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The inhibitory response of *Azadirachta indica* extract on nitric oxide production by milk leukocytes during clinical mastitis

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ABSTRACT

The somatic cell count (SCC), total bacterial count (TBC), differential leukocyte count (DLC) of milk and production of nitrite plus nitrate (NOx) by milk cells in response to hydro-methanolic extract of *A. indica* was evaluated against bovine mastitis. Thirty six lactating cows were selected and divided into four equal groups. Groups I and II consisted of 18 cows selected on the basis of somatic cell count (SCC) <0.5 million/mL of milk, and out of the 18 cows, 9 cows served as the normal healthy control and 9 healthy cows served as drug (herb) control. Eighteen cows, in groups III and IV, positive for intramammary infection (IMI) showing SCC >4.5 million cells / mL of milk, were taken for the drug trial. The cows of group II and III received 700 mg sterile hydro-methanolic extract of *A. indica* by the intramammary route twice daily for 7 days. Cows in group IV positive for IMI were left as the untreated control. Observations were made up to 15 days post treatment (PT) for changes in SCC and TBC. The SCC and TBC reduced significantly ($P<0.05$) as early as on day 3 in group III cows. Milk DLC and NOx production by milk cells were studied just prior to treatment and on day 5. In the present study the lymphocyte % in milk enhanced significantly ($P<0.05$) in group III cows on day 5 PT, however the neutrophil % and NOx production by milk cells decreased significantly ($P<0.05$) in group III cows on day 5, whereas no significant changes in the SCC, TBC, NOx and milk DLC were observed in group IV cows in the post therapeutic period. The results suggest the anti inflammatory, antibacterial potential of the herb, which activities could be due to the presence of bio-active principles that are anti-inflammatory and antibacterial in nature. The present study therefore emphasizes the use of *A. indica* as an anti-inflammatory and antibacterial drug against bovine mastitis. This is a preliminary trial indicating beneficial effect of the herb against IMI; it can be developed as an alternative therapy against bovine mastitis.

Key words: anti-inflammatory, *Azadirachta indica*, mastitis, nitric oxide, somatic cells

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Introduction

Mastitis is one of the most costly and problematic diseases of dairy animals. It is a complex multifactorial inflammatory reaction, which often results from an intramammary bacterial infection (GROHN et al., 2004). In intramammary infection (IMI), leukocytes are the predominant cell types that travel from peripheral circulation to mammary gland in response to inflammatory insults and attributed to pathophysiology of the mammary gland (BAUMAN and GAULDIE, 1994). Among the secretory products of the inflammatory cells, reactive nitrogen intermediates (RNIs) and reactive oxygen species (ROS) are the important radicals which play a complex role in inflammatory process (GOFF et al., 1996). The exact role of nitric oxide (NO) released by leukocytes during clinical mastitis (CM) has been an area of hot debate as it is a mediator of inflammation that accompanies mastitis (LACASSE et al., 1997). The possible clinical relevance of NO production associated with a rise of systemic TNF-α in acute *E. coli* mastitis has also been reported (BLUM et al., 2000). Nitric oxide produced in large amounts by inducible nitric oxide synthase (iNOS) and its derivatives, such as peroxynitrite and nitrogen dioxide, play a role in inflammation and also possibly in the multistage process of carcinogenesis (OSHIMA and BARTSCH, 1994). The inflammatory insult caused by release of highly reactive molecules is mitigated by intracellular defense mechanisms against oxidation, which might reduce mammary cell damage during acute inflammation (NDIWENI et al., 1991).

Antibiotics are used widely for treatment of mastitis, however the therapeutic success rate is poor and it can not prevent the inflammatory reaction driven by the host leukocyte against bacterial intruders (BOUCHARD et al., 1999). Inflammatory conditions can be controlled by inhibition of inflammatory mediators, several plants such as *Abies holophylla*, *Actinidia arguta*, *Artem isiaiwayomogi*, *Larix leptolepis*, *Machilus thunbergii* and *Populus davidiana* have been reported to possess inhibitory potential against NO production (RYU et al., 2003). *Azadirachta indica* is very commonly known as Neem, and owing to its immense therapeutic properties, it is also referred to as the village dispensary (BISWAS et al., 2002). The Neem plant is extensively used in the Indian system of medicine (Ayurveda). The herb is used against wounds, respiratory disorders, skin disorders, metritis and mastitis in ethnoveterinary practices (WILLIAMSON, 2002). Neem seed contains a mixture of terpenes, limonoids and polysaccharide compounds, whose bioactive principles have been reported to possess anti-inflammatory, antibacterial and other therapeutic properties (MAJUMDER et al., 1987; TEWARI, 1992; KUROKAWA et al., 1990). There is dearth of literature regarding the effect of neem seed extract on NO production during bovine mastitis. Therefore, the present research focuses on the effect of neem seed kernel extract on NO production by milk cells in bovine clinical mastitis.

