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Research Article

Amelioration of Sarcoptic Mange-Induced Oxidative Stress and Apoptosis in Dogs by Using *Calendula officinalis* Flower Extracts

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Twenty-four clinically *Sarcoptes*-infested dogs were randomly enrolled into three groups (groups A, B, and C), 8 dogs in each group. Group A was treated with ivermectin + *Calendula officinalis* flower extract (CFE). Group B was treated with ivermectin + N-acetyl cysteine (NAC) (standard positive controls), while group C (negative control) was treated with ivermectin alone. Another eight healthy dogs were used as healthy controls (group D). By day 28 after therapy, the dogs treated adjunctly with CFE as well as with N-acetyl cysteine revealed significant ($P \leq 0.001$) amelioration of the altered markers of oxidative stress towards normalcy. The dogs of these groups also revealed significant ($P \leq 0.001$) amelioration of apoptotic leukocytes towards normalcy, and values were comparable to those of the healthy controls. While by day 28 after therapy, ivermectin alone treated dogs could not achieve comparable values to those of the healthy controls. The dogs treated adjunctly with CFE and N-acetyl cysteine also revealed faster parasitological as well as clinical cure rate as compared to the ivermectin alone treated dogs. In conclusion, CFE has remarkable antioxidant and antiapoptotic potential and can constitute a potential adjunctive remedy with miticide for the therapeutic management of canine sarcoptic mange.

1. Introduction

Sarcoptic mange is a highly contagious and pruritic acariosis of the skin affecting abroad host range including more than 100 mammalian species belonging to 27 families from 10 orders [1, 2] and afflicts 300 million people globally. In many animal species, the prevalence of sarcoptic mange is very high and an untreated animal often succumbs [3, 4]. In addition to its potential to cause huge economic loss due to reduced production and increased mortality in animals, [1, 5] it is an emerging/reemerging infectious disease that threatens human and animal health globally [6–8]. In developing countries, it is a significant public health problem because it is highly prevalent and complications are frequent [9].

Emerging resistance to the currently available therapeutic permethrin and ivermectin has recently been reported from regions where previously effective acaricides have been used extensively in socially disadvantaged communities and in some developing countries [10–12]. In addition, drug residue can build up during extensive and long-term use, which poses environmental hazards and can lead to increased drug resistance in the target species [10, 13]. This emphasizes the need to identify potential targets for chemotherapeutic and/or immunological intervention.

Some of the most important predisposing factors for the development of sarcoptic mange are known to include the immune status of the animal, nutritional status, and oxidative stress [14–16]. Recently, we have also demonstrated the involvement of oxidative stress and increased apoptosis of peripheral blood leukocytes in pathogenesis of canine sarcoptic mange [17]. The current wide prevalence and decreasing treatment efficacy of sarcoptic mange necessitate the need for novel control strategies in the form of adjunctive alternative therapies.

In this context, much attention has been paid to antioxidants from natural sources, especially flavonoids and other phenolic compounds [18]. Various flavonoids (e.g., quercetin, apigenin, and tea catechins) have been shown to have anti-inflammatory activity by inhibiting cyclooxygenase-2 (COX2) and inducible nitric oxide synthase [19], which are related to antioxidant activity. Flavonoids may inhibit cytosolic and tyrosine kinase [20, 21] and neutrophil degranulation [20]. *Calendula officinalis* L. (Asteraceae) is cultivated for ornamental and medicinal purposes in Europe, China, USA, and India. It is used mainly for cutaneous and internal inflammatory diseases of several origins [22]. The main chemical constituents of *C. officinalis* include steroids, terpenoids, free and esterified triterpenic alcohols, phenolic acids, flavonoids (quercetin, rutin, narcissin, isorhamnetin, and kaempferol), and other compounds [23]. *Calendula* flowers contain large quantities of antioxidant compounds (flavonoids and polyphenols), suggesting that they may possess antioxidants to ameliorate sarcoptic mange-induced oxidative and immunological disparities. Keeping these facts under consideration, the present study was projected with the aim of determining the protective effect of *Calendula officinalis* flowers extract against sarcoptic mange-induced oxidative stress and apoptosis of leukocytes in dogs.

