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Original Article

ANTI-ULCER AND ANTIOXIDANT ACTIVITY OF *NELUMBO NUCIFERA* GAERTN STALKS IN RATS

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ABSTRACT

Objective: *Nelumbo nucifera* Gaertn. (Nymphaeceae) is a well known aquatic herb which has been extensively used traditionally for the treatment of different diseases. Therefore present study was designed to evaluate the anti-ulcer activity of methanolic extract of *Nelumbo nucifera* Gaertn. (Nymphaeceae) stalks.

Methods: Anti-ulcer activity of methanolic extract of *Nelumbo nucifera* (100 mg/kg and 200 mg/kg) were evaluated in rats using the pylorus ligation and indomethacin induced gastric ulcer models. Biochemical investigation of stomach tissues was also done to evaluate the effect of methanolic extract of *Nelumbo nucifera* on oxidant and antioxidant parameters in stomach tissue.

Results: Methanolic extract of *Nelumbo nucifera* exhibit significant ulcer protection in the pylorus ligation and Indomethacin induced ulcer model in a dose dependent manner. The results suggested that the *Nelumbo nucifera* methanolic extract increased the resistance to necrotizing agents, providing a direct, protective effect on the gastric mucous due to their potent oxidant and antioxidant activity.

Conclusion: The results obtained from the present study demonstrated that methanolic extract of *Nelumbo nucifera Gaertn* stalks possesses significant anti-ulcer activity in addition to potent oxidant and antioxidant activity.

Keywords: Anti-ulcer activity, Pylorus ligation, Indomethacin, *Nelumbo nucifera*.

INTRODUCTION

Peptic ulcer is one of the most prevalent gastrointestinal disorders with increased morbidity, which affects approximately 5-10% of people during their life [1]. Peptic ulcer disease is a disease of multiple etiologies, till date there is continuous research to elucidate the exact pathogenesis of peptic ulcer, although scientist and researcher proposed a common ground to understand the possible pathogenesis of peptic ulcer. Peptic ulcer occurs due to an imbalance between the aggressive (acid, pepsin and Helicobacter pylori) and the defensive (gastric mucus and bicarbonate secretion, prostaglandins, innate resistance of the mucosal cells) factors in the stomach [2]. Such factors could range from natural causes, infections, and lifestyle [3-5].

Various treatment options (proton pump inhibitors, histamine receptor antagonist's prostaglandins analogs, and cyto protective agents) are available for the management of peptic ulcer. But majority of these drugs generate several undesirable adverse reactions (headache, abdominal pain, bowl upset, dizziness, constipation and diarrhoea) and also may alter normal biochemical homeostasis of the body on chronic use (elevated serum aluminium levels due to antacids and sucralfate, reduced calcium absorption by proton pump inhibitors) [6]. In recent years, a lot of work has been carried out on natural drugs to elucidate their potential effectiveness in gastric ulcer prevention. Herbal medication is promising as an alternative treatment to available synthetic drugs for the treatment of ulcer probably due to availability, affordability, lesser adverse effects and proved effectiveness [7, 8]. Many natural herbs have been pharmacologically reported possessing potent anti-ulcer activity [9-12].

Nelumbo nucifera (Nymphaeceae) is perennial and aquatic herb with elongated, branched stem. It has several common names (Indian lotus, Chinese water lilly and sacred lotus) and synonyms (*Nelumbium nelumbo* and *Nymphaea Nelumbo*). It consists of model roots, concave to cup shaped leaves and petioles, flowers are white to rosy having sweet fragrance, solitary, hermaphrodites and 10-25 cm in diameter. Almost all parts of the plant are eaten as a vegetable and also used in the traditional system of medicine. Stalks of *Nelumbo nucifera* were traditionally used by tribal peoples for the treatment of bleeding gastric ulcers, haemorrhage, and wound healing [13, 14]. Previous phytochemical studies confirmed that *Nelumbo nucifera* contains a rich amount of bioactive compounds including alkaloids, flavonoids, glycosides, triterpenoid, tannins and phenolic compounds [15].

