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GASTRO PROTECTIVE EFFECTS OF *USNEA LONGISSIMA* METABOLITES ON PROBIOTIC *LACTOBACILLUS CASEI*

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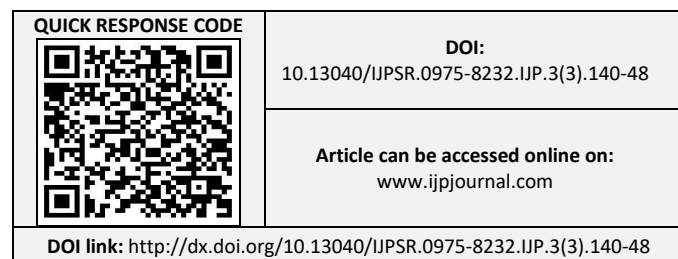
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ABSTRACT: Protective effect of the probiotic combination of usnic acid and *Lactobacillus casei* in experimentally induced ulcer in rats was investigated. Rats inward usnic acid (100 mg/kg), *L. casei* (10^{-8} con.), usnic acid (100 mg/kg) + *L. casei* (10^{-8} con.), and omeprazole (30 mg/kg) twice daily for 5 days for prevention against aspirin (ASP), ethanol (EtOH), cold restraint stress (CRS) and pylorus ligation-induced ulcer (PL). The results of the present study showed the first time that the usnic acid (100 mg/kg) + *L. casei* (10^{-8} con.) as probiotic combination significantly inhibited the ulcer index in ASP, EtOH, CRS and PL to 3.4 ± 0.12 , 6.3 ± 1.8 , 3.4 ± 0.8 and 4.3 ± 0.9 ($p < 0.001$) respectively, as compared to control group (19.2 ± 1.6 , 22.5 ± 6.3 , 24.2 ± 3.2 , 14.2 ± 2.7). Besides usnic acid (100 mg/kg) + *L. casei* (10^{-8} con.) offered protection (72%, $p < 0.001$) against ethanol-induced depletion of gastric wall mucus. The usnic acid (100 mg/kg) + *L. casei* (10^{-8} con.) showed significant inhibition of lipid peroxidation and superoxide dismutase (5.46 ± 1.30 , 120.6 ± 3.2) ($p < 0.01$, $p < 0.001$) respectively and enhance activity of catalase (32.2 ± 1.3 , $p < 0.001$) to healthy group range (34.2 ± 2.7 , $p < 0.001$). However, it elevated the decreased level of PGE₂ from 0.62 ± 0.12 ($p < 0.05$) to 2.03 ± 0.51 ($p < 0.001$) as compared to omeprazole. These results suggest that probiotic combination could attenuate the severity of gastric ulcer and prevent the toxicity level of usnic acid in the liver.

INTRODUCTION: Abnormal secretion of gastric acid and pepsin are the main reason for gastric ulcer disease. Nowadays, critical issue for gastric ulcer disease is gastric hyper secretion-linked with gastrinoma in Zollinger-Ellison syndrome, raise in parietal-cell mass, antral G-cell hyperplasia, and a physiological disproportion between the antagonistic gastric hormones gastrin and somatostatin.

It is known that cholinergic hypersensitivity and parasympathetic dominance both are related to pepsin¹. Besides having anti-inflammatory and analgesic activity, NSAID especially aspirin; significantly increase the risk of gastrointestinal infection in Asian countries. Individually those are related to gastric injury: Ulcer complications especially bleeding. NSAIDs cause an injurious effect by the inhibition of COX1 and its function in standard mucosal protection mechanisms, and also by the inhibition of thromboxane A₂, which accord platelet function and cause in gastrointestinal bleeding²⁻⁵ but simultaneous low-doses of aspirin reduces the effect². However, the interaction between microbial flora and pathogenic organisms has been reported⁶.



Probiotics have been used to decrease the colonization of intestinal pathogens in human, and these are surviving microbial food ingredients that alter the enteric flora and have a constructive effect on health⁷. *L. casei* is probably the best-known probiotic micro-organism and its ability to hold stomach wall and to grow under acidic circumstances⁸. *L. casei* as supplement improve immunity and decrease the risk of disease akin to colon cancer, allergies, diarrhoea⁹ and can prevent peptic ulcer which is caused by long term use of NSAIDs like aspirin.

