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Effect of *Flourensia cernua* Bioactive Compounds on Stability of an Oil-in-Water (O/W) Emulsion

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Abstract: The stability of O / W emulsions cannot be controlled by just the emulsifier additives; antioxidants are needed to improve the stability of the emulsified system and protect against lipid oxidation. This study aimed to evaluate the effect of the bioactive compounds of tarbush *Flourensia cernua* as natural stabilizers of an oil-in-water (O/W) emulsion based on glycerol, gum arabic, jojoba oil, and candelilla wax under refrigeration conditions. The samples were coded as EBCT (emulsion with bioactive compounds of tarbush) and CE (control emulsion). These were characterized in terms of opacity and transparency and evaluated in terms of the system's stability, antioxidant activity, and microbiological analysis during storage. EBCT showed higher transmittance and minor opacity concerning CE. Bioactive compounds of tarbush showed higher antioxidant activity in oil in water emulsion, as measured by ABTS and DPPH. The microbiological analysis results demonstrated that bioactive compounds presented a higher fungistatic effect on yeast growth in the emulsion. EBCT showed higher stability, antioxidant, and fungistatic activity relative to CE, without any significant storage differences during 4 weeks in refrigeration conditions. This research provides the agri-food industry interesting results to formulate a stabilized green emulsion with bioactive compounds of tarbush.

Keywords: natural stabilizers; antioxidants; fungistatic; transparency.

1. Introduction

Emulsified systems are thermodynamically unstable due to the surface tension between oil and water, which opposes the increase of interfacial area and can be stabilized by amphiphilic molecules that are adsorbed in the oil-water interface, decreasing the surface tension between the two phases, which is known as Pickering emulsion [1].

The stability of emulsions is conditioned by the competition between attractive (Van der Waals, hydrophobic interactions, electrostatic attractions, hydrogen bonds) and repulsive forces (electrostatic repulsion, steric repulsion) between the dispersed droplets. It depends on the constituents of the emulsion, the concentration of the emulsifier or stabilizing agent, pH and solvent type [2-3].

One of the most effective and convenient strategies to retard or prevent lipid oxidation is to add antioxidants [4]. According to the mechanisms of action, antioxidants can be broadly classified as primary antioxidants that scavenge free radicals to break chain-reactions of oxidation, or secondary antioxidants that protect lipids against oxidation mainly by chelating transition metals, quenching singlet oxygen, replenishing hydrogen to primary antioxidants, and/or scavenging oxygen [5].

Synthetic antioxidants have been used intensively to inhibit the oxidative reactions of lipids and improve the emulsions' stability [6]. However, its use has caused consumer concerns about their food safety and environment. For this reason, researchers are exploring the use of natural antioxidants to replace synthetic ones in food applications.

Tarbush *Fluorensia cernua* is abundant in arid and semi-arid regions of Mexico, where the tea brewed from the leaves of this plant is used in traditional medicine to treat digestive disorders, rheumatism, venereal diseases, herpes, bronchitis, varicella, and common cold [7]. It has been reported that bioactive compounds of tarbush extracts are biodegradable, have a low environmental impact, and have antioxidant and antifungal properties [8].

The antifungal and antioxidant activity of tarbush is due to its chemical composition mainly by compounds as methyl orsellinate, ermanin, flourensadiol, dehydroflourensic acid, long-chain hydrocarbons from tetracosane-4-olide to triacontane-4-olide, lactones, saponins, terpenes, condensed tannins equivalent to catechins, flavonoid glycosides, luteolin 7-O-rutinoside and 6-C-glucosyl-8-C-arabinosyl apigenin [8-12].

Oil-in-water (O/W) emulsion is more susceptible to oxidation than oil, promoting interactions between the lipids and water-soluble pro-oxidants. The high efficacy of antioxidants in O/W emulsions is primarily attributed to their high affinity to orient toward the oil-water interface [13].

However, since lipid oxidation in O/W emulsions cannot be controlled by just the emulsifiers and thickeners, antioxidants are needed to further protect against rancidity, which may interact with antioxidants and affect the rate of oxidation. Moreover, the oxidative stability of structured lipids in real foods has seldom been investigated in the literature [14].

In this paper, it was studied for the first time the effect of bioactive compounds of tarbush *Flourensia cernua* as natural stabilizers of an oil-in-water (O/W) emulsion, providing an alternative green method to stabilize emulsions with potential application in the agri-food industry.

2. Materials and Methods

2.1. Materials.

Glycerol, gum arabic, tween 80, and jojoba oil were supplied by Panreac (Madrid, Spain). Candelilla wax was supplied by Bioingenio Liftech S.A. de C.V. (Saltillo, México). Ultrapure water was obtained from a Milli-Q filtration system (Millipore Corp., Massachusetts, USA).