Flavonoids represent beneficial gastroprotective effects in healing gastric ulcers acting as cytoprotective, anti secretory, and antioxidant agents [16]. Tannins are used traditionally because of their astringent properties; they can also prevent the gastric mucosa by the proteolytic enzymes and toxic substances [17]. An indigenous drug having lesser side effects is the major area of the present research, looking for a better and safe formulation for the management of gastric ulcer. *Nelumbo nucifera* is reported to possess antidiarrheal, psychopharmacological, diuretic, antipyretic, antimicrobial, hypoglycemic, antioxidant and nephroprotective activity [18-25]. Proved antioxidant, antiinflammatory [26], antimicrobial [27, 28] and wound-healing activity [29] on *Nelumbo nucifera* has been the rationale for selection of plant for anti-ulcer activity and for the development of new anti-ulcer drug that can offer better protection and minimal side effects. In the present investi gation we had studied the anti-ulcer and antioxidant activity of *Nelumbo nucifera* stalks in rats.

MATERIALS AND METHODS

Collection and authentification of plant material

Nelumbo nucifera stalks during its flowering stage were collected from Sahayak Sanchalak Udhyan Samittee, Tulsinagar, Bhopal and authenticated by Dr. Zia ul Hasan, Professor, Department of Botany, Saifia Science College, Bhopal, Madhya Pradesh, India. The specimen voucher number (136/Bot/Saifia/2013) was deposited with the herbarium in the Department of Pharmacognosy (VNS Institute of Pharmacy, Bhopal, Madhya Pradesh, India) for future reference.

Extraction

The stalks of *Nelumbo nucifera* were shade dried for three weeks, pulverized to coarse powder, passed through sieve no. 18 to maintain uniformity and coarsely dried powder of stalks was first defatted with petroleum ether (60-80ºC) to remove fatty materials

and then finally extracted with methanol using soxhlet apparatus for 36 hours, after extraction extract was collected, concentrated in vacuum under reduced pressure using rotary flash evaporator and the dried crude extract was stored in air tight container at 4ºC for further study.

Phytochemical screening

Methanolic extract of *Nelumbo nucifera* stalks (MENN) was subjected to qualitative phytochemical investigation for the identification of the different phytoconstituents using standard tests and procedures [30, 31].

Preparation of test formulation of extract

A suspension formulation of MENN was prepared in 0.5% carboxy methyl cellulose (CMC) solution in distilled water stored at 2-8ºC for further studies.

Chemicals and reagents

All the drugs, solvents and chemicals used in the study were of analytical grade. Omeprazole was obtained as a gift sample from Sapience Bio-analytical Research Lab, Bhopal, Madhya Pradesh, India. All other chemicals e. g. Methanol, ether, formalin, sodium hydroxide, citric acid monohydrate, trichloroacetic acid, sodium nitrate, sodium potassium tartrate, ethylene diamine tetra acetic acid disodium salt were purchased from S. D. Fine Chemicals, Mumbai, India. Tris buffer, Topfer's reagent, Folin's Reagent and Phenolphthalein were purchased from Hi-Media Pvt. Ltd., Mumbai, India. Aneket (Ketamine Injection) was purchased from Drug Market, Bhopal, Madhya Pradesh, India.

Animal care and handling

The experiment was carried out on healthy Wistar albino rats, weighing between 150-200g Animals were provided by the authorized animal house of VNS Institute of Pharmacy Bhopal, Madhya Pradesh, India. The animals were acclimatized to the standard laboratory conditions at temperature 25±2°C relative humidity 44-56% and 12:12 hours light and dark cycles, fed with standard pellet diet and water *ad libitum* during experiment. The experiment was approved by the institutional animal ethics (IAEC) committee as per the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA) guidelines (IAEC registration number: 778/03/C/CPCSEA).

Acute oral toxicity studies

Acute oral toxicity study was evaluated as per Organization for Economic Cooperation and Development (OECD) guidelines no. 425 on Wistar albino rats, weighing between 150-200g. Before experiment rats were fasted overnight with water *ad libitum*. Three animals were selected which receives a dose of 2000 mg/kg. All three animals were received a dose of 2000 mg/kg body weight of MENN by oral gavage. Animals were observed individually for any sign of toxicity, behavioral changes, and mortality after dosing, with special attention given during the first 4 hours, and thereafter for 24 hours, for a total period of 7 days. Administered dose was found tolerable (as no death found).