Materials and methods

Collection of plant materials and preparation of herbal extract. The seed of *A. indica* was collected from the campus of Indian Veterinary Research Institute (IVRI), Izatnagar. The plant materials were identified at the National Botanical Research Institute, Lucknow (India). The seeds were washed, dried and ground to a course powder. Extraction was performed as per the method described earlier (PEACH et al., 1956). The seed powder was loaded into sox let apparatus and extracted with 70% methanol (yield 14.28% w/v), dried under Vacuo below 40 °C. The condensed herb was reconstituted in sterile phosphate buffer saline (PBS, 10 mM, pH 7.4) having 700 mg extract /5 mL PBS. The reconstituted extract was filtered through a membrane filter (0.22 μm pore size, Millipore, Bangalore Pvt. Ltd., India) and refrigerated in a sterile container for intramammary infusion. The dose of *A. indica* extract was standardized in the pilot study, by taking 4 cows in 2 batches with clinical mastitis. Phytochemical analysis of the extract was performed as per the method described earlier (KHANDELWAL, 2006).

*Screening of cows and experimental design.*Thirty-six crossbred lactating cows aged between 3-5 years in their first to third lactation were selected from the organized dairy farm at the institute (Indian Veterinary Research Institute, Izatnagar). The cows were maintained in the institute's dairy farm animal shed under identical management practices and were divided into 4 equal groups. Group I and Group II consisted of 18 clinically healthy cows, selected on the basis of somatic cell count (SCC) <0.5 million/mL of milk and negative for intramammary infection. Nine cows in Group III and 9 cows in group IV positive for intramammary infection, screened on the basis of SCC >4.5 millions/ mL of milk were taken for the drug trial. Seven hundred mg of sterile *A. indica* extract was infused per teat via the intra mammary route in Group II and Group III cows, after diluting the drug in 5 mL sterile phosphate buffer saline twice daily for 7 days, whereas, nine diseased cows of group IV were left as the untreated control.

Collection of milk samples. Hundred milliliters of milk from each cow was collected in sterile vials after cleaning the teat surface with 70% ethanol and after discarding a few streams of milk. The milk samples were collected on day 0 and thereafter on days 3, 7 and 15. Somatic cell count of milk was performed as per the method described by SCHALM et al. (1971). The total bacterial count (TBC) was carried out as per the standard method (GRIFFIN et al., 1977). The identification of causative organisms in the collected milk samples was performed by spreading 10 μL of milk over 5% bovine blood agar plate, and the growth of the organism on selective media. The organisms were identified on the basis of colony morphology, characteristic hemolytic pattern and Gram's staining and further processed for biochemical tests (BALOWS et al., 1991).

Separation of milk cells. Isolation of cells from the milk was carried out as per the method described by DALEY et al. (1991). The cell suspension was adjusted to 1×10^7 cells/mL in sterile PBS (10 mmol, pH 7.4) for nitric oxide production assay.

Production of nitrite plus nitrate (NOx) by milk cells. Nitric oxide production of milk cells was measured by nitrate reduction on copper-cadmium alloy (Cu-Cd alloy) followed by colour development with a Griess reagent as per the method described earlier (SASTRY et al., 2002). In brief, 100 µL of 1×10^7 cells/ mL in phosphate buffer saline (PBS, pH 7.4, 10 mmol) stimulated with 25 µg of lipopolysaccharide (LPS) was incubated at 37 ºC for 24 hours. After incubation, 100 µl of pre-stimulated cells were suspended in 400 µl of carbonate buffer (50 mM) with 150 mg of Cu-Cd fillings and incubated at room temperature with frequent vortexing for 1 hour. The reaction was stopped by adding NaOH (0.35 M) and $ZnSO_4$ (120 mM). Further, the mixture was vortexed and centrifuged at 400 g for 15 minutes. Finally the Griess reagent was added to the clear supernatant in equal volume and the OD was measured at 545 nm in a micro plate reader after a reaction of 10 minutes. The observations were made before treatment and thereafter on day 5.

Differential leukocyte count (DLC) of milk. The differential leukocyte count (DLC) of milk was carried out as per the method described by DULIN et al. (1982). Numbers of neutrophil and lymphocyte were counted in 100 cells and expressed in percentages. The observations were made before treatment and thereafter on day 5.

