

## Investigating the anti-sarcoptic mange activity (*in vivo*) of propolis ointment in naturally infested rabbits.

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### Abstract

**Introduction:** *Sarcoptes scabiei* (*S. scabiei*) var. *cuniculi* is the cause of sarcoptic mange in rabbits and results in considerable loss of weight, productivity, and wool quality.

**Objective:** The efficacy of 10% Propolis (bee glue) ointment on *S. scabiei* var. *cuniculi* was evaluated under *in vivo* conditions in pet rabbits with sarcoptic mange lesions compared to the currently used drug, ivermectin (400 µg/kg, S/C).

**Methods:** In the *in vivo* condition, the efficacies of 10% Propolis ointment, 1% ivermectin (400 µg/kg, S/C), and Propolis ointment in combination with ivermectin were evaluated. Serum factors of the control and treated groups were tested to evaluate the toxic effects of Propolis on the liver.

**Results:** Topical application of 10% Propolis ointment showed complete recovery from clinical signs and complete absence of mite in microscopic examination from 10-15 days of treatment.

**Conclusions:** 10% Propolis ointment has antisarcoptic mange effects under *in vivo* conditions and may be used in clinical applications.

**Keywords:** Rabbits, *Sarcoptes scabiei*, Propolis ointment, *In vivo*.

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### Introduction

Mange is common in pet rabbits; it inhabits the epidermal layer of the skin and causes irritation, hypersensitivity reaction, inflammation, hyperkeratosis, seborrhoea, and alopecia as a result of the feeding behaviour of larvae and nymphs [1]. Mange lowers animal productivity and the quality of animal products and is often fatal if not treated [2]. *S. scabiei* var. *cuniculi* causes sarcoptic mange in rabbits. Infection with *S. scabiei* var. *cuniculi* induces relative protection against subsequent infection in rabbits [3]. Elimination of *S. scabiei* in rabbits is difficult compared to other domestic animals [4]. However treatment of sarcoptic mange with various acaricides like diazinon, deltamethrin, and ivermectin have achieved success [5], many of these chemical acaricides have side effects [6] such as resistance [7], toxicity [8], environmental contamination and persistence [8,9]. These limits have suggested a search for novel alternatives [10-13]. Propolis has lots of biological and pharmacological properties and its mechanisms of action have been widely investigated *in vitro* and *in vivo* in the last years. Propolis used as a popular remedies and is available in either pure or combined form with other natural products. Researchers have been focused on the study of its constituents and biological properties [14]. Propolis is used for upper respiratory tract infections, common cold and flu-like infections, and as dermatological preparations for wound healing [15]. Canine dermatophytosis resolved with

topical propolis treatment [16]. Propolis is widely used in human and veterinary medicine as it has antimicrobial, antiviral, and antioxidant activities. The aim of the present study was to evaluate antisarcoptic mange activity of Propolis ointment under *in vivo* conditions.

### Materials and Methods

#### Source of propolis

Propolis samples were obtained by contact with beekeepers in different zones of Riyadh, Saudi Arabia. Each sample was weighed, frozen at -20°C, ground with a mortar, and then stored at 4°C until use [17].

#### 10% Propolis ointment preparation

Propolis extract was prepared as previously described [16] with modifications. Briefly, (5 g) was heated in a water bath with 50 g petroleum jelly (Vaseline) until melting and mixing. This mixture was used as the 10% propolis ointment for application twice/day to the skin lesion of each infested rabbit.

#### Experimental animals

Forty one-year-old and 2-2.4 kg weight naturally infested rabbits, of both sexes (eleven male and twenty nine female) were obtained from markets with crustaceous lesions on nose,

lips, ears, legs and surround the eyes. Infestation was identified by taking skin scraping samples with a scalpel blade and they were microscopically examined for mobility and morphology of the mites [18]. Ten healthy rabbits free from any infestations were purchased and used as negative control. Mortality was checked daily. The animals were kept in wire-bottomed cages in a room under standard conditions of illumination with a 12 hour light-dark cycle and at a temperature of  $25 \pm 1^\circ\text{C}$  for one week until the beginning of treatment. Animals were provided with tap water and a balanced diet ad libitum. The animals were divided into 5 groups, with 10 mice in each group. The five groups were group I (negative control), group II (positive control), group III (infected and treated with 10% Propolis ointment twice/day), group IV (1% ivermectin sterile solution (Jaamectin™, Jaapharm Canada Inc.) in a dose of (400 µg/kg, S/C), two injections with two weeks interval) [19], group V (infected and treated with both propolis ointment and ivermectin). Weekly, skin scrapings were collected from infected and recovered areas from each animal and were examined microscopically during the course of the experiment to detect sarcoptic mite.

