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Efficacy of topical application of carvacrol, amitraz, and combination of carvacrol-amitraz for the treatment of dogs with generalized demodicosis

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ABSTRACT

Canine demodicosis is an inflammatory parasitic skin disease caused by an increasing population of *Demodex spp*. mite. Thus, there will be an urgent need for effective, safe, and available treatment for demodicosis. This study aimed to evaluate the activity of Carvacrol %5, Amitraz %0.05, and Carvacrol-Amitraz combination on killing *Demodex canis* mites after topical pour-on use and analyzing the improvement in associated dermatologic signs. Fifteen dogs with naturally acquired generalized demodicosis were randomly allocated to 3 equal study groups. The dogs were selected from the animal shelter in Mazandaran province. Treatment groups included a pour-on solution of 50 ml of Carvacrol %5, 50 ml of Amitraz %0.05, and the combined Carvacrol and Amitraz 1:1 mixture at the same concentrations. Evaluation of clinical factors consisted of mite count by skin scraping, hair re-growth, comedones, erythema, crusts, and scales during seven-day intervals over four weeks. Mite counts significantly decreased in all groups during the study, especially in Carvacrol %5 ($p<0.05$). There was no significant difference between Amitraz and Carvacrol-Amitraz on days 14, 21, and 28 ($p>0.05$). The highest decrease in mite population was associated with Carvacrol %5 from day 14 up to %90 in demodicosis treatment. This clinical field study demonstrated that pour-on administration of Carvacrol %5 is highly effective in the reduction of mite numbers and improvement in associated dermatologic signs of demodicosis.

اثربخشی کاربرد موضعی کارواکرول، آمیتراز و ترکیب کارواکرول آمیتراز در درمان سگ‌های مبتلا به دمودیکوزیس عمومیت یافته

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چکیده

دمودیکوزیس سگ یک بیماری پوستی انگلی التهابی است که با افزایش جمعیت گونه‌های جرب دمودکس ایجاد می‌شود. بنابراین، نیاز فوری به درمان موثر، ایمن و در دسترس برای دمودیکوزیس وجود خواهد داشت. این مطالعه با هدف ارزیابی فعالیت کارواکرول ۵ درصد، آمیتراز ۰/۰۵ درصد و ترکیب کارواکرول- آمیتراز در کشتن جرب های دمودکس کنیس پس از استفاده موضعی و تجزیه و تحلیل بهبود علائم پوستی مرتبط انجام شد. ۱۵ سگ مبتلا به دمودیکوز عمومیت یافته اکتسابی طبیعی به طور تصادفی به ۳ گروه مطالعه مساوی تقسیم شدند. سگ ها از پناهگاه حیوانات استان مازندران انتخاب شدند. گروه های درمانی شامل محلول ریختنی ۵۰ میلی لیتر کارواکرول ۵ درصد، ۵۰ میلی لیتر آمیتراز ۰/۰۵ درصد و مخلوط کارواکرول و آمیتراز ۱:۱ در غلظت های یکسان بودند. ارزیابی فاکتورهای بالینی شامل شمارش کنه شامل تراشیدن پوست، رشد مجدد مو، جوش های زیرپوستی، قرمزی، میزان کبره بستن و پوسته ریزی در فواصل هفت روزه در چهار هفته بود. تعداد جرب ها در تمام گروه ها به ویژه در کارواکرول ۵ درصد به طور معنی داری کاهش یافت ($p<۰/۰۵$). تفاوت معنی داری بین آمیتراز و کارواکرول آمیتراز در روزهای ۱۴، ۲۱ و ۲۸ وجود نداشت ($p>۰/۰۵$). بیشترین کاهش جمعیت جرب با کارواکرول ۵ درصد از روز ۱۴ تا ۹۰ درصد در تیمار دمودیکوزیس همراه بود. این مطالعه میدانی بالینی نشان داد که تجویز کارواکرول ۵٪ به صورت تزریقی در کاهش تعداد جرب و بهبود علائم پوستی مرتبط با دمودیکوزیس بسیار موثر است.