Statistical analysis. The data were analyzed using one-way variance analysis. The Mean ± SE of the same group of treatment was analyzed using Duncan's Multiple Range Test as per the standard method (SNEDECOR and COCHRAN, 1994).

Results

Phytochemical analysis of plant extract. The hydro-methanolic extract of *A. indica* was brownish-black in colour, sticky in nature and bitter in odor. The chemical analysis of the extract revealed the presence of triterpene and carbohydrate.

Somatic cell count (SCC) and total bacterial count (TBC). The SCC and TBC in milk of healthy cows ranged from 3.07 ± 0.33 to $3.77 \pm 0.50 \times 10^5$ cells/mL and 0.33 ± 0.02 to $0.36 \pm 0.03 \times 10^3$/mL of milk respectively in group I cows. Intramammary infusion of *A. indica* extract did not show any significant change in SCC and TBC in group II cows. The SCC of group III cows significantly ($P<0.05$) decreased to the extent of 30.66%, 63.99% and 77.37% on days 3, 7 and day 15 respectively (Table 1).

Similarly the TBC level decreased significantly ($P<0.05$) in group III cows to the extent of 24.82% on day 3 but a non significant decrease (34.08%) was observed on day 7, however, the TBC significantly ($P<0.05$) decreased to 62.63% on day 15 by intramammary infusion of the herbal extract. However, the SCC and TBC in group IV remained significantly higher than the *A. indica* group until day 15 (Table 1). Out of the eighteen milk samples collected from diseased cows, the organism isolates were *Staphylococcus aureus* (28%), *Streptococcus agalactiae* (17%), other *Streptococcus* sp. (33%), coliform bacilli (22 %) (Table 1).

Production of nitrite plus nitrate (NOx) by milk cells. The Nox production ranged between 4.44 ± 1.14 to 4.57 ± 0.87 µmol/1 × 107 cells/24 hrs in milk cells isolated from healthy cows (group I). Intramammary infusion of *A. indica* extract did not show any significant change in Nox production in group II healthy cows. However, Nox production in the milk cells of group III cows was significantly high before treatment as compared to normal healthy cows. It significantly ($P<0.05$) decreased to 137.54 % on day 5 compared to the day 0 value. However, non significant changes of Nox production were observed in group IV untreated cows on day 5 (Table 2).

Milk differential leukocyte count (DLC). The neutrophil % and lymphocyte % in the milk did not show any significant changes on day 0 and day 5 in group I and group II cows. However, the neutrophil % decreased significantly ($P<0.05$) to 31.31% and lymphocyte % increased to 53.12% on day 5 compared to the day 0 value in group III cows treated with *A. indica* extract. There was no significant difference in milk DLC in group IV untreated cows on day 5 (Table 3).

Table 1. Somatic cell count (SCC) ($\times 10^5$ cells/mL) and Total bacterial count (TBC) ($\times 10^3$/mL) in response to the treatment with *Azadirachta indica* extract (group II and group III) compared with normal healthy cows (group I) and untreated control cows (group IV).

Parameter group		Days post treatment			
		0 day	day 3	day 7	day 15
SCC	I	$3.77 \pm 0.50^{a,x}$	$3.33 \pm 0.29^{a,x}$	$3.07 \pm 0.33^{a,x}$	$3.27 \pm 0.27^{a,x}$
	II	$3.76 \pm 0.19^{a,x}$	$3.62 \pm 0.27^{a,x}$	$3.42 \pm 0.24^{a,x}$	$3.43 \pm 0.24^{a,x}$
	III	$45.66 \pm 1.73^{a,y}$	$31.66 \pm 1.56^{b,y}$	$16.44 \pm 0.94^{b,y}$	$10.33 \pm 0.78^{c,y}$
	IV	$46.11 \pm 2.04^{a,y}$	$45.33 \pm 2.72^{a,z}$	$46.22 \pm 1.80^{a,z}$	$46.55 \pm 1.60^{a,z}$
TBC	I	$0.36 \pm 0.03^{a,x}$	$0.33 \pm 0.02^{a,x}$	$0.35 \pm 0.01^{a,x}$	$0.34 \pm 0.04^{a,x}$
	II	$0.35 \pm 0.02^{a,x}$	$0.35 \pm 0.02^{a,x}$	$0.34 \pm 0.02^{a,x}$	$0.34 \pm 0.05^{a,x}$
	III	$22.88 \pm 1.88^{a,y}$	$17.20 \pm 1.44^{b,y}$	$16.40 \pm 1.09^{b,y}$	$8.55 \pm 0.82^{c,y}$
	IV	$23.11 \pm 2.05^{a,y}$	$23.22 \pm 2.19^{a,z}$	$24.22 \pm 2.13^{a,z}$	$23.55 \pm 1.94^{a,z}$

*Value with different superscripts in each row (a, b, c) and each column (x, y, z) differ significantly ($P<0.05$)

Table 2. Changes in production of nitrite plus nitrate (NOx) (μmoles/1 × 10^7 cells/24 hrs) in response to *A. indica* treatment (group II and III) and untreated control (group IV) compared with normal healthy cows (group I).

