation (Tirzitis and Bartosz, 2012). Most of the methods of determination of total antioxidant activity characterize the ability of the tested compound or product to scavenge free radicals (Tirzitis and Bartosz, 2010). One such method that is currently popular is based upon the use of the stable free radical 1,1-diphenyl-2-picrylhydrazyl (DPPH) (Molineux, 2004; Tirzitis and Bartosz, 2010). When a solution of DPPH is mixed with that of a substance that can donate a hydrogen atom, then this gives rise to the reduced form (Figure 2) with the loss of this violet colour (Molineux, 2004).

A B

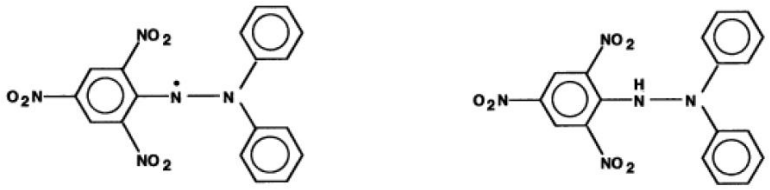


Figure 2. Diphenylpicrylhydrazyl (A, free radical) and Diphenylpicrylhydrazine (B, nonradical) (Molineux, 2004).

4.6 Phenolic content and antioxidant activity of *Tithonia diversifolia*: methods for total phenolic and flavonoids content

Secondary metabolites are compounds with a restricted occurrence in taxonomic groups, that are not necessary for a cell (organism) to live, but play a role in the interaction of the cell (organism) with its environment, ensuring the survival of the organism in its ecosystem (Verpoorte and Alfermann, 2000). Various polyphenols and carotenoids can be included among the natural antioxidants of plant origin (Trebušak *et al.,* 2014). Polyphenols are secondary metabolites of plants (Pandey and Rizvi, 2009), characterized by the presence of more than one aromatic ring with each containing at least one hydroxyl group (Hammerstone, 2000).

The total phenolic content is analyzed by using the Folin-Ciocalteau colorimetric method, based on the reaction of the reagent with the functional hydroxyl groups of phenols. This method requires extraction of the phenols from the sample, a calibration curve using a pure phenolic compound (e.g. gallic acid) and the measurement of absorbance after colour reaction (Bail *et al.*, 2008). There is a wide range of polyphenols; the main classes are phenolic acids, stilbenes, lignans and flavonoids (Scalbert and Williamson, 2000).

Flavonoids, are the most common polyphenols in the plant kingdom (Quiñones *et al.*, 2012), are compounds of low molecular weight and chemically are based upon a fifteen-carbon skeleton consisting of two benzene rings (A and B as shown in Figure 3) linked via a heterocyclic pyrane ring (C) (Kumar and Pandey, 2013).

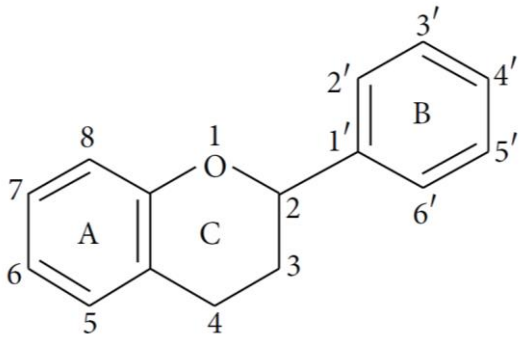


Figure 3. Basic flavonoid structure (Kumar and Pandey, 2013).

To determine the total flavonoids content, the Aluminum Chloride colorimetric method is used, the principle of this method is the formation of acid stable complexes with the C-4 keto group and either the C-3 or C-5 hydroxyl group of flavones and flavonols in addition with aluminium chloride. Aluminium chloride also forms acid labile complexes with the ortho-dihydroxyl groups in the A- or B-ring of flavonoids. For building the calibration curve, quarcetin is used as a standard material (Kalita *et al.*, 2013).

Flavonoids are able to scavenge free radicals directly by hydrogen atom donation (Procházcová *et al.*, 2011).The B ring hydroxyl configuration is the most significant determinant of scavenging of ROS and reactive nitrogen species (Kumar and Pandey, 2013). After acting as antioxidant, flavonoid phenoxyl radical is formed (Figure 4) (Procházcová *et al.*, 2011). The flavonoid phenoxyl radical may react with a second radical, acquiring a stable quinone structure (Hernández *et al.*, 2009).