واژه های کلیدی: دمودیکوزیس، کارواکرول، آمیتراز، پوست، ریختنی

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INTRODUCTION

Canine demodicosis is one of the well-known inflammatory skin parasitic diseases associated with the marked increase in *Demodex spp*. mites. The mites are predominantly present in the hair follicles and sebaceous glands [1]. These mites are resident commensal organisms in the hair follicles of many mammals. Small numbers of these mites are regarded as normal microfauna in the skin of healthy dogs, but the proliferation of mites may lead to an important disease [2]. In dogs, puppies acquire mites from their mother after birth through direct skin contact, yet puppies that are raised in isolation after cesarean section do not have any *Demodex* mites [3]. There are three recognized *Demodex* species in dogs which include: *Demodex canis*, *Demodex injai*, and *Demodex cornei*. *Demodex canis* is the most important and best-known of the three canine Demodex species [4]. *Demodex injai* is much longer than *Demodex canis*. Genetic comparisons revealed that *Demodex canis* and *Demodex injai* were two different species, but *Demodex cornei* with a shorter body than other canine Demodex mites seems to be a morphological variant of *Demodex canis*. published data indicates similar efficacy of reported treatments regardless of *Demodex* type [5]. Canine demodicosis is classified as localized and generalized demodicosis based on clinical manifestations and adult or juvenile-onset demodicosis depending on the age of the affected dog [3, 5]. The localized form appears as lesions characterized by scattered alopecia, comedones, and often mild erythema in young dogs. Lesions in localized demodicosis are considered no more than four lesions and smaller than 2.5 cm in diameter. Localized demodicosis is a benign disease and most cases are resolved spontaneously [1]. Generalized demodicosis may be a severe life-threatening disease with the evident symptoms of alopecia, redness, comedones, follicular papules to pustules, scales, furunculosis, cellulitis, and secondary bacterial infection that are characterized by five or more affected areas, or by lesions covering an entire region of the body, and/or Pododemodicosis involving two or more paws [6, 7]. The generalized form of demodicosis may develop from the localized condition or occur spontaneously in older animals with underlying disease, those who are under severe stress, and immune-compromised animals due to other diseases or undergoing immunosuppressive therapies. It can occur in the form of juvenile demodicosis in dogs aged from 2 to 18 months or as an adult-onset disease in mature dogs [8, 9]. The diagnosis is based on clinical signs, and laboratory tests, such as deep skin scrapings which is a choice diagnostic test for the detection of *Demodex* mites. A definitive diagnosis is established by the presence of numerous adult mites and/or immature forms on microscopic examination of deep skin scrapings. Trichograms, acetate tape impressions, and skin biopsy may also be performed as an alternative diagnostic test to deep skin scrapings [10, 11]. Treatment of generalized demodicosis is a challenging and time-consuming process for both dog owners and veterinarians. Only a few products, either topical or systemic, are labeled for the treatment of generalized demodicosis whereas others are off-label, and some have the potential to cause severe adverse reactions [12]. The approved mainstay treatment for canine demodicosis includes the use of amitraz as a leave-on rinse at the recommended concentration rate of 0.025–0.05%, once a week or every two weeks [13, 14]. While amitraz has shown varying effectiveness in treating canine demodicosis, it is important to note that the use of this medication can lead to adverse effects in dogs, including depression, ataxia, skin irritations, pruritus, increased thirst, hyperglycemia, slow heart rate, high blood sugar, vomiting, and

