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Research Article

ANTIULCER AND *IN VITRO* ANTIOXIDANT ACTIVITY OF *ALLIUM HOOKERII*: AN ETHNOMEDICINAL PLANT OF MANIPUR

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ABSTRACT

Objective: To evaluate the antiulcer and antioxidant activity of the methanolic extract of *Allium hookerii* (MEAH) leaves.

Methods: *In vivo* antiulcer activity of MEAH was evaluated at two dose levels of 200 mg/kg and 400 mg/kg body weight by pyloric ligation, ethanol (EtOH) induced ulcer and indomethacin-induced ulcer models through estimation of gastric contents *viz*. gastric volume, pH, free acidity, total acidity, total hexoses, hexosamine, fucose and total protein. Antioxidant activity was evaluated by 1,1-diphenyl-2-picrylhydrazyl hydrate (DPPH) assay, reducing power assay, superoxide radical scavenging activity, and hydrogen peroxide radical scavenging activity. Histopathology of the stomach was studied using hematoxylin and eosin stained sections.

Results: A significant decrease in ulcer index and increase in percentage protection in ulcerated rats. After 7 days of treatment, the pH, free acidity and total acidity decreased, thereby increased the content of total hexoses, hexosamine, fucose and total protein in the gastric content. MEAH showed a significant antioxidative effect in DPPH, hydrogen peroxide radical scavenging activity and superoxide radical scavenging but showed very low effect in reducing power assay. Histopathological studies of the stomach in ulcer and treated groups substantiate the cytoprotective action of extract in EtOH induced ulcer animals.

Conclusions: It can be concluded from the research work that *A. hookerii* has potent antiulcer activity and supports the *in vitro* antioxidative status.

Keywords: Antisecretory, Antiulcer, Cytoprotection, Antioxidants, *Allium hookerii.*

INTRODUCTION

A peptic ulcer is one of the major gastrointestinal disorders, which occur due to an imbalance between the offensive (gastric acid secretion) and defensive (gastric mucosal integrity) factors [1]. Ulceration in the mucosa can be because of either breakdown of mucosa with the development of surface defects or failure of restitution of mucosal integrity resulting in retardation or failure of healing of the ulcers. Although in most of the cases the etiology of the ulcers is unknown, it is generally accepted that ulcer results from an imbalance between aggressive factors and the maintenance of mucosal integrity through endogenous defense mechanisms [2]. The type of drugs varies from being proton-pump inhibitor to H_2 antagonist or a cytoprotective agent. At the same time, each of these drugs confers simpler to several side effects like arrhythmias, impotence, gynecomastia, enterochromaffin-like cell, hyperplasia and hemopoeitic changes [3]. There are evidences for the participation of reactive oxygen species in the etiology and pathophysiology of human disease, such as neurodegenerative disorders, inflammation, viral infections, autoimmune gastrointestinal inflammation and gastric ulcer [4]. It has been demonstrated that many drugs and formulations possess potent antioxidant action and are effective in healing experimentally induced gastric ulcers [5].

Allium hookerii Thw. Enum (Family: Liliaceae) is locally known as "Maroi napakpi." It is a herb with thick evergreen, linear with prominent midribs, basal leaves membranous, and shorter than the tall subtrigonous scape [6]. *A. hookerii* is widely used by the local people of Manipur, a North Eastern State of India to garnish cooking and as ingredients in cooking popular delicious dishes. The leaves of this plant has been used as a home remedy by the Meitei community of Manipur in their folklore medicine as an antiulcer agent [7], cardioprotective agent [8] and commonly available in the vegetables markets.

METHODS

Chemicals and apparatus

Petroleum ether, methanol, omeprazole, indomethacin, ethanol (EtOH), xylene, misoprostol, ranitidine, Mayer's reagent, Dragendroff's reagent, Hager's reagent, wagner's reagent, hydrochloric acid, pyridine, sodium nitroprusside, chloroform, sulfuric acid, pH meter, Topfer's reagent, sodium hydroxide, ascorbic acid, 1,1-diphenyl-2-picrylhydrazyl hydrate (DPPH), phosphate buffer, trichloroacetic acid (TCA), aluminum chloride, potassium ferricyanide, potassium acetate, hydrogen peroxide, nitroblue tetrazolium (NBT), oxalic acid, phenolphthalein, orcinol reagent, acetone, Ehrlich's reagent, Cystein reagent, Lowry's reagent, Leica microscope were used in this study. All the chemicals and reagents used were of analytical grade.