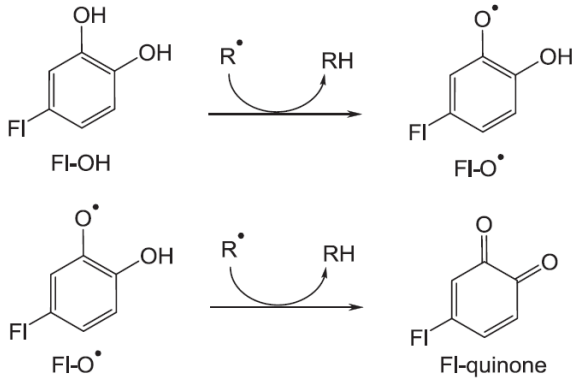
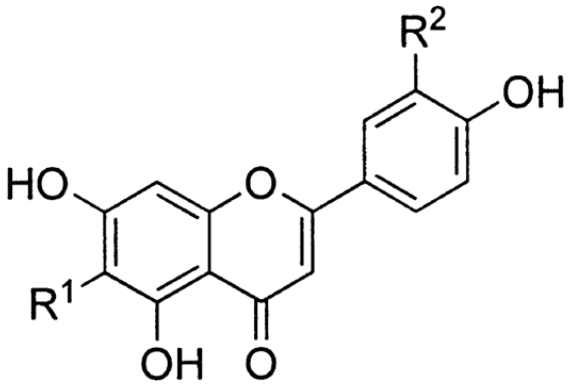


Figure 4. Scavenging of reactive oxygen species by flavonoid (Procházcová *et al.*, 2011).

The genus Tithonia comprises 13 taxa, which are distributed in eleven species. *Tithonia diversifolia* Helms. A. Gray (TD) is native to Mexico and also grows in parts of Africa, Australia, Asia, and other countries of North America (Di Giacomo *et al.*, 2015).

Kuroda *et al.* (2007) reported three flavonoids identified in the areas parts of the *Tithonia diversifolia* Helms. A. Gray: luteolin, nepetin e hispidulin (Figure 5).



	R^1	R^2
1	H	OH
2	OMe	OH
3	OMe	H

Figure 5. Flavonoids structure of *Tithonia diversifolia* Helms. A. Gray, luteolin (1), nepetin (2) and hispidulin (3) (Kuroda *et al.*, 2007).

4.7 Sunflower oil

Sunflower oil is one of the most produced oils after palm, soybean and rapeseed oils; the main producers are: Ukraine, Russian Federation, Argentina, Turkey and France (FAOSTAT, 2014). Sunflower oil has high content of polyunsaturated fatty acids (PUFA) (Table 2).

Table 2. Fatty acids composition of sunflower oil.

Fatty acid	%
Lauric acid [C12:0]	0.02
Myristic acid [C14:0]	0.09
Palmitic acid [C16:0]	6.20
Margaric acid [C17:0]	0.02
Stearic acid [C18:0]	2.80
Arachidic acid [C20:0]	0.21
Palmitoleic acid [C16:1 (n-7)]	0.12
Oleic acid [C18:1cis(n-9)]	28.00
Gondoic acid [C20:1(n-9)]	0.18
Linoleic acid [C18:2cis (n-6)]	62.20
α-Linolenic acid [C18:3 (n-3)]	0.16
Saturated fatty acids (SFA)	9.40
Monounsaturated fatty acids (MUFA)	28.30
Polyunsaturated fatty acids (PUFA)	62.40
n-3 PUFA	0.20
n-6 PUFA	62.20

Adapted from Orsavova *et al.* (2015).

The presence of high amounts of PUFA such as linoleic and linolenic acids in oils make them more susceptible to oxidation (Kamkar *et al.*, 2010).

5. MATERIALS AND METHODS

The study was carried out at campus Montecillo of the Postgraduate College and the Chapingo Autonomous University, Texcoco, Mexico.

5.1. Reagents

Folin-Ciocalteu, gallic acid, quercetin, 2,2-diphenyl-1-picrylhydrazyl (DPPH), 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), 1,1,3,3-tetraethoxypropane, butylated hydroxytoluene (BHT) were purchased from Sigma–Aldrich (St Louis, MO, USA). All other reagents used were analytical grade.

5.2. Collection of *Tithonia diversifolia* (Td)

The Td leaves were harvested in Tuxtla, Zapotitlán de Méndez, Puebla, Mexico; located at 20°00'02.726" N, 097°39'19.225" W, and 926 m of altitude (INEGI, 2019). The leaves were sun-dried on concrete slabs, until they were dry enough (2 to 3 days) for easy milling. The dried leaf powder of Td was stored in an airtight container at room temperature until the methanol and water extracts preparation.

