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EFFECT OF USNIC ACID AND *CLADONIA FURCATA* EXTRACT ON GASTROESOPHAGEAL REFLUX DISEASE IN RAT

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ABSTRACT

The study aims to understand the effect of usnic acid and standardized extract of *Cladonia furcata* on gastroesophageal reflux disease (GERD) in rats. The hydro-ethanolic extract of *C. furcata* in doses of 50, 100 and 200 mg/kg were administered orally twice daily at 10:00 and 16:00 h, respectively, for 5 days and usnic acid (100 mg/kg) and omeprazole (30 mg/kg) were given orally one hour prior to the induction of GERD disease. GERD disease was experimently induced and evaluated by levels of alkaline phosphatase, histamine, lipid peroxidation, H^+-K^+-ATPase and catalase in rats. Administration of *C. furcata* extract significantly reduced oesophageal index from 2.35±0.11 to 0.92±0.04 and usnic acid and omeprazole inhibited the oesophageal index to 1.67±0.47 and 0.43±0.09 respectively, as compared to control group (0.89±0.23). The level of alkaline phosphatase was increased (from 0.13±0.09 to 0.20±0.07) and level of plasma histamine was significantly decreased (from 260.1±14.09 to 189.6±11.25) in extract treated group. Usnic acid and omeprazole showed significantly enhance in alkaline phosphatase level (0.18±0.05 and 0.16±0.05) and decrease in levels of plasma histamine (191.5±10.11 and 181.7±17.10). Treatment with *C. furcata* at dose of 50-200 mg/kg significantly reduced the lipid peroxidation (from 0.48±0.08 to 0.39±0.02), H^+-K^+-ATPase (from 1.14±0.01 to 0.51±0.03) and SOD (from 167.2±18.1 to 103.1±7.8) and increased in levels of catalase (from 26.0±1.7 to 32.0±1.4). Usnic acid and omeprazole showed significant inhibition in lipid peroxidation (0.41±0.04 and 0.41±0.06), H^+-K^+-ATPase (0.67±0.04 and 0.47±0.02) and SOD (113.3±5.9 and 109.6±6.8) and enhanced the activities of catalase (31.8±3.3 and 31.9±1.8) as compared to GERD group. *C. furcata* extract attributed to its scavenging the free radicals and possess antioxidant activity as that of usnic acid (100 mg/kg) and proton pump blockers (30 mg/kg) to treat gastroesophageal reflux disease.

Keywords: Gastroesophageal reflux disease, Histamine, Alkaline phosphatase, Lipid peroxidation.

INTRODUCTION

Lichens are symbiotic organisms formed by fungi and algae and/or cyanobacteria. Lichens, the nonvascular cryptogams and an excellent example of symbiotic association, have a composite thallus comprising of two components, mycobiont and photobiont [1]. *Cladonia furcata* (Hudson) Schrade - a common fruticose species of the Cladoniaceae family. It is a member of the lichen genus *Cladonia* growing mainly on grassland, sand hill and woodlands. Several lichen extracts have been used in folk medicine, and some lichen metabolites have been shown to have antibiotic, antimycobacterial, antiviral, analgesic, or antipyretic properties and antioxidant activity [2]. The antioxidant property of the lichen *C. furcata* has been reported the strong relationships between total phenolic and flavonoid contents [3]. Flavonoids are considered as protective effects against gastric lesions with distinct mechanisms to inhibit acid production, histidine decarboxylase in isolated parietal cells. *Cladonia furcata* is a excellent source of usnic acid and it is one of the most common and abundant lichen metabolites [4]. Indeed, usnic acid has been reported as antiviral [5], antibiotic [6], antiprotozoal [7], antitumor [8], anti-inflammatory, antipyretic and analgesic [9,10] hepatoprotective [11] and in gastric ulcers [12]. However, there is considerable report of *Cladonia furcata* and its secondary metabolite as

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potential sources of pharmacological agents in treating gastroesophageal reflux disease (GERD). Therefore, the aim of the present study was to evaluate the effect of usnic acid (Fig 1) and *Cladonia furcata* extract against gastroesophageal reflux disease in rats.

