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Immunomodulatory and antioxidant potential of *Asparagus racemosus* Wild and *Terminalia chebula* Retz and their corresponding homeopathic mother tincture against canine demodicosis

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ABSTRACT

A study was conducted to carry out the immunomodulatory and antioxidant potential of *Asparagus racemosus* and *Terminalia chebula* extracts and their corresponding homeopathic mother tincture (MT) on clinical cases of canine demodicosis. Alopecia, erythema, pustule/papules/vesicles, scale/excoriation/crust, hyperkeratinization and general appearance were the clinical manifestations of scoring demodicosed canine. The mean clinical scores were significantly higher in demodicosed canine than healthy dogs. The oral *Asparagus racemosus* extract or its homeopathy MT, in combination with amitraz dipping responded better in respect with the cellular and humoral immunities in demodicosed canine as compared with only amitraz dipping. In demodicosed canine there was increase in the level of lipid peroxidation in terms of malondialdehyde and the activity of superoxide dismutase and decrease in the activities of catalase and reduced glutathione in erythrocytes. The oral *Terminalia chebula* extract along with amitraz dipping exhibited maximum antioxidant activity in terms of reduced level of lipid peroxidation and activity of superoxide dismutase and increase activities of catalase and reduced glutathione in erythrocyte as compared with the decreasing orders of antioxidant activities of *Asparagus racemosus* extract, homeopathy *Terminalia chebula* MT and homeopathy *Asparagus racemosus* MT. In conclusion, demodicosis produced immunosuppression and oxidative stress in canine and for the treatment, amitraz dipping in combination with oral *Asparagus racemosus* extract responded better recovery.

Key words: Antioxidant, Demodicosis, Herbs, Homeopathy, Immunostimulant

Demodicosis is a common inflammatory skin disease of dogs, characterized by excessive proliferation of *Demodex* mites in hair follicles and skin surfaces (Gortel 2006). Amitraz dips are the most common treatment with protocols ranging from whole body immersions at various concentrations at intervals of 1–2 weeks (Mueller 2004). *Asparagus racemosus* Wild alcoholic root extract enhanced humoral and cell mediated immunities of albino mice injected with sheep red blood cells as particulate antigen (Muruganadan *et al.* 2000). *Terminalia chebula* exhibited antioxidant activity at different magnitudes of potency for anti-lipid peroxidation, anti-superoxide radical formation and free radical scavenging activities (Cheng *et al.* 2003). Mother tinctures (MT), in homeopathic system of medicine, is defined as the original tincture prepared with the aid of alcohol, directly from the crude drug. They are the precursors of the corresponding potencies of the respective drug and the starting point for the production of most homoeopathic medicines (Shanbhag and Jayaraman 2008). The present study was conducted to evaluate the immunomodulatory and antioxidant potential of 70% ethanolic extracts of *Asparagus racemosus* and *Terminalia chebula* and their corresponding homeopathic MT on clinical cases of canine demodicosis.

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MATERIALS AND METHODS

All chemicals were purchased commercially. *Asparagus racemosus* roots and *Terminalia chebula* kernel were obtained from authorized dealer at Bareilly and were correctly identified as *Asparagus racemosus* Wild (Asparagaceae) and *Terminalia chebula Retz*. (Combretaceae). The herbs were dried and gound, and were exhaustively extracted using 70% ethanol (100 ml/100 g) under reflux condenser. To make the extract ethanol free it was kept in hot air-oven and the dried form of extracts were further dissolved in distilled water to provide to the dogs. *Asparagus racemosus* and *Terminalia chebula* mother tinctures were obtained from authorized homeopathy dealers.

