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Original Article

Antioxidant and Antiulcer Activity of *Curculigo orchioides* Rhizomes Extract on Experimental Animals

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ABSTRACT

Curculigo orchioides Gaertn (Hypoxidaceae) is used traditionally in Indian system of medicine as carminative, indigestion, vomiting, diarrhoea and aphrodisiac. Ulcer in either the stomach or duodenum is called as peptic ulcer, which is one of the common problem in human population. This study aimed to evaluate the antiulcer activity of *Curculigo orchioides* rhizome. *Curculigo orchioides* rhizome extract 50-200mg/kg administered orally, twice daily for 5 days for prevention from aspirin induced, ethanol, cold restraint stress and pylorus ligation induced ulcer. Estimation of antioxidant enzyme activity and various gastric secretion parameter like volume of gastric juice, acid output, pepsin and gastric pH value carried out in CRS and PL induced ulcer model respectively.COE showed dose dependent decrease in ulcer index in different ulcer model with decrease in SOD and LPO while increase in CAT level in PL induced ulcer model. COE also showed marginal decrease in volume, acid pepsin concentration and output. The result showed that COE possesses antiulcer property with significant antioxidant potential.

Keywords: *Curculigo orchioides,* Antiulcer, Antioxidant.

1. INTRODUCTION

Curculigo orchioides Gaertn also known as Kali musali in hindi is herb traditionally used in treatment of piles, fatigue, aphrodisiac and appetizer as ayurvedic medicine. Alcoholic extract show spermatogenic activity (Dixit et al., 2008), [1] Estrogenic activity (Vijayanarayana et al., 2007) [2] and effect on sexual behaviour (Rao et al., 2007). [3] Plant has to possesses hepatoprotective effect (Venukumar et al., 2002), [4] Antidiabetic activity (Madhvan et al., 2007) [5] and Antiasthmatic activity (Patil et al., 2008) [6] . Six phenolic

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compounds isolated from Curculigo orchioides, including 2,6-dimethoxybenzoicacid(1), curculigoside A (2), curculigosideB(3), curculigineA(4), curculigineD(5) and 3,30,5,50-tetramethoxy-7,90:70,9-diepoxylignan-4,40- di-O-b-D-glucopyranoside(6), together with the ethanol extract of Curculigo orchioides were evaluated for their antiosteoporotic activity (Qiao. Yan Zhang et al., 2009) [7] Significant immunostimulant activity was found in purified glycoside rich fraction isolated from ethyl acetate extract (Lakshmi et al., 2003) [8].

Phytochemical screening of methnolic extract of *Curculigo orchioides Gaertn* show the presence of alkaloid, carbohydrate and glycosides, saponin, proteins and aminoacids , phytosterols, gum and mucilage, phenolic compound and flavanoides (Agrahari et al., 2010) [9].

Now a days ulcer is one of the common problem among people due to stress, smoking, alcohol, analgesic drug .Ulcer is a lesion in mucosal lining in stomach and duodenum, commonly called peptic ulcer. Pathophysiology of ulcer explain role of offensive factor (pepsin and hydrochloric acid) and defensive factor (mucus), imbalance between them causes lesion. Autacoids play important role in regulating gastric juice and mucus secretion, like histamine increases gastric acid secretion while serotonin and prostaglandin decreases gastric acid and increases mucus secretion.

Present study is lead to established antiulcer activity of rhizome of *C.orchioides* G. because Various pharmacological activity has been established for *C.orchioides* G..but antiulcer activity has not been reported so for.

2. MATERIAL AND METHODS

2.1. Collection of the Plant

The dried rhizomes of the plant *Curculigo orchioides Gaertn.* (Family -Amaryllidaceae) were purchased from local market in Lucknow, India in month of Nov 2009. The plant materials were identified by Botany division and the voucher specimens (NAB 783) were deposited in the departmental herbarium of National Botanical Research Institute, Lucknow, India for future reference.

