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Research Article

Rubus rosaefolius Extract as a Natural Preservative Candidate in Topical Formulations

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Abstract. Even though the synthetic preservatives may offer a high antimicrobial efficacy, they are commonly related to adverse reactions and regarded as having potentially harmful effects caused by chronic consumption. The development of natural preservatives provides a way of reducing the amount of synthetic preservatives normally used in pharmaceutical and cosmetic preparations. In addition, these agents have less toxic effects and represent a possible natural and safer alternative of the preservatives. The purpose of this research was to evaluate the *Rubus rosaefolius* Smith extract efficiency as a natural preservative in base formulations. Of the extract, 0.2% (*w*/*w*) was assayed for its effectiveness of antimicrobial protection in two different base formulations (emulsion and gel). The microbial challenge test was performed following the standard procedures proposed by The United States Pharmacopoeia 33nd, European Pharmacopoeia 6th, Japanese Pharmacopoeia 15th, and the Cosmetics, Toiletries, and Fragrance Association using standardized microorganisms. The results demonstrated that *R. rosaefolius* extract at the studied concentration reduced the bacterial inocula, satisfying the criterion in all formulations, even though it was not able to present an effective preservative behavior against fungi. Thus, the investigation of new natural substances with preservative properties that could be applied in pharmaceutical and cosmetic products is relevant due to the possibility of substituting or decreasing the concentration of synthetic preservatives, providing a way for the development of safer formulas for the use of consumers.

KEY WORDS: antimicrobial effectiveness testing; challenge test; natural preservative; *Rubus rosaefolius* Sm.; topical formulations.

INTRODUCTION

The concern of the industries related to the microbiological safety of pharmaceutical and cosmetic products is extremely relevant as it may represent a risk for the consumer's health and also contribute to product degradation or infection dissemination.(1)

Even though microbiological contamination must be avoided during all processes involved in manufacturing, storage, and usage, it is responsible for most product recalls in the whole world, highlighting the importance of developing new preservative systems able to inhibit microorganism growth such as bacteria, molds, and yeasts (2). According to the United States Pharmacopoeia (USP) 33nd (3), antimicrobial preservatives should be used as a substitute for good manufacturing practices or solely to reduce the viable microbial population of non-sterile product or control the pre-sterilization bioburden of multi-dose formulations during manufacturing.

The Food and Drug Administration requires that the manufactured products must be safe for consumers' use; therefore, they must not contain significant amounts of microorganisms or toxic ingredients (4).

Preservatives are antimicrobial chemicals added to cosmetics, pharmaceuticals, foods, and industrial products to protect the formulation against microbial spoilage and the consumer against infections as well.

The traditional preservative categories include alcohols, amides and amines, carbanilides, formaldehyde donors, inorganic, metal and organic compounds, paraben esters, phenol derivatives, pyridine, and quaternary compounds. The North American Contact Dermatitis Group reports a reaction rate of 2.3% of paraben esters (5). Official methods used for evaluating the effectiveness of a preservative system have been described in the different Pharmacopoeias, and the similitude and differences among them have been commented by authors (6,7). One of the important causes of allergy from commercial products is related to the variety of preservatives

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added to maintain their microbiological stability. Such products may be in direct contact with the skin for long periods of time (as emulsions and gels), and their difference relies on the preservative type and concentration.

In this way, the aim of the formulator is to find out the safe and effective preservation system in the formulation. Therefore, new or improved preservative systems are required which cease the bacterial development and prevent allergy and skin irritations (8).

The search for new antimicrobial agents has led to a tendency of avoiding synthetic substances, which increased considerably the researches involving natural sources of this particular class with less toxic effects, representing a possible natural and safer alternative (1).

Historically, many plant oils and extracts have been used as topical antiseptics or reported to possess antimicrobial properties. It is important to investigate scientifically plants that have been used in traditional medicines as potential sources of novel antimicrobial compounds. Also, the resurgence of interest in natural therapies and increased consumer demand for effective, safe, and natural products mean that quantitative data on plant oils and extracts are required (9).