5.3. Experiment 1. Effects of solvent, light and storage time on antioxidant activity, and total phenolic and flavonoids content in dry extracts

5.3.1 Obtaining and evaluation of dry extracts

According to Olayinka *et al.* (2015), twenty g of dried leaf powder were vigorously shaken with 200 mL of distilled water or methanol. The mixtures were kept at room temperature for 48 h. After that, the solid-liquid mixture was filtered with Whatman No. 4 paper. The liquid extracts

were distributed in glass petri dishes and dried in an oven at 50 °C (Do *et al.*, 2014). Finally, the petri dishes were covered with plastic film.

The factors evaluated and their levels were: solvent (methanol or water) and light (darkness or lightness); which effects were determined at 1, 14 and 28 days of storage. Darkness was achieved by wrapping the extracts with foil and lightness by exposing the extracts to an incandescent lamp of 40 *W*. All glass petri dishes were kept in a refrigerator at 9-10 °C. The dry extracts were diluted with its corresponding solvent to perform the analyses: 2400 mg/L for antioxidant activity (AA) and 600 mg/L for total phenolic content (TPC) and total flavonoids content (TFC) determinations. The AA, TPC and TFC were evaluated at 1, 14 and 28 days of storage.

5.3.2. Determination of total phenolic content (TPC)

The TPC was determined, in triplicate, using the Folin-Ciocalteu micro-method described by Slinkard and Singleton (1977) and modified by Arabshahi-Delouee and Urooj (2007), except that fifty µL of diluted extracts were used. The absorbance was measured in a Thermo Scientific, Model 10S Vis (USA) spectrophotometer. The following standard equation was obtained: $Y = 8.5378X - 0.0823$, $R^2 = 0.9992$; where Y is the total phenolic content as gallic acid equivalents (mg/g of dry extract) and X is the absorbance.

5.3.3. Determination of total flavonoids content (TFC)

The TFC was estimated using the methodology described by Woisky and Salatino (1998) and modified by Chang *et al.* (2002). The TFC was expressed in terms of quercetin equivalents using the following equation: $Y = 13.796X + 0.1005$, $R^2 = 0.9995$; where, Y is the concentration of

flavonoids as quercetin equivalents (mg/g of dry extract) and X is the absorbance. To obtain this equation, one hundred milligrams of quercetin were dissolved in 80% ethanol and then diluted to 12.5, 25, 50, 75 and 100 μg/mL. The absorbance was measured in a Thermo Scientific, Model 10S Vis (USA) spectrophotometer.

5.3.4. Antioxidant activity (AA)

The AA of the extracts was measured in triplicate by the method of the radical 2,2-diphenyl-1-picrylhydrazyl (DPPH), according to Kamkar *et al.* (2010). Briefly, 25 μL of the diluted extracts were added to 2.5 mL of a DPPH solution (0.004% in methanol). After 30 min incubation at room temperature, the absorbance was measured in a Thermo Scientific, Model 10S Vis (USA) spectrophotometer against a methanol blank. The radical-scavenging activities of the extracts were calculated as percentage inhibition according to the following equation:

AA% = (Absorbance of the DPPH solution without extract – Absorbance of the DPPH solution with extract) × 100/Absorbance of the DPPH solution without extract.

In order to facilitate the interpretation of results, trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) was used as a standard to convert the AA% of each extract to trolox equivalents, according to the methodology of Serpen *et al.* (2012). Standard calibration curves were obtained by plotting AA% against trolox concentration according to the following equation: $Y = 0.2651X + 2.3176$, $R^2 = 0.9716$; where, Y is the AA in terms of trolox concentration (μmol/g of dry extract) and X is the AA%.

5.4. Experiment 2: Effects of the methanolic dry extracts on the oxidative stability of sunflower crude oil

5.4.1. Preparation of the extract

Based on the results of experiment 1 where methanolic dry extract (MEDEX) resulted rich in TFC, methanol was used to obtain the dry extract of this study.

5.4.2. Sunflower oil extraction

Powdered dehulled seeds of sunflower (*Helianthus annuus* L.) were mixed with diethyl ether in an Erlenmeyer flask. This mixture was kept at room temperature for 24 h with stirring every six hours. Then, it was filtered and the solvent was removed in a rotary evaporator at 70 °C. The crude oil obtained was stored at 4 °C until analyses were performed.

5.4.3. Sunflower oil plus antioxidants

Sunflower crude oil samples (100 g) were transferred into 250 mL Erlenmeyer flasks fully wrapped with foil. Three treatments were tested: Control (0 mg MEDEX and BHT/kg); BHT (200 mg butylated hydroxytoluene/kg) and MEDEX (2400 mg MEDEX/kg). Both antioxidants per kg of crude sunflower oil. The contents of all Erlenmeyer flasks were stirred for 10 min at room temperature, and the methanol content of MEDEX treatment was removed at 50 °C for 10 min, then they were subjected to an accelerated storage at 60 °C (Zhang *et al.*, 2010), after which, samples were taken periodically in triplicate to perform the correspondent analyses. The TPC (gallic acid equivalents) and MDA (malondialdehyde) concentration were evaluated at 1, 5, 9 and 13 days, and the AA (in terms of trolox) and PV (peroxide value) were determined at 1, 13 and 25 days of storage.