Collection of plant material and preliminary study of the methanolic extract

The leaves of *A. hookerii* were collected from Imphal, Manipur (India) during the month of March, 2012. The plant was identified and authenticated by Dr. W. Ingo Meitei, Department of Horticulture, Central Agricultural University, Imphal. Leaves were washed thoroughly with water and then shade dried. The dried leaves were then pulverized in a mechanical grinder to produce a coarse powder and then stored in an airtight container free from moisture. Powdered crude drug (1550 g) of *A. hookerii* was extracted by soxhletion (continuous hot extraction) with 10 L of methanol for 36 hrs after pretreatment with petroleum ether. The solvent was recovered at 50°C by distillation under reduced pressure and the extract was concentrated to obtain a brown semisolid mass. Preliminary phytochemical tests were carried out with all the extracts in order to evaluate for the presence of different phytochemical constituents [9]. Methanolic extract of *Allium hookerii* (MEAH) showed positive test for flavonoids, carbohydrates, glycosides, steroids, saponins, tannins and phenolic compounds.

Selection and maintenance of animals

Male albino rats of Wistar strain, weighing about 150-200 g were obtained from M/S Chakraborty Enterprises, Kolkata and used for the experimental study. The animal experiment was approved by the Institutional Animal Ethics Committee, Dibrugarh University; vide registration number - 1576/GO/a/11/CPCSEA dated 17/2/2012, and approval number-IAEC/DU/44 dated: 24/9/2013. The animal house was well ventilated and maintained at room temperature (25°C±2°C), 30-35% of relative humidity and 12 hrs dark/light cycle. They were housed in polypropylene cages during the course of the experimental period and were provided with standard pellet diet (Hindustan Lever, Mumbai, Maharashtra, India) and water *ad-libitum*. The place of the experiment was kept hygienic by cleansing with an antiseptic solution, and further procedures involving care of animals was conducted in conformity with the institutional guidelines.

Acute toxicity study

Healthy adult female albino mice were fasted overnight and divided into four groups containing five animals in each group (n=5). They were orally fed with MEAH dissolved in 0.3% carboxymethylcellulose in increasing dose levels of 50, 100, 800 and 2000 mg/kg. The animals were under observation for 4 hrs to see the behavioral changes viz. gross behavior, writhing, convulsion, response to tail pinching, pupil size, itching, excessive salivation, urination, faecal output, water intake, feeding behavior, sedation, etc., neurological and autonomic profiles. After a period of 24 and 72 hrs, they were observed for any lethality or death and further kept under observation up to 15 days. The effective dose of the MEAH was determined to be $1/10^{th}$ of the maximum dose which is 2000 mg/kg [10].

Antiulcer study

Dosage

In the experiment, the rats were divided into four groups (n=5). Group 1 was the control group which received suspension of 0.3% Carboxymethyl cellulose in distilled water (1 ml/kg). Groups II received standard drugs, Groups III and IV received MEAH at the doses of 200 and 400 mg/kg respectively for seven days for acute ulcer protective studies.

Pylorus ligation (PL) induced ulcer

Drugs were administered continuously for a period of 7 days and the rats were kept for 18 hrs fasting. Ranitidine was used as a standard drug at the dose of 50 mg/kg. Animals were anaesthetized using pentobarbitone (35 mg/kg, i.p.), the abdomen was opened, and pylorus ligation was done without causing any damage to its blood supply. The stomach was replaced carefully, and the abdomen wall was closed in two layers with interrupted sutures. The animals were deprived of water during the post-operative period [11]. After 4 hrs, stomachs were dissected out and cut open along the greater curvature and ulcers were scored.

