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Antimicrobial Activity, Antioxidant Properties and Phytochemical Screening of *Echinacea angustifolia, Fraxinus excelsior* and *Crataegus oxyacantha* Mother Tinctures Against Food-Borne Bacteria

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Abstract

The beneficial health effects of extracts from many types of plants have been known for centuries and the search for new natural extracts, such as mother tinctures (TMs), to be used in the food and cosmetics industry, is very important at present. In this study, the antioxidant and antimicrobial activities of *E. angustifolia, F. excelsior* and *C. oxyacantha* mother tinctures against a range of foodborne bacteria and their major components were analyzed and determined.

F. excelsior proved the highest content of polyphenols, (313.88 ± 14.30 mg GAE/100 ml), while *C. oxyacantha* and *E. angustifolia* showed similar values of polyphenols, 161.25 ± 4.05 mg GAE/100 ml and 123.27 ± 4.06 mg GAE/100 ml, respectively. *C. oxyacantha* proved the highest content of tannins and flavonoids. *E. angustifolia* and *F. excelsior* showed also a good content of flavonoids. Radical-scavenging activity was evaluated by the use of free stable radical, DPPH, and the scavenging activity of the hydroalcoholic extracts were found to be 418.23 ± 36.11 mg TE/100 ml, 266.71 ± 28.32 mg TE/100 ml and 131.33 ± 14.26 mg TE/100 ml in *F. excelsior*, *C. oxyacantha* and *E. angustifolia*, respectively. Similar results were obtained by FRAP test, that showed the highest reducing power in *F. excelsior*. As the lipid peroxidation inhibition, the BCB method evidenced in *F. excelsior* the highest inhibition (38.41 ± 3.60%), with an inhibition lower than 50% in all mother tinctures.

The antimicrobial activity and the MIC of the mother tinctures were evaluated against selected bacterial strains. All mother tinctures demonstrated no antimicrobial activity against the 50% of tested bacteria. All Gram-negative bacteria were sensitive to all mother tinctures with a middle-low antimicrobial activity, except *Escherichia coli* that proved to be no sensitive; while the antimicrobial activities observed varied with the type of tested Gram-positive bacterium.

The observed antimicrobial and antioxidant activities might be due to the synergistic actions of bioactive compounds detected in the mother tinctures. The results of this study could be applied in pharmaceutical field, establishing an important role of mother tinctures in phytotherapy, in order to adopt integrated strategies to effectively counter the excess and the effects of free radicals, and also in food preservation, alternative medicine and natural therapies.

Keywords: *Mother Tincture; Polyphenols Content; Flavonoids Content; Tannins Content; Antimicrobial Activity; MIC; Antioxidant Properties*

Introduction

Plant extracts have been used for a wide variety of purposes for many thousands of years. These purposes vary from the use in perfumery, to flavouring drinks and the application for the food preservation. In particular, the antimicrobial activity of plant extracts has formed

the basis of many applications, including raw and processed food preservation, pharmaceuticals, alternative medicine and natural therapies [1]. During the last decades, there is increasing interest to unlock the secrets of ancient herbal remedies. For this purpose, various strategies have been developed e.g., biological screening, isolation as well as clinical trials for a variety of plants. Based on the screening methodologies, the therapeutic values of many herbal medicines have already been established and are considered as safe for human beings [2]. Nowadays, the excessive use of synthetic antimicrobial compounds in food manufacture as additive agents is well known, many of which are suspected for their residual toxicity. Several plant extracts offer potential applications in food preservation, and the use of these extracts in the food industry can help reduce the addition of chemical preservatives as well as the intensity of heat treatments, resulting in foods which are more naturally preserved and richer in organic properties [3,4].

The beneficial health effects of extracts from many types of plants have been known for centuries and the search for new natural extracts, such as mother tinctures (TMs), to be used in the food and cosmetics industry, is very important at present [5]. TMs are plant extracts obtained by macerating the fresh herbal drug in the hydroalcoholic solvent in the portion of 1:10 (calculated on the dried weight of the herbal drug), according to the French Pharmacopoeia and their homeopathically diluted solutions are used to treat several ailments [6].

Because of the extraction mode, the hydroalcoholic solvent is able to extract and preserve the whole bioactive substances of plants, so the TMs contain a variety of secondary metabolites, such as flavonoids, anthocyans, saponins and tannins. The antioxidant capacity of these compounds can promote their use as natural food additives [4].

