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Antidermatophyte and antioxidant activities of *Nigella sativa* alone and in combination with enilconazole in treatment of dermatophytosis in cattle

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ABSTRACT: The purpose of this study was to comparatively assess the antidermatophyte and antioxidant activities of enilconazole, *Nigella sativa* (NS) and enilconazole with NS in the treatment of dermatophytosis in cattle. A total of 24 cattle with clinically established diagnosis of dermatophytosis were used in the study. *Trichophyton verrucosum* was isolated and identified from all of the specimens stemming from the dermatophytosis-suspected animals. The lesion areas in Groups 1, 2 and 3 were treated as follows: enilconazole (three times at 3-day intervals), NS (once a day for two weeks) and enilconazole with NS, respectively. There were significant increases ($P < 0.05$) in plasma aspartate aminotransferase, alanine aminotransferase and alkaline phosphatase levels and non-significant increases ($P > 0.05$) in creatinine and blood urea nitrogen levels after treatment in Group 1 when compared with Groups 2 and 3. After treatment, glutathione peroxidase, superoxide dismutase, glutathione levels increased ($P < 0.05$) and plasma thiobarbituric acid reactive substances levels decreased ($P < 0.05$) in Groups 1, 2 and 3 in comparison with before treatment. However, there were significant decreases ($P < 0.05$) in plasma thiobarbituric acid reactive substances levels and significant increases ($P < 0.05$) in glutathione peroxidase, superoxide dismutase, glutathione levels after treatment in Groups 2 and 3 when compared with Group 1. This study indicates that NS might have antidermatophyte and antioxidant effects in the treatment of dermatophytosis in cattle and the antidermatophyte effects of NS plus enilconazole was stronger among all groups.

Keywords: *Nigella sativa*; enilconazole; antioxidants; trichophytosis; bovine

Dermatophytosis is a fungal infection of the superficial, keratinised structures of the skin and hair of animals and humans (Iqbal et al. 2012). In cattle, dermatophytosis is most often caused by *Trichophyton verrucosum* (Swai and Sanka 2012). Treatment of clinical dermatophytosis in cattle is expensive and time-consuming and preventive measures often fail (Zahran and Abdeen 2013). Moreover, some of these treatments have been reported to be ineffective or even toxic to the host (Rochette et al. 2003).

Treatment of ringworm in cattle with older antimycotic products like thiabendazole, iodine, etisazol and thiadiazine is now quite uncommon. According to the European Consultation Conference on the availability of veterinary medicinal products only two substances, enilconazole and natamycin, will remain for the treatment of ringworm. The in-feed medication of griseofulvin is no longer licensed for food producing animals in the European Economic Community (Rochette et al. 2003). Topical use of enilconazole as a spray is completely safe for cattle (De Keyser 1981). In another study, one month after the last treatment with enilconazole, all animals were cured as assessed by clinical and mycological evaluation (Schepens and Spanoghe 1981). Some researchers reported that several systemic antifungal agents have adverse effects resulting in mild-to-severe hepatic injuries and renal syndromes in cats (Kim et al. 2003; Figg et al. 2010). Enilconazole is generally well tolerated but has also been associated with hypersalivation, anorexia, weight loss, emesis, idiopathic muscle weakness, and slightly elevated serum alanine aminotransferase (ALT) concentrations (Dejaham 1998; Hnilica and Medleau 2002). Therefore, trials of new drugs such as propolis,

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Nigella sativa etc. or alternative treatment regimens using the existing drugs, have been used in cattle with dermatophytosis (Aljabre et al. 2005; Cam et al. 2009).