Biochemical estimation

The gastric juice was collected after 4 hrs after PL and centrifuged for 5 minutes at 2000 rpm. The gastric volume and pH of the juice was measured. The supernatant was collected, and the gastric juice was expressed as ml/100 g body weight. Acid concentration and output was determined by titrating with 0.1 M NaOH using Phenolphthalein as an indicator and is expressed as µeq/ml [12]. The estimation of total protein, hexose, hexosamine and fucose was carried out as it has been taken as the mucin activity [13]. It is expressed in µg/ml.

EtOH induced gastric ulcer

The gastric ulcer was induced in rats by administering EtOH (1 ml/200 g) and the animals were sacrificed by cervical dislocation and the stomachs were incised along the greater curvature and examined for ulcers [14]. Omeprazole was used as the standard drug (10 mg/kg).

Indomethacin (IND) induced gastric ulcer

IND at the dose of 20 mg/kg was administered to all the animals and ulcers were scored after 4 hrs [15]. The stomachs were taken out and cut along the greater curvature and ulcers were scored. Misoprostol was used as the standard drug (20 mg/kg).

***In vitro* antioxidant activity**

DPPH assay

The antioxidant activity of the extract was determined on the basis of its scavenging activity of the stable DPPH [16]. Radical scavenging activity of MEAH against DPPH was determined spectrophotometrically at 517 nm. Ascorbic acid was used as the standard. The absorbance was carried out in triplicates. Percentage inhibition was calculated by the formula given by Sadhu *et al.* [16].

Reducing power assay

Reducing power assay was carried out according to [17]. It was determined on the basis of the ability of antioxidant principles to form color complex with potassium ferricyanide, TCA and ferric chloride. The reducing power was determined spectrophotometrically at 700 nm. Ascorbic acid was used as the standard and all the test samples were observed in triplicates.

Superoxide radical scavenging activity

Superoxide radical scavenging activity was observed by the NBT reduction method [17,18]. Reagent prepared in 50 mM KH_2PO_4-KOH buffer, pH 7.4 was used. The various concentrations of the extract and standard samples were treated with NBT, hypoxanthine and Na_2EDTA. Absorbance was recorded spectrophotometrically at 405 nm in triplicates for all the samples.

Hydrogen peroxide radical scavenging activity

Hydrogen peroxide (H_2O_2) radical scavenging activity was carried out according to Ruch [19]. 40 mM solution of H_2O_2 was prepared in phosphate buffer solution (PBS, pH 7.4). Various concentrations of 0.5 ml of the MEAH and ASC were added to 1ml of H_2O_2 solutions in PBS. After 10 min, the absorbance was obtained at 230 nm..

Histological study

At the end of the study, the animals were sacrificed by cervical dislocation. The stomach of the animals were collected and washed with normal saline. It was kept to 10% formalin solution for 24 hrs and dehydrated with alcohol. The tissues were embedded in paraffin wax and cleaned with xylene and alcohol [20]. The washed tissues were treated with haematoxylin and eosin dye to differentiate the nucleus and cytoplasm. Photographs were taken using Leica microsystem.

Statistical analysis

The results were expressed as mean ± standard error mean. The data was analyzed by using the software Graphpad and Prism. It was statistically analyzed using ANOVA followed by Dunnett's t-test considering $p<0.01$ as significant.

RESULTS

The phytochemical screening of the leaves of *A. hookerii* showed the presence of flavonoids, carbohydrates, glycosides, steroids, saponins and phenolic compounds.

Acute toxicity studies

Toxicity study of the MEAH was carried out in female albino mice weighing 20-25 g. No mortality was found up to 2000 mg/kg. So, the MEAH was found to be safe up to 2000 mg/kg.

Antiulcer study

Pylorus ligation ulcer

The pylorus ligated animals showed a significant ($p<0.01$) decrease in the ulcer index thereby increasing percentage protection when compared to the control animals (Table 1). The extract also significantly reduced the total and free acidity and increased the pH, total hexoses,

hexosamine, fucose and total protein content proving its antisecretory activity (Table 2). MEAH at the doses of 200 mg/kg and 400 mg/kg showed a percentage protection of 31.0% and 40.5% when standard showed 71.6%.