5.4.4. Determination of total phenolic content (TPC) in sunflower crude oil

The TPC was measured using the Folin-Ciocalteau method according to Lianhe *et al.* (2012) with the modifications of Yang *et al.* (2016). The absorbance was measured at 750 nm in a Thermo Scientific, Model 10S Vis (USA) spectrophotometer. Gallic acid was used as a standard according to the following calibration curve: $Y = 9.7258X - 1.3409$, $R^2 = 0.9989$, where Y is the concentration of total phenolic content as gallic acid equivalents (mg/kg of sunflower crude oil) and X is the absorbance.

5.4.5. Determination of antioxidant activity (AA)

The AA was expressed in terms of trolox per kg sunflower crude oil, using the same methodology described in the experiment 1.

5.4.6. Analysis of peroxide value (PV)

The peroxide value (PV) was determined according to the AOCS (1990).

5.4.7. Determination of malondialdehyde (MDA) content

The malondialdehyde (MDA) content of all oil samples was assessed by the 2-thiobarbituric acid (TBA) method according to the Chinese national standards methodology GB/T 5009.181-2003 with some modifications made by Chen *et al.* (2014) and avoiding the use of chloroform. The absorbance was measured at 532 nm in a Thermo Scientific, Model 10S Vis (USA) spectrophotometer. To know the crude oil lipid oxidation, the following standard curve of MDA (a product of lipid oxidation) was prepared in the range of 0.02 to 0.3 µg/mL, by acidification of 1, 1, 3, 3-tetraethoxypropane (which is converted into MDA): $Y = 1.0009X - 0.0208$, $R^2 =$

0.9995, where, Y is the MDA concentration (g/kg of sunflower crude oil) and X is the absorbance.

5.5. Statistical analysis

All chemical analyses were performed in triplicate, under a completely randomized design. The experiment 1, had a 2×2 factorial arrangement of treatments, TPC, TFC and AA were determined in the days 1, 14 and 28 of storage at 9-10°C. In the experiment 2, TPC and MDA were determined in the days 1, 5, 9 and 13, while AA and PV were evaluated in the days 1, 13 and 25 of accelerated storage (60 °C). The experiments had a repeated measures design over time. All statistical analyses were carried out using the MIXED procedure of SAS 2006, and means were separated using the Tukey test ($P < 0.05$).

6. RESULTS AND DISCUSSIONS

6.1. Experiment 1. Effects of solvent, light and storage time on antioxidant activity, and total phenolic and flavonoids content in dry extracts

6.1.1. Effect on total phenolic content (TPC)

Because foods are generally storage and exposed to light, studies are needed to find out whether natural antioxidants compounds are not affected by light and solvent during storage. It has been mentioned that the antioxidant activity of phenolic compounds is influenced by solvent (Sultana *et al.,* 2009), light and time of storage (Nasr *et al.,* 2016) Nevertheless, the effect of solvent and light through storage time on the stability of phenolic compounds and antioxidant activity of dry extracts from *Tithonia diversifolia* (Td) has not been reported. In plants there is a wide variety of compounds presenting a molecular structure with the presence of one or several phenolic rings, these compounds can be called polyphenols, the main groups of polyphenols are: phenolic acids, stilbenes, lignans, phenolic alcohols and flavonoids (Quiñones *et al.,* 2012). Differences in the structure of phenolic compounds determine their solubility in solvents of different polarity, therefore, type of extraction solvent may have a significant impact on the yield of extraction polyphenols from plants material (Zlotek *et al.,* 2016). Table 3, shows that TPC was not affected by any kind of solvent ($P > 0.05$), but an interaction Time × Solvent was observed (Figure 6). Previous reports on TPC from Td have not been consistent (55.92 ± 4.45 mg/g dry weight, Thongsom *et al.,* 2013; 22.185 ± 0.201 mg/g dry extract, Barboza *et al.,* 2018), these differences could be explained by the methodologies used in each study. It has been reported that methods of drying leaves affect TPC (Lin *et al.,* 2011). Roshanak *et al.* (2016) reported that green tea leaves had more TPC with oven dried at 60°C method in comparison to sun drying; this latter method was used in the current study, so that it could explain the low values of TPC (Table 3).

Table 3. Average TPC, TFC and AA in aqueous and methanolic dry extracts of Tithonia diversifolia[1].