Nigella sativa (NS), an annual herbaceous plant of the Ranunculaceae, has been used traditionally in the Middle East, Northern Africa, Far East and Asia for the treatment of various diseases for over 2000 years (Phillips 1992). NS contains 36–38% fixed oil, proteins, alkaloids, saponins and 0.4–2.5% essential oil (Lautenbacher 1997). The main compounds are thymoquinone (30–48%), p-cymene (7–15%), carvacrol (6–12%), 4-terpineol (2–7%), t-anethole (1–4%) and the sesquiterpene longifolene (1–8%; Burits and Bucar 2000). Recently, many therapeutic effects of NS extracts have been documented, including antioxidant effects in experimental studies (Yaman and Balikci 2010). NS has been rarely assessed for antifungal activity. Its ether extract inhibited the growth of the yeast *Candida albicans* in experimental animal infection (Khan et al. 2003). More recently, the antifungal activity of ether extract of NS seed and its active principle thymoquinone was tested against eight species of dermatophytes (Aljabre et al. 2005).

It was hypothesised in the present study that NS could have antifungal and antioxidant activities against dermatophytosis in cattle. The purpose of this study therefore was to comparatively assess the effects of enilconazole, NS and enilconazole together with NS in the treatment of dermatophytosis in cattle.

MATERIAL AND METHODS

Animals and treatments. In total, 24 cattle were randomly divided into three groups according to the size and distribution of the skin lesions. Each group contained eight animals, seven months to two years old, from different farms in Elazig, Turkey. In Group 1, oily liquid containing enilconazole (10%) was applied topically to areas with lesions once a day at 3-day intervals. In Group 2, NS was applied topically once a day for two weeks; in Group 3, oily liquid containing enilconazole (10%) was applied topically to areas with lesions once a day at 3-day intervals; NS was applied topically once a day for two weeks. All of the animals were kept under similar management conditions. Enilconazole (10%) (Imaverol by Janssen Animal Health) and NS oil (Copyright © 2005 Origo Gida Kimya Tarim Urn. San. and Tic. Ltd. Sti. – Gaziantep, Turkey; the company produces NS oil by cold pressing fresh seeds without the use of chemicals) were used in this study.

The shape, size, position, distribution and appearance of skin lesions and the general condition of the animals were observed daily for six weeks; the results of clinical examinations were recorded at two-week intervals (Arslan et al. 2007). Before and after the treatments, the numbers, spreads, forms and localisations of the lesions, and scurf, hair loss, pruritus, scales, keratinisation and crust were scored (3+ = severe, 2+ = moderate, 1+ = weak, 0 = absent; Arslan et al. 2007).

Mycological examination. The sites of lesions on the affected animals were thoroughly cleaned with 70% ethyl alcohol. Skin scales were collected in a sterile Petri dish by scraping the margin of the lesion with a disposable scalpel blade (Cheesbrough 1992). A small portion of the skin scale was placed in a drop of 10% potassium hydroxide solution on a microscopic slide and covered with a cover slip. The slide was gently heated over a flame for 1–2 min to bring about cleaning; the preparation was then left inside the Petri dish for 20 min. After that, the slide was examined under the microscope using the low power objective × 10 and × 40 to locate the fungal elements (i.e., yeast, hyphae, or mycelium).

Another portion of the specimens was cultured under aseptic conditions on mycobiotic agar medium (Difco), incubated at 28 °C for one month, and examined for colony formation. Fungal species were identified on the basis of culture characteristics. Microscopic examination in lactophenol cotton blue preparation was done for the positive fungal cultures (Halley and Standard 1973).

Hair and scale samples were collected for mycological examination at the beginning and end of the study period.

Sample collection and biochemical assays. Blood was collected from the jugular vein in 10 ml heparinised test tubes and centrifuged at 3000 × *g* at 4 °C for 10 min to separate the plasma from the erythrocytes. Plasma was stored at –20 °C to determine aspartate aminotransferase (AST), ALT, and alkaline phosphatase (ALP) enzyme activities and blood urea nitrogen (BUN), and creatinine concentrations. To obtain packed erythrocytes, the remaining erythrocytes were washed twice with an isotonic solution of sodium chloride. To obtain

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erythrocyte haemolysates, 500 µl of packed erythrocytes were destroyed by adding four volumes of cold redistilled water. The resulting suspension was centrifuged twice, firstly for 10 min in a tube centrifuge at 1500 × *g* at 4 °C and then in an Eppendorf centrifuge at 5000 × *g* for 5 min at 4 °C. Clear supernatant was obtained as haemolysates to determine glutathione peroxidase (GSH-Px), superoxide dismutase (SOD) and glutathione (GSH). The haemolysates were frozen at –20 °C until the time of analysis.