EtOH induced ulcer

EtOH in the gastric mucosa produced hemorrhagic gastric lesions. The MEAH produced a significant ($p<0.01$) reduction of these lesions. The MEAH at the doses of 200 mg/kg and 400 mg/kg showed a protective index of 21.4% and 51.7% when standard produced 60.7% (Table 3).

IND induced ulcer

The MEAH at the doses of 200 mg/kg and 400 mg/kg produced a percentage protection of 23.7% and 44.3% respectively when compared to standard showing 59.7% protection (Table 4).

Table 1: Effect of *Allium hookerii* on pyloric ligated rats indicating ulcer index and percentage protection

Animal groups	Ulcer index	Percentage protection
I (solvent control)	14.80±1.5	-
II (ranitidine)	4.20±0.5**	71.6
III (MEAH 200 mg/kg)	10.20±0.4**	31.0
IV (MEAH 400 mg/kg)	8.80±0.6**	40.5

Data represents mean±SEM (n=5). *p<0.05, **p<0.01 compared to normal control. SEM: Standard error mean, MEAH: Methanolic extract of *Allium hookerii*

Table 2: Effect of *Allium hookerii* on gastric juice of pyloric ligated rats

Gastric juice	Group I	Group II	Group III	Group IV
Gastric volume (ml)	2±0.3	2.7±0.1	2.2±0.2	2.5±0.2
pH	2.8±0.1	5.3±0.3**	3.1±0.10	4.1±0.2**
Free acidity (µeq/ml)	74.6±1.3	36.1±1.2**	51.9±1.1**	43.4±0.6**
Total acidity (µeq/ml)	105.0±2.0	63.9±2.3**	52.7±0.7**	58.4±0.8**
Total hexoses	12.6±3.2	42.1±0.9**	20.4±4.4	28.6±0.9**
Hexosamine	11.6±20.2	47.8±0.5**	19.1±4.1	22.8±2.4
Fucose	6.8±3.1	64.1±1.3**	10.3±1.5	28.7±1.3**
Total protein	7.3±1.4	23.2±1.5**	14±7.8	19.2±0.7**

Data represents mean±SEM (n=5). *p<0.05, **p<0.01 compared to normal control. SEM: Standard error mean

Table 3: Effect of *Allium hookerii* on ethanol-induced ulcer rats indicating ulcer index and percentage protection

Animal groups	Ulcer index	Percentage protection
I (solvent control)	11.2±1.2	-
II (omeprazole)	4.4±0.6**	60.7
III (MEAH 200 mg/kg)	8.8±0.8	21.4
IV (MEAH 400 mg/kg)	5.4±0.6**	51.7

Data represents mean±SEM (n=5). *p<0.05, **p<0.01 compared to normal control. SEM: Standard error mean, MEAH: Methanolic extract of *Allium hookerii*

Table 4: Effect of *Allium hookerii* on indomethacin-induced ulcer rats indicating ulcer index and percentage protection

Animal groups	Ulcer index	Percentage protection
I (solvent control)	19.4±2.6	-
II (misoprostol)	7.8±0.9**	59.7
III (MEAH 200 mg/kg)	14.8±2.8	23.7
IV (MEAH 400 mg/kg)	10.8±1.4**	44.3

Data represents mean±SEM (n=5). *p<0.05, **p<0.01 compared to normal control. SEM: Standard error mean, MEAH: Methanolic extract of *Allium hookerii*

Histopathology of EtOH induced ulcer

From the three ulcer models, the MEAH showed most effectiveness in EtOH induced ulcer rats. After seven days of treatment, the rats treated with EtOH showed loss of gland architecture with erosion of the epithelial layer, edema and hemorrhage of the stomach wall (Fig. 1). Standard drug treated group showed no ulceration in the gastric mucosa (Fig. 2). Pretreatment with MEAH at 400 mg/kg showed significant protection against erosion but mild inflammation (Fig. 3) but produced hemorrhage at 200 mg/kg (Fig. 4).

Antioxidant activity

DPPH assay

DPPH has an advantage over other antioxidant methods which are being unaffected by certain side reactions. Fig. 5 shows the result of MEAH on DPPH radical scavenging activity with increasing doses. The maximum dose of MEAH shows an inhibition of 85.06% when compared to ascorbic acid showing 98% inhibition. MEAH showed decreased in absorbance with increasing dose.