diarrhea, which are associated with amitraz poisoning [1]. Macrocyclic lactones, such as ivermectin, doramectin, and moxidectin demonstrated better efficacy in the treatment of canine generalized demodicosis. These off-label drugs had the potential for toxicity, particularly in collie breeds, due to the mutations for (MDR-1) multidrug resistance mutation 1 (P-glycoprotein deficiency) in these breeds [3, 15]. While milbemycin oxime is authorized for the treatment of canine demodicosis in certain countries, it is worth noting that dogs homozygous for the MDR-1 mutation may experience certain adverse effects associated with its use [16, 17]. Another treatment protocol for canine demodicosis is the use of the combination of amitraz + metaflumizone, amitraz + fipronil + Methoprene, and moxidectin + imidacloprid; these topical application products provide convenient and safer treatment with different rate of efficacy [7, 18, 19]. Recently isoxazoline class ectoparasiticides including fluralaner, sarolaner, lotilaner, and afoxolaner introduced to veterinary medicine for the treatment of canine demodicosis, with excellent therapeutic results in published data [20-23]. While adverse reactions during treatment with isoxazolines are highly uncommon, there have been reported cases of gastrointestinal symptoms like reduced appetite, vomiting, and non-bloody diarrhea, as well as neurological manifestations such as seizures [8, 24, 25]. The potential of herbal medicine has been recognized as a viable and safer alternative to conventional drugs due to its minimal side effects, low occurrence of resistance, affordability, and wide availability, particularly in developing nations [26]. Although, the skin's outer layer, the stratum corneum, acts as a barrier against enhancing the efficacy of certain components in defending against infections, there are situations where therapeutic methods must penetrate the deeper layers to increase plasma concentration and effectively eliminate microorganisms [27, 28]. The factors that increase the skin barrier penetration include surfactants [29] fatty acids/esters [30] solvents [31], and terpenes [32]. One of the most effective terpenoids is Carvacrol which has an essential role as a penetration enhancer and also has anti-parasitic effects such as dermal demodicosis [28]. Carvacrol is a monoterpene phenolic bioactive compound of various medical plant's essential oils, especially the Labiatae family, including *Origanum*, *Satureja*, *Tymbra*, *Thymus*, and *Coridothymus* species [33, 34]. Numerous articles have highlighted the diverse pharmacological properties of carvacrol, which include anti-inflammatory, antibacterial, antifungal, antioxidant, and anticancer effects [35-37]. Carvacrol has been reported to have insecticidal and acaricidal activity against agricultural, stored products, and medical arthropod pests [38]. Carvacrol is also an attractive and safe food additive in many countries because of its low toxicity and low cost of production. European Union Food Improvement Agents FAO/WHO and the Joint Expert Committee on Food Additives (JECFA) have classified carvacrol as a safe flavoring agent for human consumption [39-41]. Earlier studies have documented the remarkable acaricidal effectiveness of various herbal essential oils and their bioactive constituents, with particular emphasis on tea tree oil, in combating mites belonging to the Demodex genus (*Demodex folliculorum* and *Demodex brevis*) in humans [42, 43]. Moreover, in a few articles the in vitro acaricidal effect of tea tree oil as a herbal essential oil on *Demodex canis* mites was evaluated [44]. Thyme oil and carvacrol, recognized as the primary active constituents, have been assessed for their efficacy in eradicating *Demodex folliculorum* and *Demodex brevis* mites in humans. These studies have demonstrated superior and more potent mite-killing abilities compared to tea tree

oil, black seed oil, St. John's Wort oil, and sage oil [43]. The efficacy of carvacrol against *Demodex canis* has never been evaluated. Therefore, the present study aimed to assess the in vivo acaricidal effects of carvacrol 5%, carvacrol 5% + amitraz 0.05% (50/50 mixtures), and amitraz 0.05%, and evaluate the clinical signs and improvements of each solution on dogs with demodicosis.

MATERIALS AND METHODS

Animals

Fifteen Mongrel dogs (6 male and 9 female), older than one year old, and average 20-25 kg bodyweight, with natural infestations of *Demodex spp*. mites and presenting clinical signs of generalized demodicosis (hair loss, erythema, comedones, follicular casts, and crusts on more than five areas or the entire body, and pododemodicosis involving two or more feet) were used in the study. Deep skin scrapings performed on all dogs were positive for *Demodex spp*. Mites before starting the survey. Except for clinical signs of generalized demodicosis, the dogs were healthy on veterinary assessments and had not been treated with any miticidal product for at least two months before inclusion in the study. The dogs were allocated to three equal groups. Each dog was housed individually in kennels that conformed to accepted animal welfare guidelines for the duration of the study. Each pen was approximately 3.5 m × 2 m with an indoor/outdoor run, and the outdoor run area was covered to prevent exposure to rain. There was no contact between dogs and the cages were cleaned daily. All dogs were fed appropriate maintenance canned food and had access to water ad libitum. For animal welfare considerations there was no negative control group.

