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Food preservative based on propolis: Bacteriostatic activity of propolis polyphenols and flavonoids upon *Escherichia coli*

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Abstract

Propolis was tested as food preserver, due its recognized bactericidal and bacteriostatic properties. Furthermore, most propolis components are natural constituents of food and recognized as safe substances. Fifteen propolis from Santa Fe, Argentine in 20% w/w ethanolic extracts, were tested upon *Escherichia coli* ATCC 25922 by agar diffusion and plate culture methods. Considering propolis physicochemical characteristics and inhibitory effects, tested samples were classified in three groups. A minimum inhibitory concentration mean value of 14.3 ± 6 mg soluble compounds/ml of the most active propolis was capable of inhibiting 10^5 cfu/ml cellular concentration. Such extract had 32.31% total soluble compounds (2.1% coumaric + siringic acids, 5.16% quercetin, 0.47 apigenine, 8.15 galangine, 7.2 caffeic acid + crisine and 9.23% no-identified phenolics compounds). By relating the zone of growth inhibition with extracts concentration, a linear response was obtained. On the propolis samples tested, a single value of the minimum inhibitory concentration could not be established. Those values were strongly dependent on propolis composition and botanical origin. The propolis extracts tested, may successfully inhibit the *E. coli* development in vitro, and consequently may be useful as natural food preserver.

Keywords: Propolis; Ethanolic extract; *Escherichia coli*; Inhibition; Polyphenols; Flavonoids

1. Introduction

Food spoilage is caused by the action of microorganism among other factors. Food is preserved when the basic causes of its spoilage are controlled. The methods for preserving food are varied and, depending upon their basic approach, may be effective for either short or long periods of spoilage. Preservation of high moisture-fresh food as meat may be accomplished by low temperature, but only for a short time. Ground beef is a staple commodity and the manner in which it is processed can render it susceptible to microbiological contamination. Emswiler, Kotula, and Rough (1976) reported that when beef carcasses are fabricated into retail cuts, any microbial contamination present on carcasses is inoculated to newly exposed surfaces. When meat is grounded, their shelf life is shortened. The meat industry continues to face concerns regarding the hygiene and safety of its products. It has been shown that the use of single decontamination interventions is effective for reducing pathogens on carcass surfaces (Dickson & Anderson, 1992; Siraguza, 1995). However, since most carcass decontamination treatments do not sterilize the carcass, microorganisms remaining on carcass surfaces can easily become inoculated onto freshly cut surfaces during carcass fabrication, and subsequently carried through grinding operations. Consequently, it would be advantageous to develop meat decontamination procedures closer to the ground beef packaging operation. The use of single intervention techniques during ground beef manufacture has been relatively effective for reducing microorganisms compared to carcass decontamination (Dorsa, Cutter, & Siragusa, 1998). Therefore, researchers have only begun to study the effect of antimicrobial interventions on ground

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beef potentially contaminated during processing (Dorsa et al., 1998; Ellebracht, Castillo, Lucia, Millar, & Acuff, 1999; Pohlman, Stivarius, McElyea, & Waldroup, 2002).

The use of multiple antimicrobial treatments to decontaminating meat before grinding might provide a greater barrier to microbial survival and proliferation in ground beef by taking advantage of different weakness of differing microbial stains (Pohlman, Stivarius, McElyea, Johnson, and Johnson, 2002).

Some meat preservers have been in use from ancient times. Salt is an important ingredient in the preparation of meat emulsions for imparting the typical flavour of processed meats and contributing to keeping quality. The salt content of most processed meats ranges between 2.5% and 5.0% of the final product. A higher salt content would produce a salty taste. To function as preservative, salt concentrations in the product of about 17% would be necessary, far too high for a palatable product. Nitrates and nitrites are curing ingredients. The development of the red or pink colour is the most obvious effect. In addition, nitrites have bacteriostatic properties, sodium nitrite is an effective inhibitor of the growth of the bacterium *Clostridium botulinum*. But sodium nitrite is suspected to be a dangerous, cancer-causing ingredient. On the other hand, nowadays, an increasing awareness of the consumers for the use of synthetic preservatives needs research for more efficient antimicrobials with fewer side effects on human health. The use of various combined preserving methods and substances is under consideration. Polyphenols from various natural sources has plants, apple skin (Alberto, Rinsdahl Canavosio, & Manca de Nadra, 2006), red wine (Papadopoulou, Soulti, & Roussis, 2005) and propolis, among others, have been reported to have a variety of biological effects, including antimicrobial activities.