Reducing power assay

The reducing capacity of the compound ferricyanide complex to the ferrous form may serve as a significant indicator of its antioxidant capacity. The key of the reducing power is the existence of reductones, which exhibit their antioxidant activities through the action of breaking the free radical chain by donating a hydrogen atom. Fig. 6 shows the reducing power of MEAH which indicates the increased in absorbance

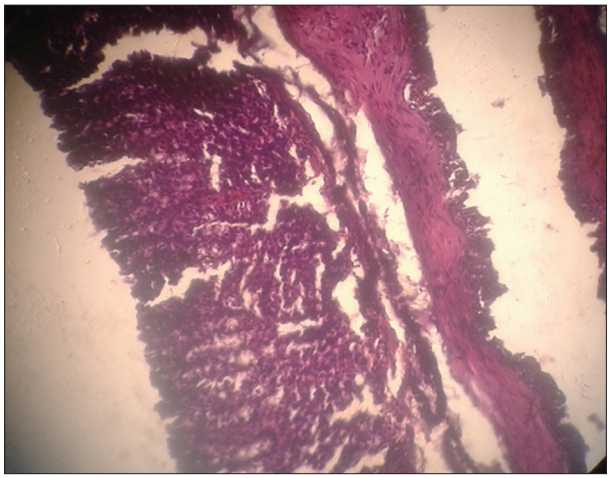

Fig. 1: Normal control

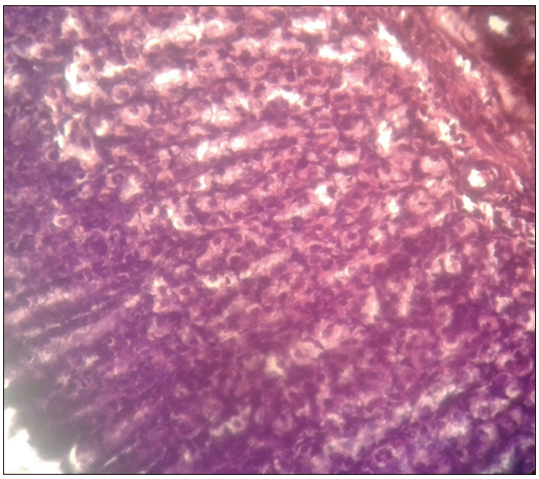

Fig. 2: Standard control

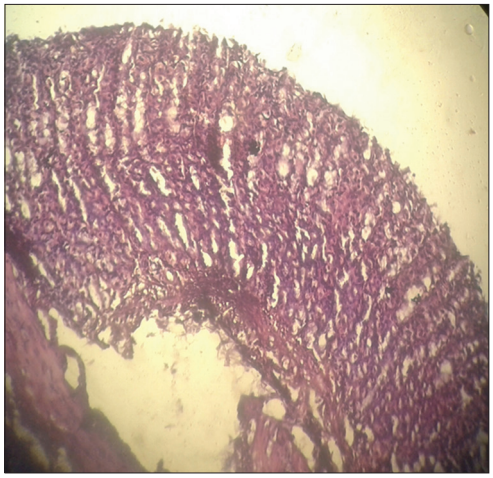

Fig. 3: Test control (low dose)

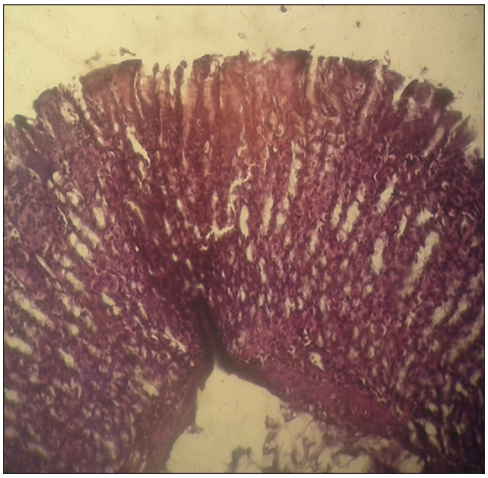

Fig. 4: Test control (high dose)

with increasing dose. The extract showed very least effect when compared to ascorbic acid.