Experimental design: Clinical cases of demodicosed dogs (without any discrimination of sex and breed, female free from heat and other stressful condition) were divided into 5 groups of 6 dogs each. Six healthy dogs were selected as control group 1. Group 2 was treated with amitraz dipping at 500 ppm concentration at weekly intervals for 6 dips. Groups 3 to 6 demodicosed canine were treated with amitraz dipping along with different oral medication. Groups 3 and 5 were treated with ethanolic extract of *Asparagus racemosus* and *Terminalia chebula* @ 25mg/kg b.wt orally daily for 28 days. Groups 4 and 6 were provided homeopathic preparation of *Asparagus racemosus* MT and *Terminalia chebula* MT @10 drops with drinking water. Dogs suffering from demodicosis, irrespective of host variables, constituted the subject for the clinical trial. The clinical scores were assessed by 6 clinical manifestations of alopecia, erythema, pustules/papules/vesicles, scale/excoriation/crusts, hyperkeratinization, general appearance (below normal) and each clinical manifestation was graded maximum 4 point on the basis of severity of signs and lesions (Muller *et al*. 1989). One cm^2 area on skin was marked at most active lesion and a deep skin scraping was collected and was digested using 10% KOH. Blood samples were collected on days 0, 14, and 28 days post therapy and were kept in sterilized test tube containing heparin and stored at 4°C for laboratory test.

Immunological study: Cell mediated immunity was determined as per Thompson *et al.* (1980). 100 µl of phytohaemagglutinin-P (conc 100 µg/ml) dissolved in phosphate buffer saline (PBS pH 7.4) was injected intradermally in one side and on the other side 100 µl of PBS was injected as a control. The difference in the skin fold thickness between PHA-P injected sites and control sites was measured with the help of vernier caliper at 24, 48 and 72 h post sensitization. To assess humoral immune response in all the 6 groups, sheep RBC was used as antigen (Allan and Gough 1974). In each group of dog 0.2 ml of sheep RBC (25×10^6 RBC) was injected and serum samples were collected on days 0 (prior to injection), 14 and 28. Antibody titre was measured using microhaemagglutination technique. 50 µl of serum was serially diluted with 50 µl of PBS. 50 µl sheep RBC (25×10^6 cells) were added to each dilution and incubated at 37°C for 1 h. The reciprocal of the highest dilution of the test serum giving agglutination was taken as the antibody titre.

Oxidant/antioxidant system (in erythrocytes): The lipid peroxidation in terms of malondialdehyde (MDA) production in 10% hemolysate was determined (Placers *et al*. 1966). The method depends on formation of a colour complex between the products of lipid peroxidation and thiobarbituric acid (TBA). Reduced glutathione (GSH) was estimated in the RBC suspension by dithio-bis-2-nitro benzoic acid (DTNB) method as per Prins and Loos (1969). Superoxide dismutase (SOD) activity of RBC hemolysate samples was measured by using nitro blue tetrazolium as a substrate after suitable dilution according to Marklund and Marklund (1974) with certain modifications suggested by Minami and Yoshikawa (1979). Catalase (CAT) was assayed in erythrocyte by the spectrophotometric method as described by Bergmeyer (1983).

Statistical analysis: Statistical analysis was carried out as per Snedecor and Cochran (1994) using one way ANOVA.

RESULTS AND DISCUSSION

Clinical study: The highest clinical score regression (2.00) was observed in group 3 on day 28 post treatment whereas, the least clinical score regression (6.00) was observed in group 2 which was significant ($P<0.01$) as compared with healthy control (Table 1). Intermediate clinical score regression in the descending order was group 5, followed by group 4 and lastly group 6. So, oral *Asparagus racemosus* extract along with amitraz dipping was highly effective to reduce the 6 clinical manifestations of demodicosis than other combination of therapy or along amitraz dipping.

Skin scraping examination of groups 3, 4 and 5 on day 28 post treatment, only 1 animal in each group was positive for mites, whereas, in group 2 and 6, two animals in each group were positive for demodex. In demodicosed dogs treated only with amitraz the clinical recovery was slow as compared with the combination therapy of amitraz and herbal extract or their corresponding homeopathic MT. In generalized demodicosis amitraz dipping at a concentration of 250 ppm once every 2 weeks is recommended, but the efficacy is increased significantly when dips are increased to once weekly and increasing the concentration to 500 ppm (Birchard and Sherding 2006). Mueller (2004) reported 693 cases of juvenile- and adult-onset generalized demodicosis, where 65% were in remission after long-term amitraz rinse therapy,

Table 1. Effect of different treatments on clinical scores on the basis of signs and lesions in demodicosed and healthy canines

