PE4.60 Antioxidant activity of rosemary, thyme and synergism with α-tocopherol in a liposome system 217.00

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Abstract— **Antioxidative effects of by-products of aromatic industry: rosemary distilled leaves (*DRL*), thyme distilled leaves (*TDL*) and the combined antioxidative effects of *DRL*, *DTL* and α-tocopherol (*TOH*), were investigated for peroxidation of L-α–phosphatidylcholine liposomes with oxidation initiated by hydrophilic azo-initiators. The results showed that *DRL* and *DTL* had a clear antioxidative effect as evidenced by a lag phase for formation of conjugated dienes. *DRL* and DTL had a similar antioxidative effect. Combination of α-tocopherol with *DRL* and *DTL* acted in both synergistically in prolongation of the lag phase. This study describes the use of complex liposomes as real membranes models to evaluate the potential benefits of natural antioxidants in relation to lipid peroxidation.**

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***Index Terms*— *Rosmarinuss officinalis*, *Thymus zygis*, liposome oxidation, Synergy.**

I. INTRODUCTION

A Wide range of methods has been described for assessing antioxidant activity. The use of liposomes (LDL) appears to be the most promising method of assessing antioxidant properties, relevant to animal feeding, since LDL allow to investigate the protection of a susbtrate by an antioxidant in a model biological membrane or a lipoprotein [1]. Moreover, the liposome system allows studying the synergism between tocopherol and phenolics compounds [1], which are antioxidant that are present naturally in combinations and they are very used in animal feeding. So a correct assessment of lipid oxidation in the meat industry is very important. The rate of lipid oxidation (that is the major quality problem by the meat industry and by the consumers) in meat products can be effectively retarded by the use of antioxidant trough dietary supplementation.

The use of synthetic antioxidants in the meat industry is tending to diminish because of growing concern among consumers about such chemical additives. This concern has led to increased of interest focused on the search of natural antioxidant. The use of natural preservatives to increase the shelf life of meat products is a promising technology since many vegetal substances show antioxidant and antimicrobial properties. Several studies have indicated that some dietary antioxidants may be absorbed and help prevent lipid and colour oxidation in meat to a limited extent [2]. Dietary supplementation with α-tocopherol and herbs of the *Labiatae* family offers a simple and convenient way to introduce a natural antioxidant into muscle food without any special processing. Natural antioxidants are incorporated into the cellular structures, where initiation of lipid oxidation is thought to occur [3]. At this location α-tocopherol and phenolics compounds in rosemary and thyme exert a strong antioxidant activity by neutralizing free radicals before lipid oxidation propagates among highly unsaturated fatty acids in cellular and subcellular membranes [4].

The search for natural antioxidants in aromatic plants by-products has become an alternative to synthetic antioxidants in meat industry [5]. Rosemary (*Rosmarinuss officinalis, L).* and thyme (*Thymus zygis* ssp. *gracilis* or red thyme) are the aromatic plants most exploited in Murcia (Spain), their use mainly being the extraction of essential oils, a process which generates an excess of distilled leaves [6]. These products are currently under-used, but could be used as potential sources of natural antioxidants and antimicrobials in the meat industry [7, 8]).

The objective of this study was to determine the antioxidant activity of distilled rosemary and distilled thyme leaf in a liposome system, and the synergism between them and α-tocopherol**.**

II. MATERIALS AND METHODS

2.1. Chemicals

L-α- phosphatidyl choline (PC) from soybean (purity 99 %) was purchased from Sigma-Aldrich Chemie Gmbh (Steinheim, Germany). α-Tocopherol from Fluka (Switzerland). 2, 2`-azobis (2-amidiopropane) dihydrochloride (AAPH) was supplied from Wako Chemicals Inc. Folin-Ciocalteu Reagent from Merk (Germany). Rosemary (*Rosmarinus officinalis*, L) and thyme (*Thymus zygis* ssp. *gracilis*) obtained from a Murcia (Spain) producer. The distillation of the leaves and dried process was carried out agree with Jordan *et al*. [9].

2.2. Polyphenols extraction of distilled leaves.

Extract were prepared by mixing 0.5g milled spices (rosemary distilled leaves –*DRL*- and thyme distilled leaves- *DTL*-) with 4 ml of acetone. The air in the tube was replaced with nitrogen and the phenolics are extracted at 20º C by shaking (200 rpm) for 20 min. The mixture was subsequently centrifuged (2 min, 1100g), and the filtrate was evaporated to dryness using a rotary vacuum evaporator and 35 ºC hot water bath. The evaporation takes between 5 and 20 min. The residue was dissolved in 6 ml 25% aqueous ethanol and adjusted to pH 2 with acetic in order to stabilize the phenolics. The extracts were covered with nitrogen and stored at -18 ºC prior to use (maximum 21 days).