2.2. Vegetal material.

Leaves of tarbush *Flourensia cernua* was provided by Bioingenio Liftech S.A. de C.V. (Saltillo, México). Vegetal material was dehydrated at room temperature for 8-10 days and using a conventional oven (Labnet, International, Inc.) at 60 ± 1 ºC for 2 days. The leaves were

stored in amber bottles or dark plastic bags at room temperature (25 ± 1 ºC) until the obtention of the bioactive compounds of tarbush.

2.3. Preparation of emulsion with bioactive compounds of tarbush.

2.3.1. Obtaining of bioactive compounds of tarbush.

The bioactive compounds of tarbush were obtained by infusion method and heating [8]. It was used one sample of 10 g of leaves of tarbush and placed in an amber flask, and then 100 mL of deionized water was added. The mixture was manually stirred and heated for 2 h at 60 ± 1 ºC. The extract was filtered with a Whatman No. 1 paper, transferred to a glass Petri plates, and then placed in a conventional oven (Labnet, International, Inc.) for 36 h at 60 ± 1 ºC. The bioactive compounds of tarbush were stored in containers covered with aluminum or amber bottles at 5 ± 2 ºC.

2.3.2. Preparation of emulsion.

Oil-in-water (O/W) emulsion was prepared using the hot high shear stirring method [15]. Briefly, gum arabic (3% w/v) was homogenized using a high shear stirrer Ultra-Turrax T25 Digital, IKA®, (Staufen, Germany with an S25N-25 G, IKA disperser element) in distilled water at 800 rpm for 1 min, and then was heated to 85 ± 2 ºC. Candelilla wax (1% w/v), jojoba oil (0.15%), glycerol (0.4%) and tween 80 (0.8%) were added. For the emulsification of components, a high shear stirrer at 10,000 rpm for 5 min was used. A concentration of 3310 mg/L of bioactive compounds of tarbush was used in the emulsion based on the antifungal activity reported in previous work [8]. The samples were coded as EBCT (emulsion with bioactive compounds of tarbush) and CE (control emulsion without bioactive compounds of tarbush). These were characterized in terms of opacity and transparency and evaluated in terms of stability of the system, fungistatic and antioxidant activity during storage.

2.4. Opacity and transparency.

The opacity of the emulsion solutions (EBCT and CE) was measured with a colorimeter Konica Minolta (Model CR-400, Minolta, Tokyo, Japan). The instrument was calibrated with a standard white plate (Y, x, Y). Measurements were performed in small Petri dishes, which contained 1 mL of a liquid sample mounted on a plate [16].

Opacity is determined by obtaining the CIE Y coordinates of the samples using five replicates on a black and white background and was calculated as follows:

$$Opacity = Y_b/Y_w \, x \, 100$$

Where Y_b, Y coordinate is measured on the black background and Y_w is the Y coordinate measurement on the white background.

The transparencies of the emulsions at wavelengths ranging from 800 and 1000 nm were investigated.

2.5. Evaluation of emulsion solutions.

The EBCT and CE were made to evaluate the effect of bioactive compounds of tarbush on the system's stability, fungistatic and antioxidant activity in the emulsified system during storage in refrigeration conditions (10 ± 1 ºC). Periodic sampling was carried out each week during 7 weeks of storage in refrigeration conditions.

2.5.1. Determination of stability.

The volumetric method was used to find out ESI for emulsion stability [17]. The emulsions were incubated at 100 ± 1 ºC for 2 h when the separated layer was formed. All samples were measured in triplicate. ESI was calculated as follows:

$$ESI = \left[1 - \frac{volume\ of\ separated\ layer}{total\ volume\ of\ emulsion}\right] x\ 100$$

2.5.2. Determination of antioxidant activity.

The cation radical ABTS was synthesized by the reaction of a 7 mM ABTS solution with a 2.45 mM $K_2S_2O_8$ solution. The mixture was kept at 23 ± 1 ºC in the dark for 16 h. Afterward, the ABTS solution was diluted with ethanol until an absorbance of 0.7 at 734 nm was achieved in a UV–Vis spectrophotometer. 10 µL of the sample (EBCT and CE) was added in the reaction cuvette immediately after 1 mL of ABTS solution was added. After 10 min, the percentage inhibition of absorbance at 734 nm was calculated for each concentration relative to the blank absorbance (ethanol). The DPPH radical is characterized by an unpaired electron, which is a free radical stabilized by resonance. A solution of DPPH radical at a concentration of 60 mM by diluting with methanol was prepared. 100 µL of the sample (EBCT and CE) was added in test tubes covered with foil, then 2.9 mL of DPPH solution was added and allowed to stand for 30 min. The absorbance was recorded at a wavelength of 517 nm. All samples were measured in triplicate. The percentage of inhibition of the radicals ABTS and DPPH was calculated as follows:

$$Inhibition\ (\%) = \frac{(A_{control} - A_{sample})}{A_{control}} \times 100$$

2.5.3. Microbiological assays.

The count of UFC was done by counting fungi and yeasts in food based on the Official Mexican Norm NOM-111-SSA1-1994. Dilutions of 1:1000 of the EBCT and CE in sterile phosphate solution were used. One milliliter of each sample was transferred and distributed using five replicates in Petri dishes. To each inoculated dish, approximately 15 mL of acidified potato dextrose agar with sterile tartaric acid was added at 45 ± 1 ºC. The samples were mixed immediately after pouring by rotating the Petri dish sufficiently to obtain evenly dispersed colonies after incubation. After complete solidification, the plates were inverted and incubated at 25 ± 1 ºC for 5 days. The count was expressed in CFU/mL of emulsion. All samples were measured in triplicate.

2.6. Statistical analysis.

The results were statistically evaluated by analysis of variance (ANOVA) and Tukey's test at 5% significance level, using the software Statistica® 7 (StatSoft Inc., Tulsa, USA).

3. Results and Discussion

3.1. Opacity and transparency.

Table 1 shows the effect of bioactive compounds of tarbush in the opacity and transmittance of the emulsion at wavelengths of 800 and 1000 nm stored during 4 weeks in

refrigeration conditions. The opacity and transmittance of the emulsions were assessed until the fourth week of storage in refrigeration since the control emulsion became unacceptable on the appearance after 4 weeks. The EBCT showed higher transmittance (88 and 97% at 800 and 1000 nm, respectively) and minor opacity (17%) with respect to CE (Table 1).

Table 1. Opacity and transmittance of the oil-in-water (O/W) emulsions stored during 4 weeks in refrigeration conditions (10 ± 1 ºC).

Parameter	Treatments	
	EBCT	CE
Opacity (%)	17 a	48 b
Transmittance (%) to 800 nm	88 a	80 b
Transmittance (%) to 1000 nm	97 a	89 b

CE: Control emulsion. EBCT: Emulsion with bioactive compounds of tarbush. Within a row, different letters represent a significant difference ($P < 0.05$).

The presence of bioactive compounds of tarbush in the emulsion decreased the opacity. It increased the transparency, which was evident in the emulsions' appearance (Fig. 1). The transmittance of the emulsion was higher with bioactive compounds of tarbush (Table 1).

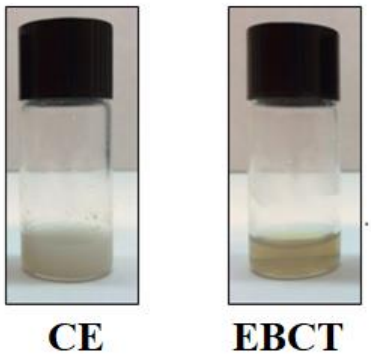

Figure 1. CE and EBCT stored for 4 weeks in refrigeration conditions (10 ± 1 ºC).

This is because bioactive compounds reduce interactions between polymer molecules, which results in a structure with visible cracks, through which light passes easily, thereby decreasing the system's opacity [18-19]. Transmittance is directly correlated with the particle size, which is attributed to the fact that small particles scatter light weakly; therefore, as the particle size increase, the light scattering is strong, and emulsions tend to be opaque [20]. , The addition of tarbush bioactive compounds in the O/W emulsion (EBCT), promotes the formation of a larger number of particles ($9x10^8$ nanoparticles/mL) with a smaller size (50 nm) in the emulsified system, in comparison with the CE ($5x10^8$ nanoparticles/mL, with a size of 100 nm) [21].

3.2. Microbiological stability.

Even though the microbial analysis accounts for fungi and yeast, visual analysis of the plates indicated that most microorganisms on the emulsions were yeast (Fig. 2). Yeasts growth in the CE began in the second week (12 CFU/mL) and continued until the seventh week (47 CFU/mL) of storage (Fig. 2).

The bioactive compounds of tarbush inhibited fungi and yeasts' growth in the system until the fourth week of refrigerated storage (Fig. 2). Yeast's growth in the EBCT began in the fifth week (2 CFU/mL) and continued until the seventh week (8 CFU/mL) of storage (Fig. 2) due to the fungistatic effect of the bioactive compounds of tarbush.