Lichens are symbiotic associations of algae and fungi. Indeed, *Usnea longissima* (family Parmeliaceae with 1000 species across the world), has been used conventionally for pain relief and fever control¹⁰. It is also effective as anti-bacterial, anti-inflammatory, detoxifying agent¹¹. Usnic acid **Fig. 1**, most common and abundant metabolite of *U. longissima*¹², has anti-viral, antiprotozoal, anti-inflammatory, anti-pyretic, anti-tumoral, liver protective and analgesic activity¹³⁻¹⁷. Indeed, the effect of *U. longissima* showed significant antiulcer activity.

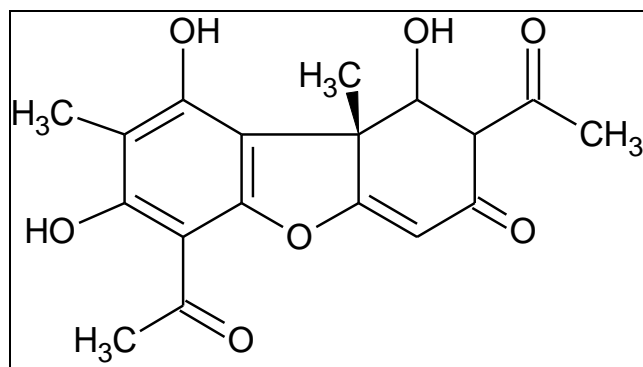


FIG. 1: STRUCTURE OF USNIC ACID

In continuation, the aim of our present study is to reduce the toxicity of usnic acid along with probiotic potential¹⁷. Therefore, in this study, we have investigated the anti-ulcer activity of *L. casei* in combination with lichen secondary metabolites.

MATERIALS AND METHODS:

Collection and Identification: Lichen material (*Usnea longissima*) was collected from Uttarakhand (India) in September 2012, known by its vernacular names *U. longissima*. The specimen has been submitted and perpetuate as the specimen in the herbarium of CSIR-National botanical research Institute, Lucknow (LWG).

Extraction and Isolation of Usnic Acid: Air-dried parts (200 g) of *U. longissima* were extracted with 300 ml of diethyl ether by Soxhlet apparatus, and usnic acid was isolated. Filtered extract was stored at 3-5 °C for 24 h to precipitate out usnic acid. A precipitate was subjected to silica gel (70-230 mesh) column chromatography eluted with chloroform and *n*-hexane (90:10). 876.5 mg of usnic acid was obtained with 0.88% (w/w) yield. UV, IR and NMR determined chemical structure of usnic acid and also confirmed by previously established spectral data¹⁸.

General Analytical Procedures: All the required chemicals were purchased from Mark (India). UV-Visible spectrum for the biochemical assays of Usnic acid was taken by using lambda 25 PerkinElmer spectrophotometer. Alpha Bruker FT-IR spectrophotometer was used to record IR spectra, in KBr pellets. NMR spectra obtained at 200MHz for 1H and 50MHz for 13C (δ) on Varian spectrometer.

Animals: Experimental animals (Wistar rats of either sex) were acquired from the National Laboratory Animal Centre, Lucknow, India. For one week all the animals were kept under conventional conditions (temp., 24 ± 5 °C, and relative humidity 40-46%) and fed traditional rodent pellet diet (Amrut, India). Light/dark cycles of 12 h respectively, were maintained for seven days before and during the experiments. The food was withdrawn 18-24 h before the experiment while water was allowed impromptu. Experiments were performed according to the guidance given by the Institutional Animal Care Committee, CPCSEA, India (Reg. No. 173/GO/Re/13/CPCSEA).

Microorganism: Bacterial strain *L. casei* (NCIM 2737) was procured from National Collection of Industrial Microorganisms at National Chemistry Laboratory, Pune, India and maintained on recommended MRS medium. To determine the suitable concentration of *L. casei* for the study of growth promoting effects of lichen metabolites, bacterial culture was serially diluted in the concentration of 10⁻², 10⁻⁴, 10⁻⁶ and 10⁻⁸ in MRS broth medium and kept at 37 °C for 24 h. The resulting 10⁻⁸ concentration having OD₅₅₀ = 0.2 was selected for the experiments

Doses: Usnic acid in dose of 100 mg/kg (p.o.), *L. casei* 10⁻⁸ con (p.o.), usnic acid 100 mg/kg (p.o.) + *L. casei* 10⁻⁸ con. (p.o.), and H⁺ K⁺ ATPase blocker, omeprazole in a dose of 30 mg/kg (i.p.) were administered twice daily at 09:00 and 15:00 h, respectively for 5 days for acute ulcer protective studies. Control group of animals received a suspension of 1% w/w carboxymethyl cellulose in double distilled water (10 ml/kg).

