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Evaluation of acute oral toxicity of a herbomineral supplement for immunity against mastitis

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Abstract
Mastitis, one of the costliest health problems faced by dairy farms, is an inflammation of the mammary gland parenchyma, which, together with physical, chemical and microbiological changes of milk, is characterized by an increase in the number of somatic cells in the milk and by pathological changes in the mammary tissue. AV/IAM/16 is a herbomineral supplement for immunity against mastitis. A study was undertaken to evaluate the acute oral toxicity potential of AV/IAM/16 (M/s Ayurvet Limited, India) according to OECD 423 guidelines. Six (3 male and 3 female) Swiss albino mice were used for the study, where each animal served as its own control. After procurement, the animals were kept in the cages for seven days for acclimatization. Thereafter, the animals were fasted overnight; food but not water was withheld for 3-4 hours. Following the period of fasting, the animals were weighed and the test substance was administered orally. Following the oral administration of the test substance, the animals were observed over a period of 14 days for manifestation of toxic effects and mortality. The observations included changes in skin, coat and eyes; and changes in respiratory, circulatory, CNS, autonomic, somatic activity and behavior. Clinical signs like muscular tremors, convulsions, salivation, diarrhea, lethargy, sleep, and coma, if observed, were recorded. At the end of 14 days, necropsy and histopathological parameters were also studied.
No toxic effects or mortalities were observed during the study and, hence, AV/IAM/16 was found to be safe for oral use.

Keywords: AV/IAM/16, limit test, mastitis, OECD 423, oral toxicity, safety

Introduction
Mastitis, an inflammatory reaction of the mammary gland, usually caused by a microbial infection, is recognized as the costliest disease of dairy cattle [1]. It is characterized by physical, chemical and, usually, bacteriological changes in the milk and pathological changes in the glandular tissues. The disease causes huge financial losses to farmers through reduced milk yield and milk quality, reduced milk sale, premature culling, and deaths of affected cow, blindness of milk teats, reduced breeding performance of animal, and also due to associated treatment costs [2]. The disease occurs due to invasion of the mammary glands by pathogenic bacteria, viruses, fungi and mycoplasma; followed by their multiplication in the milk producing tissues [3]. The most common treatment method available against mastitis is the intramammary infusion of antibiotics. However, such treatments pose concerns over the growing menace of antibiotic resistance, undesirable residues, and withholding of milk, as well as cost considerations [4]. Also, the incidence of mastitis has increased in recent years due to the widespread use of milking machines [5]. Mastitis renders milk unsuitable for human consumption and provides a medium for the transmission of diseases like tuberculosis, streptococcal intoxication, colibacillosis, streptococcal sore throat and brucellosis [6]. Public hazards associated with the consumption of antibiotic-contaminated milk and milk products involve allergic responses, changes in intestinal flora, and the development of antibiotic resistant pathogenic bacteria [7]. The prevention of the public hazards associated with the consumption of antibiotic-contaminated milk amidst increasing emergence of antimicrobial resistance demands the development and use of non-antibiotic alternatives. Several herbal extracts have shown *in vitro* antibacterial activity against major mastitis-causing pathogens [8]. AV/IAM/16 is a herbomineral supplement for providing immunity against mastitis, containing herbs like *Tephrosia purpurea* and *Glycyrrhiza glabra*, reputed for their antioxidant [9],

wound-healing [10], anti-inflammatory [11], and antimicrobial properties [12, 13]. The present study aimed at determining the acute oral toxicity potential of AV/IAM/16.

Materials and Methods

The present study was conducted at the Department of Pharmacology and Toxicology, Krantisinh Nana Patil College of Veterinary Science (KNPCVS), Shirwal, District Satara, India. The experimental protocol of the study was got approved by the Institutional Animal Ethics Committee of KNPCVS (Approval number: IAEC/16/KNPCVS/05/2019; dated: 23/08/19). Six healthy adult Swiss albino mice (3 males and 3 females), weighing 20-25g, were used. The animals were procured from a CPCSEA-registered breeding source *i.e.* National Institute of Biosciences, Pune. All animals were maintained as per the SOPs outlined in CPCSEA guidelines. The animals were identified by appropriate means. The number of animals per cage was kept at three for clear observation of each animal; housing conditions were conventional. The ambient temperature was 25°C and relative humidity was 70%. The animals were exposed to 12 hour light-dark cycle and provided with standard pelleted feed and water *ad lib* [14]. After procurement, the animals were kept in the cages for seven days for acclimatization. Thereafter, the animals were fasted overnight; food but not water was withheld for 3-4 hours. Following the period of fasting, the animals were weighed and the test substance was administered orally. After the administration of the test substance @ 2000 mg/Kg body weight, food was withheld for 1-2 hours. The animals were observed intensively for first 24 h, and then further for a period of 14 days for the manifestation of toxic effects and deaths; LD_{50} value was also assessed. The observations included changes in skin, coat and eyes; and changes in respiratory, circulatory, CNS, autonomic, somatic activity and behavior. Clinical signs like muscular tremors, convulsions, salivation, diarrhea, lethargy, sleep, and coma, if observed, were recorded. At the end of 14 days, necropsy and histopathological parameters were also studied.