2.3. *Total phenol concentration*

The amount of total phenolics in extracts was according to the Folin–Ciocalteu procedure [10]. Samples (200 µl, two replicates) were mixed with 1.0ml of Folin–Ciocalteu's reagent (diluted 1:10 with water) and 0.8ml of a 7.5% solution of sodium carbonate was added. The absorption at 765 nm was measured after 30 min with a Cary 3 UV–Vis spectrophotometer (Varian Techtron Pty. Ltd, Mulgrave, Victoria, Australia). The total phenolic content is expressed as gallic acid equivalents (GAE) in mg per litre of extract.

2.4. *Preparation of liposomes*

Liposomes were prepared, with minor modifications following the procedure described by Roberts & Gordon [1]. A solution (2 ml) containing 1.5 µmol soybean phosphatidyl choline dissolved in chloroform was mixed with 1mL pure hexane or 1 mL hexane containing α-tocopherol. The concentration of α-tocopherol is calculated as mol % of the lipid fraction by using a molecular mass of soybean PC equal to 900g/mol. The solvent is subsequently removed under reduced pressure (approximately 100 mbar) on a rotatory with water bath set at 30 C. Nitrogen is introduced to establish atmospheric pressure in the evaporation flask after the complete evaporation of the solvents. The residue in the flask is hydrated with 10 ml of 0,01 mM phosphate buffer (pH 7.4), and vortexed for 10 min and sonicated for 30s to ensure complete suspension and to yield a white homogenous suspension of multilamellar liposomes. The liposomes are stored in the rotatory evaporation flask protected from light by aluminium foil and kept under nitrogen at all times. Unilamellar liposomes are prepared from the multilamellar liposomes an Avestin Liposofast Basic small volume (500 µl) extrusion device (Avestin Europe GmbH, Mannheim, Germany). The suspension is passed 21 times through a double layer (100nm pore size polycarbonate membrane) to obtain large unilamellar liposomes. The extracts of the spices are added to the liposome system with the phosphate buffer. Different volumes of 1.000 times diluted concentrate of *DRL* and *DTL* was added to the buffer to obtain the same phenol content of 1.84×10^{-5} GAE.

2.4. *Peroxidation of liposomes*

Lipid peroxidation is followed by measuring formation of conjugated diene, monitored as the change in absorbance at 234 nm (A_{234}), using a Shimadzu UV-2101PC UV–VIS scanning spectrophotometer

Unilamellar liposome suspension (2.5 ml) is pipetted into quartz cuvettes and incubated for 10 min at 37 ºC within the water-jacket cell holder of a Shimadzu UV-2101PC UV–VIS scanning spectrophotometer (UV- 2101 PC) with automatic cell changer. Lipid peroxidation is initiated by introducing 25 µl of 75 mM AAPH in sodium phosphate buffer (pH 7.4)]. The curvettes are quickly inverted five times and then sealed to avoid evaporation. Up to six samples will be measured in each run with phosphate buffer as blank and the liposome suspension without antioxidant as control. The absorbance is measured at 234 nm (absorption maximum of conjugates dienes) every 10 min for 900 min in total. The lag phase before onset of oxidation is measured as the time in minutes

corresponding to the intercept between the tangent to the propagation phase and the tangent to the lag phase (Roberts and Gordon, 2003).

III. RESULTS AND DISCUSSION

3.1. *Phenolics compounds*

The result in ***Table 1*** showed that phenolic compounds are presents in *DRL* in a content value of 118 mg GAE/L, while *DTL* showed a content of 65.7 mg GAE/L. The presence of these compounds in the distilled lef is due to that they are water soluble and they frequently occur combined as glycosides, and they are usually located in the cell vacuole [11], so after the distillation process phenolics compounds are in the distilled leaves.

This affirmation agrees with the result published by Parejo *et al*. [5], who considered the study of the remaining distillation material potentially interesting as a result of the water-soluble properties of phenolic compounds that rarely form part of essential oils.

According to Jordan *et al*. [9] phenolic compound are present in the *DTL*, but for these authors the phenolics content values ranged from 122.2-108.5 mg of gallic acid equivalents (GAEs)/g of dry plant. The different found between our results and these authors could be due to the different solvent used for the extraction of phenolics compounds.

3.2. *Antioxidant activity of rosemary and thyme distilled leaves.*

Table 2 shows the lag phase found by spectrophotometric measurement of conjugated dienes in soybean phosphatidyl choline liposomes for *DRL* and *DTL*.