Plasma AST, ALT, and ALP enzyme activities, BUN and creatinine concentrations were determined using Olympus Kits in an Olympus AU600 autoanalyser (Olympus Corp., Tokyo, Japan).

The activities of the GSH-Px and SOD antioxidant enzymes were measured in haemolysates. GSH-Px activities were measured by the oxidation of glutathione using tert-butyl hydroperoxide. Oxidised glutathione was converted to the reduced form in the presence of glutathione reductase and NADPH, while NADPH was oxidised to NADP. The reduction in absorbance of NADPH at 340 nm was measured. The absorbance change per minute and the molar extinction coefficient of NADPH were used to calculate glutathione peroxidase activity (Paglia and Valentine 1967). SOD activity was measured in the haemolysates (Sun et al. 1988). Haemolysates were assayed for SOD activities using the xanthine/xanthine oxidase system for superoxide radical generation. This anion reduced nitroblue tetrazolium to the red formasone compound. SOD activity was measured at 560 nm by detecting the inhibition of this reaction. One unit of SOD activity was defined as the activity that caused a half-maximal inhibition of the nitroblue tetrazolium reduction rate.

Reduced glutathione concentrations were determined in the blood haemolysates by titration with 0.1 mmol/l dithiobis in a 0.1 mol/l disodium phosphate buffer solution. The formation of the reduced product, thionitrobenzene, was measured spectrophotometrically at 412 nm. The GSH content was expressed as millimoles per litre of haemolysate (Buetler et al. 1963).

Plasma levels of thiobarbituric acid reactive substances (TBARS) were analysed spectrophotometrically after extraction with nbutanol according to the optimized method of Yagi (1984) by adding 100 µl of plasma to a 0.37% thiobarbituric acid solution.

Statistical analysis. The data were expressed as mean ± SD. Differences between group means were estimated using a one-way analysis of variance (ANOVA) and the Duncan test was done for multiple comparisons using SPSS 12.0 for Windows. Results were considered statistically significant at $P < 0.05$.

RESULTS

Trichophyton verrucosum was isolated and identified from all of the specimens stemming from the trichophytosis-suspected animals ($n = 24$). Pruritus was absent in all animals. All cows exhibited hair loss zones. Scurf, scales, crusts and keratinisation areas were also generally noted in all the cattle. The average clinical scores of Groups 1, 2 and 3 before treatment were 11.4, 11.6 and 10.3, respectively. Clinical scores decreased by 0.7, 0.7 and 0 points after the treatment in Groups 1, 2 and 3, respectively. Clinical signs healed completely after 42 days in six animals in Group 1 and in five animals in Group 2 and in all animals in Group 3. At the end of the study, direct microscopic examination revealed fungal hyphae and spores in one sample from Group 1, two from Group 2, and in none from Group 3. *T. verrucosum* was isolated from one sample from Group 1, two from Group 2 and in none from Group 3.

The means (SD) of the results obtained in all the groups are given in Table 1. There were significant increases ($P < 0.05$) in plasma AST, ALT, ALP levels and non-significant increases ($P > 0.05$) in creatinine and BUN levels after treatment in Group 1 when compared with Groups 2 and 3. After treatment, GSH-Px, SOD, GSH levels increased ($P < 0.05$) and plasma TBARS levels decreased ($P < 0.05$) in Groups 1, 2 and 3 in comparison with before treatment. However, there were significant decreases ($P < 0.05$) in plasma TBARS levels and significant increases ($P < 0.05$) in GSH-Px, SOD and GSH levels after treatment in Groups 2 and 3 when compared with Group 1.