Group	0 day	day 5
I	$4.44 \pm 1.14^{a,x}$	$4.57 \pm 0.87^{a,x}$
II	$4.54 \pm 0.38^{a,x}$	$4.62 \pm 0.37^{a,x}$
III	$50.90 \pm 15.58^{a,y}$	$19.02 \pm 2.24^{b,y}$
IV	$50.43 \pm 8.25^{a,y}$	$50.66 \pm 7.97^{a,z}$

*Value with different superscripts in each row (a, b) and each column (x, y, z) differ significantly (P<0.05)

Table 3. Changes in milk DLC in response to *Azadirachta indica* extract (group II and group III) and untreated control (group IV) compared with normal healthy cows (group I).

	Neutrophils %		Lymphocyte %	
Groups	0 day	days 5	0 day	day 5
I	$20.66 \pm 1.11^{a,x}$	$20.88 \pm 1.71^{a,x}$	$20.55 \pm 1.01^{a,x}$	$21.55 \pm 1.71^{a,x}$
II	$21.22 \pm 1.47^{a,x}$	$22.11 \pm 1.54^{a,x}$	$21.88 \pm 1.24^{a,x}$	$22.22 \pm 1.62^{a,x}$
III	$64.22 \pm 2.58^{a,y}$	$44.11 \pm 3.10^{b,y}$	$10.88 \pm 0.61^{a,y}$	$16.66 \pm 0.83^{b,y}$
IV	$64.33 \pm 2.38^{a,y}$	$65.22 \pm 2.95^{a,z}$	$11.11 \pm 0.63^{a,y}$	$10.77 \pm 0.57^{a,z}$

*Value with different superscripts in each row (a, b) and each column (x, y, z) differ significantly (P<0.05)

Discussion

Clinical mastitis is commonly observed during the lactation period in high yielding dairy cows. It is accompanied by heavy influx of polymorphonuclear cells into the infected gland from the peripheral circulation (PAAPE et al., 1979). During inflammation the leukocytes travel to the inflammatory site in response to chemotactic stimuli for bacterial clearance. Leukocytes play an important role in defense of the mammary gland, similarly they are also involved in the pathophysiology of many inflammatory diseases by releasing cytotoxic molecules such as reactive nitrogen intermediates (RNIs) and reactive oxygen species (ROS) into the extra cellular space and damage surrounding tissues (SMITH, 1994). Nitric oxide is one of the important secretory reactive molecules of milk cells. It plays a complex role in inflammatory response, apart from several other physiological functions (DAWSON and DAWSON, 1995). Nitric oxide is produced by inducible and non inducible nitric oxide synthase. However, NO produced by inducible nitric oxide synthase is believed to cause tissue damage mainly through peroxynitrite, which is formed by NO and superoxide anion (BECKMAN et al., 1990). The peroxonitrite is a powerful oxidant to nitrosilate protein and the DNA of cells and initiates lipid peroxidation during inflammation (KAUR and HALLIWELL, 1994). Many researchers have

observed increased production of nitric oxide from iNOS activity during bovine mastitis, which results in enhancement of classical markers of mastitis such as raised SCC, bovine serum albumin concentration and N-acetyl-beta-D-glucosaminidase (NAGase) activity in milk (BOUCHARD et al., 1999; DE and MUKHERJEE, 2005). Natural products, including those derived from higher plants, have contributed greatly to the development of modern therapeutic drugs. Certain plants such as *Artemisia iwayomogi, Macholus thunbergii, Populus davidiana* and *Populus maximowiczii,* have been recently identified as iNOS inhibitors. The active principles of these plants, such as bisbenzyle-isoquinoline alkaloids, benzoquinones, sesquiterpenes lactones and curcumenoids (KONDO et al., 1993; NIWA et al., 1997; JANG et al., 2001) have shown inhibitory action on NO production through the inhibition of iNOS expression. *A. indica* has been extensively used in Indian and Chinese systems of medicine for the treatment of various ailments since time immemorial (CHATTERJEE and PAKRASHI, 1994).