Assessment of anti-ulcer activity

Pylorus ligation induced gastric ulcer [32]

Wistar albino rats were divided into four groups, consisting of six rats in each group. Group I (Control group) received only distilled water, group II (Standard group), treated with omeprazole 30 mg/kg (p. o.), group III (Test-1) & group IV (Test-2) received the different doses of MENN, 100 mg/kg and 200 mg/kg (p. o.). Animals were treated with standard and different doses of suspensions of MENN for a period of 7 days. On the 7th day after the last dose of MENN, animals were kept for 24 hrs of fasting and care was taken to avoid coprophagy. Using ketamine anesthesia, the abdomen was opened and pylorus was ligated without causing any damage to its blood vessels. The stomach was replaced carefully and the abdominal wall was closed with interrupted sutures. The animals were deprived of water during the post operative period. Four hours after ligation, animals were sacrificed by cervical dislocation under ether anesthesia, stomachs were dissected out and stomach contents were collected in to clean glass tubes. The volume of the total gastric content was measured then the gastric content was centrifuged at 2000 rpm for 10 minutes, filtered and subjected to titration against 0.1N sodium hydroxide using topfer's reagent and 1% phenolphthalein as indicator for determination of free acidity and total acidity. Each stomach was examined for lesions in the fore stomach portion and indexed according to severity.

Macroscopic evaluation of stomach

The stomachs were opened along the greater curvature, washed with saline and observed by a 10X Magnifier lens to evaluate the formation of ulcers. The numbers of ulcers were counted. Scoring of the ulcer will be made as follows:

Normal colored stomach. (0)

Red coloration. . (0.5)

Spot ulcer. . …. (1)

Hemorrhagic streak. (1.5)

Deep ulcers. . . … (2)

Perforation. . … (3)

Mean ulcer score for each animal will be expressed as the ulcer index. The percentage of ulcer protection was determined as follows: Ulcer index (UI) was measured by using following formula: $UI = UN + US + UP \times 10^{-1}$

Where,

UI= Ulcer Index; UN = Average number of ulcers per animal; US = Average number of severity score; UP = Percentage of animals with ulcers.

Percentage inhibition of ulceration was calculated by the given formula:

$$\%\ Inhibition\ of\ Ulceration = \frac{(Ulcer\ index_{control} - Ulcer\ index_{test})}{Ulcer\ index_{control}} \times 100$$

Determination of pH

An aliquot of 1 ml gastric juice was diluted with 1 ml of distilled water and pH of the solution was measured using a digital pH meter.

Determination of total acidity

An aliquot of 1 ml gastric juice diluted with 1 ml of distilled water was taken into a 100 ml conical flask and 2-3 drops of phenolphthalein indicator was added to it and titrated with 0.01N Sodium hydroxide until a permanent pink colour was observed. The volume of 0.01N Sodium hydroxide consumed was noted. The total acidity is expressed as mEq/L by the following formula:

$$Acidity = \frac{Volume\ of\ NaOH \times N \times 100\ mEq/L}{0.1}$$

Determination of free acidity

For the determination of free acidity, topfer's reagent was used instead of phenolphthalein indicator. Aliquot of gastric juice was titrated with 0.01N Sodium hydroxide until yellow colour was observed. The volume of 0.01N Sodium hydroxide consumed was noted. The free acidity was calculated by the similar formula for the determination of total acidity.

Indomethacin induced ulcer test [33]

Wistar albino rats were divided into five groups, each consisting of six rats. Group I (Control group) received only distilled water, group II (Standard group), treated with omeprazole 30 mg/kg (p. o.), group III (Test-1) & group IV (Test-2) received the different doses of methanolic extract of *Nelumbo nucifera* stalks, 100 mg/kg and 200 mg/kg (p. o.). Animals were treated with standard and different doses of suspensions of methanolic extract of *Nelumbo nucifera* stalks (MENN) for a period of 7 days. On 7th day after the omeprazole and MENN treatment, animals in all groups received 25

mg/kg indomethacin orally. Six hours after indomethacin administration, all animals were sacrificed by cervical dislocation under ether anesthesia. The stomach of all animals removed carefully and opened along the greater curvature. Stomach mucosa was examined macroscopically for the severity of the ulcer and ulcer index. After that all the stomach was stored at -80°C for biochemical investigation.