MATERIALS AND METHODS

Lichen sample

Lichen sample of *Cladonia furcata* (common name: Forking lichen) was collected in the month of September 2012 from Uttarkashi district of Uttarakhand, India and recorded the accession no. 28559, specimen LWG no. 12-018750 was prepared and cross identified by its vernacular names. The specimen was deposited in the Herbarium of CSIR-National Botanical Research Institute, Lucknow.

Animals

Sprague-Dawley rats (140-180 g) of either sex were purchased from the animal house of the Central Drug Research Institute, Lucknow. They were kept under controlled conditions of temperature 27 ± 2°C and relative humidity 44-56%, light/dark cycles of 12 hours respectively for one week before and during the experiments. Animals were provided with standard rodent pellet diet (Dayal Industries Limited, Barabanki, India) and the food was with drawn 18-24 h before the experiment though water was allowed *ad libitum*. All experiments were performed in the morning according to current guidelines for the care of laboratory animals and the ethical guidelines for investigations of experimental pain in conscious animals [13].

Preparation of the lichen extract

The air-dried parts (1000 g) of *C. furcata* were extracted with 50% ethanol in a Soxhlet extractor at 50^0C on a water bath for 3 hours and centrifuged at 10,000 rev/min. The extract was separated by filtration and concentrated on rotavapour (Buchi R-200, USA) at 40^0C and then freeze-dried in lyophilizer (Labconco, USA) under reduced pressure to obtain solid residue. The dry extracts were stored at −20°C until they were used in the tests. The extracts were dissolved in 5% dimethyl sulphoxide (DMSO) at a concentration of 1 mg/ml for the further experiments.

Induction of GERD and treatment

GERD model was induced in rats according to methods described by Rao and Vijayakumar [14]. According to this method rats were fasted for 24 h under pentobarbitone sodium anesthesia (50 mg/kg, i.p.), the abdomen of the animal was opened by a median incision about 2 cm. Then the transitional region between the fore stomach and corpus was then ligated very carefully with a 2-0 silk thread, and continuously the pylorus portion was ligated. A longitudinal cardiomyotomy of about 1 cm length across the gastroesophageal junction was performed to enhance reflux from the stomach into the oesophagus. Immediately the incised regions were sutured and the animal were kept in recover chamber (Medi HEAT, UK) and returned to their home cages. After 6h, the animals were sacrificed by cervical decapitation and the chest was opened with a median incision and the tissue oesophagus and stomach were removed. The tissue organs were opened along the greater curvature of the stomach, and the oesophagus was dissected out by extending the dissection line along the major axis. The tissues were washed with physiological saline and were examined for ulceration under a stereoscopic microscope (SZX12 OLYMPUS, Japan) and as graded as follows: 0, no visible lesions: 1, a few erosions: 2, total area of lesions ≤ 30 mm^2: 3, total area of lesions ≥ 30 mm^2:4, perforation. *C. furcata* in doses of 50, 100 and 200 mg/kg were administered orally twice daily at 10:00 and 16:00 h, respectively, for 5 days and usnic acid (100 mg/kg) and omeprazole (30 mg/kg) were given orally one hour prior to the induction of GERD disease. Control group received physiological saline (0.9% NaCl in double distilled water).

Estimation of lipid peroxidation (LPO)

A volume of homogenate (0.20 ml) was transferred to a vial and mixed with 0.2 ml 8.1% sodium dodecyl sulfate Solution,1.5 ml 20% acetic acid solution (adjusted to pH 3.5 with NaOH) and 1.5 ml 0.8% solution of thiobarbituric acid (TBA). The final volume was adjusted to 0.4 ml with distilled water. Each vial was tightly capped and heated in a boiling water bath for 60 min. The vials were then cooled under running water. Equal volumes of tissue blank or test samples and 10% trichloroacetic acid were transferred into a centrifuge tube and centrifuged at 1000 rev/min for 10 min. The absorbance of the supernatant fraction was measured at 532 nm. A control experiment was processed using the same experimental procedure except that TBA solution was replaced with distilled water [15]. 1,1,3,3-Tetraethoxypropan was used as the standard for calibration of the curve and results were expressed as nmol MDA (mg protein)$^{-1}$

Catalase activity

Catalase (CAT) activity assayed by the method of Claiborne [16]. Briefly, the assay mixture consisted of 1.95 ml phosphate buffer (0.05M, pH 7.0), 1.0 ml hydrogen peroxide (0.019M) and 0.05 ml homogenate in a final volume of 3.0 ml. Changes in absorbance were recorded at 240 nm. One unit (U) catalase was defined as the amount of enzyme required to decompose 1µmol H_2O_2 Min^{-1}, at 25^0C and pH 7.0. Results were expressed as CAT (mg protein)$^{-1}$.