2. Materials and Methods

2.1. Plant Materials and Preparation of Extracts. Seedlings of *C. officinalis* were obtained from an authorized herbalist and cultivated organically. After complete blooming, the whole flower pods were harvested between 9 a.m. to 3 p.m. GMT; petals were separated and subjected to the shade to dry. Dried flowers were grinded to make the powder. The powder was subjected to ethanol (80%) extraction in Soxhlet apparatus and was concentrated under reduced pressure. Finally, CFE was dispensed in capsules and given orally to the infested dogs.

2.2. Animal Selection Criteria and Design of the Study. The dogs enrolled in the study were recruited among the patients presented for clinical and dermatological examination. All diseased dogs were naturally infested with *S. scabiei* var. *canis* and were reported to be suffering from the clinical disease for at least 15 days before presentation. None of the dogs had been treated with ectoparasiticides or steroidal anti-inflammatory drugs in the last 30 days before obtaining the first blood samples. On microscopic examination, stool samples of all enrolled dogs were found negative for helminth parasite eggs by sedimentation technique. The diseased dogs were also free from ectoparasites apart from the *S. scabiei* var. *canis* mite infestation.

Clinical diagnosis of sarcoptic mange was based on the following six clinical inclusion criteria: (1) intense pruritus, (2) papular eruption (abdomen, inner leg region, and thorax), (3) self-trauma, (4) excoriations, (5) alopecia, and (6) crusting of the elbow, ear margins, and hocks. Severity of *Sarcoptes*-induced skin lesions was also scored. Seven clinical symptoms (pruritus, erythema, papules, excoriations, crusts (scabs), alopecia, and pyoderma lesions) were assessed and rated on a scale from 0 (absent) to (severe 3). The seven scores were added up and expressed as a *Sarcoptes*-induced skin lesions score (SSLS) that could have values between 0 and 21 [24]. Dogs were included in the study only if *S. scabiei* var. *canis* mites or mites with their developmental stages were found in microscopic examination of material from up to six deep skin scrapings and revealed at least three clinical inclusion criteria. Diseased dogs were examined at weekly intervals throughout the study period. The main efficacy criterion was the parasitological cure rate, which was calculated as the proportion of dogs negative for *S. scabiei* var. *canis* mites on the basis of microscopic examination of deep skin scrapings obtained on days 14, 21, and 28 after the treatments. On days 0, 14, and 28, presence and severity of *Sarcoptes*-induced skin lesions were scored and used as secondary efficacy criteria.

Twenty-four *Sarcoptes*-infested dogs (9 intact males and 15 intact females) of 12–48-month age groups were selected for the study (8 mongrels, 6 German Shepherds, 4 Labrador Retrievers, and 3 each of Pomeranians and pugs) and were allocated randomly into three groups (groups A, B, and C), with 8 dogs in each group. Another eight 12–48-month-old clinically healthy dogs (3 Mongrels, 2 Labrador Retrievers and 1 each of Pomeranians, German Shepherds, and pugs) of either sexes (5 intact males and 3 intact females) were used as controls (group D). These dogs were also free from ectoparasites and helminth parasites on microscopic examinations of skin scraping materials and stool samples, respectively. The dogs of group A were subcutaneously given a commercial formulation of ivermectin 1% w/v solution at a dose rate of 0.2 mg/kg once weekly + CFE at a dose rate of 50 mg/kg orally, once every day. (The 50 mg/kg once daily dose of CFE was selected from a preliminary study conducted on mice (unpublished). In this study, CFE was given at the doses of 50, 100, and 200 mg/kg to protect the mice against cadmium chloride-induced oxidative stress). The dogs of group B were subcutaneously given a commercial formulation of ivermectin 1% w/v solution at a dose rate of 0.2 mg/kg once weekly + N-acetyl cysteine at a dose rate of 70 mg/kg orally twice a day as a standard antioxidant and were kept as positive standard controls, while the dogs of group C were treated subcutaneously with ivermectin alone at a dose rate of 0.2 mg/kg once weekly. As the dogs were client owned, it was unfeasible to standardize the diets for all animals. However, as per the owners, all the dogs were managed on a similar diet (mainly rice, chapatti, wheat dalia, milk, eggs, and cooked chicken and goat meat).