Biochemical investigation of stomach tissues

For biochemical estimation, stomach tissue from all groups of animals of Indomethacin induced ulcer test model were frozen at -80°C before until biochemical investigations of glutathione (GSH), myeloperoxidase (MPO), and malondialdehyde (MDA) enzyme activities and levels in stomach tissues. For preparation of tissue homogenates, stomach tissues were ground with liquid nitrogen in a pestle mortar.

The 0.5g of each tissue was then separately treated with 4.5 ml of isotonic buffer to prevent the tissue hydrolysis. Finally tissues were homogenized in ice using homogenizer for 15 minutes. Tissue homogenates were filtered and centrifuged in cooling centrifuge at 4°C. Supernatants was separated out and used for the further determination of the enzymatic activities.

The amount of Glutathione (GSH) in the gastric mucosa was measured according to the method of Sedlak and Lindsay [34]. The results of the GSH level in the gastric mucosa were expressed as micromoles per milligram tissue (µmol/mg tissue). Myeloperoxidase (MPO) activity was measured according to the modified method of Bradley *et al*. [35] MPO activity in gastric tissues were expressed as minimoles per minute per milligram tissue (mmol/min/mg tissue). The concentrations of gastric mucosal lipid peroxidation were determined by estimating MDA using the thiobarbituric acid test [36]. The results were expressed as nanomoles MDA per gram wet tissue (nmol/mg tissue).

Statistical analysis

The results are expressed as the mean±SEM for each group. Statistical differences were evaluated using a One-way analysis of variance (ANOVA) followed by Dunnett's test. Results were considered to be statistically significant at *p<0.001.

RESULTS

Acute toxicity studies

There was no change in the behavioral pattern and not any sign of toxicity and mortality observed during the overall toxicity studies.

Phytochemical screening

The phytochemical screening revealed that methanolic extract of *Nelumbo nucifera* contain a rich amount of flavonoids, alkaloids, glycosides, tannins, carbohydrates and phenolic compounds.

Effect of methanolic extract of *Nelumbo nucifera* stalks (MENN) in pylorus ligation induced gastric ulcer

Effect of MENN on pyloric ligation induced ulceration is shown in table 1 and 2. Pyloric ligation has caused the accumulation of gastric secretions of 8.26±1.48 ml with pH 2.87±0.69 in group-I (control). The free acidity and total acidity of the gastric secretions was found to be 11.53±1.37 and 13.67±0.11 mEq/L respectively.

Pretreatment with the MENN significantly reduced the volume of gastric secretions 46.98±0.78 and 5.68±0.88 ml at the doses of 100 and 200 mg/kg respectively. pH of the gastric juice was significantly elevated up to 4.28±0.77 only at the higher dose of the extract. In addition, total acidity and free acidity were also reduced significantly in a dose dependant manner. Further it is observed that the percentage inhibition of ulceration was found to be 43.93 and 64.13% at 100 and 200 mg/kg respectively. The gastro-protection revealed by the MENN was comparable to that of the standard drug, omeprazole (30 mg/kg).

Effect of methanolic extract of *Nelumbo nucifera* stalks (MENN) in indomethacin induced gastric ulcer

Effect of MENN on ulcer index and percent protection in indomethacin induced gastric ulcer is shown in table 3. It is clear from the results of the study that the MENN (100mg/kg and 200 mg/kg); significantly reduces the formation of gastric ulcer in indomethacin induced gastric ulcer in rats. Percentage inhibition of ulceration was found to be 45.13 and 62.07% at 100 and 200 mg/kg respectively. The gastro-protection revealed by the MENN was comparable to that of the standard drug, omeprazole (30 mg/kg).

Results of biochemical study are shown in table 4. The level of Glutathione (GSH), in control animals was found to reduce whereas Myeloperoxidase (MPO) and malondialdehyde (MDA) level was found to increase in control animals causing the damage of gastric tissues in the formation of an ulcer. Administration of standard drug Omeprazole (30mg/kg) plays a therapeutic role in the reduction of severity of ulcer by increasing the level of GSH as well as reducing the level of MPO and MDA. Administration of MENN (100 mg/kg/p. o. and 200 mg/kg/p. o.) showed a significant reduction in lipid peroxidation and MPO activity in a dose dependant manner and an increase in the antioxidant enzyme activity of GSH.