Aspirin-Induced Ulcers (ASP): Aspirin was administered at a dose of 200 mg/kg and ulcers were created after 4 h. Animals were sacrificed, and the abdomen was then excised¹⁹. It was cut on the larger curvature and washed thoroughly with 10.0 ml of 0.9% NaCl. Ulcers were scored by the person unaware of the experimental protocol. Lesion index was calculated after recording the full severity of ulcers per abdomen. Total severity of the ulcers resolved by the technique of Sanyal *et al.*, 1982.²⁰

Ethanol-Induced Ulcers (EtOH): Gastric ulcers were induced in rats by administering ethanol (1ml/200 g, 1 h)²¹. After cervical dislocation of animals, the stomach was cut along the greater curvature and inspected for ulcers. Ulcer index (mm²/rat) was calculated by multiplying length and breadth of ulcers present in the glandular portion of the stomach. Unpaired Student's *t*-test was used for Statistical analysis.

Cold-Resistant Stress-Induced Ulcers (CRS): About 18 h before the experiment food was withdrawn, but the water was allowed. On the sixth day, experimental animals were immobilized by fastening the fore and hind limbs on a wooden board and left for 2 h, at 4-6 °C temperature²². After 2 h, the animals were sacrificed. The degree of lipid peroxidation (LPO) was also calculated under the stress condition using the standard method of Jamall and Smith, 1985.²³ Inhibition of the auto-oxidation of pyrogallol was taken as a measure of the activity of superoxide dismutase (SOD)²⁴. Enzyme-catalyzed, the decomposition of hydrogen peroxide by potassium permanganate was used to determine CAT activity²⁵.

Pylorus-Ligation- Induced Ulcers (PL): For ulcer induction in rats by administration of drugs for a period of 5 days the last dose was given on the

6th day. The rats were kept for 18 h fasting. Animals were anesthetized in the anesthetic chamber by using pentobarbitone 35 mg/kg (i.p.). pylorous ligation was done by the opening of the abdomen without causing any damage to the blood supply. With interrupted sutures stomach was replaced and the abdomen wall was closed in two layers. Animals were deprived of water during the post-operative period²⁶ after 4 h stomachs were dissected and cut open along the greater curvature. Contents were collected for estimation of biochemical parameters. The ulcers were scored as described under ASP- induced ulcers. Gastric secretion volume, pH and HCl concentration were measured.

Measurement of Ulcer Index: Aspirin model of rats was used to create the ulcers. These were scored according to the arbiter scoring system and graded as Shedding of epithelium = 10; petechial and frank hemorrhages = 20; one or two ulcers = 30; more than two ulcers = 40; and perforated ulcers = 50. Area of ulcer base was measured by the help of biovis image analysis software in the ulcer model, which is considered as the ulcer index. Ulcer index is calculated from scorings described as under:

$$UI = U_s + U_p \times 10^{-1}$$

Where, U_s is the severity of ulcer score; U_p is the percentage of animals with ulcer incidence.

For anti-ulcer studies Percentage protection index is calculated as under:

$$C-T / C \times 100$$

Where, C is ulcer index for the control group; T is ulcer index for the treated group.

Determination of Gastric Wall Mucus: Gastric wall mucus was determined according to the method of Mizui *et al.*, 1983.²⁷ The glandular segments from stomachs were removed, weighed and incubated in tubes containing 1% alcian blue solution (0.16 M sucrose in 0.05 M sodium acetate, pH 5.8) for 2 h. The alcian blue binding extract was centrifuged at 3000 rpm for 10 min, and the absorbency of the supernatant was measured at 498 nm. The quantity of alcian blue extracted (gram per gram of glandular tissue) was then calculated.