Propolis is a resinous natural product, produced by bees (*Apis mellifera*), from vegetable secretions. Bees use propolis mainly to cover the hive interior and the breeding cells and also to repair cracks and fissures. These uses are significant, because propolis avoids hive colonization with diseases. Propolis is increasingly recognized by their antibacterial and antifungal properties. Propolis chemical composition is complex and varies according to its botanical and phytogeographical origin (Bankova & Marcucci, 2000; Bonvehi, Coll, & Jorda, 1994; Greenaway, May, Scaysbrook, & Whatley, 1991). Propolis is constituted by a wide variety of substances such as polyphenols, quinones, coumarins, steroids, aminoacids and inorganic compounds. Most propolis components are of phenolic nature, mainly flavonoids. It is known that simple phenols, phenolic acids and polyphenols are active antimicrobial agents (Cowan, 1999). Similarly, gallic acid derivatives have shown inhibitory effects against gram (+) and gram (−) bacteria (Kayser & Kolodziej, 1997). Flavonoids are synthesized by plants as a response to microbial infections and are recognized to have effective antimicrobial effects against a wide range of microorganisms (Recio, Ríos, & Villar, 1989). It was demonstrated that the three –OH group substitution in ring B, and a third –OH group in ring C constitute the necessary structures to *E. coli* antibacterial activity of flavonoids, as is the case of myricetine, present in propolis (Farré, Frasquet, & Sánchez, 2004; Mori, Nishino, Enoki, & Tawata, 1989; Puupponem-Pimiä et al., 2001). The bactericidal or bacteriostatic effects depend on the concentration of propolis extract, and are influenced by the extraction method (Obregón Fuentes & Rojas Hernández, 1990). Propolis antioxidants, antibacterial and antifungal properties make it useful in food technology. Substances, which are identified in propolis, generally are typical constituents of food and/or food additives, and are recognized as GRAS (Generally Recognized As Safe) (Burdock, 1998) substances. As a result of the lack of acceptability for synthetic preservatives, there is a growing interest of introducing natural additives to food, and propolis is an interesting alternative to be considered in new applications of food technology. It has been proposed as a chemical preservative in meat products (Han & Park, 1995) and as germicide and insecticide for food packaging (Mizuno, 1989a, 1989b). Donadieu (1979) reported an extension of frozen storage life of fish by 2–3 times.

The aim of the present in vitro study was to screen a number of ethanolic extracts of propolis (EEP) for potential antimicrobial activities against *E. coli*, and to determine the potential usefulness of ethanolic extracts of propolis as food preserver.

2. Materials and methods

2.1. Ethanolic extracts of propolis

Fifteen samples of propolis were tested. Table 1 shows their composition, in phenolic compounds of its ethanolic extract. Propolis samples were taken from hives in Santa Fe province, Argentine Republic. Propolis was collected with propolis traps to minimize their contamination with foreign substances. Propolis samples were frozen up to −18 °C and milled in a refrigerated mill IKA A-10 (IKA Labortechnic. Janke & Kunkel GmbH & Co. KG, Staufen, Germany). Extracts were made by mixing 20 g crude propolis with 80 g 96% ethanol, with intermittent shaking, at room temperature in the dark for a week. The insoluble fraction was separated by filtration. The filtrate was named ethanolic extract of propolis (EEP). The solubilized fraction was determined by weight difference. EEP at 20% (w/w) referred to crude propolis were used. Extracts were maintained in caramel flask in dark at room temperature.

2.2. Propolis phenolic compounds identification and quantification

Phenolic compounds from EEP were identified and quantified by reverse phase HPLC (Markham & Bloor, 1998; Waksmundzka-Hajnos, 1998). A C18 reverse phase

Table 1
Ethanolic propolis extract (EPE) composition

Propolis group and sample number		Phenolic and flavonoid compounds[a]						
		Coumaric acid + Siringic acid	Quercetin	Apigenine	Galangine	Caffeic acid + crisine	Non-identified[b]	Total[c] % (w/w)
Group 1	1	2.1	5.16	0.47	8.15	7.2	9.23	32.31
	15	0.6	9.8	0.1	0.01	8.45	11.9	30.86
	11	0.62	7.95	0.21	–	12.1	4.4	25.28
	4	0.65	3.27	0.41	5.36	4.78	10.3	24.77
	2	0.67	4.62	2.7	5.51	4.86	5.48	23.84
	10	0.35	2.92	0.24	–	6.47	13.64	23.62
Group 2	3	0.44	3.07	–	6.2	5.56	6.0	21.27
	12	0.69	2.39	–	–	4.39	9.37	16.84
	7	0.67	4.23	1.92	–	4.61	5.1	16.53
	13	0.43	7.4	–	–	6.0	1.6	15.43
Group 3	8	0.3	2.75	2.1	2.15	1.53	4.92	13.75
	14	0.4	1.58	0.1	0.1	0.59	9.1	11.87
	6	0.25	1.83	–	3.53	2.33	2.10	10.04
	9	1.13	1.63	–	–	2.31	4.8	9.87
	5	0.05	2.03	0.22	–	2.46	3.65	8.41

Phenolic and flavonoid compounds.