Groups	Particulars	Days of post treatment		
		0	14	28
1	Healthy control	0.33 ±0.21^{b}	0.50 ±0.22^{b}	0.33 ±0.21^{d}
2	D^++Amitraz	17.33 ±0.88^{aA}	11.50 ±0.76^{aB}	6.00 ±0.68^{aC}
3	D^++Amitraz+ AR25mg	17.50 ±0.76^{aA}	10.50 ±1.76^{aB}	2.00 ±0.36^{cdC}
4	D^++Amitraz+ AR MT	17.17 ±0.83^{aA}	10.00 ±0.58^{aB}	3.83 ±0.31^{bcC}
5	D^++Amitraz+ TC25mg	17.00 ±0.63^{aA}	9.17 ±0.60^{aB}	3.17 ±0.60^{bcC}
6	D^++Amitraz+ TC MT	17.50 ±0.76^{aA}	10.67 ±0.71^{aB}	4.67 ±0.42^{abC}

Values with superscript A, B, C differ significantly ($P<0.01$) in a row; values with superscript a, b, c, d differ significantly ($P<0.01$) in a column.

9% were maintained in remission with ongoing treatment, and 25% exhibited no response, or relapsed, or therapy was stopped because of adverse effects. Better clinical recovery in *Asparagus racemosus* extract group might be due to the better restoration of immunity as compared with other treated groups though the skin scraping examination scores were same as in *Terminalia chebula* extract group.

Cellular and humoral immunity evaluation

There was significant (P<0.01) decrease in skin fold thickness in demodicosed canine as compared with healthy control (Table 2) after 24 h of PHA-P intradermal injection. The increased skin fold thickness was significantly higher in group 3 as compared with group 2 on day 28 post treatment. Reddy and Rao (1992) studied the skin reactions to phytohaemagglutinin P and concanavalin A in healthy controls and demodectic dogs and found that the mean diameter of skin reaction to both the lectins was significantly reduced in generalized demodicosis at 24 h and almost completely reduced at 48 h whereas skin reaction persisted in all controls as well as in dogs with localized demodicosis at the end of 48 h, indicative of cell-mediated immune deficiency in generalized demodicosis. The lowered response in infested group might be a state of severe T-cell suppression in infected dogs (Tarpataki and Kadocsa 2004). *Asparagus racemosus* extract (group 3) and its homeopathic MT (group 4) responded better recovery of cellular immunity in demodicosed canine as compared with only amitraz therapy (group 2), might be due to the immunostimulative effect of this plant. The alcoholic extract of *Asparagus racemosus* enhanced both, humoral and cell mediated immunity in albino mice injected with sheep RBC as particulate antigen (Muruganadan *et al.* 2000). *Asparagus racemosus* extract exhibited significant proliferative effects on T and B lymphocytes in presence of antigenic stimulus, which further supports its adjuvant role to activated lymphocytes (Shive *et al*. 2000).

In the present study, there was lower antibody titre in demodicosed canine as compared with healthy control animals. So, the affected animals were in a state of humoral immunosuppression. Castellano and Portionsky (1988) demonstrated that sera from dogs with demodicosis significantly reduced the phagocytic capacity of macrophages and interfered with the macrophage-T lymphocyte interaction

Table 2. Effect of treatment on cellular and humoral immune response in demodicosed canine

Groups	Particulars	Cellular immunity (thickness in mm)			Humoral immunity (titre dilution)	
		Day 0	Day 14	Day 28	Day 14	Day 28
1	Healthy control	2.47 ^{a}±0.18	2.53 ^{a}±0.15	2.50 ^{a}±0.14	33.33 ^{a}±7.28	45.33±8.68
2	D^{+}+Amitraz	1.02 ^{bB}±0.09	1.38 ^{bAB}±0.09	1.62 ^{cA}±0.09	13.33 ^{b}±1.69	28.00±4.00
3	D^{+}+Amitraz+AR25mg	1.00 ^{bC}±0.11	1.60 ^{bB}±0.06	2.10 ^{abA}±0.10	21.33 ^{ab}±3.37	42.67±6.75
4	D^{+}+Amitraz+AR MT	1.0 ^{bB}±0.11	1.43 ^{bA}±0.08	1.80 ^{bcA}±0.10	18.67 ^{ab}±2.67	34.67±6.42
5	D^{+}+Amitraz+TC25mg	1.02 ^{bC}±0.09	1.50 ^{bB}±0.06	1.92 ^{bcA}±0.09	20.00 ^{ab}±4.00	38.67±8.86
6	D^{+}+Amitraz+TC MT	1.02 ^{bB}±0.09	1.40 ^{bAB}±0.13	1.73 ^{bcA}±0.13	16.00 ^{ab}±3.58	32.00±7.16

Values with superscript A, B, C differ significantly (P<0.01) in a row; values with superscript a, b, c differ significantly (P<0.01) in a column.

