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Antimicrobial effect and enzymatic activity of extract of *Zingiber officinale* Roscoe and stability in topical preparations

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ABSTRACT

The rhizomes of common ginger (*Zingiber officinale* Roscoe) contain substances with antimicrobial activity and proteolytic enzymes and thus may have various pharmaceutical applications. The aim of the present study was to prepare *Zingiber officinale* Roscoe rhizome extracts for pharmaceutical use, preserving the proteolytic enzyme activity and testing the antimicrobial activity, in order to develop topical formulations. Two extracts were obtained - aqueous and glycolic – and assessed for their physical, physicochemical and organoleptic characteristics. To measure their proteolytic activity, the extracts were assayed for enzymatic hydrolysis of 1.2% casein solution, at pH 6.0 and 37°C; papain was used for comparison. The antimicrobial activity of the glycolic extract of *Zingiber officinale* Roscoe was tested by microdilution; the inoculants were prepared from cultures of *Escherichia coli, Staphylococcus aureus, Staphylococcus epidermidis* and *Pseudomonas aeruginosa*. There was no growth of S. aureus and S. epidermidis at concentrations of 150 mg/mL or more of extract, whereas *P. aeruginosa* was inhibited from 100 mg/mL and *E.coli* from 75 mg/mL. Emulsion formulations were prepared as vehicles for the extracts and their stability was tested. The results showed proteolytic activity in both *Z. officinale* rhizome extracts, the glycolic extract having 691.68 PU/g juice and the aqueous extract, 338.14 PU/g juice. The formulations were stable, especially the one that contained the glycolic extract. In sum, the formulations showed satisfactory stability and the *Z. officinale* extract showed bactericidal activity against the cultures tested; the results are promising for the use of the extract in foods, medicine and cosmetics.

Keywords: Antimicrobial. Cosmetic. Enzyme. Medicine. *Zingiber officinale*.

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INTRODUCTION

The rhizomes of *Zingiber officinale* contain substances with several properties of interest, including bactericidal, fungicidal, antiviral, antiulcerative and antioxidant activity; they also contain enzymes with proteolytic activity (Millar, 1998; Kim et al., 2008; Ali et al., 2008; Takara et al., 2005).

These enzymes are called zingibain and exhibit collagenase activities (Choi and Laursen, 2000; Shukla and Singh, 2007). Their activities are similar to those of the protease papain, which is associated with the ripening of fruit and tenderizing of meat. These characteristics make the enzyme a promising alternative for culinary and industrial applications (Kim and Lee, 1995). In the pharmaceutical field, papain, collagenase and other protease activities may be used for wound healing, necrotic tissue removal and reepithelialization (Traversa et al., 2007; Saarialho et al., 1993; Thompson et al., 1973; Choi and Laursen, 2000; Su et al., 2009). These enzymes may also be used in the debridement of necrotic tissue in burn patients (Kim, 2007). According to Imokawa (2008), an extract of *Zingiber officinale* (L.) Roscoe has a safe and potent inhibitory activity against fibroblast elastases. In other words, it helps maintain the young and healthy aspect of the skin.

The similarities of the enzymes in *Zingiber officinale* and papain suggest that *Z. officinale* may also have pharmaceutical applications; its extract may be mixed with other extracts, such as curcumin, as suggested by Bhagavathula et al. (2009), to give a promising combination of enzymes for cicatrization.

The antimicrobial activities suggest the use of ginger extract against skin diseases caused by microorganisms, such as acne (a chronic inflammation of pilosebaceous follicles). The onset of acne may be associated with several factors, such as hyperactivity of the sebaceous glands and overproduction of sebum; this, in turn, may lead to obstruction of the follicles and local irritation, causing the release of inflammatory mediators. Microbial colonization may contribute to the deterioration of this situation (Hassun, 2000).

The aim of the present study was to prepare rhizome extracts from *Zingiber officinale* for application in the pharmaceutical industry. The goal was to preserve

the activities of the proteolytic enzymes, study the antimicrobial activity and develop an emulsion-based formulation. The stability of the formulation was to be assessed, to investigate the viability of the enzyme as an active ingredient in topical formulations.