Treatment

On day 0, dogs of group 1 were treated with 50ml of a formulation containing carvacrol 5% for each dog, group 2 was treated with 50ml of a formulation containing carvacrol 5% + amitraz 0.05% (50-50) for each dog, and group 3 were treated with 50ml of a formulation containing amitraz 0.05% for each dog. Carvacrol 98% (Lot: MCDK6589), and Amitraz (EC number: 251-375-4) purchased from Sigma Aldrich, St. Louis, USA. All formulations were prepared on the day of treatment and were applied to the skin as a single pour-on at the region of the vertebral column between the shoulders to the tail.

Mite assessments

Mite counts were performed on days 0, 7, 14, 21, and 28, to assess Demodex mite infestations. Deep skin scrapings from five sites of skin lesions were performed with a blade, in which the 2×2 cm^2 of skin was squeezed and scraped until capillary oozing occurred. The scraping samples were transferred onto a separate labeled microscope slide containing mineral oil and observed under a microscope for the presence of *Demodex spp*. mites. The numbers of mites (immature and adult) and mite eggs in each scraping were counted and recorded separately. The same sites of initial skin scrapings and/or new lesions were scraped at each subsequent examination.

Skin and hair clinical scores

To evaluate the effects of treatments on demodectic lesions and clinical signs in each dog. All dogs were assessed on the days when skin scrapings were made. The clinical signs such as body areas with alopecia, erythema, comedones, and skin with scales and crusts

were assessed for each dog and sketched on a silhouette (left and right-hand side) diagram of a dog. The dogs were also photographed before treatment administration and at various times during the study to illustrate the extent of lesions and their resolution of lesions after treatment. The success rate was defined as the percentage of dogs in each group that had a great reduction in mite numbers or were negative for live mites and eggs. The secondary efficacy was based on the resolution of clinical signs of demodectic mange (erythema, comedones, and crusts/scales), and was calculated from the percentage of dogs that resolved each sign, after treatment during the assessment period of 28 days.

A semi-quantitative assessment of hair regrowth was made, comparing alopecia and hair coat before, within, and after the study duration. Hair regrowth was assessed as a percentage and divided into three categories as < 50% hair regrowth occurred, 50-90% hair regrowth occurred, and > 90% hair regrowth took place.

Statistical analysis

The primary efficacy was based on a decrease in mite counts on dogs in each treatment group on each assessment day compared with pre-treatment mite counts. The average percentage reduction in mite counts was derived from geometric means (gm) and calculated by using Abbott's formula as follows: %Efficacy (%mite reduction) = (gm pre-treatment – gm post-treatment)/ gm pre-treatment ×100 [17]. To investigate the effect of treatment groups (Carvacrol, Amitraz, and combination groups) and the days elapsed since treatment on the average number of mites in clinical conditions, repeated measures ANOVA and Tukey's post-hoc test were applied to compare pairwise medians were used. This test was used to compare treatment groups during 0, 7, 14, 21, and 28 days of study. Statistical analysis was performed using SPSS version 26 (SPSS Inc., Chicago, IL, USA). All results were expressed as Mean ± SD. A significant level of statistical analysis is considered by p-value<0.05.