2.3. Blood Samples. Approximately a 5 mL of blood sample was obtained from each *Sarcoptes*-infested dog in EDTA containing tubes at day 0 before the start of the therapy and at day 28 after treatment. Further, blood samples were subjected to assays of oxidative stress and apoptosis. Single 5 mL of blood sample was also obtained from each healthy dog to determine the studied parameter and was used as a comparative standard.

2.4. Assays of Oxidative Stress. In order to evaluate the ameliorative potential of CFE adjunct therapy on sarcoptic

mange-induced oxidative stress, the markers of oxidative stress, for instance, levels of lipid peroxides, malondialdehyde (MDA), and potential antioxidant reduced glutathione (GSH) as well as the activities of antioxidant enzymes: glutathione peroxidase (GSH-Px), glutathione-S-transferase (GST), superoxide dismutase(SOD), and catalase (CAT) were determined in erythrocytes of dogs.

2.4.1. Preparation of Haemolysate and RBC Suspension. For assays of oxidative stress, 3 mL of blood samples from each dog were centrifuged at 200 ×g for 10 min to harvest the erythrocytes. Erythrocytes were washed thrice with normal saline solution, and finally, 10% haemolysate was prepared by adding chilled distilled water. For estimation of reduced glutathione, RBC suspension was prepared by adding equal volume of erythrocytes and normal saline solution. Haemolysate and RBC suspension were kept at −70°C and were used for antioxidant assay within 6 h. Haemoglobin concentration was estimated by cyanomethaemoglobin method [25].

2.4.2. Lipid Peroxides (LPO) Assay. The concentration of MDA, one of the markers of lipid peroxidation, was estimated in haemolysate following the method suggested by Placer et al. [26]. MDA contents were calculated on the basis of molar extinction coefficient of MDA-TBA complex at 548 nm, that is, 1.56×10^{-5}/mol/cm, and expressed in terms of μmol of MDA/mg of haemoglobin.

2.4.3. Full Thiol Content Assay. The concentration of total thiol in RBC suspension was estimated by 5,5-dithiobis-(2-nitro-benzoic acid) (DTNB) method as per the procedure of Prins and Loos [27]. Thiol concentration in the test sample was calculated by employing the molar extinction coefficient of DTNB-GSH conjugate (ηmol/mg of haemoglobin), 13600/M/cm.

2.4.4. Glutathione Peroxidase (GSH-Px) Assay. GSH-Px activity was determined by the method of Paglie and Valentie [28]. Activity was determined spectrophotometrically by coupling the oxidation of glutathione and NADPH using glutathione reductase. GSH-Px activity was standardized against protein concentrations and expressed as NADPH ηmol oxidized per min per mg of haemoglobin (mU/mg Hb) by using the molar extinction coefficient (ε) of 6200 at 340 nm.

2.4.5. Glutathione-S-Transferase (GST). The GST activity in erythrocytes was determined according to the standard procedure of Habig et al. [29]. The specific activity of GST is expressed as mmol of GSH-CDNB conjugate formed/min/mg of haemoglobin using an extinction coefficient of 9.6/mM/cm.

2.4.6. Superoxide Dismutase (SOD) Assay. SOD activity in haemolysate was measured by using nitro blue tetrazolium as a substrate after suitable dilution as per the method suggested by Minami and Yoshikawa [30]. One unit of SOD activity was defined as the amount of enzyme that inhibited autooxidation by 50% under the given experimental condition, and the values were expressed as U/mg of haemoglobin.

2.4.7. Catalase (CAT) Assay. CAT activity in haemolysate was estimated by using H_2O_2 as a substrate as per the method of Bergmayer [31]. One unit of activity is equal to mmol of H_2O_2 degraded per min and is expressed as units/mg of haemoglobin.

2.5. Apoptosis Assay

2.5.1. Peripheral Blood Leukocytes (PBL) Isolation. The aliquot of 400 μL of whole blood samples was added into appropriate tubes and centrifuged at 200 ×g. The supernatant plasma was discarded, and sediment cells containing buffy coat were washed with phosphate-buffered saline (PBS; pH 7.4). After removing the PBS, the samples were incubated in NH_4Cl buffer (0.15 M NH_4Cl, 10 mM $NaHCO_3$ [pH 7.4]) for 7–10 min at 4°C to destroy the erythrocytes. For complete lysis of erythrocytes, the treatment was repeated once again, followed by two washes in Ca^{2+}–Mg^{2+}-free PBS (PBS-A), and supplemented with 0.035% (w/v) EDTA, and centrifugation was at 400 ×g for 10 min at 4°C. Finally, isolated PBL were kept on ice until further processing.

