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Sensitization Properties of Propolis and Balsam of Peru in Guinea Pig Maximization Test (GPMT)

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Abstract

Introduction: Propolis is used in Poland as an active ingredient of some drugs administered externally, dietary supplements and cosmetics. According to the literature, propolis is a non-toxic and safe substance, although it may cause allergic contact dermatitis. Aim: The aim of this study was to assess the allergenic properties of propolis and Balsam of Peru. Material and methods: The study was conducted according to the *OECD Guideline for testing of chemicals-Skin sensitization* with use of Guinea pig maximization test (GPMT). Guinea pigs have similar sensitivity to allergens as human body. Sensitization properties of propolis were tested in comparison with sensitization properties of Balsam of Peru because of the possibility of cross-reaction between those two substances. Results: The skin of guinea pigs in the propolis group showed no visible change compared to the control group during the first (48 h) and second observation (72 h). The skin of guinea pigs from Balsam of Peru group showed discrete erythema in only one case at the first observation (8.33% of the animals). During the second observation, no visible changes were observed compared to the control group. Conclusions: Guinea pig maximization test showed no sensitizing properties of propolis and weak sensitizing properties of Balsam of Peru in concentration of 5%.

Keywords

Propolis, Balsam of Peru, Guinea Pig Maximization Test, Sensitization Properties, Allergic Contact Dermatitis

1. Introduction

Propolis is a substance collected by worker bees from the resin of trees, mainly

from poplar buds and conifer. It is used by them as a multifunctional material in constructing and maintaining hives and also as a protection from microbiological contamination. Propolis is widely used in Poland as an active substance in some drugs administered externally for healing wounds, burns, chilblains, and also as dietary supplements and an ingredient of some cosmetics. The most important properties of propolis are an antibacterial, antivirus and antifungal activity, but also anti-inflammatory and regenerative action [1] [2] [3] [4].

Propolis is known to cause contact allergy, but according to data found in the literature, the hypersensitivity to propolis more often appears in dermatological patients, treated earlier for allergic dermatoses 1.2% - 6.7% (average 3.82%) than in healthy population. In healthy population, allergy to propolis seems to be very rare phenomenon (0.64% - 1.3%) [5] [6] [7] [8] [9]. Taking into account the data from human population, the data from animal population from the literature is puzzling. The study conducted on guinea pigs by Petersen in 1977 indicated that propolis is a strong allergen because it sensitized 19 out of 25 animals (76% of propolis group) [10]. Today, it occurs that the method use by Petersen-GPMT of Magnusson and Kligman [11] may cause false positive results. This method was criticized by Kligman-one of the authors of this method [12]. Nowadays, for testing skin sensitization, it is recommended to use updated maximization test according to the *OECD Guideline for the Testing of Chemicals-Skin sensitization* [13]. Moreover, the allergic potential of propolis is very often compared with the allergic potential of Balsam of Peru, because those two substances are cross-reactivity allergens. Data indicate that Balsam of Peru can sensitize two to three times more frequently than propolis [13].

2. Aim

The aim of the paper is to assess the sensitizing potential of propolis and Balsam of Peru and to compare those two substances.

3. Material and Methods

3.1. Ethical Approval

The study was submitted to The First Local Ethical Committee on Animal Testing at the Jagiellonian University in Krakow, Poland. The agreement was approved on the 17th October, 2012 (number of agreement 142/2012).

3.2. Animals

Guinea pigs, all males, weight: 260 - 530 g, were procured from Animal Breeding Laboratory number 0055 according to the List of units eligible for breeding experimental animals approved by the Polish Ministry of Science and Higher Education. Animals were acclimatized to the laboratory conditions for 7 days prior to the test. They were kept at constant temperature of 22˚C and 50% of the humidity, under artificial illumination (12 h of light, 12 h of dark) and were nourished and watered ad libitum. They received 4 drops of 100 mg/ml ascorbic acid solution. Before the study started, guinea pigs were grouped randomly and were weighed. In

the propolis group (P) there were 11 animals, in the Balsam of Peru group (B) there were 12 animals and in the control group (C) there were 6 animals. At the beginning the propolis group consisted of 12 animals, but before the study starts one animal was excluded from the study because of its health condition.

3.3. Chemicals

Balsam of Peru and Freund's Adjuvant Complete (FCA) were purchased from Sigma-Aldrich. Ethanol extract of propolis (EEP) was obtained from Przedsiębiorstwo Pszczelarsko-Farmaceutyczne "Apipol-Farma" Sp. z o.o. Its quality was in accordance with the Polish Norm PN-A-77627:1996 The Concetrate of propolis.