[a] No kaempferol or acacetine were found in the propolis samples, so they were not included in table.

[b] Non-identified compounds, the percentage concentration of these compounds was evaluated by a medium integration factor from those obtained from identified flavonoids, and equal to 3×10^{-8}.

[c] Referred to crude propolis.

column (Supelco Inc. Supelco Park, Bellefonte, USA) 250 × 4.6 mm with a 5 μm particle size, and a pre-column of the same material were used. Chromatography was performed in isocratic way at 50 °C, with a (60:75:5 by volume) mixture of water/methanol/acetic acid as elution solvent, at 0.7 ml/min. The measurement was performed at $\lambda = 275$ nm, by using an external calibration (Bankova, Popov, & Marekov, 1985). A Shimadsu LC 10AS (Shimadsu Co. Kyoto, Japan) chromatograph, with a visible–UV Shimadsu SPD-10A detector was used. For identification and quantification purposes, HPLC analytic quality standards Sigma (Sigma Chemical Company, St. Louis, MO, USA) and Merck (Darmstad, Germany) of caffeic acid, coumaric acid, syringic acid, apigenine, acacetine, crisine, quercetin, kaempferol and galangine were used.

2.3. Antimicrobial activity determined by the agar diffusion method

Activity was measured using the agar diffusion method (Kirby–Bauer method). Inocula were prepared from *E. coli* ATCC 25922 pure cultures, incubated in nutritive agar for 24 h. One ml of the obtained cellular suspension (3×10^8 cfu/ml) was added to 10 ml Mueller–Hinton agar previously melted, mixed, poured in Petri dishes and left 1 h to solidify. On solidified agar, 6 mm-diameter wells with a staggered arrangement were made using a sterile punch. Forty microliter 20% EEP was added in peripheral holes and 40 μl 80% ethanol was added in the central hole for negative control. A 35–37 °C, 24 ± 2 h aerobic incubation was performed. After incubation, the inhibition zones were measured to 1 mm accuracy and the effect was calculated as the mean of the duplicate experiments.

2.4. Antimicrobial activity and minimum inhibitory concentration (MIC) determined by plate culture

Solid media containing different concentrations of EEP were used (0.025–2.0% v/v). Ten milliliter melted Mueller–Hinton culture medium was mixed at 45 °C together with 20% EEP aliquots and a given volume of cellular suspension to obtain a 10^2, 10^3, 10^4, 10^5 and 10^6 cfu/ml concentration range. The mixture was poured in Petri dishes. As control, a plate with 80% ethanol was used. A 35–37 °C, 24 ± 2 h aerobic incubation was performed. To verify, after a 24 h observation, culture plates were kept at 30 °C for 5 days to establish the inhibition degree and the absence of growth after the 24 h culture. Propolis samples were classified into three groups according to their composition and antimicrobial activity.

2.5. Zone of growth inhibition (ZGI) vs. propolis concentration

To establish the ZGI as regards propolis concentration, methodological steps according to Section 2.3 were followed. Three sets of tests, with a single EEP for each propolis group, selected as the most active EEP from the group, with five replications each were performed. To compare, another set was carried out using pure quercetin (Table 2). In each set three different EEP or quercetin concentrations were used. In every group, concentrations were

Table 2
Zone of growth inhibition or inhibition halo diameter (D) as regards of ethanolic propolis extract (EPE) and quercitine concentration, linear regression curve and r^2

Propolis group[a] (sample number)	EPE[b] added (mg)	Zone of growth inhibition or inhibition halo diameter (D)[c] (mm)	Linear regression curve equation	r^2 Curve fitting
Group 1 (1)	0.5	Non-observable	$D = 25.4$ EPE $- 6.29$	0.98
	0.6	9.5 ± 0.2		
	0.8	13.8 ± 0.5		
Group 2 (3)	0.8	Non-observable	$D = 9.25$ EPE $- 1.5$	0.99
	1.0	7.6 ± 0.1		
	1.4	11.5 ± 0.2		
Group 3 (8)	1.4	Non-observable	$D = 3.90$ EPE $+ 0.47$	0.99
	2.0	8.1 ± 0.4		
	2.5	10.3 ± 0.6		
Quercetin	0.03	Non-observable	$D = 192.11$ EPE $+ 0.04$	0.97
	0.045	8.2 ± 0.3		
	0.055	10.9 ± 0.7		