MATERIALS AND METHODS

Preparation of *Zingiber officinale* extracts

Specimens of ginger "root" (*Zingiber officinale* rhizomes) were collected and identified at the Pontifícia Universidade Católica de Campinas, Campinas, SP, Brazil. After disinfecting with 70% ethanol, the rhizomes were peeled and the juice was extracted in a juicer. The juice was passed through a nylon filter and diluted to several concentrations, as described below. The filtered juice was diluted 50% with distilled water or propylene glycol, yielding the aqueous and glycolic extracts. These were stored in amber glass and frozen until the moment of use, when they were thawed and centrifuged at 3000 rpm for 5 min, the supernatants being used as the extracts in all tests [method adapted from Azu and Onyeagba (2007)].

Evaluation of proteolytic activity of aqueous and glycolic extracts of *Zingiber officinale*

Proteolytic activity was determined by the enzymatic hydrolysis of casein (Bergmeyer, 1965, Iaderoza and Baldini, 1991). Initially, 5.0 mL of 1.2% casein solution at pH 6.0 was incubated at 37.0o C until temperature equilibrium. Next, the reaction was started by adding 1.0 mL of the sample to each tube. The samples consisted of *Zingiber officinale* juice diluted with 50.0% distilled water or propylene glycol; papain (Sigma ™) was diluted to 0.0025 g/mL in distilled water for comparison.

After 10 minutes, 5.0 mL of trichloroacetic acid solution at 5.0% (w/v) was added to interrupt the reaction. Next, tubes were shaken to homogenize the mixture and left to rest for 20 minutes in a warm water bath. The solution was then filtered through qualitative filter paper and the amount of soluble peptides was read at 280 nm.

One unit of proteolytic activity (PU) is defined as the amount of enzyme that, by acting on casein for 10 min under specified conditions, releases an amount of peptides equivalent to 1.0µg of tyrosine, as determined by reading the absorbance at 280 nm.

The analytical curve for tyrosine was plotted at the concentrations 10, 20, 40, 60, 80 and 100 µg/mL and a linear equation derived. The activity is defined as the proteolytic activity per gram of enzyme sample (PU/g).

Assessment of antimicrobial activity of the glycolic extract of *Zingiber officinale*

The antimicrobial activity of the glycolic extract of *Zingiber officinale* was assayed by microdilution; the tests were carried out in triplicate (Brasil, Ministério da Saúde, 2005). The glycolic extract and 9 dilutions were prepared with sterilized materials. The inoculants were prepared from 24-h cultures of *Escherichia coli* [ATCC 259220, *Staphylococcus aureus* (ATCC 29213)], *Staphylococcus epidermidis* ATCC 12228 and *Pseudomonas aeruginosa*. Suspensions were prepared in sterile saline solution (0.85% NaCl) at about 1.5x108 CFU/mL, with turbidity of the 0.5 McFarland standard (Brasil, Ministério da Saúde, 2005).

U-bottom, 200-µL microdilution plates were used for the assays. Aliquots of *Z. officinale* glycolic extract, Muller Hinton Broth (Oxoid™) at a pH of 7.3 and bacterial inoculum were mixed, to give a final volume of 180 µL, in each well. The positive and negative controls were prepared with inoculated culture medium and culture medium alone, respectively.

Next, plates were incubated at 35.0°C ± 2.0°C for 24 hours; triphenyltetrazolium chloride (TTC) was added to each well to identify microbial growth by its reduction to a red dye and colorimetric readings were carried out in a plate reader. Where necessary, a well was subcultured by transferring a portion to a solid medium; the subculture was incubated under the same conditions and used to confirm bactericidal or bacteriostatic activity.

Formulation for the extracts

The vehicle for *Zingiber officinale* aqueous and glycolic extracts was a 20.0% O/W emulsion prepared with 18.0 wt% anionic self-emulsifying base (Cetearyl Alcohol & Ceteareth 20 & Mineral Oil & Lanolin Alcohol & Petrolatum), 5.0% propylene glycol, 2.0% isopropyl myristate and 0.8% preservatives (phenoxyethanol, methylparaben, ethylparaben, propylparaben, butylparaben, isobutylparaben); finally, distilled water was added to complete 100 %.