### Biochemical analysis

To assess the toxic effects of propolis in the livers of the rabbits, serum samples were collected from the rabbits. Alanine Aminotransferase (AST), Aspartate Aminotransferase (AST), and Alkaline Phosphatase (ALP) were measured [19] using commercial kits (Roche) with a Reflotron® Plussy system machine (Roche, Mannheim, Germany) as well as serum cortisol levels [20] using commercial, coated tube radioimmunoassay kits (Pantex, Santa Monica, CA), and the results were statistically analysed for assessment before and after treatment.

### Histopathological study

Tissue samples were fixed in 10% neutral formalin for 24 hours, and paraffin blocks were obtained and routinely processed for light microscopy. Slices of 4-5 µm thickness were obtained from the prepared blocks and stained with haematoxylin-eosin. The preparations were visualized using a Nikon microscope at a magnification of x400 [21].

### Scanning electron microscope

Specimens of parasites were collected from skin lesions scraping in tubes, and fixed in (2.5%) buffered glutaraldehyde overnight at 4°C. The tubes were checked frequently, and then centrifuged for 5 min at 1500 rpm. The supernatant was removed, and the specimens were fixed in (1%) osmium tetroxide in the same buffer for one hour at 4°C. Dehydration was carried out in graded series of acetone from (30-100%). Specimens were dried at a critical point-drying apparatus, mounted on stubs and were coated with gold according to standard procedures [22]. The prepared specimens were examined by Zeiss DSM 940 electron microscope in the Electron Microscope Unite of King Saud University.

### Statistical analysis

Data are presented as the means and standard errors of the mean or standard deviations using the Statistical Package for the Social Sciences (SPSS v.22, Chicago, IL, USA). The results are expressed as the means  $\pm$  Standard Errors of the Mean (SEM). All statistical comparisons between the control and the treated groups were performed using One-Way Analysis of Variance (ANOVA) followed by Dunnett's post-hoc test for multiple comparisons. Significance was assigned at the level of ( $P < 0.05$ ) [23]. Receiver Operating Characteristics (ROC) curve analysis was performed. Area Under the Curve (AUC), cut-off values, and degree of specificity and sensitivity were calculated.

## Results

### Macroscopic evaluation

The healing from clinical signs was observed after 7 days of treatment by 10% propolis ointment either alone (Figures 1a and 1b) or in combination with ivermectin (Figures 1c and 1d). Itching, scale formation, and anxiety were vanished. Moreover, smooth skin and new hair growth were observed after 15 days of treatment. Recovery was late till the 28<sup>th</sup> day of the experiment in ivermectin-treated group (Table 1). Untreated control rabbits exhibited signs of sarcoptic mange throughout the study (Figure 1e). Seven days post treatment; skin scrapings from group III and group V were contained dead mites. Fifteen days post treatment; the skin scrapings were negative for the presence of eggs, larvae and adult parasites. In group IV, dead mites were found in the microscopic examination until the end of the 28<sup>th</sup> day and eggs of sarcoptic mite were detected in scraping at the end of the experiment (Figure 1f).

**Table 1.** Mean time on days for the treatment of infested rabbits with mites by 10% propolis ointment, ivermectin (400 µg/kg, S/C), and propolis ointment in combination with ivermectin.