Fraxinus (F.) excelsior, locally known as "l'ssane l'ousfour", is a native shrub belonging to the Oleaceae family widely distributed throughout the south-eastern region of Morocco (Tafilalet). Ash (*F. excelsior*) is known worldwide to possess several biological activities including antioxidative, anti-inflammatory, anti-rheumatic, analgesic and antipyretic [7]. Plant parts used for mother tincture include leaves, bark, new branches and buds, aerial part of plant rich in different bioactive compounds, such as flavonoids.

Hawthorn, *Crataegus (C.) oxyacantha* (Rosaceae), is a perennial plant, usually a shrub or tree 1 to 6 m in height, and is found in deciduous forests and underbrush in the regions of Southeast Serbia. Hawthorn is well known in phytotherapy for the treatment of many cardiovascular diseases, but it is used also in the food industry for the production of jam and various beverages including wine, juice, compote and herbal tea [8,9]. Plant parts used for mother tincture include flowers, fruits and bark, that contain flavonoids, considered to be the main groups of active constituents in hawthorn extracts and natural antioxidants to be applied in nutrition and medical treatments since they contribute to the prevention of oxidative stress [8].

Echinacea (E.) angustifolia is a native herb in both North America and Europe belonging to Asteraceae family and widely accepted for its immuno-stimulant medicinal usage [10]. Plant parts used for mother tincture include leaves, flowers and roots, rich in naturally occurring phenolic acids and flavonoids, that are known to be important antioxidants [10].

The purpose of the study was to gain insight on the antioxidant and antimicrobial activities of *E. angustifolia, F. excelsior* and *C. oxyacantha* mother tinctures against a range of foodborne bacteria, including gram-negative and gram-positive bacteria. Moreover, the major components of the extracts were analyzed and determined.

Materials and Methods

Hydroalcoholic extracts

Hydroalcoholic extract (55% ethanol solution) of *Echinacea (E.) angustifolia, Crataegus (C.) oxycantha* and *Fraxinus (F.) excelsior* were purchased by Laboratories Boiron.

Total polyphenol content

The content of polyphenols of investigated mother tinctures was measured by using Folin-Ciocalteu assay [11]. Briefly, 50 µl of hy-

droalcoholic extract and 450 µl of distilled water was added to 500 µl Folin Ciocalteu reagent and 500 µl of Na_2CO_3 (10% w/ v). The mixture was mixed and after 1h of incubation in the dark at room temperature the absorbance was measured at 723 nm using a UV-Vis spectrophotometer (UV 640 Spectrophotometer). The total phenolic content was expressed as mg Gallic Acid Equivalent (GAE)/100 ml of hydroalcoholic extract. The experiment was carried out in triplicate.

Flavonoid Content determination

The aluminium chloride colorimetric method was used to determine flavonoids content in the sample [12]. Mother tincture (150 µl) was added to 45 µl of 5 % NaNO3 into a microcentrifuge. After 5 minute, 90 µl of 10 % $AlCl_3$ was added. At the 6th minute, 300 µl of 1 M NaOH solution and 915 µl of water were added. The solution was mixed well and the absorbance was measured after 10 minutes at 510 nm. Results, carried out in triplicate, were expressed as mg Quercetin Equivalent (QE)/100 ml of hydroalcoholic extract.

Tannin Content determination

The content of total tannin was measured by protein precipitation assay as reported by Armentano., *et al.* [12] An aliquot of hydroalcoholic extract (250 µl) was added to 500 µl of BSA solution (1.0 mg/ml in 0.2 M acetic buffer, pH 5.0 with 0.17 M NaCl, buffer A) in a microcentrifuge tube. After 15 min, the solution was centrifuged for 15 min at 5000 g. The surface of the pellet and the walls of the tube were washed with buffer A and the precipitate was dissolved in in 1 ml of SDS-triethanolamine solution. Ferric chloride reagent (1 ml) was added, and the solutions were mixed immediately. The absorbance at 510 nm was taken after 30 min. Results were expressed as mg Tannic Acid Equivalent (TAE)/100 ml of hydroalcoholic extract. Experiments were carried out in triplicate.

***In-vitro* antioxidant activity**

2,2-diphenyl-1-picrylhydrazyl (DPPH) test

Radical-scavenging activity was evaluated by using DPPH test [13]. Briefly, 120 µl of mother tincture or dilutions were added to 1380 µl of 100µM DPPH solution in a microcentrifuge tubes. After 60 min in the dark, the absorbance was monitored at 515 nm by using spectrophotometer (UV 640 Spectrophotometer). Trolox was used as standard and results were expressed as mg Trolox/100mL of hydroalcoholic extract. Experiments were carried out in triplicate.