For the purpose of comparison, three formulations were prepared: (A) emulsion base, (B) emulsion with *Z. officinale* aqueous extract and (C) emulsion with *Z. officinale* glycolic extract.

The formulations were stored in 30-g plastic bottles for lotions. These were closed with a screw top and stored at room temperature, 4°C, 40°C and 60°C. Formulations were analyzed periodically for their organoleptic properties and pH, as specified in the guidelines for stability of cosmetics published by the Brazilian Health Surveillance Agency (Brasil, Ministério da Saúde, 2003).

RESULTS

Initially, both samples of extracts showed similar characteristics (Tables I and II). During freeze storage, to preserve the sample, especially from microbial growth, there was a gradual darkening of the extract and possible decomposition of polyphenols, causing the inactivation of enzymes. The pH during storage shows that the glycolic extract was the more stable of the extracts. Storing in a freezer was important to maintain the pH fairly constant. In preliminary studies, we noted a fall in pH when the extracts were kept under refrigeration but not frozen.

The results suggest that freezer storage is not the most viable option for the commercial use of extracts. We also carried out lyophilization of the aqueous extract in a preliminary study. No enzymatic activity remained after lyophilization; therefore, lyophilization was discarded.

Assay of proteolytic enzyme activity is showed in Table III. A preservative mechanism or agent should be used to avoid loss of activity.

A preliminary study of antimicrobial activity showed that the glycolic extract of *Z. officinale* gave better results than the aqueous extract. The former inhibited microbial growth of all bacterial strains tested, while the latter did not inhibit microbial growth of any of the strains. Therefore, we report only the results for the glycolic extract (Table IV).

Tables V and VI show the results of stability tests on the topical formulations.

Table I - pH of rhizome extracts from *Zingiber officinale* during refrigerated storage.

Time	pH aqueous extract	pH glycolic extract
Initial	6.80	7.00
7 days	6.57	6.84
12 days	6.44	6.66
21 days	6.44	6.85

Table II - Odor and color of rhizome extracts from *Zingiber officinale* during storage at -20°C.

Time	Odor*	Color*
Initial	Characteristic	Yellow
7 days	Characteristic	Yellow and slight darkening
12 days	Characteristic	Dark yellow
21 days	Characteristic	Dark yellow

* Color and odor were the same for the aqueous and glycolic extracts during the period of storage.

Table III - Proteolytic enzyme activity in *Zingiber officinale* rhizome extracts and aqueous solution of papain assayed by hydrolysis of casein (linear equation y=0.00741x + 0.0236 was found for tyrosine standard curve). Molar absorption coefficient $\varepsilon=1300M^{-1}cm^{-1}$

	Aqueous extract	Glycolic extract	Papain
Enzyme activity	169.07 PU/g*	345.84 PU/g*	1,108.25 PU/g*

1 PU = amount of enzyme that, after 10 min of reaction, releases an amount of peptides equivalent to 1.0µg of tyrosine, determined by reading the absorbance at 280 nm; PU/g = no. of proteolytic enzyme units per gram of juice.

Table IV - Microbial growth in the presence of glycolic extract of *Zingiber officinale* rhizome at 10 concentrations (microdilution method); triplicates.

Extract concentration	Microorganisms			
mg/mL	S.aureus	S.epidermidis	P.aeruginosa	*E.coli*
25	(+) (+) (+)	(+) (+) (+)	(+) (+) (+)	(+) (+) (+)
50	(+) (+) (+)	(+) (+) (+)	(+) (+) (+)	(+) (+) (+)
75	(+) (+) (+)	(+) (+) (+)	(+) (+) (+)	(-) (-) (-)
100	(+) (+) (+)	(+) (+) (+)	(-) (-) (-)	(-) (-) (-)
125	(+) (+) (+)	(+) (+) (+)	(-) (-) (-)	(-) (-) (-)
150	(-) (-) (-)	(-) (-) (-)	(-) (-) (-)	(-) (-) (-)
200	(-) (-) (-)	(-) (-) (-)	(-) (-) (-)	(-) (-) (-)
300	(-) (-) (-)	(-) (-) (-)	(-) (-) (-)	(-) (-) (-)
400	(-) (-) (-)	(-) (-) (-)	(-) (-) (-)	(-) (-) (-)
500	(-) (-) (-)	(-) (-) (-)	(-) (-) (-)	(-) (-) (-)