2.5.2. Annexin V Binding Assay to Detect Apoptotic Cells. For quantification of apoptotic and dead cells, dual parameter analysis of Annexin V-EGFP Detection kit (GenScript, Centennial Avenue, Piscataway, USA) was used [32]. Briefly, isolated PBL (0.5×10^6) were washed twice with PBS by centrifugation at 2000 rpm for 5 min, and cells were resuspended in 500 μL binding buffer and kept at 4°C for 30 min. Finally, 5 μL of annexin V-EFGP and 5 μL of propidium iodide (PI) were added, and samples were incubated at room temperature for 10–15 min, away from light after thorough mixing. Final analysis of Annexin V-EGFP was performed in flow cytometer (Ex = 488 nm; Em = 530) using the FITC signal detector (FL 1) and PI staining by the phycoerythrin emission signal detector (FL 2). For fluorescence balance, normal unstained cells were used as controls.

2.5.3. Assay of Depolarization Mitochondrial Membrane Potential ($\Delta\Psi m$) to Detect Apoptotic Cells. For determination of depolarization of the mitochondrial membrane potential, Mitochondrial Apoptosis Detection Kit (JC-1) (GenScript, Centennial Avenue, Piscataway, USA) was used [33]. Depolarization of $\Delta\Psi$m was measured by using the lipophilic cationic dye (5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolylcarbocyanine iodide, commonly known as JC-1) which selectively enters mitochondria. Briefly, isolated PBL (0.5×10^6) were washed twice with PBS by centrifugation at 2000 rpm for 5 min. One μL of JC-1 was added to 500 μL of prewarmed 1X incubation buffer, vortexed and mixed thoroughly, and used as a working solution. After mixing, the working solution was centrifuged at 13000 g for 1 min, and the supernatant was transferred carefully for removing the particles. The cells were suspended with 500 μL of JC-1 working solution and incubated at 37°C in a humidified

incubator containing 5% CO_2 for 15–20 min. Further, the cell suspensions were centrifuged at 2000 rpm for 3 min at 37°C, and the supernatant was discarded. Sediment cells were resuspended with 500 μL of 1X incubation buffer and subjected to centrifugation at 2000 rpm for 3 min at 37°C. This step was repeated again, and finally, cells were suspended with 500 μL of prewarmed 1X incubation buffer and analyzed immediately by flow cytometer. Mitochondria containing JC-1 aggregates in the healthy cells were detected in the propidium iodide channels (FL 2), and JC-1 monomer in the apoptotic cells was detected in the FITC channels (FL 1).

2.6. Statistical Analysis. Comparisons between the groups were performed using the MANOVA and post-hoc Tukey's tests. The level of statistical significance for all the comparisons made was established at $P \leq 0.05$.

3. Results

Microscopical examination of skin scrapings of dogs with sarcoptic mange revealed the presence of very high numbers of eggs, developing and adult mites per microscopic field before the start of the therapy. Partial anorexia was noted in all diseased dogs. By day 14, the parasitological cure rates in groups A, B, and C were 75%, 87.5%, and 25%, respectively. A 100% parasitological cure rate was found in groups A and B by day 21 after therapy, while in ivermectin alone treated dogs (group C), only 50% and 75% parasitological cure could be achieved by days 21 and 28 after therapy, respectively.

Percent improvements of SSLSs for clinical recovery by days 14 and 28 are depicted in Table 1. By day 14, ivermectin + CFE treated dogs (group A) revealed 73.56% improvement of SSLSs, while by day 28, the improvement could be achieved up to 90.08%. The ivermectin + NAC treated dogs (group B) revealed 72.75% improvement in SSLSs by day 14 after therapy, while by day 28, it was 91.73%. However, by days 14 and 28, the percent improvements of SSLSs for ivermectin alone treated dogs (group C) were 40.95% and 61.37%, respectively (Table 1).