Solvent	TPC	TFC	AA
Methanol	6.30[a]	5.55[a]	30.17[a]
Water	6.81[a]	1.32[b]	28.45[a]
SEM	0.19	0.16	1.03
Source of variation		*P*-value	
Time	0.1767	0.8571	0.8661
Solvent	0.0711	<0.0001	0.2459
Light	0.9961	0.7872	0.1573
Time × Solvent	0.0129	0.0414	0.7732
Time × Light	0.2647	0.0976	0.9694
Solvent × Light	0.0614	0.7542	0.7200
Time × Solvent × Light	0.2444	0.0985	0.3670

[a,b] Different superscripts within each variable (column) are significantly different at $P < 0.05$. [1]Data are presented as least squares means (LSM) and their standard errors of the mean (SEM). TPC=Total phenolic content (mg/g), TFC=Total flavonoids content (mg/g), AA=Antioxidant activity (µmol/g). All per g of dry extract.

In Figure 6 it is observed that TPC was not different ($P > 0.05$) through storage time when water was used as solvent, however, with methanol, TPC decreased ($P < 0.05$) from 1 to 28 d of storage. Nevertheless, at 28 days of storage, the TPC was the same ($P > 0.05$) with water and methanol. Nasr *et al.* (2016) reported that exposing the extract to light increased the destruction of anthocyanins (flavonoids). On the contrary, in the current study TPC, TFC and AA were not

affected ($P > 0.05$) by light (Table 3). Likewise, Pedreño and Escribano (2001) did not find effect of light on AA and the concentration of betanin (a natural phenolic antioxidant, Kanner *et al.*, 2001).

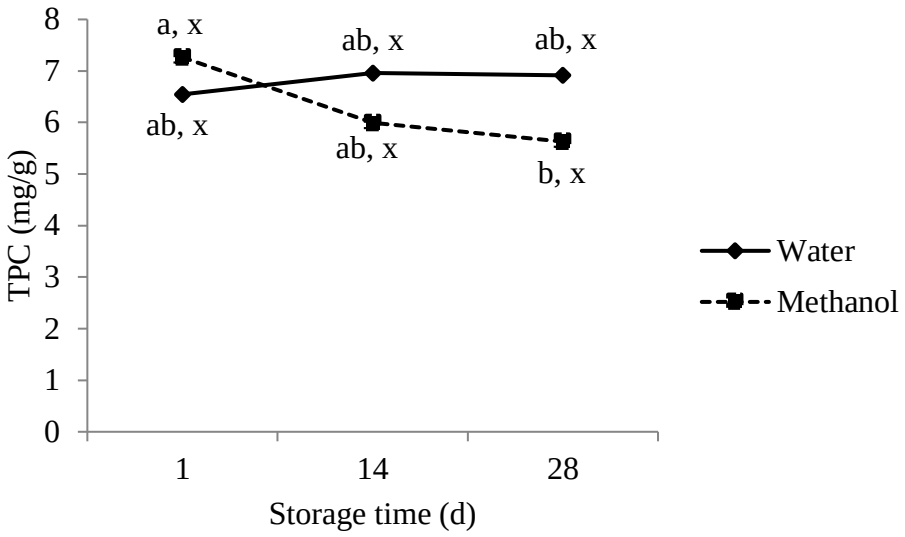


Figure 6. Effect of the interaction Time × Solvent in the total phenolic content (TPC) of Tithonia diversifolia.

[a, b] Different superscripts within each kind of solvent through storage time are significantly different at $P < 0.05$.

[x, y] Different superscripts within each time are significantly different at $P > 0.05$.

6.1.2. Effect on total flavonoids content (TFC)

Flavonoids are widespread plant secondary metabolites, including flavones, flavanols, and condensed tannins. Epidemiological studies suggest that the consumption of flavonoid-rich foods protects against human diseases associated with oxidative stress (Xu and Chang, 2007), given that flavonoids show antioxidant activity (Pourmorad *et al.*, 2006). Flavonoids are the most