Natural products such as active ingredients isolated from plants or herbal preparations with potent medical use are a relevant group of substances for the pharmaceutical industry. Nowadays, up to 40% of the modern drugs are obtained from natural sources, and Brazil possesses one the world's greatest biological diversity, therefore holding great potential to provide substances of pharmaceutical and cosmetic interest (10–12). *Rubus* is a larger genus in the family Rosaceae that has been found to have different pharmacological activities. *Rubus brasiliensis* has anxiolytic (13), hypnotic, anticonvulsant, and muscle relaxant effects (14), and *Rubus rosaefolius* has an analgesic effect (15). With regard to antimicrobial activity, *Rubus urticaefolius* (16) and *R. rosaefolius* (17) have presented that effect. The known plant *R. rosaefolius* is a shrub with compound leaves, recurvate prickles, with flowers, and aggregate fruit popularly known as sylvan strawberry (18).

The aim of this study was to verify *R. rosaefolius* extract efficiency as a natural preservative in topical formulations. The study refers to the preservative effects in different types of base formulations, emulsion and gel, which were evaluated using antimicrobial effectiveness testing.

MATERIALS AND METHODS

R. rosaefolius Smith Extract Preparation

R. rosaefolius Smith leaves were dried and protected from direct exposure to sunlight at room temperature. The dried material was extracted with ethanol 92°GL using an automatic extractor (ASE 300, Dionex) at 70°C in extraction cycles of 15 min. The extract was then concentrated under vacuum in a rotary evaporator and dried in a steam bath at 50°C in order to remove the residual water.

Test Organism Preparation

The selected microorganisms were *Pseudomonas aeruginosa* (ATCC 9027), *Burkholderia cepacia* (ATCC 25416), *Staphylococcus aureus* (ATCC 6538), *Escherichia coli* (ATCC 10536), *Candida albicans* (ATCC 10231), and *Aspergyllus brasiliensis* (ATCC 16404), formerly *Aspergillus niger (A. brasiliensis* Varga *et al.* deposited as *A. niger* van Tieghem, anamorph).

The bacteria were cultured on tryptone soya agar (TSA, Difco™) for 24 h at 37°C, while the fungi were grown on Sabouraud dextrose agar (SDA, Difco™) at 25°C for 48 h up to 5 days.

The microbial growth was recovered using 5 mL of physiological saline solution for bacteria and yeast, and 5 mL of the same solution was added to 0.05% (*w*/*v*) polysorbate 80 for the mold. The number of colony forming units (CFU/mL) of each suspension was determined by the pour plate count method. The concentration of microorganisms was suitable to provide 10^5–10^6 CFU/mL in the test immediately after the inoculation. The standardized suspensions were used to inoculate the formulations (19,20).

Developed Formulations

The base formulations containing the *R. rosaefolius* extract were prepared, corresponding to gel and emulsion. All components were of pharmaceutical or cosmetic grade, obtained from commercial sources, and the selection was based on their commercial availability and wide usage.

The emulsion was composed of 10% (*w*/*w*) emulsifying wax NF (Polawax® NF), 2% (*w*/*w*) paraffin liquid (mineral oil), 1% (*w*/*w*) cetyl acetate (and) acetylated lanolin alcohol (Crodalan® LA), 3% (*w*/*w*) cyclomethicone (Dow Corning® 245), 2% (*w*/*w*) methyl gluceth-20 (Glucan® E 20), 5% (*w*/*w*) propylene glycol, fragrance (Cosmetic® 35), and distilled water.

The gel contained 0.8 (*w*/*w*) carbomer (Carbopol® Ultrez 10), 3% (*w*/*w*) PPG-5-ceteth-20 (Procetyl® AWS), 5% (*w*/*w*) propylene glycol, 0.8% (*w*/*w*) PEG-7 glyceryl cocoate (Cetiol® HE), sodium hydroxide, fragrance (Cosmetic® 35), and distilled water.

The formulations were preserved with *R. rosaefolius* extract 0.2% (*w*/*w*), previously diluted in ethanol 99.9% (Merck, Darmstadt, Germany) and irradiated in Gammacell with 25 KGy at Nuclear and Energetic Research Institute (IPEN)–São Paulo, Brazil. Control formulations lacking the preservative were prepared using the same components and submitted to the same procedures.