(+) presence of microbial growth
(-) absence of microbial growth

DISCUSSION

Water was used as a solvent to extract the *Zingiber officinale* juice. Imokawa (2008) indicated that the substances responsible for the spicy characteristics of *Z. officinale* are, for the most part, insoluble in water, whereas the desired proteases are extracted with water. Therefore, the aqueous extract is non-irritating and safe to use on human skin (Thompson, 1973). Propylene glycol was also used as an extraction solvent (Grégio, 2006); the goal was to maintain the antimicrobial activity and to compare the stability of the two extracts.

During storage in the freezer, we observed a gradual darkening of the extract, suggesting that freeze storage is not the most viable option for the commercial use of the extracts. As a possible alternative, we carried out lyophilization of the aqueous extract in a preliminary study. This showed that the enzymatic activity was lost after freeze-drying; therefore, this storage method was discarded.

The results for the *Z. officinale* rhizome extracts suggest their proteolytic activity is inferior to that of papain. The calculation was carried out using the mass of the rhizome used in the replicates, the dilution, and the volume used in the reaction. The results are expressed in PU/g or mL of the sample; thus, one gram of papain showed 443.30 PU, whereas the aqueous extract of the rhizome showed 338.14 PU/g of juice, and the glycolic extract showed 691.68 PU/g of juice.

The activity was lower than that of papain under the same experimental conditions. The papain used was of high quality and highly purified, whereas the ginger extracts were of crude quality. The results corroborate Ha et al. (2012); those authors assessed papain, bromelain, actinidin and zingibain. The activity of papain was approximately twenty times higher than that of zingibain, but the results indicated that the zingibain protease preparation was the most effective at hydrolyzing meat connective tissue proteins.

Moreover, the cost of *Zingiber officinale* juice is considerably lower than that of papain, as reported by Adulyatham and Owusu-Apenten (2005); moreover, the papain used in the study had a high level of purity.

The results for *Z. officinale* may thus be considered satisfactory and are consistent with the concentrations used by Naveena et al. (2004), who compared the effects of 5.0% (w/v) *Zingiber officinale* and 0.2% papain solutions on the tenderization of buffalo meat. Those authors reported excellent results for the *Zingiber officinale* solution, suggesting that it may be a viable alternative to papain and an option for new formulations.

The activities of the glycolic extract of *Zingiber officinale* may be associated with a variety of constituents in *Zingiber officinale*, which include: [6]-gingerol, zingiberene, geranial, β-sesquiphellandrene, farnesene, cineole, geraniol, and curcumin. These constituents have had successful pharmacological use in the treatment of certain diseases, including chronic inflammations (El-Baroty et al., 2010).

The results in the antimicrobial assay corroborate Jagetia et al. (2003), who administered *Zingiber officinale* intraperitoneally and observed antimicrobial activity against *Pseudomonas aeruginosa*, *Salmonella typhimurium*, *Escherichia coli* and Candida albicans.

Other studies show that hydroethanolic ginger extract exhibits potent antibacterial activity against both

Table V - Tests of stability (color, odor, appearance and pH) of the emulsion formulated with 20.0% aqueous extract of *Zingiber officinale* rhizome over time at 4 different temperatures

Time	Color				Odor				Appearance				pH			
(days)	RT	40ºC	60ºC	4ºC	RT	40ºC	60ºC	4ºC	RT	40ºC	60ºC	4ºC	RT	40ºC	60ºC	4ºC
Initial	W	W	W	W	C	C	C	C	H	H	H	H	5.5	5.5	5.5	5.5
7	W	D	Be	W	C	I	I	C	H	L	L	H	5.5	5.5	5.5	5.5
19	W	D	Be	W	C	I	I	C	H	HT	HT	H	5.5	5.5	5.4	5.5
25	W	D	Be	W	C	I	I	C	H	HT	HT	H	5.5	5.4	5.5	5.5
39	W	D	Be	W	C	I	I	C	H	HT	L	H	5.5	5.6	5.1	5.5
46	W	D	D	W	C	I	I	C	H	HT	L	H	5.4	5.4	5.0	5.5

RT: Room temp, W: White; D: Darkening; Be: Beige; C: Characteristic; I: Intense; H: Homogeneous; HT: Heterogeneous; L: Liquid.