Both studied adjunctive therapies (CFE and NAC) revealed significant ($P \leq 0.01$) improvement in the primary and secondary clinical evaluation scores in comparison with ivermectin alone treated dogs at the time point of evaluations. The improvements in CFE treated dogs were also comparable to those in the NAC (standard) treated dogs, and no significant difference existed for all clinical evaluation parameters at the time point of evaluations. No adverse drug reactions were observed in any group of dogs treated with CFE and NAC along with ivermectin or with ivermectin alone.

The means ± S.D for oxidative stress markers of diseased and healthy dogs before and after receiving therapy are depicted in Table 2. On day 0, dogs with sarcoptic mange revealed significantly ($P \leq 0.001$) higher lipid peroxides contents, lesser total thiol levels, and lowered glutathione peroxidase, glutathione-S-transferase, superoxide dismutase, and catalase activities in comparison with the healthy dogs. By day 28 after therapy, the altered oxidant/antioxidant balance was remarkably ameliorated towards normalcy in dogs of groups A and B. The increased erythrocytes MDA contents of these groups were significantly lowered ($P \leq 0.001$) in comparison with the values pertaining on day 0. Erythrocytes MDA contents of these dogs on day 28 also achieved a level comparable to that of the healthy dogs. A significant enhancement ($P \leq 0.001$) of the total thiol contents and lowered activities of GSH-Px, GST, SOD, and CAT was also revealed in these groups when compared with the values pertaining on day 0 and was found to be comparable to that of healthy dogs. However, the dogs treated only with ivermectin (group C) could not achieve the comparable values to those of healthy dogs by day 28 after therapy.

Table 1: Comparison groups A, B, and C for secondary clinical efficacy criterion (SSLS) on days 0, 14, and 28 of therapy.

Groups	Days	SSLS	Improvement (%)
Group A (Ivermectin + CFE)	0	15.13 ± 1.46	—
	14	4.00 ± 1.06[a,b]	73.56
	28	1.50 ± 0.75[a,b]	90.08
Group B (Ivermectin + NAC)	0	15.12 ± 2.58	—
	14	4.12 ± 1.45[a,b]	72.75
	28	1.25 ± 1.03[a,b]	91.73
Group C (Ivermectin)	0	15.87 ± 1.35	—
	14	9.37 ± 2.19	40.95
	28	6.13 ± 1.95[a]	61.37

[a]Statistically significant differences ($P \leq 0.01$), when compared with the day 0 values.
[b]Statistically significant differences ($P \leq 0.01$), when compared with the same days values of a group.

The means ± S.D for apoptotic markers of all groups before and after therapy are depicted in Table 3. On day 0, the percentage of peripheral leukocytes with externalization of phosphatidylserine on cell membrane (apoptotic cells) was significantly ($P \leq 0.001$) increased in all dogs with sarcoptic mange in comparison with the healthy dogs. These dogs also revealed a significantly ($P \leq 0.001$) higher percentage of leukocytes with depolarized mitochondria (%$\Delta\Psi$m). By day 28, the increased percentage of apoptotic leukocytes (both the leukocytes with externalization of phosphatidylserine and depolarized mitochondria) of groups A and B was significantly ameliorated ($P \leq 0.001$) in comparison with the values pertaining on day 0 and became comparable to that of healthy dogs. However, the dogs treated only with ivermectin (group C) could not achieve the comparable values to those of healthy dogs by day 28 after therapy. Further, no adverse effects have been recorded in both CFE and NAC adjunctively supplemented dogs, and the administered dose regimen of these supplementations was found to be safe.

4. Discussion

In the present study, we found that the dogs with sarcoptic mange were in a state of significant oxidative stress. The results of the present study are in agreement with the earlier scientific reports where altered antioxidant systems with states of oxidative stress have been demonstrated with various

Table 2: The markers of oxidative stress (lipid peroxides, reduced glutathione, glutathione peroxidase, glutathione-S-transferase, superoxide dismutase, and catalase) in peripheral blood of dogs with sarcoptic mange and healthy control dogs at days 0 and 28 of therapy (mean ± SD).