2.2. Preparation of 50% MeOH extract of Curculigo orchioides

The dried rhizome (1000g) of *Curculigo orchioides* were washed with distilled water and air-dried at 30 ± 2^0C. Then dried it in tray drier under the control conditions and powdered. The powdered plant materials (750g) was macerated with petroleum ether to remove fatty substances, the marc was further exhaustively extracted with of 50% methanol for 3 days (3 X 5L). The extract was separated by filtration and concentrated below 50°C in rotavapour (Buchi, USA) and then dried in lyophilizer (Labconco, USA) under reduced pressure obtain 65.0 g of solid residue (yield 8.66 % w/w). The extracts obtained was further subjected to toxicological and pharmacological investigations.

2.3. Animals

Studies were carried out using Swiss albino mice weighing 25-30 g. They were obtained from the Central Animal House Facility of Central Drug Research Institute, Lucknow. The mice were group housed in polyacrylic cages (38×23×10cm) with not more than six animals per cage and maintained under standard laboratory conditions (temperature 25 ± 2 ^{0}C) and relative humidity 44 –56 %, with a dark and light cycle of 12 ± 1 h. They were allowed free access to standard dry pellet diet (Amrut, India) and water *ad libitum*. All procedures described were reviewed and approved by the institutional committee for ethical use of animals (Zimmerman, 1983) [10].

3. In-Vitro Antioxidant Activity

Estimation of total phenolic content (TPC)

TPC was analysed by the Folin–Ciocalteu colorimetric method using gallic acid as standard developed by Ragazzi and Veronese (1973) and expressed as mg/g gallic acid equivalent (GAE). 1.0 ml of extracts is mixed with 1.0 ml of Folin's Reagent (1N) and 2.0 ml of Na_2CO_3 (20 %) subsequently. The test mixture were mixed properly on cyclomixer, left at room temperature for 30 min and maintained to 25 ml with water. The absorbance of test mixture was measured at 725 nm.

Reducing power (RP) activity

Reducing power determined using ferric reducing-antioxidant power assay and ascorbic acid as reference standard (S. Hemalatha, 2010**).** Different concentrations of the plant extracts in 1.0 ml of deionized water were mixed with 2.5 ml phosphate buffer (0.2 M, pH 6.6), and 2.5 ml potassium ferricyanide (1% w/v),). The mixture was incubated at 50ºC for 20 min. After incubation aliquots of 2.5 ml trichloroacetic acid (10%w/v) were added to the mixture, which was then centrifuged at 3000 rpm for 10 min whenever necessary. The upper layer of solution (2.5 ml) was mixed with distilled water (2.5 ml) and a freshly prepared 0.5 ml ferric chloride solution (0.1% w/v). The absorbance was measured at 700 nm. A blank was prepared without adding extract. Ascorbic acid at various concentrations was used as standard. Increased absorbance of the reaction mixture indicates increase in reducing power.

Free radical scavenging activity

- ***DPPH (1,1-diphenyl-2-picrylhydrazyl) radical scavenging activity***

The DPPH radical scavenging activity of *C.orchioides* was determined by using the method proposed by Yen and Duh (1994). Different aliquot was added to 2.9ml of freshly prepared solution of DPPH (6 x 10^{-5} M in MeOH). The Absorbance was recorded at 517 nm at 0 time & after 1 hour of incubation and inhibitory concentration (IC_{50}) were calculated as described by Kroyer (2004) [11]. IC_{50} value is defined as the concentration of sample required to scavenge 50% of free radical (Yu et al., 2002) [12].

- ***Hydroxyl radical scavenging activity***

The deoxy ribose method was used for determining the scavenging effect on hydroxyl radicals as describe by

Halliwell, Gutteridge, & Aruoma (1987). The reaction mixture contained ascorbic acid (50 µM), $FeCl_3$ (20 µM), EDTA (2 µM), H_2O_2 (1.42mM), deoxyribose (2.8mM), with different concentration of *C.orchioides* extract in a final volume of 1ml in potassium phosphate buffer (10mM, pH 7.4). It was incubated at 37 ºC for 1 h and then 1ml of 2.8 % TCA and 1ml of 1 % TBA were added. The mixture was heated in a boiling water bath for 15 min. It was cooled and absorbance was taken at 532 nm.