Ferric Reducing Ability Power test (FRAP)

The reducing power was determined by FRAP assay [14]. The stock solution included 300 mM acetate buffer (3.1 g $C_2H_3NaO_2 \cdot 3H_2O$ and 16 ml $C_2H_4O_2$), pH 3.6, 10 mM TPTZ (2, 4, 6-tripyridyl-s-triazine) solution in 40 mM HCl, and 20 mM $FeCl_3 \cdot 6H_2O$ solution. The working solution was prepared by mixing acetate buffer, TPTZ and $FeCl_3 \cdot 6H_2O$ (10: 1: 1). Hydroalcoholic extracts (150 µl) were allowed to react with 2850 µl of the FRAP solution for 40 min at 37°C. Readings of the colored product (ferrous tripyridyltriazine complex) were taken at 593 nm. Trolox was used as standard and results were expressed in mg Trolox/100 ml of hydroalcoholic extract.

Beta Carotene Bleaching Assay (BCB)

The antioxidant activity was evaluated by the β-carotene-linoleic acid bleaching method as reported by Milella., *et al* [14]. β-carotene (0.2 mg) dissolved in 0.2 ml of chloroform, linoleic acid (20 mg) and Tween 20 (200 mg) were mixed. Chloroform was removed by using rotary evaporator at 37°C and distilled water (50 ml) was added. Four milliliters of the emulsion were transferred into several tubes containing 0.2 ml of mother tinture or ethanol as control. BHT was used as positive control. The tubes were placed at 50°C for 3h and the absorbance was measured at 470 nm by using spectrometer. The results are expressed as percent of antioxidant activity (%AA).

Antibacterial activity assay

Bacterial strains and growth conditions

The three mother tinctures were tested against a panel of bacterial strains shown in table 1.

Strain	Bacterial species	Growth conditions	
		Temperature	Medium
9P	*Carnobacterium maltaromaticum*	20°C	Tryptone Soya Yeast Extract Medium
H02	*Carnobacterium divergens*	20°C	Tryptone Soya Yeast Extract Medium
6P2	*Pseudomonas fragi*	20°C	Tryptone Soya Yeast Extract Medium
53M	*Hafnia alvei*	30°C	Tryptone Soya Yeast Extract Medium
42M	*Pseudomonas proteamaculans*	30°C	Tryptone Soya Yeast Extract Medium
7R1	*Brochothrix thermosphacta*	20°C	Tryptone Soya Yeast Extract Medium
32	*Escherichia coli*	37°C	Tryptone Soya Yeast Extract Medium
LMG6399	*Enterococcus hirae*	37°C	M17 Medium
ATCC14434	*Enterococcus faecium*	37°C	M17 Medium
ATCC14433	*Enterococcus faecalis*	37°C	M17 Medium
ATCC14436	*Enterococcus casseliflavus*	37°C	M17 Medium
ATCC11576	*Enterococcus durans*	37°C	M17 Medium
LMG13129	*Enterococcus gallinarum*	37°C	M17 Medium
DSM 20410	*Weissella viridescens*	30°C	MRS Medium
DSM 20196	*Weissella confusa*	30°C	MRS Medium
DSM 7378	*Weissella hellenica*	30°C	MRS Medium
DSM20014	*Weissella minore*	30°C	MRS Medium
DSM 15878	*Weissella cibaria*	30°C	MRS Medium
DSM20288	*Weissella paramesenteroides*	30°C	MRS Medium
DBPZ0062	*Lactobacillus sakei*	30°C	MRS Medium
DBPZ0416	*Lactobacillus sakei*	30°C	MRS Medium
DBPZ0329	*Lactobacillus sakei*	30°C	MRS Medium
DBPZ0338	*Lactobacillus sakei*	30°C	MRS Medium
DBPZ0098	*Lactobacillus sakei*	30°C	MRS Medium
DBPZ0224	*Staphylococcus xylosus*	30°C	Tryptone Soya Yeast Extract Medium
DBPZ0248	*Staphylococcus equorum*	30°C	Tryptone Soya Yeast Extract Medium
DBPZ0044	*Staphylococcus equorum*	30°C	Tryptone Soya Yeast Extract Medium
DBPZ0251	*Staphylococcus succinus*	30°C	Tryptone Soya Yeast Extract Medium
DBPZ0241	*Staphylococcus equorum*	30°C	Tryptone Soya Yeast Extract Medium
BL/26	*Listeria innocua*	30°C	Tryptone Soya Yeast Extract Medium