Lipid Peroxidation (LPO): The thiobarbituric acid test was used to determine the concentration of gastric mucosal LPO by estimating malondialdehyde (MDA) ²⁸. After that, rat stomachs were excised without delay and washed with cold saline. The mucosa was cleansed adequately of the blood, to reduce the interference of hemoglobin with free radicals. The corpus mucosa was scratch out, weighed and homogenized in 10mL of 100 g/l KCl. The homogenate (0.5 ml) was mixed with 1.5 ml of 8 g/l 2-thiobarbiturate, 1.5 ml of 200 g/l acetic acid, 0.2 ml of 80 g/l sodium lauryl sulfate and 0.3 ml distilled water. The mixture was incubated at 98°C for 1 h. This mixture was cooled and 5 ml of *n*-butanol: pyridine (15:1) was added to it. First the mixture vortexed for 1 min and then centrifuged for 30 min at 4000 rpm. Supernatant was collected and absorbance was measured at 532 nm. The standard curve was constructed by using 1,1,3,3-tetramethoxypropane. The recovery was about 90%. The results were expressed as nanomole MDA per gram of wet tissue (nmol g tissue⁻¹).

Superoxide Dismutase Activity (SOD): SOD activity was measured according to Sun *et al.*, 1988. ²⁹ For SOD estimation xanthine and xanthine oxidase activity was measured based on the generation of superoxide radicals. These superoxide radicals react with nitro blue tetrazolium (NBT) to form formazan dye. The degree of inhibition of the reaction at 560 nm was taken as a measure of SOD activity and was expressed as millimole per minute per milligram tissue (mmol min⁻¹ mg tissue⁻¹).

Catalase Activity (CAT): Catalase decomposed H₂O₂ and its activity were recorded at 240 nm ²⁵. CAT activity is defined as the amount of enzyme needed to decompose 1 nmol of H₂O₂ per minute, at 25°C and pH 7.8. Results were expressed as millimole per minute per milligram tissue (mmol min⁻¹ mg tissue⁻¹).

Estimation of Mucosal PGE₂: Frozen gastric mucosal tissue (1 g) was added to 5 ml homogenization buffer 0.1 M phosphate (pH 7.4), containing 1 mM EDTA and 10 mM indomethacin. The lysate was then centrifuged in a microcentrifuge at 16,000 × g for 15 min at 2°C to 8°C. The supernatant was transferred to a new tube,

and total protein content was analyzed using the sophisticated protein assay. PGE₂ concentrations were investigated using the PGE₂ ELISA Kit CS0200S (Sigma Aldrich) ³⁰.

Statistical Analysis: All the results were presented as mean ± SEM for six rats and analyzed by Wilcoxon Sum Rank Test and unpaired Student's *t*-test used for determination of the level of significance between various groups. Value of p < 0.05, 0.01 and 0.001 was considered statistically significant.

RESULTS: Usnic acid showed significant ulcer protective effect, and it showed better positive effect when it was given with *L. casei* twice daily for 5 days against aspirin (ASP), ethanol (EtOH), cold restraint stress (CRS) and pyloric ligation (PL) induced gastric ulcer. Usnic acid supplemented *L. casei* showed significant (p < 0.001) decrease in ulcer index (UI) against ulcers induced by: (1) ASP (control UI: 19 ± 1.6), % curative ratio levels of *L. casei*, usnic acid, omeprazole, usnic acid + *L. casei* are 23.95%, 70.8%, 67.7% and 82.3% respectively (p < 0.05 to p < 0.001); (2) EtOH (control UI: 22.5 ± 6.3), % curative ratio levels of *L. casei*, usnic acid, omeprazole, usnic acid + *L. casei* 20.8%, 67.11%, 59.55% and 72% respectively (p < 0.05 to p < 0.001); (3) CRS (control UI: 24.2 ± 3.2), % curative ratio levels *L. casei*, usnic acid, omeprazole, usnic acid + *L. casei* 17.35%, 79.75%, 80.99% and 85.95% respectively (p < 0.05 to p < 0.001); (4) PL (control UI: 14.2 ± 2.7), % curative ratio levels *L. casei*, usnic acid, omeprazole, usnic acid + *L. casei* 28.16%, 63.38%, 59.83% and 69.17% respectively (p < 0.05 to p < 0.001) **Table 1**.

Secretion of mucus and bicarbonate by the surface epithelial constitute mucus-bicarbonate barrier, which is regarded as the first line of defense against potential ulcerogenic. The gastric wall mucus was significantly enhanced (UI: 225.7 ± 14.6, % curative ratio: 72%, p < 0.001) by probiotic combination of usnic acid + *L. casei* as compared to usnic acid 100 mg/kg (UI: 199.1 ± 8.3, % curative ratio: 67%, p < 0.01) and omeprazole 30 mg/kg (UI: 213 ± 10.1, % curative ratio: 60%, p < 0.001) administered twice daily and is regarded as a first line of defense against EtOH-induced gastric ulcers showing cytoprotective property **Table 2**.