Table VI - Tests of stability (color, odor, appearance and pH) of the emulsion formulated with 20.0% glycolic extract of *Zingiber officinale* rhizome over time at 4 different temperatures

Time	Color				Odor				Appearance				pH			
(days)	RT	40ºC	60ºC	4ºC	RT	40ºC	60ºC	4ºC	RT	40ºC	60ºC	4ºC	RT	40ºC	60ºC	4ºC
Initial	W	W	W	W	C	C	C	C	H	H	H	H	5.8	5.8	5.8	5.8
7	W	Be	Be	W	C	I	I	C	H	L	L	H	5.8	5.8	5.8	5.8
19	W	Be	Be	W	C	I	I	C	H	H	H	H	5.8	5.8	5.6	5.8
25	W	D	D	Be	C	I	I	I	H	H	L	H	5.5	5.6	5.3	5.8
39	W	D	D	Be	C	I	I	I	H	HT	HT	H	5.5	5.6	5.6	5.8
46	W	D	D	Be	C	I	I	I	H	HT	HT	H	5.5	5.6	5.6	5.8

RT: Room temp, W: White; D: Darkening; Be: Beige; C: Characteristic; I: Intense; H: Homogeneous; HT: Heterogeneous; L: Liquid.

Gram-positive and Gram-negative bacteria, including *Staphylococcus aureus*, *Streptococcus pyogenes/pneumonia* and *Haemophilus influenzae* (Akoachere et al., 2002). Yet others have shown antibacterial activity against the Gram-negative bacteria *Pseudomonas aeruginosa*, *Salmonella typhimurium* and *Escherichia coli*, for which the minimum inhibitory concentration (MIC) of ginger extracts ranged from 0.0003µg/mL to 0.7µg/mL, while the minimum bactericidal concentration (MBC) ranged until 2.04µg/mL for ginger (Jagetia et al., 2003). Comparison of those concentrations with those obtained in the present study shows that their concentrations were much lower. However, in their study, ginger powder was extracted with 50% ethanol, differently from the present study. Therefore, the substances extracted may have been quite different. It is important to note that Jagetia et al. (2003) probably extracted spicy substances, thus rendering the extract unviable for topical use.

The glycolic extract gave the more stable topical formulation (Tables V and VI). This extract showed less change in appearance and pH during the assessment period. Adulyatham and Owusu-Apenten (2005) prepared a *Z. officinale* rhizome extract by a method similar to that of the present study. The authors investigated how to stabilize the protease activity, which decays rapidly, mainly by modifying storage conditions. They concluded that addition of ascorbic acid may enhance the stability of the extract, thus increasing its shelf life. In the present study, the data suggest that enzymatic activity may improve if the extract is stabilized; also, the activity may improve with the addition of antioxidants.

We attempted to assay the enzymatic activity in samples of the formulations. However, owing to the interference of other components, the assays failed; thus, another method of analysis will have to be employed.

Studies of the topical efficacy of *Zingiber officinale* extract have identified several types of activity. Guahk et al., (2010) prepared a water extract of ginger rhizomes and examined the effects of the whole extract, gingerol, and shogaol on cell viability and cytokine and chemokine production in UV-irradiated HaCaT cells. The results show that these compounds were effective in protecting UV-damaged skin against inflammation. Treatment with *Z. officinale* also attenuated UVB-induced hyperplasia, infiltration of leukocytes and dilation of blood vessels in the dermis of mice. The authors concluded that *Z. officinale*, gingerol and shogaol show potential as anti-inflammatory agents to protect skin against UVB irradiation damage.

Tsukahara et al. (2006) investigated the topical use of an extract on hairless mouse skin. The results showed a decrease in photoaging and a significant lessening of the decrease in skin elasticity. The extract was prepared by mixing 200g of ginger rhizome with 1L of 20.0% aqueous ethanol for 7 days at room temperature (RT) and then filtering. Next, in a batch method, the filtrate was stirred for 2h at RT with 30g activated charcoal and filtered again. The filtrate was concentrated under reduced pressure, to yield a residue of about 3.2% wt/vol. The extract was applied at concentration of 1.0% in a hydroalcoholic vehicle, before UV irradiation.