The antioxidant activity of *DRL* and *DTL* were investigated by studying the ability to inhibit lipid oxidation of liposomes made of soybean phosphatidyl choline initiated by the azo compound AAPH, which is a hydrophilic radical initiator. The extent oxidation was monitored by following the formation of conjugated dienes, which began immediately upon addition of AAPH to the liposomes. However, a lag phase was observed before conjugated dienes were formed when *DRL* and *DTL* were added to the liposomes solution, demonstrating that both distilled leaves are efficient antioxidant towards lipid oxidation [12].

The observed lag phase showed in ***Table 2*** in *DRL* (65.5 ± 3 min) and *DTL* (65.7 ± 6 min) indicates that the antioxidant effect for both distilled leaves is similar. This phenomenon could be attributed to that both plants are from the same family (*Labiatae*) and the phenolics compounds containing in *DRL* and *DTL* are quite similar.

According to our results Altunkaya *et al*. [13] showed that lettuce was found to be a rich source of antioxidants as was shown for lipid oxidation in a liposome system.

Rosemary (*Rosmarinus officinalis* L.) extract contains antioxidant compounds, such as carnosol, carnosic acid, rosmanol, epirsomanol, isorosmanol, methyl carnosate and other phenolic acids, such as rosmarinic acid [14]. Carnosic acid is the major phenolic constituent present in rosemary leaves with an antioxidant activity approximately three times higher than carnosol and seven times higher than the synthetic antioxidants butylated hydroxytoluene and butylated hydroxyanisole [15].

Jordan *et al*. [9] showed that the phenolic component quantified at the highest concentrations in the *DTL* were: rosmarinic acid, followed by apigenin, ferulic, carnosic and caffeic acids.

The phenolics compounds in *DRL* and *DTL*, containing conjugated ring structures, hydroxyl group and stabilizes free radical; moreover these compounds containing the carboxylic acid groups that inhibit lipid oxidation by metal chelation [16].

Several studies have showed the antioxidant effect of *Labiatae's* plant family used as food additives [17,18,19], and added to the animal feed [20]. In this line, Nieto *et al*. [2] reported that feeding pregnant sheep with *DRL* reduced lipid and pigment oxidation on fresh lamb meat, as determined by *TBARS* analysis and CIElab coordinates, over time compared to the control.

3.3. *Synergism of rosemary and thyme distilled leaves with α-tocopherol*

Synergism is, in general, the phenomenon in which a number of compounds when present together in the same system have a more pronounced effect that that which would be derived from a single concept [21].

The observed lag phase (Table 2) for 1 mol% *TOH* was 100 ± 2 min. The calculated lag phase for *TOH* + *DRL* and *TOH* + *DTL* was 165 and 166 min, respectively, while the experimental lag phase was 186 ± 2 and 187 ± 2, respectively. So these results showed

clearly a lag phase that was longer than the sum of lag phases of each component. Thus, a synergistic effect between *DRL*, *DTL* and *TOH* was demonstrated. This finding is in agreement with other studies where synergistic effects were observed for α-tocopherol and polyphenols in homogeneous solutions [22,13], in heterogeneous systems, such as in o/w emulsions [23] and liposomal suspensions [1]. These synergistic effects may be understood by different solubilities and localisations of the antioxidants in different phases in the heterogeneous systems.

IV. Conclusion

DRL and *DTL* were found to be a rich source of antioxidants as was shown by the inhibition of the formation of conjugated dienes in a liposome system. The major concentration of phenolics compounds were found in rosemary distilled leaf. A synergistic effect between *DRL*, *DTL* and *TOH* was demonstrated. Taking into accounts everything, rosemary and thyme distilled leaves are a good alternative to using synthetic additives antioxidant in animal diets.

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Table 1. Phenolics compounds in rosemary distilled leaves and thyme distilled leaves.

Sample	**Phenolics compounds Folin-Ciocalteu mg GAE/L**
DRL	118
DTL	65.7

The values are expressed as means of two determinations
DRL**:** distilled rosemary leaves; ***DTL***: thyme distilled leaves

Table 2. Lag phase found by spectrophotometric measurement of conjugated dienes in soybean phosphatidyl choline liposomes with free radical initiation of oxidation in the aqueous phase AAPH with pH 7.4 at 37 °C

Sample	**Lag phase time (min.)**
Control	4 ± 3
TOH	100 ± 2
DRL	65.5 ± 3
***TOH* + *DRL* (calculated)**	165
***TOH* + *DRL* (experimental)**	186 ± 2
DTL	65.7 ± 6
***TOH* +*DTL* (calculated)**	166
***TOH* + *DTL* (experimental)**	187 ± 2

The values are expressed as means of two determinations ± SD.
DRL**:** distilled rosemary leaves; ***DTL***: thyme distilled leaves; ***TOH***: α-tocopherol. α-tocopherol= 1 mol % of the lipid fraction. The liposomes samples contain DRL or DTL with a phenol content of 1.84×10^{-5} GAE.