Table 3. Effect of different treatment on antioxidant parameters in demodicosed canine

Gr	GSH (µmol/g Hb)			LPO (nmol MDA/mg Hb)			SOD (U/mg Hb)			CAT (U/mg Hb)		
	Day 0	Day 14	Day 28	Day 0	Day14	Day28	Day 0	Day 14	Day 28	Day 0	Day14	Day28
1	9.68 ^{a} ±0.52	9.52 ^{a} ±0.56	9.59 ^{a} ±0.53	1.28 ^{b} ±0.05	1.29 ^{d} ±0.03	1.27 ^{d} ±0.05	0.307^{b} ±0.022	0.309 ^{b} ±0.025	0.312 ^{b} ±0.025	2.61 ^{a} ±0.09	2.62 ^{a} ±0.09	2.64 ^{a} ±0.09
2	4.23 ^{bC} ±0.31	5.61 ^{cB} ±0.33	7.04 ^{cA} ±0.15	2.67 ^{aA} ±0.07	2.21 ^{aB} ±0.05	1.92 ^{aC} ±0.05	0.892 ^{aA} ±0.059	0.730 ^{aAB} ±0.043	0.573 ^{aB} ±0.028	0.82 ^{bC}± 0.04	1.20 ^{cB} ±0.04	1.87 ^{cA} ±0.04
3	4.30 ^{bC} ±0.23	6.61 ^{bcB} ±0.12	8.46 ^{abA} ±0.23	2.65 ^{aC} ±0.04	1.97 ^{bcB} ±0.06	1.64 ^{bcA} ±0.06	0.880 ^{aA} ±0.033	0.641 ^{aB} ±0.063	0.465 ^{aB} ±0.027	0.88 ^{bC} ±0.05	1.61 ^{bB} ±0.04	2.10 ^{bcA} ±0.07
4	4.32 ^{bC} ±0.15	6.05 ^{bcB} ±0.11	7.23 ^{bcA} ±0.26	2.65 ^{aA} ±0.07	2.18 ^{abB} ±0.04	1.87 ^{abC} ±0.07	0.884 ^{aA} ±0.041	0.689 ^{aAB} ±0.056	0.533 ^{aB} ±0.056	0.92 ^{bC} ±0.06	1.35 ^{cB} ±0.05	1.98 ^{cA} ±0.06
5	4.26 ^{bC} ±0.27	6.88 ^{bcB} ±0.21	8.83 ^{aA} ±0.28	2.64 ^{aA} ±0.05	1.82 ^{cB} ±0.07	1.49 ^{cdC} ±0.05	0.893 ^{aA} ±0.054	0.618 ^{aB} ±0.043	0.420 ^{abC} ±0.021	0.89 ^{bC} ±0.07	1.78 ^{bB} ±0.05	2.27 ^{bA} ±0.04
6	4.30 ^{bC} ±0.23	6.22 ^{bcB} ±0.17	7.45 ^{bcA} ±0.26	2.64 ^{aA} ±0.08	2.13 ^{abB} ±0.05	1.78 ^{abC} ±0.07	0.900 ^{aA} ±0.040	0.672 ^{aB} ±0.066	0.507 ^{aB} ±0.045	0.91 ^{bC} ±0.05	1.29 ^{cB} ±0.05	1.95 ^{cA} ±0.07

Values with superscript A, B, C differ significantly (P<0.01) in a row in concerned antioxidant parameter; values with superscript a, b, c differ significantly (P<0.01) in a column.