Results and Discussion

Individual body weights of mice were recorded on days 0, 7 and 14 of the study and body weights in both the groups (I and II) continued to increase throughout the study period (Table 1).

Table 1: Individual body weights of experimental mice

Formulation and Dose	Mice No.	Body Weight (g) on Day		
		0	7	14
AV/IAM/16@ 2000 mg/Kg b.wt. orally (Group I: Females)	1	22	24	25
	2	20	22	25
	3	21	22	25
AV/IAM/16@ 2000 mg/Kg b.wt. orally (Group II: Males)	1	22	22	23
	2	21	24	24
	3	23	24	24

No mortality was observed throughout the period of observation in the mice after receiving the limit dose of AV/IAM/16 @ 2000 mg/Kg body weight, *i.e.* the maximum dose which can be administered by oral route, and, hence, the LD_{50} was inferred to be beyond this limit. Similarly, no abnormal symptoms, including lethargy, tremor, abdominal breathing, piloerection were observed up to 14 days of AV/IAM/16 administration. Necropsy and histological investigations after day 14 did not show any remarkable changes in the gross or microscopic appearance of liver, kidney, spleen, heart, lungs, and genital organs in any of the animal.

Besides minerals like selenium, copper AV/IAM/16 contains parts of plants like *Tephrosia purpurea*, *Glycyrrhiza glabra*, *etc*. that fall under the category of Generally Regarded as Safe (GRAS). *Tephrosia purpurea* is well-known for its healing, anti-ulcerative and antibacterial properties. It is credited with the abilities to stabilize mast cells and enhance the integrity of erythrocyte membranes in various animal models [15]. Histopathological study of wounds in rats showed significant increase in fibroblast cells and collagen fibers, and blood vessel formation with the use of *Tephrosia purpurea*-based ointment [16]. *Glycyrrhiza glabra* contains glycyrrhizin (GL), reputed for immunomodulatory functions in animals such as the stimulation of lymphocytes and induction of interferons (IFN) to enhance killer activities of the natural killer cells (NK) [17]. In addition, GL is known to facilitate activation of the extra-thymus differentiated T-cells including $\gamma\delta^+$ and $CD8^+$ T-cells that are selectively distributed in the intestinal tract and mucosal organs independently of the thymus of the mouse [18]. Previously, the cold aqueous extract of *Glycyrrhiza glabra* roots exhibited potent antimicrobial action against both Gram positive (*Staphylococcus aureus*) and Gram negative (*Escherichia coli*) microorganisms implicated in bovine subclinical mastitis [19]. In another study supporting the anti-inflammatory role of GL in mastitis control, it was observed that intramammary infusion of GL alone resulted in significant improvements in swelling and firmness of the mammary glands, and decreased the somatic cell count (SCC) and number of clots in milk. The percentage of neutrophils decreased significantly (to less than 30%) by two days after administration; lactoferrin, a marker of inflammation in mammary glands, decreased in concentration, whereas α-lactalbumin, a marker of recovery, increased significantly. Accompanying these anti-inflammatory effects, a decrease in the concentration of histamine in milk was observed and the decrease in histamine production by milk leukocytes was concentration-dependent [20].

AV/IAM/16 can exert diverse benefits, including protection against mastitis by immunomodulation, decreasing bacterial load in udder tissue, improvement in swelling, redness and firmness of mammary gland, and improvement in milk quality by decreasing somatic cell count in milk and number of clots in milk, in turn preventing farmers from huge economical losses from mastitis and, thus, maximizing their economic returns.

Conclusion

AV/IAM/16 did not produce acute oral toxicity, evident as absence of mortality, toxic clinical symptoms, and gross and histopathological alterations, when administered up to limit dose (2000 mg/Kg) in mice. Based on this study, the formulation was found safe for oral use.

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