RESULTS

Mite counts

All the enrolled dogs were confirmed to have live *Demodex* mites before the treatment (Figure 1). The geometric mean number of mites before treatment administration indicates relatively extreme pre-existing *Demodex* mites in each enrolled group (Table 1). There were no significant differences in the geometric mean number of mites between groups before treatment administration. Treatment with Carvacrol 5% resulted in a reduction in the geometric mean mite number from pre-treatment levels following skin scrapings. The Carvacrol 5% + Amitraz 0.05% (1:1) resulted in a reduction of the geometric mean mite number from pre-treatment levels following the skin scrapings. Treatment with Amitraz 0.05% resulted in a reduction of the geometric mean mite number from pre-treatment levels following the skin scrapings during 28 days of study time with seven days intervals (Table 1). There was no significant difference between treatment groups at day 0 ($p>0.05$). On day 7 after treatment, there was a significant difference between Carvacrol 5% and other groups ($p<0.05$). Mite counts decreased in all groups during the study ($p<0.05$) (Figure 1). In Amitraz 0.05% there was no significant difference between days ($p>0.05$). In both groups Carvacrol and Carvacrol + Amitraz there was a significant difference from 14 days in mite counts ($p<0.05$) but there were no significant differences during days 21 and 28

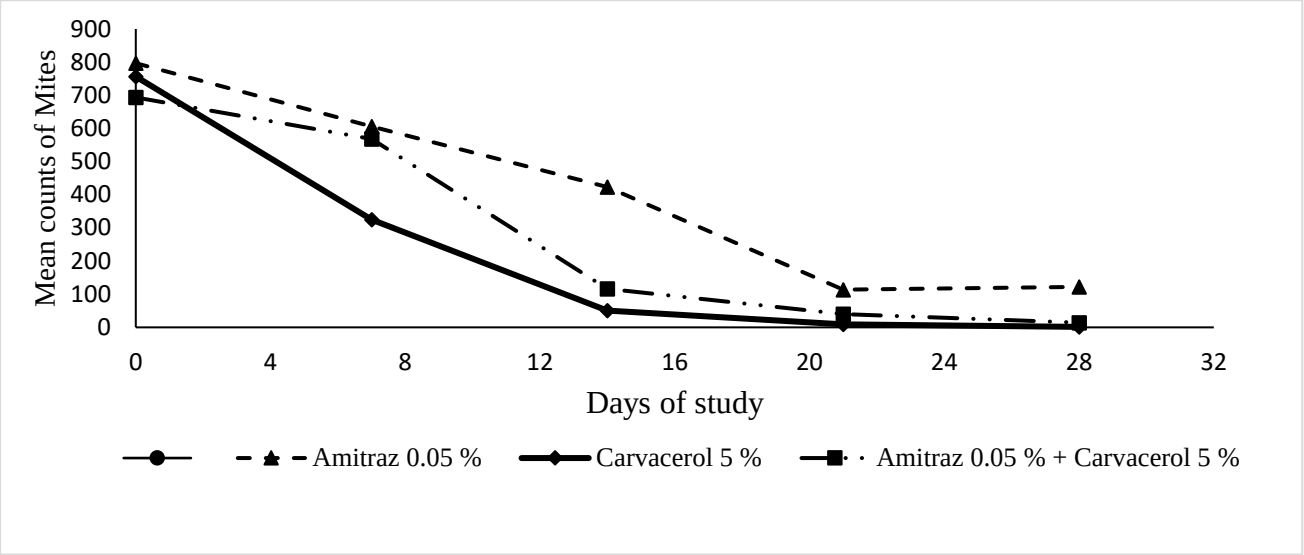

Figure 1: The trend of changes in the average number of Demodex spp. mites in dogs with demodicosis when exposed to different compounds during days of study

Table 1. The Mean ± SD of the number of mites with demodicosis and the percentage of mange reduction in response to different compounds. The numbers of *Demodex* spp. mites in treated groups based on geometric mean reductions

Group 1 - Carvacrol 5%				**Group 2 - Carvacrol 5%+Amitraz 0.05%**				**Group 3 – Amitraz 0.05%**			
Day	Geo mean	Reduction (%)	SD ±	Day	Geo mean	Reduction (%)	SD ±	Day	Geo mean	Reduction (%)	SD ±
0	$756.60^{a,A}$	-	73.57	0	$693.60^{a,A}$	-	89.70	0	$796.80^{A,a}$	-	98.69
7	$324.80^{b,B}$	57	68.88	7	$568.00^{a,C}$	18.1	71.20	7	$605.60^{a,AC}$	23.9	139.59
14	$50.40^{c,B}$	93.3	21.38	14	$115.80^{b,B}$	83.3	40.99	14	$423.20^{b,A}$	46.8	86.20
21	$8.60^{c,B}$	98.8	7.40	21	$40.40^{c,B}$	94.1	12.21	21	$113.80^{c,A}$	85.7	61.53
28	$1.40^{c,B}$	99.8	2.19	28	$14.20^{c,B}$	97.9	12.37	28	$121.80^{c,A}$	84.7	48.85

*Capital letters in each row shows significant difference ($p<0.05$), and lowercase letters in each column shows significant difference ($p<0.05$).