4. Pharmacological Evaluation

To study effect of 50% MeOH extract of *Curculigo orchioides* on ulcer rats were divided into following groups (n=6)

Group I- Ulcer Control

Group II- 50% MeOH of *Curculigo orchioides* (50 mg/kg. body wt p.o.) in ulcer rats

Group III- 50% MeOH of *Curculigo orchioides* (100 mg/kg. body wt p.o.) in ulcer rats

Group IV - 50% MeOH of *Curculigo orchioides* (200 mg/kg. body wt p.o.) in ulcer rats

Group V- Ranitidine (50 mg/kg. body wt p.o.) in ulcer rats

50 % methanolic (50% MeOH) extract of *Curculigo orchioides* and Ranitidine were administered orally twice daily at 10:00 and 16:00 hrs respectively for five days before gastric ulcers were induced. The drug samples were prepared in 1 % carboxymethyl cellulose (CMC). Control group of animals received suspension of 1 % carboxymethyl cellulose in distilled water (10 ml/kg).

4.1. Pylorus ligated (PL)-induced ulcers

Gastric ulcers were produced in rats by following method as describe earlier by Sanyal *et al.* (1971). Briefly, the rats were fasted for 24 h before pylorus-ligation but water was allowed *ad libitum*. At the end of 24 h starvation, rats were anaesthetized with pentobarbitone sodium (35 mg/kg). Abdomen was opened by a midline incision and a ligature was placed at the pyloric end of the stomach taking care not to exclude any blood vessels. The abdomen was then closed in two layer and rats were left in a cage with a false bottom of wide mesh wire gauze to prevent coprophagy. Water was withheld from one hour before pylorus ligation and till the end of 4 h period when the rats were sacrificed by overdosing with ether. Immediately afterwards abdomen was again opened, cardiac end of stomach was ligated and the stomach was taken out. The stomach was then cut open along the greater curvature and the mucosa was washed under slow running tap water. The ulcer index was calculated by adding the total number of ulcers per stomach and the total severity of ulcers per stomach. The total severity of the ulcers was determined by recording the severity of each ulcer after histological confirmation as follows: 0, no ulcer; +, pin point ulcer and histological changes limited to superficial layers of mucosa and no congestion; ++, ulcer size less than 1 mm and half of the mucosal thickness showed necrotic changes; +++, ulcer size 1-2 mm with more than two-thirds of the mucosal thickness destroyed with marked necrosis and congestion, muscular is remaining unaffected; ++++, ulcer either more than 2 mm in size or perforated with complete destruction of the mucosa with necrosis and hemorrhage, muscular is still remaining unaffected. The pooled group ulcer score was then calculated according to the method of Sanyal *et al* (1982) [13].

4.2. Aspirin (ASP)-induced ulcers

Aspirin was administered orally on the day of experiment at about 10 AM with the help of an orogastric tube in the form of an aqueous water suspension (200 mg/kg, p.o.) and animals were sacrificed after 4 h of administration (Goel *et al*., 1985) [14]. The stomach was incised along with the greater curvature and examined for ulcers as described earlier.

4.3 Cold-restraint stress (CR Stress)-induced ulcers

Rats of either sex weighing 120-150 g were immobilized for 2 h at 4 ^{o}C following the method of immobilization as describe earlier by Amar and Sanyal (1981) [15]. Briefly the animals were starved for 24 h with free access to water and 60 min after receiving the corresponding treatment they were fully stretched and strapped to a wooden plank with adhesive tape after securing each limb to the plank individually. The animals were killed after 2 h and ulcer were scored as described above.

4.4. Ethanol (EtOH)-induced ulcer

The gastric ulcer was induced in rats by administering Ethanol (EtOH, 100%, 1ml/200 g, 1 h). EtOH were administered on the day of the experiment and the animals were sacrified by cervical dislocation and stomach was incised along with greater curvature and examined for ulcers (Hollander *et al*., 1985) [16]. The ulcer index was scored, based upon the product of length and width of the ulcer present in the glandular portion of the stomach (mm^2/rat).