In the present study, intramammary infusion of Neem extract significantly ($P<0.05$) reduced the SCC and TBC as early as 3 days after initial treatment, milk neutrophil % and NOx on day 5. Reduction of SCC, milk neutrophil % and NOx and TBC reflects the anti-inflammatory and antimicrobial activities of the herb. The phytochemical analysis of the extract revealed the presence of triterpene and carbohydrate. It has been recorded that triterpene possesses anti inflammatory and antimicrobial effects (HUNTER et al., 1997; KAUR et al., 2004; SIDDIQUE et al., 1992). These active bioactive components of the plant are reported to be potently anticarcinogenic (FUJIWARA et al., 1982), anti-inflammatory (BHARGAVA et al., 1970; FUJIWARA et al., 1984; PILLAI and SANTHAKUMARI, 1981) and antibacterial (JAIN et al., 1987). Similarly, WILLIAMSON (2002) observed the anti-inflammatory and antimicrobial activities of the polysaccharide fraction of neem extract in murine model. Moreover, BISWA et al. (2001) reported that the neem extract exerts its bactericidal action by inhibiting cell membrane synthesis against pathogenic microorganisms. In the present study a significantly increased level of nitric oxide was observed in mastitic cows. Intramammary infusion of *A. indica* seed kernel extract significantly reduced NO production on day 5.

It seems that the bioactive principles of *A. indica* may have synergistically exerted their antibacterial property and anti iNOS activity in diseased cows. NO is one of the important mediators of inflammatory reaction, causing severe inflammatory changes and tissue damage during clinical mastitis, and it can serve as an important biomarker. Plant products such as *A. indica* could be used as an anti-inflammatory and antibacterial arsenal against the disease to reduce the burden of antibiotics. Further larger trials as well as isolation of the active principles is going on in the laboratory for effective drug formulation. This is a preliminary trail indicating the beneficial effect of the herb against IMI; it can be developed as an alternative therapy where the use of antibiotics is normally not recommended.

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SAŽETAK

Određivan je broj somatskih stanica, ukupan broj bakterija, diferencijalni broj leukocita u mlijeku te proizvodnja nitrita i nitrata od strane mliječnih stanica kod liječenja upale vimena krava iscrpkom biljke *Azadirachta indica*. Istraživanje je provedeno na 36 krava u laktaciji, koje su bile podijeljene u četiri jednake skupine. U prvu i drugu skupinu bilo je uvršteno 18 krava na osnovi broja somatskih stanica u mlijeku koji je iznosio <0,5 milijuna/mililitar. Te su krave bile podijeljene u dvije podskupine po devet krava. Jedna podskupina bila je sastavljena od devet zdravih kontrolnih krava dok je u drugoj bilo devet zdravih krava koje su dobivale iscrpak. Osamnaest krava svrstanih u skupine III i IV s upalom vimena i brojem somatskih stanica >4,5 milijuna/mL mlijeka uzete su u pokus s lijekom. Kravama druge i treće skupine bilo je intramamarno primijenjeno 700 mg sterilnoga iscrpka *A. indica* dvaput dnevno tijekom sedam dana. Krave četvrte skupine s infekcijom vimena ostale su kao nedirana kontrola. Broj somatskih stanica i broj bakterija u mlijeku promatran je tijekom 15 dana nakon liječenja. Broj somatskih stanica i ukupan broj bakterija značajno se smanjio ($P<0,05$) već treći dan u krava III. skupine. Diferencijalni broj leukocita i proizvodnja dušikova monoksida bili su određeni prije liječenja i peti dan nakon liječenja. Ustanovljeno je značajno povećanje postotka limfocita ($P<0,05$) u krava III. skupine petoga dana nakon liječenja, ali se istodobno značajno smanjio postotak neutrofila kao i količina nitrita i nitrata ($P>0,05$) u krava III. skupine. Broj somatskih stanica, broj bakterija, količina nitrita i nitrata te diferencijalni odnos leukocita u krava IV. skupine nije se promijenio. Rezultati upućuju na protuupalni i protubakterijski učinak iscrpka biljke, što bi se moglo pripisati bioaktivnim tvarima protuupalne i protubakterijske prirode. Naglašava se da bi se iscrpak mogao rabiti kao alternativni lijek u liječenju mastitisa u krava.

Ključne riječi: protuupalni učinak, *Azadirachta indica*, mastitis, dušikov oksid, somatske stanice