($p>0.05$). There was no significant difference between Amitraz and Carvacrol + Amitraz on days 14, 21, and 28 ($p>0.05$). The most significant decrease in mite population belongs to the Carvacrol 5% from day 14 up to 90% in *Demodex spp.* mites.

Clinical efficacy (mange and hair re-growth)

Clinical signs were reduced following all three treatments (Table 2). All enrolled animals had clinical signs of demodectic mange especially alopecia and erythema before the treatment. Following treatment in the Carvacrol 5%, the occurrence of erythema and scales/crusts were completely resolved from 80% to 0%. The occurrence of erythema and scale/crusts were reduced in both Carvacrol 5% + Amitraz 0.05% and Amitraz 0.05%, but there was no complete clearance in all cases (Table 2). There was only one dog with signs of comedones on Carvacrol 5% before the start of treatment which was resolved after 14 days (Table 2). At the end of the study, a marked hair re-growth was observed in the Carvacrol 5% treatment group. Although, dogs in both Carvacrol 5%+ Amitraz 0.05% and Amitraz 0.05% showed signs of hair re-growth, dogs in Carvacrol 5% + Amitraz 0.05% had better re-growth compared to the Amitraz 0.05% (Table 3).

Health observations

Table 2. Reduction of the clinical signs in dogs after treatment

Day	Clinical signs	Carvacrol 5% Number of dogs (%)	Carvacrol 5% + Amitraz 0.05% Number of dogs (%)	Amitraz 0.05% Number of dogs (%)
0	Erythema	4/5 (80)	5/5 (100)	5/5 (100)
	Comedones	1/5 (20)	0/5 (0)	0/5 (0)
	Scales or Crusts	4/5 (80)	3/5 (60)	5/5 (100)
7	Erythema	4/5 (80)	4/5 (80)	5/5 (100)
	Comedones	1/5 (20)	0/5 (0)	0/5 (0)
	Scales or Crusts	3/5 (60)	3/5 (60)	5/5 (100)
14	Erythema	3/5 (60)	3/5 (60)	4/5 (80)
	Comedones	0/5 (0)	0/5 (0)	0/5 (0)
	Scales or Crusts	2/5 (40)	3/5 (60)	4/5 (80)
21	Erythema	2/5 (40)	3/5 (60)	4/5 (80)
	Comedones	0/5 (0)	0/5 (0)	0/5 (0)
	Scales or Crusts	1/5 (20)	2/5 (40)	4/5 (80)
28	Erythema	0/5 (0)	1/5 (20)	3/5 (60)
	Comedones	0/5 (0)	0/5 (0)	0/5 (0)
	Scales or Crusts	0/5 (0)	1/5 (20)	3/5 (60)

*Number of the dogs in each group that showed the clinical signs.

Table 3. Evaluation of the hair re-growth on dogs after treatments

Day	Estimated percent hair re-growth								
	Carvacrol 5%			Carvacrol 5% + Amitraz 0.05%			Amitraz 0.05%		
	<50%	50-90%	>90%	<50%	50-90%	>90%	<50%	50-90%	>90%
7	4/5	1/5	0/5	5/5	0/5	0/5	5/5	0/5	0/5
14	2/5	1/5	2/5	2/5	1/5	2/5	3/5	2/5	0/5
28	0/5	2/5	3/5	1/5	2/5	2/5	2/5	2/5	1/5

Four dogs (one in Carvacrol + Amitraz and three in Amitraz) showed signs of mild redness in the skin in the region of treatment administration. All dogs returned to their normal state after 24 hours on their own. The mild skin redness was not persistent and had no adverse effect on the treatment groups during the study.