Treatment	Mean time (days) of face, nose, feet and body mange recovery
10% Propolis ointment	7-15
Ivermectin (400 µg/kg, S/C)	21-28
10% Propolis ointment+ivermectin (400 µg/kg, S/C)	7-15

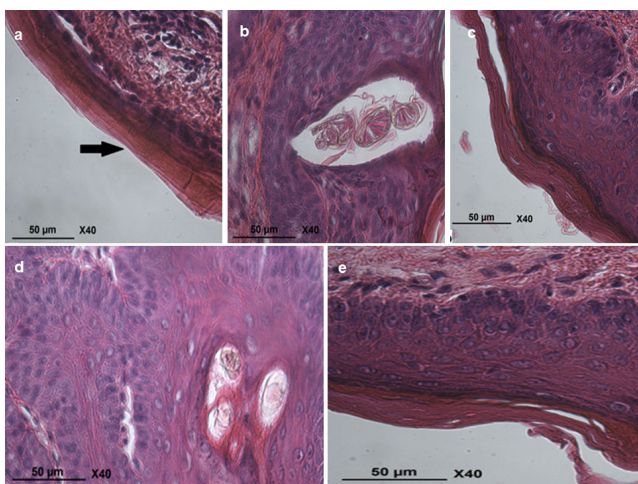
### Histopathological studies

The histopathological studies on healthy control group skin revealed non infested epidermis (Figure 2a). The positive control group skin revealed heavy infiltration of eosinophil's, lymphocytes, and mast cells in the epidermis and dermis of skin. There was hyper keratinization and sloughing of the epidermal layer. Section of embedded mites in the damaged tissue was also seen (Figure 2b). The skin of propolis group revealed complete healthy skin formation (Figure 2c), ivermectin group skin revealed egg in cornified epithelium

(Figure 2d), and rabbit's skin in 10% propolis ointment with ivermectin (400 µg/kg, S/C) treated group showed stratum corneum free from mites (Figure 2e).



**Figure 1.** Rabbits infested with *S. scabiei* var. *cuniculi* (a) sarcoptic mange in the skin of nose, ears, and around the eye before treatment. (b) Recovery from mange 10 days after treatment with propolis ointment, the skin had no scales and hair growth appeared. (c) Infested rabbit's body surface with *S. scabiei* before treatment. (d) Recovery 10 days after treatment with propolis ointment in combination with ivermectin. (e) Control untreated rabbits: alopecia all over body surface. (f) Light microscopy of *S. scabiei* egg detected in scrubbing from rabbits treated with ivermectin (x400).

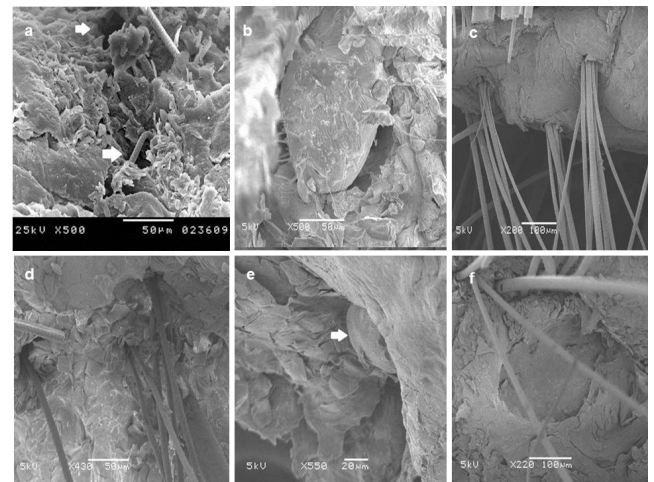


**Figure 2.** Light microscopy of rabbit skin. (a) Non-infested epidermis of negative control group. (b) Sections of mites embedded in the skin of the infested rabbit. (c) 10% propolis ointment treated group, showing non infested epidermis. (d) Ivermectin (400 µg/kg, S/C) treated group, showing regeneration of the stratum corneum and eggs of sarcoptic mite in the cornified epithelium. (e) Propolis ointment in combination with ivermectin treated group, showing stratum corneum free from mites (H and E, x40).

### Scanning electron microscopy (SEM)

The skin of group I showed numerous cracks of mite penetration (white arrow) (Figure 3a) in the epidermis and embedded mites in the keratinized layer (Figure 3b). Group II showed completely formed stratum corneum (Figure 3c).