Antitumoral activity is another desirable goal for such formulations, especially those containing [6]-gingerol, a substance that can be used effectively for the treatment of skin cancer Nigam et al. (2009). [6]-gingerol is a potent antioxidant and may be used to prevent the disease (Jagetia et al., 2003).

One of the advantages of these extracts is their low cost of production. In terms of applicability, it should be noted that proteolytic enzymes must be purified to eliminate any compounds that may interfere in their final activity. Further studies should seek new means to stabilize the extract, to facilitate its handling and conservation. It is important to investigate further the impact of lyophilization on enzymatic activity.

In each study, extracts are obtained differently: aqueous and alcoholic solvents are used. Thus, the substances extracted may vary with the polarity of the solvent and the method of extraction. In the present study, we decided to use the juice of the rhizome directly, to reduce the time of extraction and to maintain the integrity of the enzymes. If maceration had been carried out, the results would probably have been different. Considering the variety of possible compositions of the extracts, based on the range of possible methods and solvents, it is always important to assess the safety of the extracts. Some extracts may irritate the skin, depending on their composition. The extracts prepared in the present study could be used in emulsions; it is our contention that the daily use of such formulations may bring positive effects for the skin, including prevention of photoaging and antimicrobial activity. The topical use of the formulation shows promise. The formulation should thus be tested under real conditions of use, in other words, on human skin and for prolonged periods of time.

In conclusion, the extracts of *Z. officinale* rhizome showed proteolytic activity; only the glycolic extract showed antimicrobial activity. The activity differed between the extracts, the glycolic extract exhibiting 691.68 PU/g and the aqueous, 338.14 PU/g. The emulsion formulations were stable, especially the formulation that contained the glycolic extract. The ginger rhizome is a promising source of active substances for use as the active ingredient in pharmaceutical formulations; one of its advantages is the low cost of production.

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RESUMO

Efeito antimicrobiano e atividade enzimática de extrato de Zingiber officinale Roscoe e estabilidade em preparações tópicas

Os rizomas de *Zingiber officinale* contém substancias com ação antimicrobiana e enzimas proteolíticas e por essa razão podem ter aplicações farmacêuticas. O objetivo deste trabalho foi obter o extrato de rizomas de *Zingiber officinale* para emprego na área farmacêutica, de forma a preservar a atividade das enzimas proteolíticas presentes, e avaliar também sua ação antimicrobiana, visando-se o uso no desenvolvimento de formulações tópicas. Dois extratos foram obtidos, aquoso e glicólico, que foram avaliados em relação as suas características físicas, físico-química e organolépticas, além da sua atividade proteolítica, por meio de reação de hidrólise enzímica da caseína a 1,2%, em pH 6,0 e a 37ºC, utilizando-se papaína como forma de comparação da atividade. O estudo da ação antimicrobiana do extrato de *Zingiber officinale* foi realizado por método de microdiluição, utilizando-se *Escherichia coli, Staphylococcus aureus, Staphylococcus epidermidis* e *Pseudomonas aeruginosa*. Houve ausência de crescimento de S.aureus e S.epidermidis a partir a concentração de 150 mg/ml de extrato, enquanto que para P.aeruginosa a partir de 100 mg/ml e para *E.coli* a partir de 75 mg/ml. Foram elaboradas formulações para veiculação dos extratos, e sua estabilidade foi avaliada. Constatou-se a atividade proteolítica em ambos os extratos, sendo o glicólico superior quanto à manutenção desta atividade, pois o extrato glicólico apresentou 691.68 UP/g e o extrato aquoso 338.14 UP/g. As formulações apresentaram estabilidade satisfatória e o extrato de *Zingiber officinale* obtido mostrou atividade bactericida frente aos microrganismos testados e sua utilização em alimentos, medicamentos e cosméticos é muito promissora.

Palavras-chave: Antimicrobiano. Cosmético. Enzima. *Zingiber officinale*.

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