3.4. The Guinea Pig Maximization test (GPMT)

The test was carried out according to the *OECD Guideline for the Testing of Chemicals-Skin sensitization.*

A) Induction: Intradermal injections

On the 0 day three pairs of 0.1 ml injections were given in the shoulder region which was cleared of hair. The propolis group received 3 injections, six in all, on each side as follows: 1) a 1:1 mixture FCA/water for injection, 2) ethanol extract of propolis, 3): ethanol extract of propolis in a 1:1 mixture FCA/water for injection. The Balsam of Peru group received 3 injections, six in all, on each side as follows: 1) a 1:1 mixture FCA/water for injection, 2) Balsam of Peru in ethanol and 3) Balsam of Peru in ethanol in a 1:1 mixture FCA/water for injection. The control group received as follows: 1) a 1:1 mixture FCA/water for injection, 2) ethanol and 3) ethanol in a 1:1 mixture FCA/water for injection.

B) Induction: Topical application

On the 7^{th} day, which means 24 hours before the topical induction application, the test area was shaved. Then on the 8^{th} day a filter paper with appropriate test substance (EEP or Balsam of Peru) or vehicle only (ethanol) was applied and held in contact by an occlusive dressing for 48 hours.

C) Challenge: Topical application

On the 21^{th} day test areas were cleared of hair again. A patch loaded with test substance or vehicle only was applied to one flank. The patches were held in contact by an occlusive dressing for 24 hours.

D) Observations:

21 hours after removing the patch the challenge area was cleared of hair. 3 hours later (approximately 48 hours from the start of the challenge application) the skin reaction was observed and recorded according to the grades below:

0—no visible change

1—discrete or patchy erythema

2—moderate and confluent erythema

3—intense erythema and swelling.

At the end (24 hours after the first observation) the second observation (72 hours) was made.

After the test, the animals were weighed again. Their weights ranged from 362

to 594 g.

3.5. Histopathology

Animals were humanely euthanized at the end of the study and the skin samples from the challenge site were collected. Tissue samples were fixed in AFA (alcohol, formalin, and acetic acid) fixative, dehydrated and then routinely embedded in paraffin and sliced in 6 μm thick sections. For histopathological evaluation following stains were performed: hematoxylin and eosin, trichromatic Masson Goldner (TGM), May-Grünwald Giemsa. Samples were examined by light microscopy at magnifications of 20×, 40× and 100× to evaluate the various targets.

3.6. Preparation of Blood Smears

Peripheral smears were prepared from a freshly drawn blood samples into S-Monovette(R) system with Potassium EDTA as anticoagulant. Blood smears were fixed according to May-Grunwald-Giemsa staining method. Microscopic observations were carried out with magnification lens 100×. Percentage of eosinophils were examined in the blood smears in triplicate and counted with hematologic adder.

3.7. Determination of IFN-γ Level

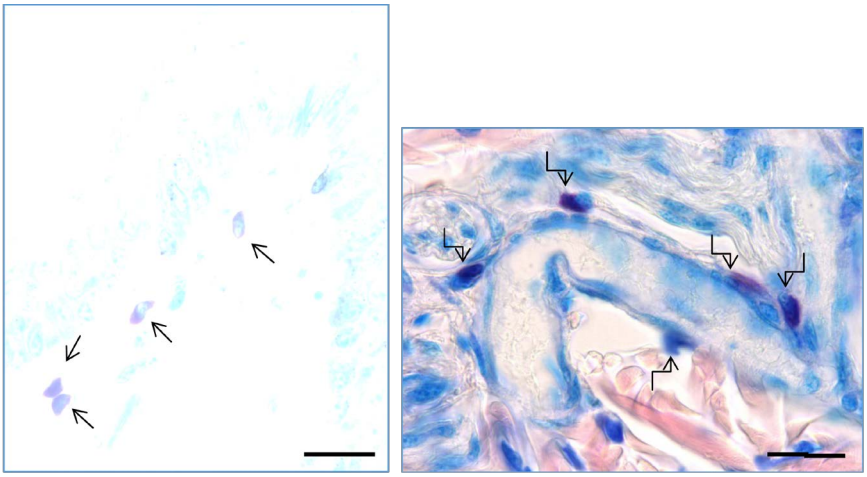
Plasma samples were prepared from the blood collected before animals were euthanized. Blood samples were centrifuged for 10 minutes at 1000× g. The supernatant was collected in a tube and stored at −20˚C. IFN gamma concentration was measured by IFN gamma PIG ELISA kit (ABCAM).