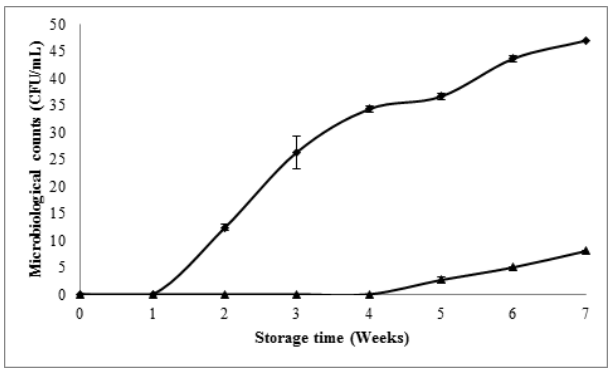

Figure 2. Microbial growth in the EBCT (▲) and CE (♦) stored during 7 weeks in refrigeration conditions (10 ± 1 ºC).

The comparison of growth rates in the emulsions indicated that EBCT showed significantly lower ($p < 0.05$) CFU values, unlike CE (Fig. 2). The concentration used of bioactive compounds of tarbush in the emulsion (3,310 mg/L) contains from 4.24 to 5.80 mg of gallic acid and glucosides of flavonoids as luteolin 7-O-rutinoside and apigenin galactoside arabinoside with antifungal activity against *R. stolonifer*, *B. cinerea*, *F. oxysporum*, and *C. gloeosporioides* [8].

The fungistatic effectiveness of tarbush bioactive compounds (Fig. 2) is attributable to the phenolic compounds present as hydroxyl groups of hydrolyzable tannins equivalents to gallic acid and glucosides of flavonoids [8].

These compounds can form complexes with the microorganism's proteins and polysaccharides, inhibiting the electron transport through membranes, causing cell lysis [22-23].

The ability of these microbes to penetrate the oil-water interface was related to their cell surface properties. As lactic acid bacteria and yeasts that live and grow in aqueous environments, they naturally show a predominantly hydrophilic behavior [24]. This behavior type shows that yeasts can grow in the aqueous phase of the emulsion and consume the available sugars of glucosides present in the bioactive compounds of tarbush, therefore destabilizing the emulsified system interfering between the aqueous and oil phase of the emulsion. This is largely due to the dominance of carbohydrates in yeast cell walls (94%) [25]. Yet, yeasts have been shown to attach to the oil-water interface via hydrophobic interactions. Such behavior is broadly determined by the composition and conformation of surface-bound proteins, polypeptides, and polysaccharides [26-27].

Based on the above, possibly the yeasts interacted in the aqueous phase of the emulsion and may have consumed sugars present in the glucosides of flavonoids of the bioactive compounds of tarbush, destabilizing the emulsified system between the aqueous and oil phase, affecting the stability (Fig. 3) the antioxidant activity (Table 2) and fungistatic of the bioactive compounds in the emulsion until the fifth week of storage in refrigeration (Fig. 2).

Table 2. Antioxidant activity of the oil-in-water (O/W) emulsions stored during 7 weeks in refrigeration conditions (10 ± 1 ºC).

Storage time (weeks) in refrigeration		**0**	**2**	**4**	**5**	**6**	**7**
CE	ABTS (%)	5 a	7 a	9 a	1 b	0 b	0 b
	DPPH (%)	0 a	0 a	0 a	0 a	0 a	0 a
EBCT	ABTS (%)	53 a	55 a	56 a	30 b	24 b	22 b
	DPPH (%)	46 a	48 a	50 a	20 b	16 b	12 b

CE: Control emulsion. EBCT: Emulsion with bioactive compounds of tarbush. Within a row, different letters represent a significant difference ($P < 0.05$).

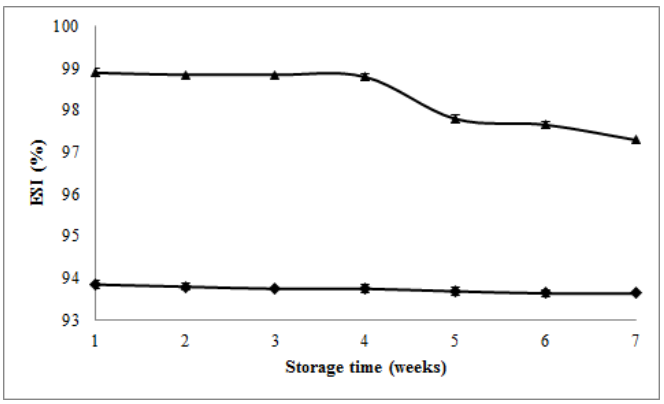

Figure 3. Effect of time (weeks) in the stability of the EBCT (▲) and CE (♦) stored during 7 weeks in refrigeration conditions (10 ± 1 ºC).