abundant polyphenols within the plant kingdom (Quiñones *et al.,* 2012). Table 3, shows lower TFC than TPC values, which could mean that flavonoids are one of the subgroups of phenolic compounds (Quiñones *et al.,* 2012). The values of TFC of this study were 5.55 and 1.32 mg/g dry extract for methanol and water (Table 3), respectively. These numbers are in the range of values reported by Barboza *et al.* (2018), who reported 3.220 ± 1.085 mg/g for saline dry extract of leaves of Td. Figure 7, shows that there were differences ($P < 0.05$) in TFC within each period of time. Methanol extracted the highest ($P < 0.05$) TFC in all periods with respect to water; this could be explained by the low solubility of flavonoids in water which is a very polar solvent. In contrast, flavonoids are low polar molecules, therefore, they are more soluble in organic solvents and little and medium polar solvents (Gaye *et al.,* 2019). This result is similar to that of Parthasarathy *et al.* (2009) who reported that methanol was more efficient to extract flavonoids from leaves of *Mitragyna speciosa* in comparison to water. Ghasemzadeh *et al.* (2011) also reported that methanol was more efficient than acetone or chloroform to extract flavonoids from leaves, stems and rhizomes of ginger. No significant differences ($P > 0.05$) were observed in TFC for both solvents measured through storage time (Figure 7), which mean that flavonoids are stable for at least 28 days. Chu *et al.* (2000) reported that flavonoids from potato leaves during 9 d of storage were higher at 4-10°C than at 25°C. Therefore, storage temperature in the current study (9-10°C) could explain the lack ($P > 0.05$) of differences in TFC through storage time.

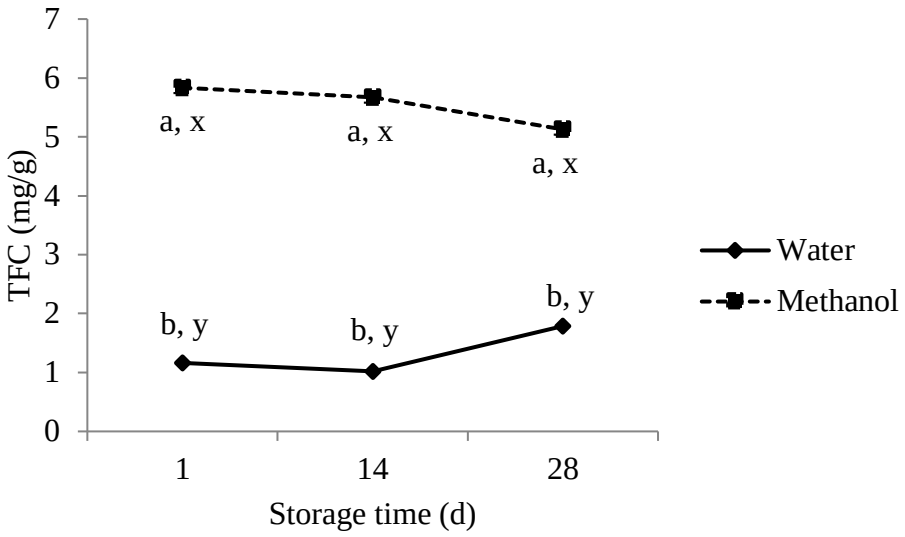


Figure 7. Effect of the interaction Time × Solvent in the total flavonoids content (TFC) of Tithonia diversifolia.

[a, b] Different superscripts within each kind of solvent through storage time are significantly different at $P < 0.05$.

[x, y], Different superscripts within each time are significantly different at $P > 0.05$.

6.1.3. Effect on antioxidant activity (AA)

The values of AA expressed in terms of trolox were 30.17 and 28.45 mg/g of dry extract for methanol and water, respectively (Table 3). There was not found any information about AA of Td dry extracts expressed in terms of trolox. The percentage inhibition values of DPPH obtained in this study were 14.73 and 13.54% for methanol and water, respectively, using 2400 mg of dry extracts/L of solvent. In contrast, Tania *et al.* (2016) used a concentration of 2500 mg of dry extracts/L of solvent of Td and reported values of 65±1.61 and 95±2.65% for aqueous and ethanol dry extracts, respectively. The possible explanation for this discrepancy lies on the DPPH technique, Tania *et al.* (2016) took 0.3 mL of diluted extract, whereas in this study only

0.025 mL were used. No differences ($P > 0.05$) were found in AA by type of solvent, light, storage-time or interactions (Table 3). There is evidence of a good correlation between total phenolic content and antioxidant activity of plant extracts (Parthasarathy *et al.,* 2009).Therefore, because there were not differences ($P > 0.05$) in TPC (Table 3) by kind of solvent, light and storage time, could explain the lack of differences in AA by kind of solvent, light and storage time.