TABLE 1: EFFECT OF USNIC ACID AND *L. CASEI* ON ASPIRIN (ASP), ETHANOL (EtOH), COLD- RESTRAINT STRESS (CRS) AND PYLORUS LIGATION (PL) INDUCED ULCER

| Treatment | Ulcer index | | | |
|------------------------------|---------------|-----------------------------|---------------|--------------|
| | ASP | EtOH (mm ² /rat) | CRS | PL |
| Healthy Group | 0.0 ± 0 | 0.0 ± 0 | 0.0 ± 0 | 0.0 ± 0 |
| Control | 19.2 ± 1.6 | 22.5 ± 6.3 | 24.2 ± 3.2 | 14.2 ± 2.7 |
| Usnic acid | 5.6 ± 0.35** | 7.4 ± 2.1*** | 4.9 ± 1.3* | 5.2 ± 1.3** |
| <i>L. casei</i> | 14.6 ± 1.2 | 17.8 ± 4 | 20.0 ± 2.7 | 10.2 ± 2.3 |
| Usnic acid + <i>L. Casei</i> | 3.4 ± 0.12*** | 6.3 ± 1.8** | 3.4 ± 0.8*** | 4.3 ± 0.9*** |
| Omeprazole | 6.2 ± 0.54*** | 9.1 ± 2.4*** | 4.6 ± 0.9 *** | 5.7 ± 1.7*** |

Data are represented as mean ± S.E.M. for six rats. *p<0.05, **p<0.01, ***p<0.001 compared to respective control group

TABLE 2: EFFECT OF USNIC ACID AND *L. CASEI* ON ETHANOL-INDUCED ULCER (EtOH) AND GASTRIC WALL MUCUS IN RATS

| Treatment | EtOH (mm ² /rat) | | |
|------------------------------|-----------------------------|--------------------|---|
| | Ulcer Index | Percent protection | (Gastric wall mucus gram per gram wet glandular tissue) |
| Healthy Group | 0.0 ± 0 | - | 279.8 ± 16.3* |
| Control | 22.5 ± 6.3* | - | 171.6 ± 12.7* |
| Usnic acid | 7.4 ± 2.1* | 67% | 199.1 ± 8.3** |
| <i>L. casei</i> | 17.8 ± 4.1* | 21% | 177.2 ± 13.1** |
| Usnic acid + <i>L. Casei</i> | 6.3 ± 1.8* | 72% | 225.7 ± 14.6*** |
| Omeprazole | 9.1 ± 2.4* | 60% | 213.6 ± 10.1*** |

Data are represented as mean ± S.E.M. for six rats. *p<0.05, **p<0.01, ***p<0.001 compared to respective control group.