5. Biochemical Estimation

The fundic part of the stomach is homogenized (5%) in ice cold 0.9% saline with a Potter – Elvehjem glass homogenizer for 30 sec. the homogenate were centrifuged at 800 x g for 10 min followed by centrifugation of the supernatant at 12,000 x g for 15 min and the obtained mitochondrial fraction (Das and Banerjee, 1993) [17]

5.1Measurement of Lipid peroxidation (LPO)

Lipid peroxidation product malondialdehyde (MDA) was estimated according to the method of Ohkawa *et al*., (1979). Briefly 1.0 ml of sample was mixed with 0.2 ml 4 % (w/v) sodium dodecyl sulfate, 1.5 ml 20 % acetic acid in 0.27 M hydrochloric acid (pH 3.5) and 15 ml of 0.8 % thiobarbituric acid (TBA, pH 7.4). The mixture was heated in a hot water bath at 85 ^{0}C for 1 h. The intensity of the pink color developed was read against a reagent blank at 532 nm following centrifugation at 1200 g for 10 min. Tetraethoxypropane was used as a standard.

5.2 Superoxide dismutase (SOD)

SOD was estimated by following the procedure of Kakkar *et al*., (1984). Assay mixture contained sodium pyrophosphate buffer (0.052 M, pH 8.3), phenazine methasulfate (PMS, 6.2 M), Nitroblue tetrazolium (NBT, 30 M), potassium cyanide

(KCN, 10 µm, pH 7.0) and 0.2 ml of sample fraction. Samples were preincubated for 5 min at 36^0C prior to the addition of reduced nicotinamide adenine dinucleotide (NADH, 52 µm). Mixture was further incubated for 120 sec at 37^0C in a water bath and the reaction was stopped by adding 1 ml glacial acetic acid (17.4 M). The violet color developed was extracted in 0.4 ml of n-butanol reagent blank. The activity was measured at 560 nm and the result have been expressed as unit (U) of SOD activity/mg protein.

5.3 Catalase (CAT)

Decomposition of H_2O_2 in the presence of catalase was followed at 240 nm (Aebi, 1983). A 50 µm sample was added to buffered substrate (50 mM phosphate buffer, pH 7.0 containing 10 mM H_2O_2) to make total volume 3 ml and decrease in the absorbance was monitored at 37^0C for 2.5 min at an interval of 15 sec. the activity was calculated using extinction coefficient of H_2O_2, 0.041/µmole/cm^2 at 240 nm. Results are expressed as units (U) of CAT activity/mg protein.

6. Histopathological Studie

Gastric tissue sample from each group were fixed in 10% formalin for 24 h. The formalin fixed specimens were embedded in paraffin and sectioned (3-5µm) and stained with hematoxylin and eosin dye. These were examined under the microscope for histopathological changes such as congestion, hemorrhage, erosion and necrosis.

7. Statistical evaluation

Data are expressed as mean ± SEM (standard error of mean) for six rats. The difference among means has been analysed by one-way ANOVA followed by Newman-Keuls multiple comparison test using Prism pad software. Differences were considered statistically significant at the value of probability less than 5% ($p<0.05$).

3. RESULTS

TPC was expressed as gallic acid equivalent (GAE) mg/gm and the TPC values of the rhizome of *C.orchioides* extracts by using various solvent, methanol, chloroform, ethyl acetate and butanol are 192.56, 3.66, 39.27, 31.38 mg/g GAE plant material. (Fig.1)

As the absorbance increases reducing power of the extract also increases. The absorbances of extract at different conc 50, 100,150, 200, 250 µg/ml, are 0.4679, 0.6000, 0.6824, 0.707, and 0.9808 respectively. (Fig 2)