6.2. Experiment 2: Effects of the methanolic dry extracts on the oxidative stability of sunflower crude oil

6.2.1. Effect on total phenolic content (TPC)

Phenolic compounds are commonly found in both edible and nonedible plants, these substances have multiple biological effects (Kähkönen, 1999), for example the antioxidant activity. This effect is given by their ability to donate a hydrogen atom or an electron in order to form stable radical intermediates (Mohdaly *et al.,* 2011). Table 4 shows that MEDEX had higher ($P < 0.05$) TPC respect to Control treatment, but there were not significant differences ($P > 0.05$) between BHT and MEDEX. Given the lack of information in *Tithonia diversifolia* we contrasted our results with those of Yang *et al.* (2016) who reported higher TPC in oils with rosemary extract in comparison with their control treatment. However, in the current study, the control treatment also exhibited certain level of TPC (957 mg/kg of oil, Table 4). This is because sunflower seeds are rich in phenols (De Leonardis *et al.,* 2003). The main phenolic compounds identified in sunflower seeds are caffeic, feluric and coumaric acids (Weisz *et al.,* 2009). Pedrosa *et al.* (2000) reported values from 1.04 to 1.30 mg/g of whole sunflower seeds. These values are similar to the results of this study, which varied from 0.957 to 1.045 mg/g of oil (957 to 1045 mg/kg of oil).

6.2.2. Effect on antioxidant activity (AA)

The highest AA ($P < 0.05$) was observed in MEDEX (Table 4). No information was found on the effect of MEDEX in the oxidative stability of oils. So that, the results of this study are compared with Yang *et al.* (2016) who reported that the addition of rosemary extract to oils result in higher antioxidant activity in comparison to oils without the extract. Given that antioxidant activity is positively correlated with total phenolic content (Wojdyło *et al.,* 2007), the results of AA are explained by the TPC values MEDEX showed in Table 4.

Table 4. Average TPC, AA, MDA and PV of sunflower crude oil added with different antioxidants[2].

Treatment	TPC	AA	PV	MDA
Control	957[b]	1842[b]	14.999[a]	0.693[a]
BHT	1037[a]	2013[b]	14.333[ab]	0.445[b]
MEDEX	1045[a]	2383[a]	11.972[b]	0.570[ab]
Source of variation		*P*-value		
Ti	0.0004	<0.0001	<0.0001	0.0413
Tr	0.0030	0.0005	0.0001	0.0328
Ti × Tr	0.9790	0.1767	0.0044	0.9968

[a,b] Different superscripts within each variable (column) are significantly different at $P < 0.05$.

[2]Data are presented as least squares means (LSM) and their standard errors of the mean (SEM).

TPC = Total phenolic content (mg/kg), AA = Antioxidant activity (µmol/kg), MDA = malondialdehyde content (g/kg), PV = Peroxide value (meq/kg). BHT = butylated hydroxytoluene, MEDEX = methanolic dry extract, Ti = time, Tr = treatment.

The reports of the AA (in terms of trolox) of sunflower oil have not been consistent. Weisz *et al.* (2013) reported values from 624.5 to 37585.9 µmol/L of oil, the values of AA of this study fall between this range (1842 to 2382 µmol/kg of oil).

6.2.3. Effect on peroxide value (PV)

Sunflower is one of the most important oil crops in the world due to the oil favorable fatty acid composition: palmitic, stearic, oleic and linoleic (Baydar and Erbas, 2005). The presence of high amounts of polyunsaturated fatty acids such as linoleic and linolenic acids in oils and fats make them more susceptible to oxidation (Kamkar *et al.,* 2010). The process of lipid peroxidation is initiated by removing a hydrogen atom in an unsaturated fatty acyl chain and propagated as a chain reaction (Shyamala *et al.,* 2005). Hydroperoxides are produced during the propagation phase constituting the major primary product of lipid peroxidation process (Ayala *et al.,* 2014). Peroxide value (PV) is a measure of the concentration of peroxides and hydroperoxides and is one of the most widely-used tests for the measurement of oxidative rancidity in oils and fats (Zhang *et al.,* 2010). The PV values are shown in Table 4, MEDEX and control treatments showed the lowest and highest ($P < 0.05$) values, respectively. However, there were not significant differences ($P > 0.05$) between BHT and MEDEX. Chen *et al.* (2014) also reported lower PV values in the control treatment than in sunflower oil containing rosemary extract (RE), furthermore, the antioxidant effects of RE were better than those of BHT and BHA. Results of the current study also are in accordance with Yang *et al.* (2016), who reported that oils with RE showed significantly lower PV values than oils without antioxidants (blank) or oils with synthetic antioxidants. The PV values of this study ranged from 11.976 to 14.999 meq/kg of oil at d 25 of storage; Shyamala *et al.* (2005) using dry extracts of coriander, spinach, hongone and

cabbage in sunflower oil, reported similar results at 21 d of storage (3.5 to 18.5 meq/kg of oil, approximately). Figure 8 shows a continuous increase ($P < 0.05$) in PV values for all the treatments with the increase in storage period, which means that there is a continuous hydroperoxides production. Therefore, methanolic dry extract of leaves from Td was effective to decrease the hydroperoxides production in sunflower oil, along the time.