Table 1: Effect of methanolic extract of *Nelumbo nucifera* stalks (MENN) on pH, volume of gastric juice, free acidity and total acidity in pylorus ligation induced gastric ulcer

Group	Treatment	pH of gastric juice	Volume of gastric juice (ml)	Free acidity (meq/L)	Total acidity (meq/L)
I	Control	2.87±0.69	8.26±1.48	11.53±1.37	13.67±0.11
II	Omeprazole (30 mg/kg)	5.72±0.91	4.54±0.46	4.59±1.23***	6.48±0.51***
III	MENN (100 mg/kg)	3.81±0.46	6.98±0.78	8.45±1.41**	9.81±0.37**
IV	MENN (200 mg/kg)	4.28±0.77	5.68±0.88	6.32±2.63***	7.12±0.16***

Values are represented as mean ± SEM, n = 6. Data were analyzed by one-way ANOVA, followed by Dunnett's test and *p<0.05,**p<0.01,***p<0.001, when compared to group-I (control)

Table 2: Effect of methanolic extract of *Nelumbo nucifera* stalks (MENN) on ulcer index and percent protection in pylorus ligation induced gastric ulcer

Group	Treatment	Ulcer index	% Protection
I	Control	12.77± 0.88	-
II	Omeprazole (30 mg/kg)	2.64±1.18***	79.32
III	MENN (100 mg/kg)	7.16±0.66**	43.93
IV	MENN (200 mg/kg)	4.58±0.32***	64.13

Values are represented as mean ± SEM, n = 6. Data were analyzed by one-way ANOVA, followed by Dunnett's test and *p<0.05,**p<0.01,***p<0.001, when compared to group-I (control)

Table 3: Effect of methanolic extract of *Nelumbo nucifera* stalks (MENN) on ulcer index and percent protection in Indomethacin induced gastric ulcer

Group	Treatment	Ulcer index	% Protection
I	Control	14.29± 1.53	-
II	Omeprazole (30mg/kg) + Indomethacin (25 mg/kg, p. o.)	3.16±0.72***	77.88
III	MENN (100mg/kg) + Indomethacin (25 mg/kg, p. o.)	7.84±0.93**	45.13
IV	MENN (200mg/kg) + Indomethacin (25 mg/kg, p. o.)	5.42±0.46***	62.07

Values are represented as mean ± SEM, n = 6. Data were analyzed by one-way ANOVA, followed by Dunnett's test and *p<0.05,**p<0.01,***p<0.001, when compared to group-I (control)

Table 4: Effect of methanolic extract of *Nelumbo nucifera* stalks (MENN) on GSH, MPO and MDA levels in the stomach tissues of rats in Indomethacin induced gastric ulcer

Group	Treatment	GSH (µmol/mg)	MPO (mU/mg)	MDA (nmoles/mg)
I	Control	0.26 ± 0.12	24.37 ± 1.32	19.86 ± 1.78
II	Omeprazole (30mg/kg) + Indomethacin (25 mg/kg, p. o.)	0.82 ± 0.072***	12.46 ± 0.75***	6.71 ± 0.46***
III	MENN (100mg/kg) + Indomethacin (25 mg/kg, p. o.)	0.43 ± 0.069***	19.84 ± 0.56*	12.79 ± 1.35**
IV	MENN (200mg/kg) + Indomethacin (25 mg/kg, p. o.)	0.69 ± 0.032***	15.21 ± 0.61***	8.64 ± 0.38***

Values are represented as mean ± SEM, n = 6. Data were analyzed by one-way ANOVA, followed by Dunnett's test and *p<0.05,**p<0.01,***p<0.001, when compared to group-I (control)

DISCUSSION

Various factors that have been implicated in the pathogenesis of gastric ulcers are an increase in gastric acid secretion, pepsin activity and oxidative stress in the gastric mucosa, and a decrease in mucous and bicarbonate secretion [37, 38]. MENN (100 mg/kg/p. o. and 200 mg/kg/p. o.) showed dose-dependent, ulcer-protective effect in rats against both ulcer models (pylorus ligation model and indomethacin induced gastric ulcer model).