Group III showed healthy skin layers (Figure 3d). Group IV skin revealed the existence of *S. scabiei* eggs (Figure 3e). Group V showed destructed stratum corneum (Figure 3f).



**Figure 3.** SEM of rabbit skin (a) Epidermis of infested control covered with crusts, white arrows point to skin tunnels. (b) *S. scabiei* mite embedded in tunnels (x500). (c) Healthy skin of negative control (x200). (d) Recovery of sarcoptic mange lesions after propolis ointment application (x430). (e) Eggs of sarcoptic mite (white arrow) (x550) after ivermectin (400 µg/kg, S/C). (f) Destructive skin occupied by burrowing tunnels empty from mites after treatment with propolis ointment combined with ivermectin.

### Biochemical analysis

The levels of total protein of rabbits in ivermectin (400 µg/kg, S/C) treated group were significantly ( $P \leq 0.05$ ) increased compared to the corresponding values of healthy control. No significant differences were noted between the healthy control group and the treated groups in the levels of albumin and globulin. ALP, ALT, AST and cortisol were the most affected parameters. ALP was significantly decreased in the treated groups ( $P \leq 0.05$ ) compared to the healthy control group. AST and ALT levels were significantly increased in the positive control group ( $P \leq 0.05$ ) compared to those of the healthy control group. AST was significantly decreased ( $P \leq 0.05$ ) in ivermectin (400 µg/kg, S/C) treated group. ALT levels were significantly decreased ( $P \leq 0.05$ ) in the treated groups compared to those of the healthy control group. Cortisol levels were significantly increased ( $P \leq 0.05$ ) in the positive control and ivermectin treated groups compared to those of the healthy control group while it was significantly decreased ( $P \leq 0.05$ ) in 10% propolis ointment groups (groups III and V). The percentage change of the measured parameters in treated groups compared to the healthy control (Table 2). ROC analysis showed satisfactory values of area under the curve, sensitivity and specificity (Table 3). Result revealed that the application of propolis had no aggressive effects on liver factors.

**Table 2.** Mean  $\pm$  S.D and independent t-test for total protein, albumin, globulin, ALP, AST, ALT and serum cortisol in control positive, 10% propolis ointment, ivermectin (400 µg/kg, S/C) and propolis ointment in combination with ivermectin groups compared to healthy control.

Parameters	Group	N	Mean ± S. D.	% Change compared to (-ve control)	P-value
Total protein (g/dl)	(-ve) Control	10	5.40 ± 0.84	100.00	
	(+ve) Control	10	5.80 ± 0.42	107.41	0.556
	10% Propolis ointment	10	5.00 ± 0.94	92.59	0.556
	ivermectin	10	6.60 ± 0.52	122.22	0.002*
	Propolis +ivermectin	10	4.80 ± 0.79	88.89	0.215
Albumin (g/dl)	(-ve) Control	10	3.80 ± 0.42	100.00	
	(+ve) Control	10	3.40 ± 0.52	89.47	0.151
	10% Propolis ointment	10	3.60 ± 0.52	94.74	0.705
	ivermectin	10	4.00 ± 0.00	105.26	0.705
	Propolis +ivermectin	10	3.40 ± 0.52	89.47	0.151
Globulin (g/dl)	(-ve) Control	10	1.60 ± 0.52	100.00	
	(+ve) Control	10	2.40 ± 0.52	150.00	0.066
	10% Propolis ointment	10	2.20 ± 0.79	137.50	0.227
	ivermectin	10	2.40 ± 0.52	150.00	0.066
	Propolis +ivermectin	10	2.00 ± 1.15	125.00	0.570
Alkaline phosphatase	(-ve) Control	10	114.20 ± 30.77	100.00	
	(+ve) Control	10	29.60 ± 11.64	25.92	0.001*
	10% Propolis ointment	10	30.00 ± 5.96	26.27	0.001*