Antimicrobial Effectiveness Testing

Preliminary studies were performed in order to assure the effectiveness of the neutralizing medium in the inocula recovery. Each inoculum was added to each 10% formulation in the Dey Engley (D/E) neutralizing medium (the recovery diluents), in physiological saline plus the recovery diluents, and in the physiological saline. The pour plate technique was used in order to quantify the viable microorganism amount in these experiments. Therefore, the assessment of the preservative system neutralization was determined by comparing the recovery in physiological saline suspension to that in each formulation plus the D/E neutralizing medium (recovery diluents) suspensions. It has also verified neutralizer toxicity by comparing the physiological saline suspensions to the

Table I. Log_{10} Reduction from Initial Calculated Count (CFU/grams) of Antimicrobial Effectiveness Test of *R. rosaefolius* Extract in Emulsion

Strains	Products	Time (day)					
		0	2	7	14	21	28
Escherichia coli ATCC 10536	Emulsion preserved with *Rubus* 0.2% (*w*/*w*)	6.17	<1	<1	<1	<1	<1
	Physiological saline	6.36	6.59	5.5	5	4.71	5.78
Pseudomonas aeruginosa ATCC 9027	Emulsion preserved with *Rubus* 0.2% (*w*/*w*)	6.07	<1	<1	<1	<1	<1
	Physiological saline	6.27	6.28	6.53	6.15	6.59	6.64
Burkholderia cepacia ATCC 25416	Emulsion preserved with *Rubus* 0.2% (*w*/*w*)	5.62	<1	<1	<1	<1	<1
	Physiological saline	5.59	6.11	6.48	6	6.54	6.36
Staphylococcus aureus ATCC 6538	Emulsion preserved with *Rubus* 0.2% *w*/*w*	5.99	<1	<1	<1	<1	<1
	Physiological saline	6.3	5.99	6.49	6.85	6.77	6.61
Candida albicans ATCC 10231	Emulsion preserved with *Rubus* 0.2% (*w*/*w*)	4.72	4.64	4.43	4.87	4.92	5.04
	Physiological saline	4.72	5.41	5.41	5.62	4.72	4.53
Aspergillus brasiliensis ATCC 16404	Emulsion preserved with *Rubus* 0.2% (*w*/*w*)	5.54	5.4	4.84	4.17	4.18	3.81
	Physiological saline	5.4	5.4	5.7	5.04	5.65	5

The Log results are the average of two different experiments

physiological saline plus recovery diluents. Both of the determinations have been according to Chapter <1227> Validation of Microbial Recovery and Chapter <1227> Microbiological Examination of Non-sterile Product: Microbial Enumeration Tests from the 33nd (3).

The efficacy of the preservative test was performed following the standards proposed by USP 33nd (3), European Pharmacopoeia 6th (21), Japanese Pharmacopoeia 14th (22), and Cosmetics and Toiletry and Fragrance Association (CTFA) (23), as described beyond.

The formulations (20-g samples) were placed in sterile containers and separately inoculated with 0.2 mL of each bacterial and fungal suspension in order to obtain a final concentration of approximately 10^5–10^6 CFU g^{-1}. The samples were gently shaken to ensure a homogeneous microorganism distribution and incubated at 25°C. Samples of 1 g were removed and placed into 9 mL of D/E neutralizing medium, and serial decimal dilutions were performed before inoculation in microbial plates. The same procedure was performed after 2, 7, 14, 21, and 28 days of contact. Cell viability was determined by the plate count method in TSA or SDA, and the CFU were counted after 2 or 5 days of incubation at 37°C and 25°C for bacteria and fungi, respectively. All determinations were performed in duplicate, and the results represent an average of two different experiments.

The viability and growth ability of the inoculated cells were evaluated by a growth control which consisted of 20 mL physiological saline samples separately inoculated with 0.2 mL of each bacterial and fungal suspension.

DISCUSSION

According to the standards proposed by USP 33nd (3), Chapter <51>, the criteria for antimicrobial effectiveness of category 2, topically used product made with aqueous base vehicle, non-sterile nasal products, and emulsions, including those applied to mucous membranes, is: "for bacteria" not <2.0 log reductions from initial count at 14 days and no increase from the 14 days "count at 28 days"; for yeast and molds, "no increase from the initial calculated count at 14 and 28 days." The Japanese Pharmacopeia 14th (18) presents the similar criteria and time of USP 33nd (3), however in "per cent reduction" that has been considered less accurate than the term logarithm.