Groups	Days	LPO μmol MDA/mg Hb	Total thiol ηmol/mg Hb	GSH-Px mU/mg Hb	GST mU/mg Hb	SOD U/mg Hb	CAT K/mg Hb
Group A (Ivermectin + CFE)	0	1.945 ± 0.15[a]	13.62 ± 1.6[a]	6236.12 ± 192[a]	0.602 ± 0.06[a]	0.737 ± 0.10[a]	89.25 ± 11[a]
	28	0.843 ± 0.09[b]	33.90 ± 3.9[b]	8823.12 ± 206[b]	0.931 ± 0.06[b]	1.082 ± 0.06[b]	153.25 ± 9[b]
Group B (Ivermectin + NAC)	0	1.875 ± 0.12[a]	14.78 ± 2.5[a]	6153.15 ± 159[a]	0.618 ± 0.06[a]	0.697 ± 0.12[a]	89.62 ± 13[a]
	28	0.808 ± 0.12[b]	34.11 ± 2.5[b]	8792.75 ± 309[b]	0.908 ± 0.07[b]	1.117 ± 0.05[b]	144.50 ± 12[b]
Group C (Ivermectin)	0	1.947 ± 0.09[a]	14.99 ± 2.0[a]	6235.12 ± 197[a]	0.592 ± 0.06[a]	0.747 ± 0.11[a]	95.25 ± 7.2[a]
	28	1.270 ± 0.05	21.33 ± 1.7	7587.15 ± 364	0.712 ± 0.08	0.866 ± 0.07	117.75 ± 4.2
Group D (Healthy)	0	0.720 ± 0.07	36.83 ± 2.5	8963.62 ± 113	0.987 ± 0.05	1.188 ± 0.03	176.25 ± 8.2

[a]Statistically significant differences ($P \leq 0.001$) when compared with group D.
[b]Statistically significant differences ($P \leq 0.001$) when compared with the values pertaining at day 0.

Table 3: Flow cytometric analysis of apoptotic leukocytes with externalization of phosphatidylserine (PS) and depolarized mitochondria ($\Delta\Psi$m) in peripheral blood of dogs with sarcoptic mange and healthy control groups at days 0 and 28 of therapy (mean ± SD).

Groups	Days	Leukocytes with external PS (%)	Leukocytes with $\Delta\Psi$m (%)
Group A (Ivermectin + CFE)	0	21.87 ± 2.78[a]	24.67 ± 2.34[a]
	28	3.83 ± 1.50[b]	7.69 ± 1.88[b]
Group B (Ivermectin + NAC)	0	21.38 ± 3.10[a]	29.00 ± 1.46[a]
	28	3.00 ± 1.01[b]	7.09 ± 1.38[b]
Group C (Ivermectin)	0	22.92 ± 1.31[a]	23.74 ± 2.17[a]
	28	13.89 ± 1.67	17.40 ± 1.47
Group D (Healthy)	0	2.13 ± 0.56	5.15 ± 1.04

[a]Statistically significant differences ($P \leq 0.001$) when compared with group D.
[b]Statistically significant differences ($P < 0.001$) when compared with the values pertaining at day 0.

parasitic skin infestations in animals, including sarcoptic mange [14–16, 34–36]. Recently, we have demonstrated that the dogs with sarcoptic mange are in a state of severe oxidative stress, and the percentage of apoptotic leukocytes in peripheral blood of these dogs is remarkably higher as compared to the healthy controls [17]. Amelioration of the altered oxidant/antioxidant balance towards normalcy in ivermectin + CFE treated dogs indicates potential antioxidant action of the adjunctive therapy.

Lipid peroxidation can be harmful for skin due to alteration in the structure and permeability [37]. Various scientific reports have demonstrated the versatile medicinal activities of *Calendula* extracts including antitumoral [38], anti-inflammatory, wound healing [39], hepato- and renoprotective [40], and antioxidant activities [22, 41]. In clinical studies, *Calendula* is highly efficacious in the prevention of acute dermatitis in cancer patients undergoing postoperative irradiation [42]. *In vitro* free radical quenching of potential *Calendula* flower extracts has been also reported [43]. Bilia et al. [44] identified narcissin, rutin, isoquercitrin, quercetin-3-O-rutinosylrhamnoside, isorhamnetin-3-O-rutinosylrhamnoside, isorhamnetin-3-O-glucosylglucoside, and isorhamnetin-3-O-glucoside in *C. officinalis* flowers. In the last decade, *C. officinalis* is used in tinctures and creams and is valuable for positive effect on skin diseases [22, 45]. The antioxidant potential of *C. officinalis* extracts against lipid peroxidation of rat liver microsomes by acting as a potent free radical scavenger and an antioxidant has been reported by earlier works [46, 47].