Superoxide radical scavenging activity

Superoxide radicals are generated from numerous biological reactions which are highly toxic species. They cannot directly initiate lipid oxidation, superoxide radical anions are potential precursors of highly reactive species such as hydroxyl radical, and thus the study of the scavenging of this radical is important. Superoxide radicals were generated in a phenazine methosulfate-nicotinamide adenine dinucleotide system and assayed by the reduction of NBT. Fig. 7 shows the superoxide anion scavenging activity of MEAH. The extract shows a dose response inhibition of the superoxide anion radicals. The MEAH extract shows a maximum inhibition of 61.53% when compared to ascorbic acid producing 96.44%.

Hydrogen peroxide radical scavenging activity

The H_2O_2 radical scavenging activity was carried out according to the method used by Ruch. Ascorbic acid was used as standard. The MEAH showed a maximum percentage inhibition of 81.06% as shown in Figure 8.

DISCUSSION

Peptic ulcers are thought to be due to an imbalance between offensive acid-pepsin secretions versus impaired mucosal resistance [21]. The defense mechanism of the gastrointestinal mucosa against aggressive

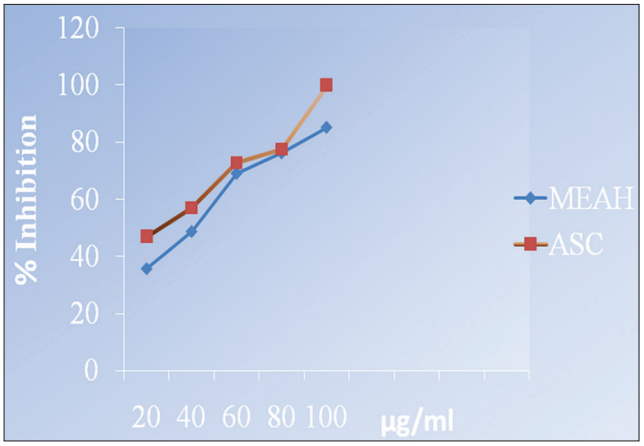

Fig. 5: 1,1-diphenyl-2-picrylhydrazyl hydrate scavenging activity

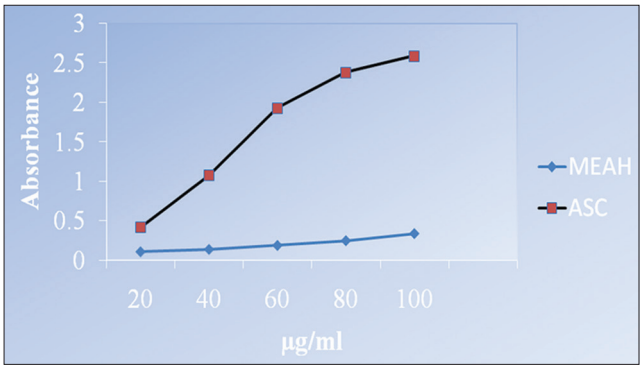

Fig. 6: Reducing power

factors such as HCl, bile acid and non-steroidal anti-inflammatory drugs (NSAIDs), mainly consists of functional, humoral and neuronal factors. Mucus-alkaline secretion, mucosal microcirculation and motility act as functional factors, while prostaglandins and nitric oxide act as humoral factors and capsaicin-sensitive sensory neurons act as neuronal factors [22]. Gastric ulcers have multiple etiopathogenesis. Ulcers caused by pyloric ligation are due to increased presence of acid and pepsin in the stomach and damage by IND are due to decrease in PG synthesis which is essential for the integrity of mucosa [23]. EtOH-induced gastric lesions are thought to arise as a result of direct damage of gastric mucosal cells [24]. Gastric acid is an important factor for the genesis of ulceration in pylorus ligated rats. The activation of the vagus-vagal reflux by stimulation of pressure receptors in the antral gastric mucosa in the hypersecretion model of pylorus ligature is believed to increase gastric acid secretion [25]. The current data clearly demonstrates that *A. hookerii* dose-dependently decreases the gastric acid, which clearly exhibit the anti-secretory activity of *A. hookerii* leaves. Further investigations on offensive and defensive factors were carried out in the gastric juice of pyloric ligated rats. Mucus serves as first line of defense against ulcerogens. Mucus is secreted by the mucus neck cells and covers the gastric mucosa thereby preventing physical damage and back diffusion of hydrogen ions [26]. *A. hookerii* significantly increased mucus secretion as observed from the increase in mucopolysaccharides like hexose, hexosamine and fucose. Further, strengthening of the gastric mucosa is evident from the decrease in the leakage of protein into the gastric juice. This increase was due to increase in mucopolysaccharides, the major constituent of mucus and also which are responsible for viscous nature and gel-forming properties of the mucus. The gel is reported to be resistant to a number of ulcerogens including acid, EtOH and NSAIDs [27]. Hence, increase in synthesis of mucus may be one of the