Table II. Log_{10} Reduction from Initial Calculated Count (CFU/grams) of Antimicrobial Effectiveness Test of *R. rosaefolius* Extract in Gel

Strains	Products	Time (day)					
		0	2	7	14	21	28
Escherichia coli ATCC 10536	Emulsion preserved with *Rubus* 0.2% (*w*/*w*)	6.18	<1	<1	<1	<1	<1
	Physiological saline	6.36	6.59	5.5	5	4.72	5.78
Pseudomonas aeruginosa ATCC 9027	Emulsion preserved with *Rubus* 0.2% (*w*/*w*)	6	<1	<1	<1	<1	<1
	Physiological saline	6.28	6.28	6.53	6.15	6.59	6.64
Burkholderia cepacia ATCC 25416	Emulsion preserved with *Rubus* 0.2% (*w*/*w*)	5.83	<1	<1	<1	<1	<1
	Physiological saline	5.59	6.11	6.48	6	6.54	6.36
Staphylococcus aureus ATCC 6538	Emulsion preserved with *Rubus* 0.2% (*w*/*w*)	6.32	<1	<1	<1	<1	<1
	Physiological saline	6.3	5.99	6.49	6.85	6.77	6.61
Candida albicans ATCC 10231	Emulsion preserved with *Rubus* 0.2% (*w*/*w*)	4.61	4	1.84	3.81	Uncountable	6.04
	Physiological saline	5.77	5.41	4.8	5.62	4.72	4.53
Aspergillus brasiliensis ATCC 16404	Emulsion preserved with *Rubus* 0.2% (*w*/*w*)	5.48	5.48	4.74	4.7	4.18	4.18
	Physiological saline	5.4	5.4	5.7	5.04	5.65	5

The log results are the average of two different experiments

In European Pharmacopoeia 6th (21), there are two sets of criteria for each product category—a target (the "A" criteria) and an acceptable level (the "B" criteria). The preservation efficacy at the level of the "B" criteria is acceptable only if there are strong reasons why the "A" criteria cannot be met. The "A" criteria for bacteria should be "not less than 2.0 log reductions at 2 days, 3.0 at 7 days, and no increase until 28 days"; for fungi, "not less than 2.0 log reductions from initial count at 14 days and no increase from the 14 days count at 28 days." Those criteria are more potent than the USP; however, it is less potent when the "B" criteria for bacteria are considered: "not less than 3.0 log reductions from initial count at 14 days and no increase from the 14 days count at 28 days." For fungi, the criteria are also more potent: "not less than 1.0 log reductions from initial count at 14 days and no increase from the 14 days count at 28 days."

In relation to cosmetic, a 3.0 log reduction is required for bacteria at 7 days after the inoculation and no increase afterwards for adequate preservation; for fungi, a 1.0 log reduction at 7 days and no increase during the 28-day tests period, in accordance with the CTFA (23) preservative efficacy test for water miscible products and CTFA M-3. In the CTFA M-4, the method for preservation testing of eye area cosmetic bacterial challenge test criteria is divided between vegetative and spore-forming bacteria. For vegetative bacteria, the preservative is proven effective in the assayed products if the concentration of the viable inoculated bacteria shows a logarithmic ($\log_{10}$) reduction of 3 by the seventh day and continue reduction by the end of the test. For spore-forming bacteria, bacteriostatic activity is requested throughout the entire test. There must be a 1.0 log reduction in fungi counts in 7 days after inoculation.

The bacterial challenge test performed in the emulsion and gel formulations preserved with *R. rosaefolius* at 0.2% concentration has satisfied the criteria for microbial effectiveness, considering the official and non-official criteria presented (23). In particular, *R. rosaefolius* reduced the bacterial suspension by a factor of 10^5 for Gram-negative and Gram-positive within 7 days, as described in Tables I (emulsion) and II (gel). The preservative in the assayed emulsion and gel was found particularly effective against Gram-negative and Gram-positive strains, presenting a lethal effect at 2 days after inoculation. The Gram-positive bacteria possess a less complex morphologic structure and cell wall which may be more permeable to the extract compounds and therefore explain the higher sensitivity to the tested preservative in all formulations (24).

The challenge test performed in the emulsion and gel formulations for *Aspergillus brasiliensis* ATCC 16,404 has shown no increase from the initial count at 14 and 28 days and therefore approved by USP 33nd criteria. However, the result did not comply with the criteria of CTFA and European Pharmacopoeia 6th due to there being 0.7 log reductions on the seventh day and 1.4 log reductions on the 14th day. Therefore, it is an evident that the criteria of the preservative effectiveness test should have worldwide harmonization.