4. Results

The skin of guinea pigs in propolis group shows no visible changes in comparison with the control group during the first and the second observation. The skin of guinea pigs from the Balsam of Peru group show discrete erythema only in one case and in other animals no visible changes in comparison to the control group during the first observation. After the second observation no visible changes were observed in any case. The results of the sensitization experiment are shown in **Table 1**.

Histopathologically, macroscopic evaluation of the collected parts of the skin showed no changes. The skin was clear, no swelling, blisters or erosions. In the studied parts of the skin there were no skin lesions typical for inflammation. Neutrophil mobilization was observed mainly in the dermis. In three cases (1 in the propolis group, 2 in the Balsam of Peru group) numerous mast cells in the field of view in the dermis were observed. Most of them were situated below the epidermis and around the capillary (**Figure 1(a)**) and around the blood vessels in the deeper layers of the dermis (**Figure 1(b)**).

In other cases, the granulocytes were few or a single in the field of view. Only in one case of the propolis group, numerous neutrophils and monocytes infiltrated epidermis (**Figure 2**).



(a) (b)

Figure 1. (a) (b) Cross section through the skin of guinea pig (group of propolis) with numerous mast cells (↓) under epidermis (a) and around capillaries (b). Staining with May-Grunwald Giemsa. Scale bar 20 μm.

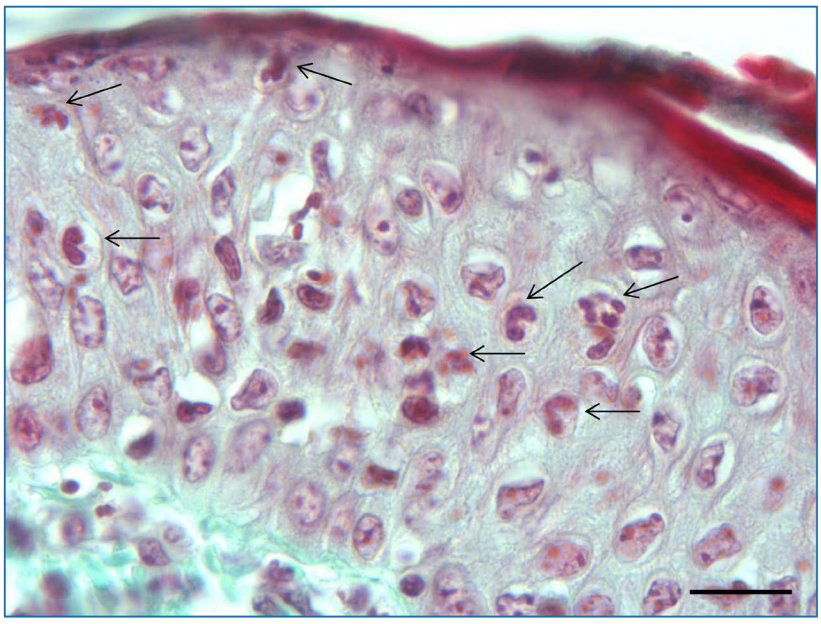


Figure 2. Longitudinal section through the epidermis. Visible plurality of granulocytes (neutrophils) and monocytes infiltrating the epidermis. Trichromatic staining by Masson Goldner. Scale bar 20 μm.

Table 1. The results of GPMT of propolis and Balsam of Peru.

Group	Observation 1		Observation 2	
	(48 h from the start of the challenge application)		(72 h from the start of the challenge application)	
	Number. of animals with positive reaction	% of animals with positive reaction	Number of animals with positive reaction	% of animals with positive reaction
Propolis	0/11	0%	0/11	0%
Balsam of Peru	1/12	8.33%	0/12	0%
Control	0/6	0%	0/6	0%

In the studied material researchers noticed also:

- the damage of the epidermis with the infiltration of eosinophils in one case of the Balsam of Peru group;

- thin, atypical epidermis; cells of the cuboidal shape with pyknotic nuclei, lack of the proper basal layer in one case of the propolis group;

- a large area of regenerating epidermis and the presence of granulocytes in the dermis under that epidermis in two cases of the Balsam of Peru group.

The observed changes could indicate the early stages of sensitization. In the skin of the control animals no changes within the epidermis were observed and there were only few granulocytes in the field of view in the dermis. The results are shown in Table 2.