DISCUSSION

In cattle, dermatophytosis is a disease most often caused by *Trichophyton verrucosum* (Swai and Sanka 2012). In this study, *Trichophyton verru-*

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Table 1. Plasma AST, ALT, ALP, BUN and creatinine levels and antioxidant enzyme and TBARS levels in dermatophytosis-affected cows (n = 24) treated with enilconazole (Group 1), NS (Group 2) and eniconazole + NS (Group 3), before and after treatment

Parameters		Group 1 (n = 8)	Group 2 (n = 8)	Group 3 (n = 8)
AST (μkat/l)	BT	2.17 ± 0.42	2.11 ± 0.34	2.04 ± 0.45
	AT	2.36 ± 0.55^{a}	1.97 ± 0.27^{b}	2.11 ± 0.40^{b}
ALT (μkat/l)	BT	0.55 ± 0.10	0.58 ± 0.07	0.48 ± 0.08
	AT	0.70 ± 0.13^{a}	0.51 ± 0.06^{b}	0.55 ± 0.08^{b}
ALP (μkat/l)	BT	2.69 ± 0.45	2.67 ± 0.40	2.64 ± 0.42
	AT	2.76 ± 0.49^{a}	2.46 ± 0.35^{b}	2.50 ± 0.32^{b}
BUN (mmol/l)	BT	8.57 ± 2.35	8.96 ± 2.57	8.78 ± 2.28
	AT	9.71 ± 2.64	8.85 ± 2.43	8.92 ± 2.46
Creatinine (μmol/l)	BT	113.12 ± 25.27	114.25 ± 23.24	118.26 ± 22.68
	AT	124.82 ± 32.36	112.13 ± 20.26	119.23 ± 19.36
GSH-Px (U/gHb)	BT	41.24 ± 5.64^{a}	43.28 ± 8.16^{a}	38.35 ± 6.34^{a}
	AT	70.14 ± 7.56^{Ab}	81.25 ± 6.84^{Bb}	82.86 ± 7.96^{Bb}
SOD (U/gHb)	BT	792 ± 106^{a}	696 ± 79^{a}	754 ± 96^{a}
	AT	1016 ± 115^{Ab}	1102 ± 89^{Bb}	1118 ± 102^{Bb}
GSH (mmol/l)	BT	1.17 ± 0.20^{a}	1.26 ± 0.18^{a}	1.36 ± 0.21^{a}
	AT	1.97 ± 0.15^{Ab}	2.51 ± 0.14^{Bb}	2.45 ± 0.25^{Bb}
TBARS (nmol/l)	BT	3.92 ± 0.40^{a}	4.12 ± 0.36^{a}	4.18 ± 0.48^{a}
	AT	1.99 ± 0.40^{Ab}	1.82 ± 0.46^{Bb}	1.76 ± 0.43^{Bb}

AT = after treatment, BT = before treatment

^{a,b}Means with different superscript in the same columns significantly differ ($P < 0.05$)

^{A,B}Means with different superscript in the same line significantly differ ($P < 0.05$)

cosum was isolated and identified from all of the specimens stemming from the trichophytosis-suspected animals. Clinical signs healed completely in all animals in Group 3, while six animals in Group 1 and five animals in Group 2 healed in 42 days in the present study. At the end of the study, direct microscopic examination revealed fungal hyphae and spores in one sample from Group 1, two samples from Group 2, and in none from Group 3. *T. verrucosum* was isolated from one sample from Group 1, two from Group 2 and none from Group 3. In this study, NS application alone cured five of eight animals in Group 2 and NS in combination with enilconazole resulted in complete recovery in all animals in Group 3. These results suggest that NS exerts considerable antifungal effects. Application of NS plus enilconazole was more effective than application of either NS or enilconazole alone in the treatment of dermatophytosis in cattle.