Table 1: *Bacterial strains and growth conditions used for antimicrobial activity assay.*

A total of thirty strains of the culture collection of the Dipartimento di Scienze, Università degli Studi della Basilicata, Potenza, Italy, were employed as screening microorganisms for this study. All strains were maintained as freeze-dried stocks in reconstituted (11% w/v) skim milk, containing 0.1% w/v ascorbic acid and routinely cultivated in optimal growth conditions (table 1). These bacteria were chosen in order to represent the diversity of species of food-borne gram positive and gram negative.

Agar well diffusion assay and Minimum Inhibitory Concentration

Antimicrobial activities of tested mother tinctures were determined by standard agar well diffusion assay [5]. For each strain, a subcul-

ture in a specific broth was obtained from the active stock culture by 1% (v/v) inoculum and incubated overnight at the corresponding culture temperature. 200 µl of each subculture was used to inoculate the agar media (to achieve a final concentration of 10^6 CFU/ml) and distributed into Petri plates. 60 µl of each extract was poured into wells (6 mm diameter) bored in the agar plates and then the plates were incubated at optimal growth conditions for each strain. Organic solvent was used as negative control while antibiotic was used as positive control. The experiment was performed in triplicate and the antimicrobial activity of each extract was expressed in terms of zone of inhibition diameter mean (in mm) produced by the respective extract after 24h of incubation. A inhibition zone < 9 mm indicated a low antimicrobial activity; 10 < zone of inhibition < 14 mm, a middle antimicrobial activity; a zone of inhibition >15 mm, an high antimicrobial activity. Extracts producing an high inhibition zone were screened to determine minimum inhibitory concentrations (MICs) in order to evaluate the antimicrobial effectiveness of each extract against different bacterial strains by the agar well diffusion method [5]. Each specific medium inoculated with the strain subculture was distributed into Petri plates and different concentrations of extracts, ranging from 1 µg/ml to 100 µg/ml, were poured into wells bored in the agar plates and the plates were incubated for 24h. After incubation, the MIC was calculated as the lowest concentration of the extract inhibiting the growth of bacterial strains. The MIC values were done in triplicate.

Results and Discussion

Plants produce diverse types of metabolites, some produced during secondary metabolism and referred to as secondary metabolites. Important among these are alkaloids, terpenoids, steroids and polyphenolic compounds. These chemicals (phytochemicals) are distributed in various parts of the plants. The exact role of these metabolites is not fully understood, however, most of these metabolites are of significance for plants in terms of preventing herbivores, pathogens and insects, attraction of pollinators, coping with abiotic stress etc.

Besides, these phytochemicals are known to exhibit a range of bioactivities such as antimicrobial, anticancer, antioxidant, antiherbivore and insect repellent activity. These plant secondary metabolites have a great potential for medicine, industry, agriculture and food sciences. It is of profound importance to detect the phytochemicals in plants so as to relate their presence with bioactivity observed and to know their possible therapeutic role [15].

In this study, the antioxidant potential and the content of secondary metabolites of three mother tinctures were evaluated by *in vitro* assays.

The content of polyphenols, flavonoids and tannins were determined using a gallic acid, quercetin and tannic acid standard curve and results were expressed as mg GAE/g, mg QE/g and mgTA/g, respectively. As shown in Table 2, *F. excelsior* proved the highest content of polyphenols, (313.88 ± 14.30 mg GAE/100 ml), while *C. oxyacantha* and *E. angustifolia* showed similar values of polyphenols, 161.25 ± 4.05 mg GAE/100 ml and 123.27 ± 4.06 mg GAE/100 ml, respectively.

C. oxyacantha proved the highest content of tannins (342.76 ± 19.73 mg TA/100 ml) and flavonoids (1052.60 ± 22.65 mg QE/100 ml). *E. angustifolia* and *F. excelsior* showed also a good content of flavonoids (Table 2). The flavonoid compounds and oligomeric procyanidins are key constituents of *C. oxyacantha*, that contains heptahydroxy flavan glycoside, flavan polymers [6,16]; whereas in *E. angustifolia*, it has been reported the presence of lipophilic compounds including alkamides and ketoalkenes, and the hydrophilic phenolic compounds, mainly caffeic acid derivatives [17]. Coumarins, secoiridoids and phenylethanoids are characteristic features of *Fraxinus* species; classes of compounds as lignans, flavonoids and simple phenolic compounds are also common, but they appear to have more limited distribution [18]. Phenolic compounds present in the plant mother tincture have been considered to be responsible for *in vitro* antioxidant properties.