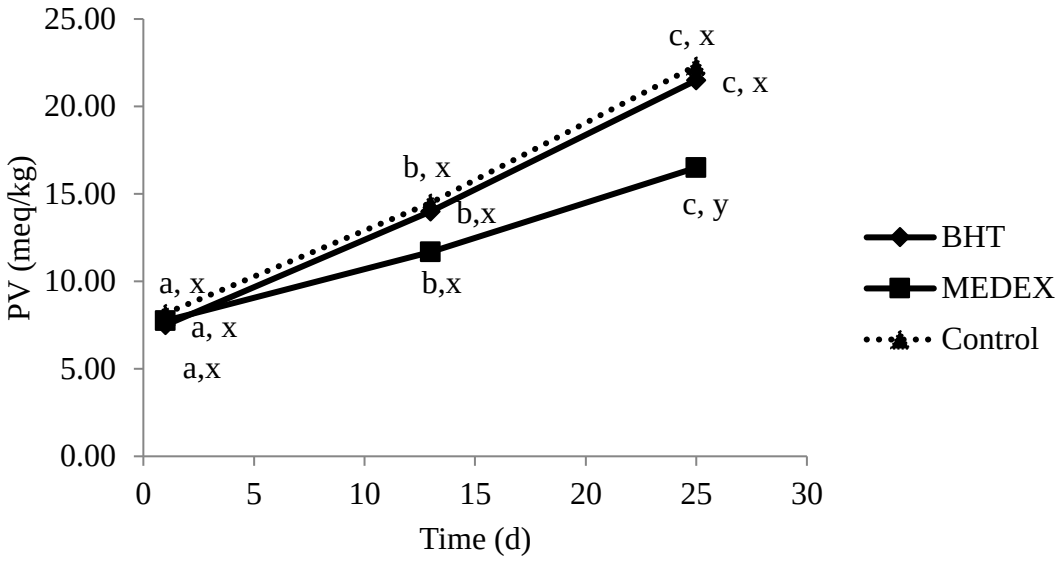


Figure 8. Peroxide value (PV) of sunflower crude oil with different antioxidnats.

BHT = butylated hydroxytoluene (200 mg/kg), MEDEX = methanolic dry extract of Tithonia diversifolia (2400 mg/kg), Control = control treatment (0 mg MEDEX and BHT/kg). All per kg of sunflower oil.

[a, b] Different superscripts within each kind of treatment through storage time are significantly different at $P < 0.05$.

[x, y] Different superscripts within each time are significantly different at $P < 0.05$.

6.2.4. Effect on malondialdehyde (MDA) content

During lipid oxidation, hydroperoxides are the primary products. They are unstable and break down to a wide range of secondary oxidation products (Shahidi and Zhong, 2010), such as malondialdehyde (MDA). The thiobarbituric acid (TBA) assay gives a measure of lipid oxidation development, in terms of MDA (Taghvaei *et al.*, 2014). The method is based on the spectrophotometric quantification of the pink complex formed after reaction of one molecule of MDA with two molecules of TBA, at an absorbance of 532 nm (Zhang *et al.,* 2010). The MDA level is reported per kg of oil (Chen *et al.*, 2014). Table 4 shows that control and BHT treatments had the highest and the lowest ($P < 0.05$) MDA values, respectively. This result is in accordance to PV values (Table 4), which means that control treatment had more MDA values due to higher hidroperoxides production. Kamkar *et al.* (2010) reported similar results; they observed that control treatment showed the higher values of MDA respect to BHT treatment in sunflower oil emulsion over at 7 day incubation at 60 °C. MEDEX and BHT treatments did not show differences ($P > 0.05$); therefore, the methanolic dry extract of leaves from Td was also effective to reduce the MDA production in sunflower oil, although it was not better that BHT, as reported by Chen *et al.* (2014), who observed that rosemary extract had better inhibitory effects than BHA and BHT. The results of the current experiment were compared with rosemary (Chen *et al.,* 2014) due to the lack of information on *Tithonia diversifolia.*

7. CONCLUSIONS

Based on the results of this investigation, we concluded: total phenolic content, total flavonoids content and antioxidant activity of the dry extracts were not affected by lightness condition. Methanol extracted more flavonoids than water, but total phenolic content and the antioxidant activity were not affected. Phenols and flavonoids are stable at least for 28 days. Methanolic dry extract of leaves from *Tithonia diversifolia* increased the total phenolic content and the antioxidant activity of sunflower crude oil. This resulted in an inhibition of primary and secondary oxidation products (hydroperoxides and malondialdehyde, respectively) in sunflower crude oil during storage.

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