Prolonged duration of treatment, drug toxicity and interactions, fungal resistance and high costs are among the issues with currently available antifungal drugs (Alexander and Perfect 2000; Mugnaini et al. 2013). These factors necessitate the development of new more efficient and safer antifungal drugs. NS has been rarely assessed for antifungal activity. Its ether extract inhibited the growth of the yeast *Candida albicans* in experimental animal infection (Khan et al. 2003). More recently the antifungal activity of ether extract of NS seed and its active principle thymoquinone was tested against eight species of dermatophytes (Aljabre et al. 2005). Similar results have been reported on the antidermatophytic action of a 1% oil-petroleum jelly formulation of the essential oil of *Chenopodium ambroisoides* which cured *Trichophyton mentagrophytes*-induced dermatophytosis in guinea pigs in 15 days (Aghel et al. 2007). Also, similar effects

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have been reported with *Phellodendron amurense* (Xiao et al. 2014) and *Eucalyptus camaldulensis* (Thippeswamy and Naidu 2005). In the present study, NS application alone healed five of eight animals in 42 days.

Some studies have reported clinical adverse effects after therapy with various systemic anti-fungal agents (Kim et al. 2003; Figg et al. 2010). Several case reports of mild-to-severe hepatic injury, including icteric and fatal cases, have been published (Kim et al. 2003; Figg et al. 2010). For instance, the adverse effects of griseofulvin include gastrointestinal symptoms, allergic reactions, photodermatitis, hepatic and renal dysfunctions. Similarly, the adverse effects of ketoconazole include hepatic dysfunction or asymptomatic increases in the activities of serum transaminases (Figg et al. 2010). In addition, nausea and vomiting are observed in response to itraconazole treatment and flucanozole treatment can trigger severe gastro-intestinal complications (Gupta and Gregurek 2001). Some researchers have reported significant increases in plasma AST, ALT, ALP, creatinine and BUN levels in cats treated with enilconazole (Dejaham 1998; Hnilica and Medleau 2002). In this study, there were significant increases ($P < 0.05$) in plasma AST, ALT, ALP levels and non-significant increases ($P < 0.05$) in creatinine and BUN levels after the treatment in Group 1 when compared with Groups 2 and 3. Although these increases elicited by enilconazol (Group 1) resulted in values slightly above the physiological range (Smith 2002), these were not sufficient to cause damage as has been previously reported in the liver and kidney in cats (Dejaham 1998; Hnilica and Medleau 2002). There were no important changes after treatment compared to before application in any of the groups. Therefore, enilconazole can be used in cattle with dermatophytosis. Moreover, there were decreases in those parameters in Groups 2 and 3 after treatment, which supports the conclusions of other studies that reported positive and protective effects of NS on the liver and kidney (Burits and Bucar 2000; Yaman and Balikci 2010).

Under conditions of oxidative stress, there is insufficient antioxidant capacity leading to excessive production of free radicals which damage molecules such as lipids, proteins, and DNA with the accumulation of final products of lipid peroxidation such as TBARS (Sayari et al. 2014). During inflammation, reactive oxygen species and free radicals with many physiological and pharmacological adverse effects, including skin irritation, are produced (Sarma et al. 2010). In this study, GSH-Px, SOD and GSH levels increased ($P < 0.05$) and TBARS levels decreased ($P < 0.05$) in all groups after the treatment compared with values before treatment. These results suggest an imbalance between lipid peroxidation processes and the antioxidant defence system in cattle with dermatophytosis. However, in the present study, there were significant decreases ($P < 0.05$) in plasma TBARS levels and significant increases ($P < 0.05$) in GSH-Px, SOD, GSH levels after treatment in Groups 2 and 3 when compared with Group 1. This may be because of the ameliorative effect of NS on oxidative stress. NS was reported to prevent oxidative stress by eliciting a rise in the levels of antioxidant enzymes (Khattab and Nagi 2007; Yaman and Balikci 2010; Yildiz and Balikci 2016). The antioxidant activity of NS seeds and oil was found to be largely dependent on its active constituents thymoquinone and carvacrol (Burits and Bucar 2000).

In conclusion, this study indicates that NS might have antidermatophyte and antioxidant effects in the treatment of dermatophytosis in cattle. The antidermatophyte effects of NS plus enilconazole was stronger among all groups. Owing to its antifungal effects, NS may be used as an adjunctive or alternative agent in the treatment of dermatophytosis. However, further studies, using different concentrations and periods of treatment, are required to determine the exact effect of NS in dermatophytosis.

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