In this study, three different approaches were used to determine the antioxidant activity; results were reported in Figure 1. Radical-scavenging activity was evaluated by the use of free stable radical, DPPH, and the scavenging activity of the hydroalcoholic extracts were found to be 418.23 ± 36.11 mg TE/100 ml, 266.71 ± 28.32 mg TE/100 ml and 131.33 ± 14.26 mg TE/100 ml in *F. excelsior*, *C. oxyacantha* and *E. angustifolia*, respectively. Similar results were obtained by FRAP test, that showed the highest reducing power (1972.53 ± 42.01 mg TE/100 ml) in *F. excelsior*.

	TPC (mgGAE/100 ml)	TTC (mgTA/100 ml)	TFC (mgQE/100 ml)
Echinacea angustifolia	123.27 ± 4.06	23.01 ± 0.89	371.30 ± 4.83
Fraxinus excelsior	313.88 ± 14.30	41.53 ± 1.48	631.73 ± 19.67
Crataegus oxyacantha	161.25 ± 4.05	342.76 ± 19.73	1052.60 ± 22.65

Table 2: *Total polyphenol content (TPC), total tannin content (TTC) and total flavonoid content (TFC) of Echinacea angustifolia, Fraxinus excelsior and Crataegus oxyacantha mother tincture*

mgGAE/100ml = milligrams of gallic acid equivalent per 100 milliliters of mother tincture;

mg TA/100ml= milligrams of tannic acid equivalent per 100 milliliters of mother tincture;

mgQE/100ml= milligrams of quercetin equivalent per 100 milliliters of mother tincture.

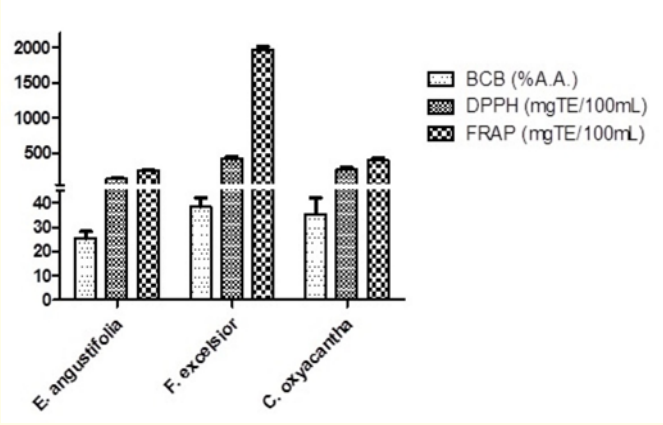

Figure 1: *Antioxidant activity of Echinacea angustifolia, Fraxinus excelsior and Crataegus oxyacantha mother tinctures by DPPH, FRAP and BCB assays.*

As the lipid peroxidation inhibition, the BCB method evidenced in *F. excelsior* the highest inhibition (38.41 ± 3.60 %), with an inhibition lower than 50% in all mother tinctures.

Moreover, the antimicrobial activity and the MIC of the mother tinctures were evaluated against selected bacterial strains of significant importance for human health by using the agar well diffusion assay. A total of thirty gram-negative and gram-positive bacteria were employed as screening microorganisms to determine the antimicrobial effect, the action spectrum and the antimicrobial effectiveness of each extract. Results showed that all mother tinctures demonstrated no antimicrobial activity against the 50% of tested bacteria. All Gram-negative bacteria were sensitive to all mother tinctures with a middle-low antimicrobial activity (inhibition zone ranging from 9.24 to 12.45 mm) except *Escherichia coli* that proved to be no sensitive to all; while the antimicrobial activities observed varied with the type of tested Gram-positive bacterium.

E. angustifolia mother tincture was more effective against *Enterococcus* spp. strains, with an high antimicrobial activity, except for *Enterococcus hirae*, while the inhibition of *Staphylococcus* spp. strains was different according to species tested. For *Staphylococcus xylosus* and two *Staphylococcus equorum* strains a low inhibition zone was observed, while the other strains were sensitive to this extract with a high antimicrobial activity, ranging from 18.78 to 21.83 mm. Moreover, also *Carnobacterium maltaromaticum* and *Brochothrix thermosphacta* were sensitive to *E. angustifolia* with an high activity (inhibition zone of 17.77 and 19.32 mm, respectively).