Superoxide dismutase (SOD) activity

The assay consisted of EDTA 0.1mM, sodium carbonate 50mM and nitro blue tetrazolium 96mM: the

inhibition of nicotinamide adenine dinucleotide (reduced)-phenazine methosulphate- nitro blue tetrazolium reaction system [17,18]. One unit of the enzyme was equivalent to 50% inhibition in formazan formation in 1 min at room temperature (25 ± 2°C) and the results were expressed as U SOD (mg protein)$^{-1}$.

Measurement of alkaline phosphate activity

The gastric mucosal specimen of each rat was scrubbed off, weighed and dissolved in 2.0 ml saline at 4°C. After sonication, the homogenates were centrifuged at 3000 rev/min for 10 min to separate particulate from the soluble fraction. Alkaline phosphatase was estimated by using a biochemistry kit (Merck Ltd, India). Protein concentrations were determined using the Lowery method [19], with alkaline phosphatase activity expressed in international units (IU) (mg protein)$^{-1}$.

Estimation of H^+K^+ATPase activity

The H^+-K^+-ATPase activity was assayed in medium consisting of 70 mM Tris-HCl buffer, pH 6.8, 5 mM $MgC1_2$ and enzyme solution in the presence of 10 mM KCl in a total volume of 1 ml, and incubated for 1 h. The reaction was initiated by adding 2 mM ATP Tris salt and further incubated for 20 min at 37°C. The reaction was terminated by adding 10% trichloroacetic acid and after centrifugation, 2.5 ml ammonium molybdate and 0.5 ml 1-amino-2-naphthal-4-sulfonic acid were added to the supernatant and the absorbance was read at 620 nm [20]. Results were expressed as mmol of Pi liberated/min/mg protein.

Estimation of Histamine content*:*

The animals were sacrificed by cervical dislocation and the abdomen was opened with a median incision and blood was collected from the supraorbital plexus using the microcapillary technique and plasma was separated. The plasma was treated with 0.2 M perchloric acid and centrifuged at 10,000 rev/min for 30 min at 4^0C. The clear supernatant was then used for the determination of histamine content by the high performance liquid chromatography [21] and expressed as IU/milligram protein.

Statistical analysis

All the data were presented as mean ± SEM and analyzed by Wilcoxon Sum Rank Test [22] and unpaired Student's t-test for the possible significant interrelation between the various groups. A value of $P < 0.05$ was considered statistically significant.

RESULTS

Gastroesophageal reflux disease (GERD) developed 6 h after the surgery in 100% of the animals.

Administration of *C. furcata* extract significantly reduced oesophageal index from 2.35±0.11 to 0.92±0.04 and usnic acid and omeprazole inhibited the oesophageal index to 1.67±0.47 and 0.43±0.09 respectively, as compared to control group (0.89±0.23)

Effects of *C. furcata* at dose of 50-200 mg/kg, twice a day for 5 days prevented the Gastroesophageal reflux disease (GERD) in a dose related manner. Table 1 showed that GERD group resulted in the decrease in alkaline phosphatase (0.11±0.03) and increase in level of plasma histamine (280.9±13.13). The level of alkaline phosphatase was increased (from 0.13±0.09 to 0.20±0.07) and level of plasma histamine was significantly decreased (from 260.1±14.09 to 189.6±11.25) in extract treated group (Table 1). Usnic acid and omeprazole showed significantly enhance in alkaline phosphatase level (0.18±0.05 and 0.16±0.05) and decrease in levels of plasma histamine (191.5±10.11 and 181.7±17.10) (Table 1).