In an attempt to evaluate the microbial contamination behavior during the assay, the samples were also analyzed at 2, 14, 21, and 28 days after the preparation. The adoption of such strategy could provide fundamental information concerning a so-called rebound or Phoenix phenomenon, which

Table III. Neutralize (Recovery) Efficacy Test and Neutralize Toxicity (Grow Promotion) Efficacy Test of the Recovery Diluents

	E. coli		*P. aeruginosa*		*B. cepacia*		*S. aureus*		*C. albicans*		*A. brasiliensis*	
	μ (CFU)	% Recovery	μ (CFU)	% Recovery	μ (CFU)	% Recovery	μ (CFU)	% Recovery	μ (CFU)	% Recovery	μ (CFU)	% Recovery
Emulsion												
Product[a]	80.8	83.9	74.4	84.2	83.69	78.66	64.6	73.57	106.8	73.15	79.5	89.02
Physiological saline[a]	93.4	97.1	80.4	90.49	93.4	87.78	74.4	84.73	174.2	87.53	80.5	90.14
Physiological saline	96.2	100.0	88.4	100.0	106.4	100.0	87.8	100.0	146.0	100.0	89.3	100.0
Gel												
Product[a]	89	91.0	80.3	89.82	79.0	78.53	69.9	73.89	112.5	86.67	75.3	84.7
Physiological saline[a]	92	94.07	99.6	111.41	97.4	96.82	78.6	83.09	123.0	94.76	87.2	98.86
Physiological saline	97.8	100.0	89.4	100.0	100.6	100.0	94.6	100.0	129.8	100.0	88.9	100.0

[a] In D/E neutralizing medium (recovery diluent)

corresponds to a decrease in microorganism survival followed by an increase during the experiment period (25).

This phenomenon was observed for *C. albicans* in the gel formulation (Table II) probably due to the used preservative concentration, which is near the minimum inhibitory concentration (2 μg/mL), as well as the reduction of its available concentration resulting from an interaction with the formulation components or a resistant microorganism selection (25).

Related to the microorganisms used during the test, it must be guaranteed that they have been challenged against the preservative system for 28 days to substantiate the results of the experiments. Thus, the control was used to prove the viability of the inoculated cells and their growth ability during the test period (Tables I and II).

In order to confirm that the microorganisms have not been prevented from growing in the pour plate method by residual antimicrobial activity of the product, neutralizer efficacy and neutralizer toxicity assays were performed (26). The results obtained for the entire studied microorganism and formulations have indicated over 70% recovery of the inoculated microorganisms (Table III). Therefore, it was confirmed that there is no presence of interferences caused by the neutralizing agent over the plate count, in accordance with USP 33nd (3). The potential toxicity of the neutralizing medium (recovery diluent) has also been in compliance with the same Pharmacopoeia. Thus, the method used has been validated, and it has been demonstrated that the viable microorganisms have been recovered in the experimental system.

The formulation components have a considerable effect on the antimicrobial efficacy taking into consideration that pharmaceutical and cosmetic products are genuinely complex vehicles and are developed with a variety of raw materials involving surfactants, emollients, fatty acid derivatives, humectants, silicone fluids, viscosity and chelating agents, fragrance, colorants, and salts. The suitable combination of the ingredients has guaranteed consumer adherence and the efficacy and safety of the products.

CONCLUSION

The extract of *R. rosaefolius* 0.2% (*w*/*w*), natural preservatives, in the assayed emulsion and gel was effective against *P. aeruginosa* (ATCC 9027), *B. cepacia* (ATCC 25416), *S. aureus* (ATCC 6538), and *E. coli* (ATCC 10536), presenting a lethal effect at 2 days after inoculation. That behavior was maintained for 28 days, satisfying the Antimicrobial Effectiveness Test criteria of the USP 33nd, European Pharmacopoeia 6th, Japanese Pharmacopoeia 14th, and CTFA, even though the extract of *R. rosaefolius* 0.2% (*w*/*w*) was not able to present an effective preservative behavior against fungi. However, the investigation of natural substances with preservative properties is relevant due to the possibility of decreasing or substituting the concentration of synthetic preservatives applied in pharmaceutical and cosmetic products, providing a way for the development of safer formulas for consumers' use.

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