[a] For each group of propolis samples (Table 1), a representative one was used.
[b] Soluble solids in the aliquot of ethanolic propolis extract 20% (w/w).
[c] Well diameter 6 mm. Average zone of growth inhibition diameter ± standard deviation from five replications.

adjusted according to the plate culture assay results. In all tests the cellular concentration was 10^4 cfu/ml.

3. Results and discussion

A negligible inhibition effect was observed for all EEP by the inhibition halos measurement after 24 h incubation. No differences were obtained for the cellular and EEP concentrations tested. This effect was attributed to the high cell concentration (3×10^7 cfu/ml) which corresponds to a high level contamination, no longer admissible in foods. The control sample shows no inhibition effect.

From the results obtained by plate culture, the following considerations can be made: tested propolis may be classified in three well-differentiated groups, according to their composition (Table 1) and inhibitory effect.

Samples 1, 15, 11, 4, 2 and 10 inhibit a 1×10^5 cfu/ml cellular concentration with a MIC mean value of 14.3 ± 2.6 mg of propolis soluble compounds/ml. All these EEP contain a high percentage of coumaric acid + siringic acid, quercetin, galangine, caffeic acid + crisine, no-identified soluble compounds and total soluble compounds.

Samples 3, 12, 7 and 13 inhibit a 1×10^4 cfu/ml cellular concentration with a MIC mean value of 19.2 ± 3.5 mg/ml. Although, the EEP comprised in this second group contain an important percentage of quercetin, galangine, caffeic acid + crisine, no-identified soluble compounds and total soluble compounds, but they present lower maximum values, thus causing a lower MIC effect.

EEP from the remaining samples 8, 14, 6, 9 and 5 comprised in the third group can inhibit a much lower cellular concentration of 1×10^2 cfu/ml with a high MIC mean value of 30.2 ± 3.7 mg/ml. This group was the only one in which a development in plates kept at 30 °C for 5 days was observed, being indicative of an insufficient inhibitory effect on *E. coli*. These samples, which show very low inhibitory effect, have the lowest content of compounds being recognized as responsible of the propolis effect.

Both the total soluble compounds and the high percentage of quercetin, galangine, caffeic acid + crisine and some non-identified soluble compounds from the propolis samples used have a remarkable influence on the inhibitory effect. These factors make MIC strongly dependent on the propolis used.

The ZGI vs. propolis concentration tests (Table 2) shows a close agreement with plate culture results. According to the decrease in polyphenols compounds content from group 1 to 3 of propolis samples, an increase in the amount of soluble solids was used as a way to produce measurable ZGI values. Pure quercetin was used to compare results; its concentration was comparable with that in active propolis. A linear ZGI response with increasing concentration of propolis was found for all propolis samples tested. The linear correlation of experimental values was high, being r^2 between 97 and 99.

ZGI values of 9.5 and 13.8 mm for 0.6 and 0.8 mg propolis soluble solids/ml, respectively (Table 2) were in close accordance with the ZGI value of 12 mm (*E. coli*) for 0.59 mg of gallic acid equivalent total phenolics/ml from red wine extracts reported by Papadopoulou et al. (2005). Furthermore, Alberto et al. (2006) reported 0.4 mm ZGI (*E. coli* ATCC 25922) for 0.14 mg total phenolics/ml from skin extracts of Granny Smith apples. *E. coli* was 50% inhibited by a 0.45 mg/ml of a commercial EEP (Mirzoeva, Grishanin, & Calder, 1997).

From the consumer standpoint, a safe dose for human consumption would be 1.4 mg/kg body weight/day, or approximately 70 mg/day in adults (Burdock, 1998). As reference, benzoic acid and sodium benzoate are included by the FDA in the list of generally recognized as safe (GRAS) food additives. An acceptable daily intake (ADI) of both substances has values between 0 and 5 mg/kg body

weight. Suitable levels of propolis as food preserver must be established by a consumer acceptance test by a trained tasting panel.

Considering the results, it may be concluded that, the EEP tested, in the performed experimental conditions may successfully inhibit the *E. coli* development in vitro, at safe levels for human consumption and, consequently, they could be useful as ground fresh beef natural preserver or as unspecific antibacterial food preserver.

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