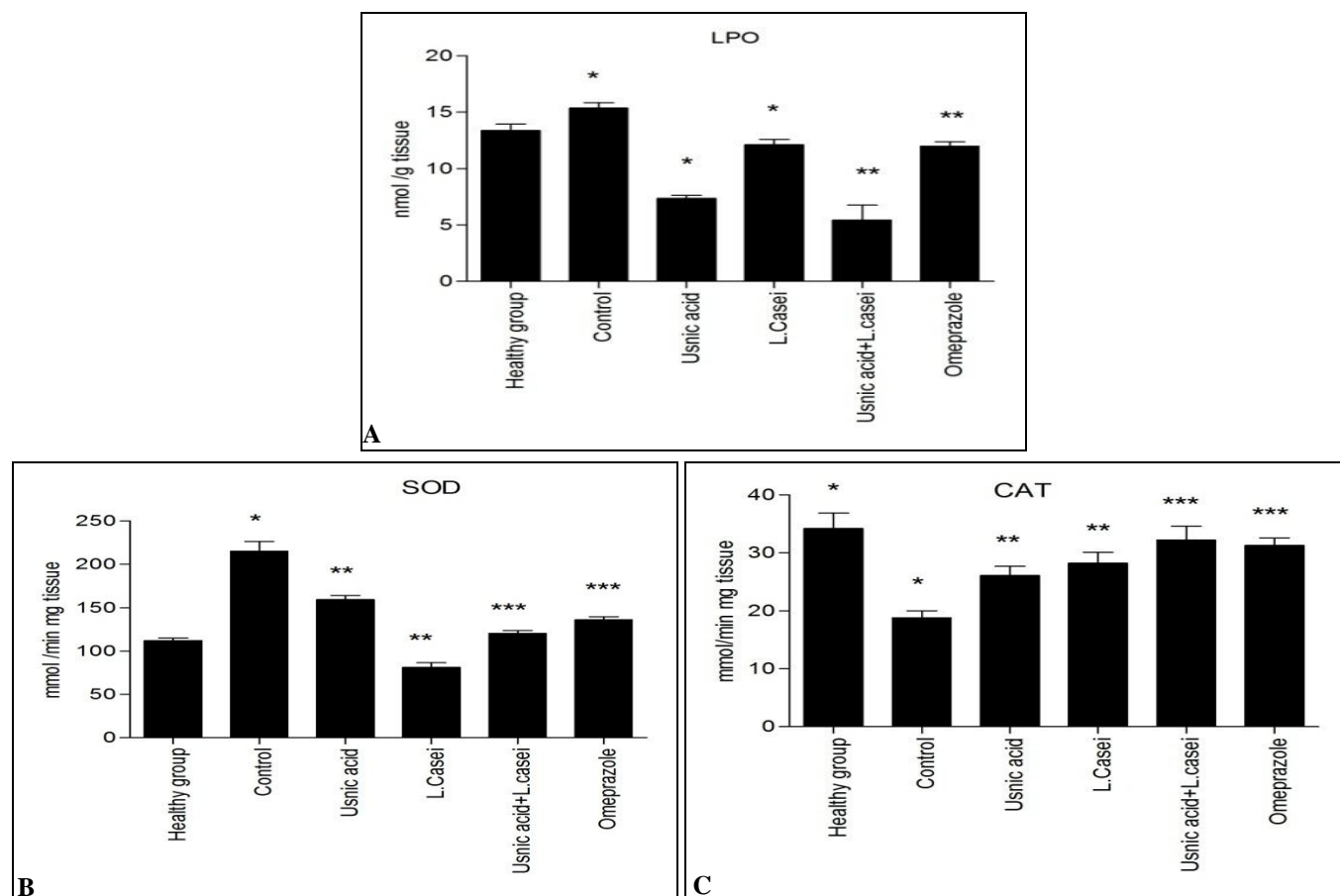


FIG. 2: EFFECT OF USNIC ACID AND *L. CASEI* ON (A) LIPID PEROXIDATION LPO, (B) SUPEROXIDE DISMUTASE SOD, (C) CATALASE CAT IN COLD-RESTRAINT STRESS (CRS)-INDUCED ULCER. Data are represented as Mean ± S.E.M. for six rats. *p<0.05, **p<0.01, ***p<0.001 compared to respective control group.

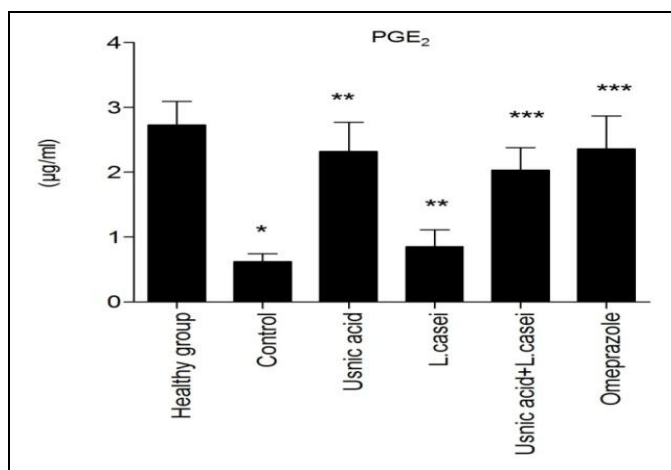


FIG. 3: EFFECT OF USNIC ACID AND *L. CASEI* ON PGE₂. Data is represented as Mean \pm S.E.M. for six rats. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ compared to respective control group.