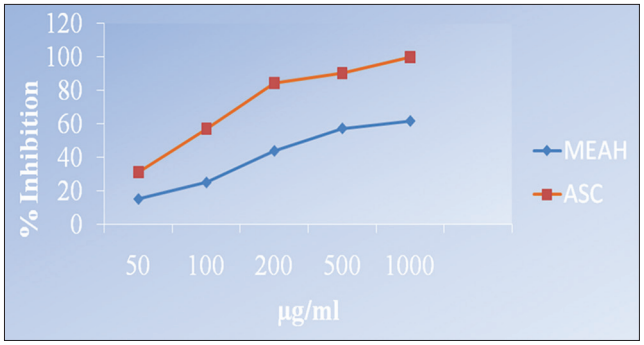

Fig. 7: Superoxide anion scavenging activity

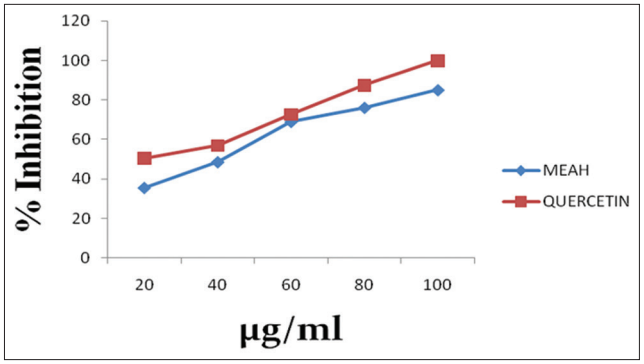

Fig. 8: Hydrogen peroxide radical scavenging activity

important contributing factors for ulcer protective role of *A. hookerii* leaves. The MEAH extract is also highly effective in blocking gastric lesions in the IND induced ulcer model. IND is known to inhibit cyclooxygenase activity of prostaglandin synthetase and causes gastric damage by decreasing the level of prostaglandin, the master molecule for gastroprotection. The anti-ulcer activity of methanolic extract of *A. hookerii* leaves was detected in absolute EtOH-lesions in rats (Table 3). These models evaluate the drug's capacity to protect the gastric mucosa, differentiating only the severity of gastric lesions. EtOH-induced gastric damage may be due to stasis in gastric blood flow, which contributes to the development of the hemorrhagic and necrotic aspects of tissue injury. In addition, EtOH also induces solubilization of the mucus constituents, decreases the difference of potential in mucosa thus increasing the flow of Na+ and K+ to the lumen and pepsin secretion, and also increases H+ ions and histamine [28]. The result shows that the tested extract have an important protective activity for gastric mucosa. The leaves extract of *A. hookerii* was found to be most effective in reducing ulcer lesion induced by EtOH. Thus, the significant antiulcer activity of *A. hookerii* could be due to the presence of various phytoconstituents detected in the phytochemical screening. It possesses potential to impart therapeutic effect on ulcer. Further studies are necessary to elucidate in detail the mechanism of action of this medicinal plant at cellular and molecular level. It is also necessary to isolate responsible active constituents to establish its mechanism of action.

CONCLUSION

It can be concluded from the research work that *A. hookerii* has potent antiulcer activity and supports the *in vitro* antioxidative status.

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