The percentage of animals in which mobilization of granulocytes occurred during the GPMT, which may suggest the early stage of an allergic reaction, is shown in Table 3. The evaluation of the number and the percentage composition of white blood cells do not clearly show the increase in the number of eosinophils in cases where histopathological study indicated the mobilization of granulocytes. Elevated levels of basophils compared to the control group were found in only one case in the propolis group. Increased levels of eosinophils compared to the control group were found in 3 cases in the propolis group and in 2 cases in the Balsam of Peru group. Levels of interferon-gamma (IFN-γ) in guinea pigs in all the samples, both in the treatment groups and the control, were below the level of quantification and did not differ between all groups.

5. Discussion

Although propolis is considered to be a safe and nontoxic substance, there are some reports about sensitizing properties of propolis. Allergic reactions to propolis usually occur as contact dermatitis after topical administration. For assessing the sensitizing potential of a substance it is recommended to apply the Gui-

Table 2. The results of histopathological studies.

GROUP	EPIDERMIS		DERMIS		
	Changes in morphology	Presence of leukocytes	Presence of leukocytes	Vascular Congestions	Oedema
Propolis	1/11	1/11	4/11	0/11	0/11
Balsam of Peru	3/12	0/12	6/12	0/12	0/12
Control	0/6	0/6	0/6	0/6	0/6

Table 3. Animals in which mobilization of granulocytes occurred.

Group	No. of animals with granulocytes mobilization	% of animals with granulocytes mobilization
Propolis group	3/11	27.3
Balsam of Peru group	5/12	41.7
Control	0	0

nea pig maximization test (GPMT) [13] [14]. The results of our study indicate that none of guinea pigs in the propolis group developed allergic contact dermatitis to propolis and in only one case in the Balsam of Peru group slight erythema was observed during the first observation. After the second observation none of guinea pigs from the Balsam of Peru group developed changes characteristic for the allergic contact dermatitis. This may indicate the lack of allergenic potential of propolis at the concentration of 5% and low sensitizing potential of Balsam of Peru at the same concentration.

Additionally, guinea pigs were weighed before and after the study. The loss in weight of 10% or more may indicate systemic toxicity of the substance [15]. During this study, all guinea pigs from each group put weight. The increase in weight ranged from 12.1% to 39.2%, which demonstrates the good health of animals and the lack of toxicity of tested substances.

The histological examination of animal skin biopsies also showed no sensitization, and only in a few cases, mobilization of leukocytes. Mechanical damage could explain the atypical and regenerating epidermis of animals in one case in the propolis group and in three cases in the Balsam of Peru group. However, in the control group, there were no such cases; therefore, these changes may indicate the early stages of an allergic reaction in animals. The examination of blood smear did not clearly indicate the possible occurrence of allergy, either. Only in 5 cases the elevated level of eosinophils was observed, namely in 3 cases in the propolis group and in 2 cases in the Balsam of Peru group. However, it is worth noting that only in one guinea pig from the Balsam of Peru and only in one guinea pig from the propolis group some changes in morphology of the epidermis during histopathological examination were observed.

The occurrence of allergy was confirmed only in one case of the Balsam of Peru group. During the first observation erythema occurred (severity point 1 according the Magnusson and Kligman scale). It disappeared during the second observation about 72 h after the induction phase. In addition, histopathological examination of the skin revealed the epidermis damage and infiltration of eosinophils. The examination of blood film showed high levels of eosinophils (8%). It can be concluded that Balsam of Peru sensitized one guinea pig of 12 (8.33% of the examined animals), and based on the result of this study it can be qualified as weak contact allergen.

The data from this animal study differ from the results obtained from the guinea pig maximization test conducted by Petersen in 1977 due to the Kligman and Magnusson method (1969). It was shown that 5% propolis in propylene glycol injected intradermally and 50% propolis in ethanol administered topically sensitized 19 out of the 25 animals used in the experiments (76% positive reaction to propolis). However, this experiment was performed using an old version of the maximization test. This method has been criticized, as giving false positive results, by Kligman one of the authors of this method [12].

This study was conducted according to the OECD Guidelines for the Testing

of Chemicals-Skin Sensitization from 1992 included the updated GPMT method. First of all the most potent sensitizer, dinitrochlorobenzene (DNCB) was no longer recommended. For induction exposure, the concentration of test substance should be well-tolerated systematically and should be the highest to cause mild to moderate skin irritation. For the challenge exposure, the concentration of test substance should be the highest nonirritant dose. The modified version of the maximization test has the same sensitivity as the original protocol, but do not lead to positive false results [16].