and both properties returned to normal after recovery. During a primary humoral response, IgM is secreted initially, often followed by a switch to a primary response which can last from only a few days to several weeks. The capacity to develop a secondary humoral response depends on the existence of population of memory B cells as well as memory T cells (Goldsby *et al*. 2000). *Asparagus racemosus* extract (group 3) and its homeopathic MT (group 4) exhibited better response of humoral immunity in demodicosed canine as compared with only amitraz treated animals (group 2) might be due to immunostimulatory effect of this plant. *Asparagus racemosus* extract also exhibited better improvement of humoral immunity as compared with *Terminalia chebula* extract. Extracts and formulations prepared from *Asparagus racemosus* exhibited various immunopharmacological actions such as increase in white cell counts, haemagglutinating and haemolytic antibody titres in cyclophosphamide (CP) treated mouse ascetic sarcoma (Diwanay *et al.* 2004). *Asparagus racemosus* produced higher antibody titres, cytoprotection and increased host resistance to tumours in experimental models (Thatte and Dahanukar 1988,Yun and Lee 2005). There was *in vivo* effects of *Asparagus racemosus* extract on effector T cell immunity and suggested its use in conditions where broader stimulation of Th1 and Th2 immunity is required (Gautam *et al*. 2009).

Antioxidant evaluation: In demodicosed canine there was significant (P<0.01) decrease in erythrocyte's reduced glutathione (GSH) level as compared with healthy control (9.68 µmol/g Hb) on day 0. On day 28 post therapy there was significant (P<0.01) increase in GSH level in groups 3 and 5 as compared with group 2. Griffith (1999) reported that GSH protects the erythrocyte membrane from oxidative damage. GSH can react with different electrophilic compounds and can effectively scavenge free radicals either directly or indirectly through enzymatic reactions (Fang *et al*., 2002). Dimri *et al*. (2008) reported that lower levels of GSH in localized demodicosis and generalized demodicosis might be the enhanced utilization for neutralization of excess free radicals generation in these conditions. *Terminalia chebula* extract (Gr. 5) exhibited highest recovery in GSH level within the therapeutic groups might be the protection of sulphydryl groups in glutathione from oxidative damages. Suchalatha and Shyamala Devi (2005) also reported that *Terminalia chebula* administration increased glutathione peroxidase activity.

Lipid peroxide (LPO) in terms of malondialdehyde (MDA) production was significantly (P<0.01) increased in demodicosed canine as compared with healthy control on day 0. LPO was gradually in decreasing level in therapeutic groups on day 14 and the highest decrease was noticed in *Terminalia chebula* extract treated canine (Gr. 5). On day 28 post treatment, there was a significant (P<0.01) decrease in the level of LPO in group 5 (1.49 nmol MDA/mg Hb) as compared with groups 2, 4 and 6. In demodicosed animals there was significant increase in the level of LPO as compared with healthy control, suggests an enhanced oxidative damage to erythrocytes, either due to compromise in antioxidant defense or excess production of free radicals. *Terminalia chebula* extract exhibited best reduction of LPO level might be due to the ability of plant in scavenging the hydroxyl and superoxide radicals. Cheng *et al.* (2003) also reported that *Terminalia chebula* exhibited antioxidant activity at different magnitudes of potency for anti-LPO, anti-superoxide radical formation and free radical scavenging activities.

In the present study, there was significant increase and decrease of erythrocytic SOD and CAT activity respectively, in demodicosed canine, might be associated with overproduction of reactive oxygen species or their accumulation due to dysfunction of the antioxidant system. SOD and CAT are the primary antioxidant enzymes present in mammalian cells. SOD catalyses the formation of O_2 from reactive oxygen species. In enzymatic antioxidant system SOD, CAT and glutathione peroxidase counteract the free radical and reduce oxidative stress. SOD removes superoxide (O^-_2) by converting it to H_2O_2 which can be rapidly converted to water by CAT (Halliwell *et al*., 1992). Dimri *et al*. (2008) showed significant increase in erythrocytic SOD activity in demodicosed canine. The highest improvement of SOD and CAT activity was observed in *Terminalia chebula* extract treated canine, might be the ability of plant to reduce the accumulation of free radical generation. Its aqueous extract of *Terminalia chebula* was reported to have free radical scavenging and radio-protector properties (Naik *et al*. 2004). It exhibited maximum inhibition in the TBA formation, restoration of antioxidant enzyme SOD from radiation-induced damage (Naik *et al.* 2003).

In conclusion, demodicosis produces immunosuppression and oxidative stress in canine and for the better recovery the combination of amitraz dipping along with oral *Asparagus racemosus* extract responded well as compared with the combination of amitraz dipping along with *Terminalia chebula* extract or homeopathy *Asparagus racemosus* mother tincture or homeopathy *Terminalia chebula* mother tincture.

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