(IU/L)	ivermectin	10	37.20 ± 13.80	32.57	0.001*
	Propolis +ivermectin	10	62.60 ± 5.06	54.82	0.001*
AST (IU/L)	(-ve) Control	10	48.60 ± 1.94	100.00	
	(+ve) Control	10	63.06 ± 2.30	129.76	0.001*
	10% Propolis ointment	10	46.60 ± 3.80	95.88	0.479
	ivermectin	10	38.30 ± 4.51	78.81	0.001*
	Propolis +ivermectin	10	45.80 ± 3.45	94.24	0.200
ALT (IU/L)	(-ve) Control	10	45.00 ± 2.05	100.00	
	(+ve) Control	10	50.68 ± 2.77	112.62	0.001*
	10% Propolis ointment	10	12.10 ± 1.73	26.89	0.001*
	ivermectin	10	11.80 ± 1.04	26.22	0.001*
	Propolis +ivermectin	10	13.80 ± 2.25	30.67	0.001*
Serum cortisole (ng/ml)	(-ve) Control	10	2.73 ± 0.56	100.00	
	(+ve) Control	10	5.18 ± 0.95	190.02	0.001*
	10% Propolis ointment	10	1.92 ± 0.15	70.36	0.030*
	ivermectin	10	3.92 ± 1.05	143.65	0.001*
	Propolis +ivermectin	10	1.84 ± 0.17	67.57	0.015*

\*Significant level between the studied groups compared to healthy control when P ≤ 0.05.

**Table 3.** ROC-curve of all parameters in control positive, 10% Propolis ointment, Ivermectin (400 µg/kg, S/C) and propolis ointment in combination with ivermectin groups.

Parameters	Group	Area under the curve	Cut-off value	Sensitivity%	Specificity%
Total protein (g/dl)	(+ve) Control	0.620	5.500	80.0%	40.0%
	10% Propolis ointment	0.620	5.500	60.0%	60.0%
	ivermectin	0.880	6.500	60.0%	100.0%
	Propolis+ivermectin	0.700	5.500	80.0%	60.0%
Albumin (g/dl)	(+ve) Control	0.700	3.500	60.0%	80.0%
	10% Propolis ointment	0.600	3.500	40.0%	80.0%
	ivermectin	0.600	3.500	100.0%	20.0%
	Propolis+ivermectin	0.700	3.500	60.0%	80.0%
Globulin (g/dl)	(+ve) Control	0.820	2.500	40.0%	100.0%
	10% Propolis ointment	0.720	2.500	40.0%	100.0%
	ivermectin	0.820	2.500	40.0%	100.0%

	Propolis+ivermectin	0.560	3.000	20.0%	100.0%
Alkaline phosphatase (IU/L)	(+ve) Control	1.000	57.500	100.0%	100.0%
	10% Propolis ointment	1.000	56.000	100.0%	100.0%
	ivermectin	1.000	63.500	100.0%	100.0%
	Propolis + ivermectin	1.000	69.500	100.0%	100.0%
AST (IU/L)	(+ve) Control	1.000	55.620	100.0%	100.0%
	10% Propolis ointment	0.640	46.900	60.0%	80.0%
	ivermectin	1.000	45.295	100.0%	100.0%
	Propolis+ivermectin	0.760	45.950	60.0%	100.0%
ALT (IU/L)	(+ve) Control	0.920	48.405	80.0%	100.0%
	10% Propolis ointment	1.000	28.460	100.0%	100.0%
	ivermectin	1.000	27.870	100.0%	100.0%
	Propolis+ivermectin	1.000	29.510	100.0%	100.0%
Serum cortisole (ng/ml)	(+ve) Control	1.000	3.930	100.0%	100.0%
	10% Propolis ointment	1.000	2.195	100.0%	100.0%
	ivermectin	0.880	2.840	100.0%	80.0%
	Propolis+ivermectin	1.000	2.190	100.0%	100.0%

AUC: Area Under the Curve; AUC=0.5-0.65 (useless biomarker); AUC=0.7-0.85 (good biomarker); AUC=0.86-1 with satisfactory sensitivity and specificity (excellent biomarker).