In pylorus ligation model, ulcers occur because of an increase in acid-pepsin accumulation due to pylorus ligation and subsequent mucosal digestion [39]. Effect of MENN in pylorus ligation model, marked by increased gastric pH and a significant reduction in volume, total acidity and free acidity of gastric juice (as shown in table 1) when compared to the control group. Previous studies have reported that the tannins may prevent development and expansion of gastric ulcer via their astringent, protein precipitating (forming an impermeable layer over the gut lining that hamper gut secretions and protecting gut mucosa from toxins and irritants) and vasoconstriction effects at the site of ulcer [40, 41].

In present study tannins present in extract may be responsible for significant reduction in ulcer index (as shown in table 2). Previously studied also stated that flavonoids are responsible for the reduction of deleterious effects of free radicals and reactive oxygen species in gastrointestinal lumen [42]. Phytochemical studies of extract confirmed the presence of flavonoids and tannins thus on the basis of findings of phytochemical studies and biochemical parameters we can conclude that the protective mechanism produced by MENN in pylorus ligation model may be due to the cytoprotective, anti-secretory mechanism provoked by the flavonoids [43] and inhibition of gastric acid secretion.

In Indomethacin induced ulcer model, Non-steroidal anti-inflammatory drug Indomethacin damage gastric mucosa by inhibiting the synthesis of prostaglandins via the arachidonic pathway [44, 45]. Prostaglandins play a key role in the protection of gastric mucosal injury by maintaining gastric microcirculation and improving gastric secretion of mucus and bicarbonates [46, 47]. In Indomethacin induced ulcer model, pretreatment with the MENN protected rats from ulcers induced by Indomethacin (as shown in table 3). Thus, the effect of the MENN in Indomethacin induced gastric ulcer model suggests that extract may possess cytoprotective action probably by increasing synthesis of prostaglandins in a dose dependent manner. Biochemical study of stomach tissue of rats in Indomethacin induced gastric ulcer model also supported the ulcer-protective effect of MENN. GSH and GSH-related enzymes (glutathione peroxidase, glutathione-S transferase and gamma-glutamyl transpeptidase), protect the gastric mucosa and acknowledged as vital protective mediators due to their antioxidant properties [48]. GSH detoxifies hydrogen peroxide and organic acids chemically; in the absence of GSH, hydrogen peroxide accumulates causing tissue damage [49]. Pretreatment of rats with MENN increased the GSH level in gastric tissue significantly; in a dose dependent manner in comparison to control and reduce the gastric damage. Our results and earlier published literatures signify that there is an important association between gastric GSH levels and ulcer severity. Non-steroidal anti-inflammatory drugs treatment causes gastric damage by initiating the lipid peroxidation in stomach tissue [50]. Indomethacin produces gastric damage by increasing mucosal MPO and MDA levels [51]. MPO present in polymorph nuclear leukocyte cells (PNL) catalyses the formation of toxic hypochlorous acid from hydrogen peroxide [52].

Together, polymorph nuclear leukocytes (PNLs) produce superoxide anion and hydroxyl radical, as free oxygen radicals [53]. Lipid peroxidation is an important etiological factor for cell membrane damage, elevated level of MPO and MDA causes oxidative damage of the gut mucosal membrane via lipid peroxidation [54, 55]. Gastric MPO and MDA level in stomach tissue of rat's decreases by pretreatment with MENN showing ulcer-protective effect of *Nelumbo nucifera* stalks.

MENN exhibited anti-ulcer activity in indomethacin induced ulcer model which is supported by the result obtained in this study that there is a marked and significant increase in the level of antioxidant enzyme GSH and reduced level of peroxidase enzyme MPO and MDA.

CONCLUSION

The methanolic extract of *Nelumbo nucifera Gaertn* stalks (MENN) possesses significant anti-ulcer activity in addition to potent oxidant and antioxidant activity. In conclusion, our results showed that the anti-ulcer activity of the methanolic extract of *Nelumbo nucifera* stalks was a result of the probable gastric ulcer healing mechanism (anti-secretory, cytoprotective and the antioxidant properties) of its active phytoconstituents. These findings suggest the potential for use of *Nelumbo nucifera* stalks as an adjuvant in the treatment of gastric ulcer. Further, studies are needed for the isolation of active constituents responsible for the anti-ulcer activity and to elucidate the exact mechanism of action in gastric ulcer healing.

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CONFLICT OF INTEREST

The authors declare that there are no conflicts of interest.

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