The lipid peroxidation is an indicator for the generation of reactive oxygen species in the oesophageal tissue in rats. Animals subjected to gastroesophageal reflux disease (GERD) showed elevation in lipid peroxidation (0.53±0.09), H^+-K^+-ATPase (1.49±0.06) and SOD (188.1±14.2) and decrease in catalase (22.4±1.2) in GERD group. Treatment with *C. furcata* at dose of 50-200 mg/kg significantly reduced the lipid peroxidation (from 0.48±0.08 to 0.39±0.02), H^+-K^+-ATPase (from 1.14±0.01 to 0.51±0.03) and SOD (from 167.2±18.1 to 103.1 ±7.8) and increased in levels of catalase (from 26.0±1.7 to 32.0±1.4). Usnic acid and omeprazole showed significant inhibition in lipid peroxidation (0.41±0.04 and 0.41±0.06), H^+-K^+-ATPase (0.67±0.04 and 0.47±0.02) and SOD (113.3±5.9 and 109.6±6.8) and enhanced the activities of catalase (31.8±3.3 and 31.9±1.8) as compared to GERD group (Table 2).

Table 1. Effect of *C. furcata* on Oesophageal Index, histamine and alkaline phosphatase in GERD rats

Treatment and dose (mg/kg)	Oesophageal Index	Histamine content	Alkaline phosphatase
Control	0.89±0.23	185.4±10.13	0.22±0.02
GERD	3.11±0.23^z	280.9±13.13^z	0.11±0.03^z
C. furcata 50	2.35±0.11	260.1±14.09^a	0.13±0.09
C. furcata 100	1.70.±0.08^a	203.6±12.15^a	0.17±0.10^a
C.furcata 200	0.92±0.04^b	189.6±11.25^b	0.20±0.07^a
Usnic acid 100	1.67±0.47^a	191.5±10.11^b	0.18±0.05^b
Omeprazole 30	0.43±0.09^b	181.7±17.10^b	0.16±0.05^b

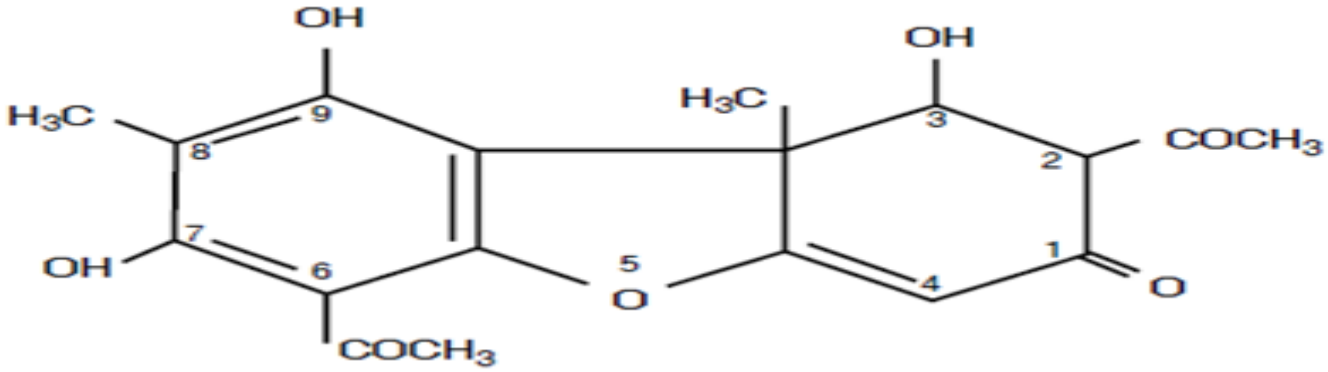
Values are mean ± SEM for six rats. P: z<0.001 compared to respective control group, P: a<0.01 and b<0.001 compared to GERD group

Table 2. Effect of *C. furcata* on LPO, CAT, SOD and H^+-K^+-ATPase activities in GERD rats