## Discussion

The drugs currently used for sarcoptic mange possess several limitations, such as the development of resistance [7], toxicity [8], and management difficulties increasing the need for safer and more effective drugs. Further studies on the treatment of sarcoptic mange with natural and herbal elements are needed. Topical application of 10% propolis ointment indicated that using this compound on *S. scabiei* effectively killed the mites. Complete recovery was observed 15 days after treatment. However, in ivermectin (400 µg/kg, S/C) treated rabbits recovery was observed at the end of the experiment. Our result of treatment success was formerly applied by [13,24,25] who registered the same factors in treatment of mange in rabbits but by using oils.

Pathological investigation revealed infiltration of inflammatory cells in epidermis and dermis and cross sections of embedded mites similar to those previously described in wild mammals [26], rabbits [3,27], and in sheep [28]. The histological structure of the epidermal and dermal layers was normal at the 2<sup>nd</sup> week of treatment with 10% propolis ointment. Skin sections from ivermectin-treated group showed slow improvement and somewhere few inflammatory cells and eggs were observed at the end of the experiment.

SEM of the skin samples taken from the 10% propolis-treated group revealed improvement in stratum corneum. This may be due the keratolytic effect of propolis. Similar result was recorded in ivermectin-treated group in addition to detection of

*S. scabiei* eggs. Oppositely, the SEM image in the skin of positive control-group rabbits revealed adult mites and eggs in tunnels. These findings were similar to those reported by other investigators [29,30].

The results revealed that the level of total protein of rabbits in ivermectin-treated group was significantly ( $P \leq 0.05$ ) increased compared to values in the healthy control. This result is in conflict with a previous study [14], in which total protein level was significantly decreased in the ivermectin groups. The administration of ivermectin in rabbits revealed non-significant increase in serum albumin. Our findings are not consistent with another study in rats [13] and in rabbits [31] in which ivermectin led to significant decrease in serum albumin. The results revealed no significant differences between the treatment and control groups in the globulin levels [32]. In addition, our findings are not consistent with [13], in which serum globulin of rabbits in positive control and ivermectin groups was significantly ( $P \leq 0.05$ ) decreased compared to values in the negative control. On the other hand, the total protein and albumin are not significantly affected [32]. ALP levels in groups II, III, IV, and V were significantly decreased compared to the negative control group. Adverse effect of scabies in liver has been indicated by [33,34]. The mechanism of action behind the low ALP level has been partially attributed to weight loss in many rabbits which could be due to the “energy demands” of mange, such as production of parakeratotic scale and scratching, as well as to the lowered food intake [35]. AST and ALT levels were significantly increased in the positive control compared to values in the

healthy control. AST was significantly decreased in ivermectin group which indicates the negative effects on hepatic factors. In addition, ALT was significantly decreased in ivermectin, Propolis, and propolis combined with ivermectin groups compared to the healthy control. This result is in conflict with a previous study of rabbits [13,36], rats [31], and rams and bucks [37] in which ivermectin-treated groups experienced inflammation of liver cells and impaired liver functions. The serum AST and ALT levels were significantly increased after 28 days of treatment with ivermectin in swine and cattle [38]. Cortisol is an important hormone released in response to stress [39]. Serum cortisol is often used in stress and welfare assessments [40,41]. The levels of cortisol in rabbits were significantly increased in the positive control and ivermectin-treated groups compared to values in the healthy control [20]. The levels of cortisol in propolis and propolis combined with ivermectin groups were significantly decreased compared to positive control and they are less significantly different compared to healthy control.

High values of specificity, sensitivity and Area Under the Curve (AUC) measured by ROC analysis suggested that the studied biomarkers could be used as predictive tools in testing the side effects of ivermectin and the potency of 10% propolis ointment in treating mange in rabbits. Among the measured parameters ALP, AST, ALT, and cortisol markers were the most predictive recording high specificity and sensitivity and AUC of almost 1.

Collectively, the results of this study demonstrated that propolis displays an antiscarptic effect under *in vivo* conditions. Various concentrations and application methods of propolis are required to further examine the effectiveness of propolis in the treatment and healing of sarcoptic mange lesions. We recommend future molecular studies to further examine the apoptotic pathway of this molecule.

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