F. excelsior mother tincture showed an high antimicrobial activity against 26.7% of tested bacteria that include all *Enterococcus* spp. strains (except for *Enterococcus hirae*), a *Staphylococcus equorum* strain and *Carnobacterium maltaromaticum* and *Brochothrix thermos-*

phacta. Moreover, this mother tincture had a middle antimicrobial effect on *Staphylococcus xylosus* and a *Staphylococcus equorum* strain, and also on *Carnobacterium divergens, Weissella cibaria* and *Listeria innocua* with an inhibition zone ranging from 11.22 and 13.79 mm.

C. oxyacantha mother tincture showed an high antimicrobial activity against 16.7% of tested bacteria, including only two *Enterococcus* spp. strains (*E. faecalis* and *E. durans*), *Staphylococcus succinus, Carnobacterium maltaromaticum* and *Brochothrix thermosphacta*. Furthermore, the mother tincture provided a middle antimicrobial effect against 30% of sensitive bacteria, consisting of the other *Enterococcus* spp. strains, the two *Staphylococcus equorum* strains and *Listeria innocua*.

Moreover, none of the extracts exhibited the antimicrobial effect on *Lactobacillus* strains tested, that is a result that possibly indicate, in case of oral administration, the respect of the intestinal flora.

In addition, the mother tinctures producing an high inhibition zone were screened to determine MIC by the agar well diffusion method against respective susceptible bacterial species (Table 3).

Bacteria	**MIC (μg/ml)**		
	Echinacea angustifolia	***Crataegus oxyacantha***	***Fraxinus excelsior***
Carnobacterium maltaromaticum	40 ± 0.54	60 ± 0.74	40 ± 0.99
Brochothrix thermosphacta	40 ± 0.88	60 ± 1.03	40 ± 1.07
Enterococcus faecium	10 ± 0.98		100 ± 0.89
Enterococcus faecalis	1 ± 0.09	1 ± 0.06	40 ± 0.23
Enterococcus casseliflavus	10 ± 0.66		10 ± 1.03
Enterococcus durans	1 ± 0.03	1 ± 0.53	1 ± 0.35
Enterococcus gallinarum			60 ± 0.66
Staphylococcus equorum	40 ± 0.77		60 ± 0.59
Staphylococcus succinus	100 ± 1.23	120 ± 1.76	

Table 3: *MIC (μg/ml) of tested mother tinctures.*

Different concentrations of mother tinctures (from 1 μg/ml to 120 μg/ml) were tested by agar well diffusion assay (Russo., et al. 2012).

Value are expressed as mean ± standard deviation.

Enterococcus durans was inhibited at a low concentration (1 μg/ml) of all mother tinctures, while *Enterococcus faecalis* required a low inhibitory concentration (1 μg/ml) of *E. angustifolia* and *C. oxyacantha* and an inhibitory concentration of 40 μg/ml of *F. excelsior*.

E. angustifolia showed a MIC of 10 μg/ml for other *Enterococcus* spp. strains, resulted sensitive to the mother tinctures, while these strains required an inhibitory concentration of 10 μg/ml and 100 μg/ml of *F. excelsior*. In addition, *F. excelsior* showed a MIC of 60 μg/ml for *Enterococcus gallinarum. Carnobacterium maltaromaticum* and *Brochothrix thermosphacta* required an inhibitory concentration of 40 μg/ml of *E. angustifolia* and *F. excelsior* mother tinctures and of 60 μg/ml of *C. oxyacantha*. Moreover, *Staphylococcus equorum* required an inhibitory concentration of 40 μg/ml and 60 μg/ml of *E. angustifolia* and *F. excelsior* mother tinctures, respectively.

Conclusion

E. angustifolia, F. excelsior and *C. oxyacantha* displayed antimicrobial and antioxidant potential. The observed antioxidant and biological activities might be due to the synergistic actions of bioactive compounds detected in the mother tinctures. The results of this study could be applied in pharmaceutical field, establishing an important role of mother tinctures in phytotherapy, in order to adopt integrated strategies to effectively counter the excess and the effects of free radicals, and also in food preservation, alternative medicine and natural therapies.

Further studies are needed to elucidate mechanisms that contributes to these extract properties and also a phytochemical investigation is also proposed to isolate the active fraction and eventually the pure compound(s) with a vital role for these activities.

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