DISCUSSION

In this study, the efficacy of Carvacrol 5%, Carvacrol 5% + Amitraz 0.05%, and Amitraz 0.05% on canine demodicosis was evaluated during 28 days. Mite reduction count and demodicosis clinical signs changed significantly after pour-on administration in all treatment groups. Repetition of skin scrapings for checking the remission was not ascertained in this study. Following the result of this study, carvacrol had a promising potential to be introduced as an efficacious acaricide against Demodex spp. mites alone or in combination with other substances. For decades, scientists have been trying to find better and safer treatments for demodicosis [45]. All treatment protocols for demodicosis sometimes depending on the conditions showed side effects and were usually time-consuming [8]. Recently, new drugs have been introduced to treat demodicosis, which have had positive results in many cases. These drugs are not easily available or are highly priced, especially in developing countries. Amitraz which is an FDA-approved drug for the treatment of canine demodicosis is still used in many countries. Many cases of

human and animal poisoning with Amitraz have been reported [8, 46]. Carvacrol is a terpenoid that could increase skin barrier penetration [28, 32]. One of the goals of this study was to investigate whether the dosage of Amitraz can be decreased with the contribution of carvacrol. This clinical field study demonstrates that treatment with a pour-on formulation of carvacrol 5% at the dose rate of 50 ml/dog resulted in the reduction of mite numbers and improvement in clinical signs of demodicosis in dogs. Also, the combination of carvacrol 5% + amitraz 0.05% (1:1) and amitraz 0.05%, both at the dose rate of 50 ml/dog resulted in a reduction of mite numbers and improvement in clinical signs but in comparison with carvacrol 5% both were less effective. This indicated that carvacrol can be effective in killing demodex canis mites. Our data are consistent with the reports of studies concerning investigating the effects of some essential oils especially thyme oil on Demodex folliculorum, The results showed that one percent concentration of thyme oil had a more effective killing time than a 5% concentration of tea tree oil and sage oil, and a significant difference was found ($p<0.0001$) [43]. Carvacrol has been identified as the most prevalent active ingredient of thyme essential oil at a concentration of 59.93%, analyzed by the gas chromatography–mass spectrometry (GC/MS) method in this study [43]. In group 2 (carvacrol 5% + amitraz 0.05% (1:1)) in addition to the miticidal activity of carvacrol, it was expected that carvacrol would help to increase the entry and penetration of amitraz to the skin and hair follicles and the combination of carvacrol 5% + amitraz 0.05% will show the best results but, in this study, it had a lower effect on demodex mites in comparison with the group 1 (Carvacrol 5%). It was considered that the lesser volume of carvacrol 5% was the reason for the lower effect on demodex canis mites. In group 3 (Amitraz 5%) improvement in associated dermatologic signs and mite numbers was even lesser than in groups 1 and 2. This indicates the more powerful miticidal activity of carvacrol than amitraz in this In vivo study. Complete remission from demodicosis should be determined if two consecutive skin scrapings remain negative at a one-month interval after the cessation of treatment [47]. Based on health observations during the study, there were no apparent adverse reactions to treatment in the carvacrol 5% group which confirms carvacrol as a safe treatment in dogs suffering from demodicosis.

CONCLUSION

A high level of efficacy was achieved with the Carvacrol 5% following pour-on application. Carvacrol alone, or together with other drugs, can be introduced as an effective and safe therapeutic combination at a reasonable price in the field of veterinary medicine. In this study, Carvacrol effectively reduced the number of Demodex canis mites in dogs with demodicosis. It is suggested to conduct more studies on the therapeutic properties of carvacrol in veterinary medicine.

ETHICS

The study was performed under review and guidelines by the Ethics Committee of animal welfare, and standards for conducting Medical Research in Iran. The study was approved by the ethics committee of the Islamic Azad University of Tehran, Science and Research Branch (SRBIAU). Approval ID: IR.IAU.SRB.REC.1402.013.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

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