3.3. Emulsion stability.

Figure 3 shows that the absence of bioactive compounds of tarbush in the emulsified system (CE) promoted a significantly lower ($p < 0.05$) stability index (93.84 to 93.63%), unlike to EBCT during storage in refrigeration, where EBCT is stable until four weeks.

The bioactive compounds of tarbush promote greater stability on the emulsion (Fig. 3), due to the content of tannin and glycosides of flavonoids, which are adsorbed on the surface of the oil globules present in the emulsion and once adsorbed at the interface is very difficult to remove making it more stable emulsion, due to steric and/or electrostatic repulsions between the droplets covered by adsorbed tannins [28].

When the polymerization degree of the tannins adsorbed is higher, the stability of the emulsion increases due to the formation of thicker layers leading to a stronger steric repulsion between the droplets preventing coalescence or flocculation [28]. Antioxidants such as all-trans-retinol improve the stability of solid lipid nanoparticles [29]. And flavonoids, which are neither soluble in water nor soluble in oils, can adsorb at the oil-water interface and stabilize O/W emulsions [30]. Food-grade particles, including rutin hydrate and naringin as flavonoid particle stabilizers, can form green and surfactant-free stable W/O and O/W emulsions [31]. Quercetin and curcumin as natural polyphenols can act as W/O Pickering stabilizers [32].

From the fifth week, a decrease was observed in the stability of EBCT during storage. The decrease stability (Fig. 3) may be related to microbiological analysis results (Fig. 2), because in the fifth week of storage was observed the yeast's growth in the system.

3.4. Antioxidant stability.

The concentration used of tarbush extract in the emulsion (3,310 mg/L) contains from 4.24 to 5.80 mg of gallic acid and flavonoid glucosides such as luteolin 7-O-rutinoside and apigenin galactoside arabinoside with antioxidant activity (6.07 to 7.62 µMol/g of TEAC) [8]. The greater antioxidant (Table 2) effectiveness of bioactive compounds of tarbush is attributable to the phenolic compounds present as hydroxyl groups of hydrolyzable tannins equivalents to gallic acid and flavonoid glucosides [8].

Tannins and flavonoids in their chemical structure include a variable number of hydroxyl groups involved in neutralizing free radicals by donating electrons and thus the influence of the antioxidant activity.

The values obtained in the capture of ABTS radical are higher than those obtained by DPPH radical (Table 2) due to the ABTS radical's sensitivity because it is a structure that easily

reacts with hydrophilic and lipophilic compounds and reducing agents [33]. However, DPPH radicals react with hydrophilic compounds such as gallic acid and flavonoids.

The values of antioxidant activity by ABTS and DPPH remained stable until the fourth week of refrigerated storage, protecting at an emulsified system of the lipid oxidation (Table 2) due to the neutralization of free radicals by a large number of hydroxyl groups present in the bioactive compounds of tarbush which remain between the aqueous and oil phase of the emulsion.

The antioxidant activity of bioactive compounds of tarbush is mainly due to its redox properties, which play an important role in the absorption and neutralization of free radicals [8]. Its antioxidant potential depends on the number of hydroxyl groups and the structure's conjugation degree because the activity improves when the number of hydroxyl groups increases as the gallic acid and flavonoids, which are phenolic compounds with OH groups and carboxylic acids [34]. The results obtained are related to the part of the emulsion's stability (Fig. 3) because lipid oxidation can be retarded by strengthening the interfacial layer and by adsorbing tannins in the oil and aqueous phase, which may affect oxygen transfer and oxidation products [35].

4. Conclusions

This is the first report publicizing the use of bioactive compounds of tarbush as natural stabilizers of an oil-in-water emulsion. Emulsion stabilization would be due to steric repulsions and/or stabilization by tannin aggregates. Bioactive compounds of tarbush promoted higher transparency on the emulsion. For the first time, it was demonstrated that bioactive compounds of tarbush presented a good antioxidant and fungistatic activity in oil in water emulsion during 4 weeks in refrigeration conditions. This research shows the potential use of high added-value tarbush bioactive compounds of tarbush distributed in Mexico's semi-arid regions that could be used as antioxidants emulsifiers for potential use as natural stabilizers emulsions with potential application in the agri-food industry.

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Conflicts of Interest

The authors declare no conflict of interest.

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