Treatment and dose (mg/kg)	LPO	CAT	SOD	H^+-K^+-ATPase
Control	0.38±0.04	32.6±1.0	96.2±7.6	0.45±0.09
GERD	0.53 ± 0.09^{y}	22.4 ± 1.2^{x}	188.1 ± 14.2^{y}	1.49 ± 0.06^{y}
C.furcata 50	0.48 ± 0.08^{a}	26.0±1.7	167.2 ± 18.1^{a}	1.14±0.01
C.furcata 100	0.46 ± 0.09^{b}	28.9 ± 1.2^{b}	126.1 ± 12.2^{b}	0.83 ± 0.08^{b}
C.furcata 200	0.39 ± 0.02^{c}	32.0 ± 1.4^{c}	103.1 ± 7.8^{c}	0.51 ± 0.03^{c}
Usnic acid 100	0.41 ± 0.04^{b}	31.8 ± 3.3^{c}	113.3 ± 5.9^{c}	0.67 ± 0.04^{c}
Omeprazole 30	0.41 ± 0.06^{c}	31.9 ± 1.8^{c}	109.6 ± 6.8^{c}	0.47 ± 0.02^{c}

Values are mean ± SEM for six rats, P: x<0.05 and y<0.001 compared to respective control group, P: a<0.05, b<0.01 and c<0.001 compared to respective GERD group

Fig 1. Structure of Usnic acid



IUPAC Name: 2, 6-Diacetyl-7, 9-dihydroxy-8, 9b-di-methyl-1, 3(2H, 9bH)-dibenzofurandione

DISCUSSION

The present study demonstrates that hydro-ethanolic extract of *C. furcata* have suppressive effect on gastric acid secretion by opposition to the action of histamine and blocking of H^+-K^+-ATPase. Histamine is widely distributed in the gastrointestinal tract in various cells and involves in the pathogenesis of gastroduodenal ulceration, gastric inflammation and gastric acid secretion [23]. Whereas, a significant increase in plasma histamine concentration was observed after development of GERD. The H^+K^+ATPase is the dimeric enzyme responsible for H^+ secretion by gastric parietal cells. The high level of H^+K^+ATPase stimulate parietal cells to hypersecretion of acid, which in turn causes the GERD and is selectively blocked by the action of omperazole, an acid blocker used to treat gastric ulcer. Our observation indicated that treatment with extract caused the reduction in histamine and H^+K^+ATPase in GERD models, indicating the gastric defensive effect. In general, the aggressive factors including gastric acid back diffusion and oxy radical generation plays a factors pivotal role in GERD [24]**.** In the disease state, oxidative stress of the stomach may occur, resulting in an elevation of mucosal lipid peroxides that are generated from the reaction of oxyradicals and cellular polyunsaturated fatty acids. It has been found that oxygen-derived free radicals are implicated in the mechanism of GERD and scavenging these free radicals can play an appreciable role in curing GERD. In this paper we have showed antioxidant activity of hydro-ethanolic extract of *C. furcata*. The role of free radicals is reported to cause GERD. Reactive oxygen species attack and damage many biomolecules, finally to increase lipid peroxides in the membrane [25]. In addition, omperazole scavenges the free radicals and has antioxidant activity [26].

The level of alkaline phosphate, a brush border enzyme, in GERD was significantly decreased compared with the control. The illustrated the increased mucosal permeability and a marked disturbance of the continuity of the gastric tissue [27]. Nevertheless, *C. furcata* extract showed its activity by its returning the alleviated mucosal alkaline phosphate activity to the pre-GERD level and repairing the gastric mucosa damage. The release of alkaline phosphate suggested a role in tissue necrosis associated with polymorph neutrophil infiltration to the site of injury [28]. Further, more recent data have shown that neuro-endocrine immune axis is crucial during surgery [29,30]. SOD converts the reactive superoxide radical to H_2O_2, which if not scavange by CAT can by itself cause lipid peroxidation by generation of hydroxyl radicals. Hence decrease in CAT levels has led to increase in accumulation of these ROS and thus, has caused increased lipid peroxidation and tissue damage [31]. This study suggested that hydro-ethanolic extract of *C. furcata* protected against GERD by its antioxidant activity and

scavenging the free radicals. Antioxidant activities of the extract had beneficial effect on the use of *C. furcata* in the GERD treatment.

CONCLUSION

The results of our study prove that hydro-ethanolic extract of *C. furcata* scavenges the free radicals and possess antioxidant activity. Hence, it can be suggested that the antioxidant activity of the extract may be attributed to its antisecretory and justify the traditional ethnic usage of this herb to treat gastroesophageal reflux disease (GERD).

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