Cold-restraint stress significantly caused ulceration with concomitant increase in LPO (healthy group 13.4 ± 0.56 , stress 15.37 ± 0.48 nmol g tissue⁻¹, $p < 0.05$) and SOD (healthy group 112.10 ± 0.35 , stress 215 ± 11.3 nmol g tissue⁻¹, $p < 0.05$) and decrease in CAT (healthy group 34.2 ± 2.7 , stress 18.8 ± 1.2 nmol g tissue⁻¹, $p < 0.05$). When the animals were treated with usnic acid + *L. casei* there is significant reversal the ulcer index, LPO, SOD, and CAT levels near to normal values when compared to the stress group (LPO 5.46 ± 1.30 , $p < 0.01$; SOD 120.6 ± 3.2 , $p < 0.001$, CAT 32.2 ± 2.4 , $p < 0.001$) **Fig. 2**. There was increase in PGE₂ (control: 0.62 ± 0.12 , $p < 0.05$ to 2.03 ± 0.35 , $p < 0.001$) when usnic acid + *L. casei* administered **Fig. 3**.

DISCUSSION: Peptic ulcer disease is the result of an imbalance between aggressive factors and the maintenance of the mucosal integrity through endogenous defense mechanism. In the present study, we have investigated the effect of lactobacillus in combination with usnic acid on peptic ulcer disease -synthetic NSAIDs like aspirin cause mucosal damage by inhibition of COX enzyme.

COX (cyclo-oxygenase) enzyme comprises COX-1 and COX-2. COX-2 is especially linked to inflammation. Aspirin is an example of classical NSAIDs; it inhibits both COX-1 and COX-2 enzymes. COX-1 is responsible for sustaining baseline levels for prostaglandins (PGE₂), responsible for maintaining the defense mechanism in the stomach against ulcerogenic content³¹⁻³². The combination of usnic acid and *L. casei*, act as a

protectant against ulcer-causing substances by increasing the thickness of the mucosal barrier. Mucosal barrier thickened as *L. casei* increased the expression of mucin and levels of PGE₂, which simultaneously acted to enhance the levels of mucous secretion in the stomach. Ethanol-induced ulcers are more common in the glandular part of stomach. Ethanol is reported to initiate the synthesis of leukotriene C4 (LTC₄), mast cell secretory products³³ and reactive oxygen species (ROS), which causes damage to gastric mucosa³⁴.

Combination of usnic acid and *L. casei* increased the levels of prostaglandins which in turn led to an increase in mucous secretion. A profuse amount of gastric mucus is secreted during the superficial mucosal damage which creates a favourable microenvironment for restoration for the injury. Ethanol-induced depletion of gastric wall mucous was significantly prevented by the given combination.

Stress plays an essential role in the pathology of gastro-duodenal ulceration. A stress-induced ulcer is possibly mediated by histamine release which reduces the production of mucus. Other factors leading to stress-induced ulcers are a decrease in gastric mucosal blood flow³⁵, decreased synthesis of prostaglandins³⁶, an increase in gastric motility, increased vagal activity³⁷. Accordingly, the protective action of the given combination against the cold stress could be due to an increase in mucin expression, suppression of bacterial overgrowth, the rise in the synthesis of antioxidants and stimulation of mucosal immunity.

Oxidative damage is a frequently reported cause in the pathogenesis of ulcer by different experimental and clinical models. A stress-induced ulcer is due to an increase in the generation of free radicals, apart from acid pepsin factor³⁸. Stress significantly initiate lipid peroxidation as it is clear from the increased levels of LPO. This was the result of an enhanced generation of reactive oxygen species due to stress. Generally, the damage due to ROS is contained by dismutation with SOD, which converts reactive oxygen to H₂O₂³⁹. If the CAT does not scavenge it, it can cause lipid peroxidation by an increase in the generation of hydroxyl radicals⁴⁰. Therefore a decrease in CAT levels favored the accumulation of these reactive oxygen species and hence, caused the increase in lipid peroxidation and tissue damage.

The effect is further worsened by the alleviated activity of gastric peroxidases during stress. Treatment by the combination of usnic acid and *L. casei* reversed the oxidative damage caused by stress. Pyloric ligation caused the accumulation of gastric acid and pepsin, leading to autodigestion of gastric mucosa⁴¹. When combination was administered; it increased the synthesis of prostaglandins leading to enhanced production of mucous which maintained the thickness of the gastric mucosal wall. Increase in the levels of PgE₂ after the administration of the combination reveals that this given combination protects the gastric wall mucosa by enhancing the synthesis of prostaglandins which in turn increases the production of mucus.

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CONFLICT OF INTEREST: Nil

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