Results of the present study signify the potential triumph of CFE against *Sarcoptes*-induced lipid peroxidation. Amelioration of increased MDA content might have endorsed faster remedy of injured skin in the infested dogs. The current study also demonstrates that CFE adjunctive treatment has significantly improved the lower total thiol content and reduced GSH-Px, GST, SOD, and CAT activities towards normalcy by day 28 in dogs with sarcoptic mange. Findings of the current study indicate the potential protective activity of CFE against *Sarcoptes*-induced oxidative stress. The encompassment of a large quantity of antioxidant compounds (flavonoids and polyphenols) in the *Calendula* flowers might have resulted in remarkable amelioration of sarcoptic mange-induced oxidative stress. Recently, it has been reported that the antioxidant action of nutritional antioxidants is conferred not only by the direct free radicals quench but also by the generation of signals for the induction of protective enzymes via their paradoxical oxidative activation of the Nrf2 (NF-E2-related factor 2) signaling pathway [48]. Thus, the possibilities of activating the Nrf2 signaling pathway in the significant ameliorations of the protective antioxidant enzymes activities in CFE administered dogs cannot be overlooked.

Resistance to diseases is a multigenic trait governed mainly by the immune system and its interactions with many physiologic and environmental factors. In the adaptive immunity, T-cell and B-cell responses, the specific recognition of antigens, and interactions between antigen presenting cells, T cells, and B cells are essential. These interactions occur through a network of mediator proteins such as the molecules

of the major histocompatibility complex (MHC), T-cell receptors, and immunoglobulins and secreted proteins such as the cytokines and antibodies. The results of the current study demonstrate remarkable deterrence of *Sarcoptes*-induced apoptosis in peripheral leukocytes in CFE treated dogs and suggest the possible immunorestorative potential of CFE against hasty immunoaging. This phenomenon may be elucidated by the free radicals scavenging and immunomodulatory potential of CFE. By ameliorating the oxidative stress, CFE may be enhancing the compromised immune status of the *Sarcoptes*-infested dogs, leading to the inhibition of proliferation of mites.

As we found that the clinical recovery and parasitological cure rates were faster in ivermectin + CFE treated dogs, the supplementation of CFE along with ivermectin might have shifted the altered oxidant/antioxidant balance towards normalcy with a faster rate. Recently, we have demonstrated a faster clinical recovery and shifting of oxidative imbalance toward normalcy by adjunct therapy with antioxidants in *Psoroptes cuniculi*-infested rabbits [49]. The restoration of antioxidants and amelioration of lipid peroxidation in CFE treated dogs might be involved in faster clinical and parasitological cure. Lipid peroxidation can be harmful for skin due to alterations in the structure and permeability [37]. CFE might have a complementary effect with ivermectin as compromised antioxidant defense systems of infested dogs and healing effect of ivermectin remarkably improved by day 28 after therapies. Findings of the present study indicate a potential role of CFE in faster clinical recovery in *Sarcoptes*-infested dogs. Previous scientific reports have demonstrated the skin healing activities of *Calendula* in various skin disorders due to its multitude of properties; for instance, anti-inflammation, stimulation of epithelialization, and an increase in gelatinase activities [22, 41]. Results of the present study signify the faster parasitological cure; in this context, CFE might have indirectly augmented the miticidal effect of ivermectin. This may be an outcome of various activities including immunomodulatory and antioxidant actions of CFE, promoting the animal body to combat against the *Sarcoptes* mites.

In conclusion, *Calendula officinalis* flower extracts have remarkable antioxidant potential contributing to the deterrence of *Sarcoptes*-induced oxidative discrepancy in dogs. They also have accomplishments against *Sarcoptes*-induced immunological misbalance, can avert and/or ameliorate the hasty aging of leukocytes, and consequently can augment miticidal activity of ivermectin by improving compromised immunity. Clinicians may recommend CFE as an adjunctive remedy along with the miticide to manage clinical canine sarcoptic mange. Additionally, large-scale studies are needed for these assumptions to be confirmed.

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