The GPMT is used to assess whether the substance has sensitizing properties and more specifically, whether it can cause allergic contact dermatitis (ACD), which is an immune skin reaction to the substance. In humans, ACD can be characterized by itching, erythema, edema, papules, vesicles, filled with transparent, aqueous liquid etc. In other species the reactions may vary and only erythema and edema can be observed according to Magnusson and Kligman grading scale, that is: 0-no visible change, 1-discrete or patchy erythema, 2-moderate and confluent erythema and 3-intense erythema and swelling. The method consists of the induction and the challenge exposure to the test substance. Between the phase of induction and challenge there must be a period of minimum one week during which the hypersensitive state may develop. The concentration of test substance used for each induction exposure should be well-tolerated systemically and should be the highest to cause mild-to-moderate skin irritation. Petersen during the induction exposure for intradermal administration used 5% solution of propolis in propylene glycol and for topical application 50% propolis ethanol solution , which itself may cause skin irritation. As it was shown, irritation threshold for propolis occurs at concentrations higher than 20% [17]. Therefore, the 50% propolis ethanol solution causes strong irritation. It has also been shown that the propylene glycol is permeation enhancer for other substances. One proposed mechanism for it is the modification of the skin barrier by changing the structure of keratin and increasing the concentration of the solution in the *stratum corneum* [18]. Furthermore, one and the same vehicle should be used for this study to eliminate the risk of causing irritation or sensitization by this substance. Petersen used two different vehicles: ethanol and propylene glycol. The OECD recommends using the concentration for the challenge exposure the highest non-irritant dose. Petersen used 25% ethanol solution of propolis, which may cause irritation from a weak to moderate. In our study we used 5% ethanol solution of propolis, as it is the highest concentration used in medicinal products with propolis authorized in Poland and it is the concentration causing no irritation. The 10% concentration of propolis is used in the patch test to induce contact sensitization. The guinea pig maximization test (GPMT) is a widely used test with Freunds Complete Adjuvant (FCA). Adjuvant tests are more accurate in predicting the sensitizing properties of substances than methods not using complete adjuvants. Although several other methods can be used to determine the potential of substance to induce skin sensitization,

as for example the Buehler test, GPMT is considered to be the preferred technique. Petersen in his study did not use FCA. Furthermore, the observation in accordance with the recommendations of the OECD is done 48h and 72h from the start of the challenge application. In the Petersen study only one observation is done with not specified time since the challenge application. The differences between the two tests performed by us and Petersen are shown in **Table 4**.

It should also be noted that in predisposed persons almost any substance may cause contact allergy at high concentrations during the long and frequent exposition [12]. Even anti-inflammatory drugs such as corticosteroids may sensitize some people, particularly if used in inflammatory conditions of the skin [19]. Negative result in the GPMT allows predicting the safety of the substance. Generally, no more testing is needed to determine the possibility of the substance to cause sensitization of the skin. However, there are some exceptions. Nickel is one of the most well-known contact allergens, but in the GPMT it is very hard to induce allergy to nickel [20]. False-negative results, as the one described above, are very rare, but do happen. Just as the reverse case, the substance, which is a potent allergen in the GPMT should not be rejected from the use, especially if it has the advantageous properties [12]. It is always possible to determine the threshold concentration that rarely would sensitize people. 5% propolis ethanol solution does not show sensitization with respect to a healthy adult population. The problem is, that propolis formulations with much more higher concentration (up to 50%) are present on the market.

Table 4. The comparison of GPMT from 1969 (Magnusson and Kligman) and the GPMT currently recommended by the OECD.

Differences		GPMT according to Magnussona and Kligmana from 1969	Updated GPMT according to the OECD *Guideline for the Testing of Chemicals-Skin sensitisation*
Use of Dinitrobenzene		Yes	No
Propolis concentration		5%, 50%, 25%	20%, 20%, 5%
Vehicle		Propylene glycol in intradermal injection and ethanol in topical application	Ethanol in intradermal injection and topical application
Induction	Intradermal application	5% propolis solution in propylene glycol	20% propolis solution in ethanol
	Topical application	50% propolis solution in ethanol	20% propolis solution in ethanol
Challenge		25% propolis solution in ethanol	5% propolis solution in ethanol
FCA		NO	YES
Observation		I observation (time not mentioned)	48 h and 72 h after challenge exposure

6. Conclusion

Propolis, if used as recommended by the Office for Registration of Medicinal Products, Medical Devices and Biocidal Products in concentrations up to 5% (used in medicinal products registered in Poland) shows no allergenic potential, as confirmed by this research. The guinea pig maximization test showed weak sensitizing properties of Balsam of Peru.

Conflict of Interest

Authors report no conflict of interest.

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