DPPH, which is a radical itself with a purple color changes in to a stable compound with a yellow color by reacting with an antioxidant and the extent of the reaction depends on the hydrogen donating ability of the antioxidant. IC_{50} value is the ability to reduce the free radical to 50% so lower the IC_{50} higher will be the reducing power of an antioxidant. Comparing the IC_{50} value of standard Ascorbic acid (62.49µg/ml) and plant methanolic (176.36µg/ml) extract it may be concluded that the plant have less reducing power in comparison to that of standard Ascorbic acid. (Table.1)

As the H_2O_2 concentration is decreased by scavenger compounds, the absorbance value at 230nm is also decreased. Higher the scavenging activity lower will be the IC_{50} values.IC_{50} values of standard Ascorbic acid (197.02µg/ml) and that of plant extract (258.56µg/ml) are comparable it may have significant H_2O_2 scavenging activity (Table.1)

Effect of COE at dose of 50-200mg/kg, twice a day for 5 days prevented the acute gastric ulcer in a dose dependent manner. The range of percent protection were ASP 28.81-67.05%, PL 32.69-63.66%, CRS 19.26-57.98% and EtOH 27.71-63.01% , respectively. The range of percentage protection of ranitidine was 73.70-81.31% in various gastric ulcer models. (Fig.3)

The effect of MeOH extract of *Curculigo orchioides* Gaertn. (200 mg/kg) when administered orally, twice daily for 5 days was studied for their effect on volume, acid and pepsin secretion in 4 h pylorus ligation rats. *Curculigo orchioides* Gaertn showed a tendency to decrease in gastric juice volume, total acidity simultaneously increase in gastric pH. (Table. 2)

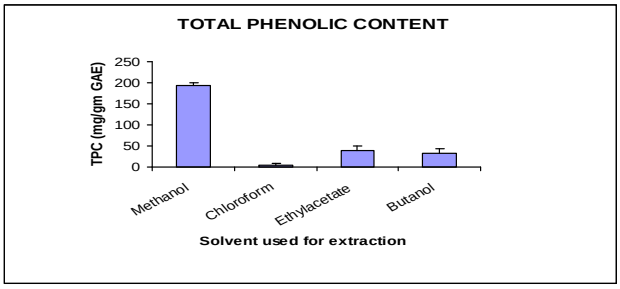

Fig 1: TPC of *Curculigo orchioides G.* Rhizome in different solvent

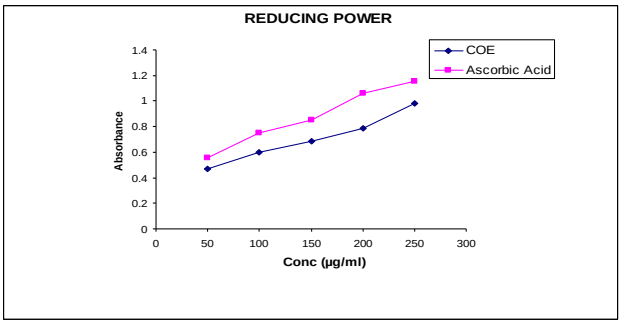

Fig 2: Reducing Power of methanolic extract of *C.orchioides* and std Ascorbic acid

Table 1: In vitro antioxidant activity of *C.orchioides* rhizome

S.No.	Methods	IC_{50} Values in µg/ml	
		Std. Ascorbic acid	Plant Methanolic extract
1	DPPH	62.49	176.36
2	H_2O_2	197.02	258.56

Table 2 : Effect of methanolic extract of *Curculigo orchioides* on gastric secretion in 4 h PL rats : effect on volume, Gastric pH, acid and pepsin

Treatment	Dose (mg/kg)	Volume (ml/100g)	Gastric pH	Toal Acidity (µEq/ml)	Peptic (µmol/ml)
Control		4.21±0.12	2.16 ±0.95	112+3.92	292.4±33.4
COE	**200**	2.02+0.70b	3.96+0.02c	39.0+2.86a	218.2+25.3
Ranitidine	**50**	1.75+0.31b	4.83+0.11b	28.74+2.31a	197.3+27.4

Data are mean±S.E.M. n=6 in each group

[a]P < 0.001 compared to respective control group.

[b]P < 0.01 compared to respective control group.

[c]P < 0.05 compared to respective control group.

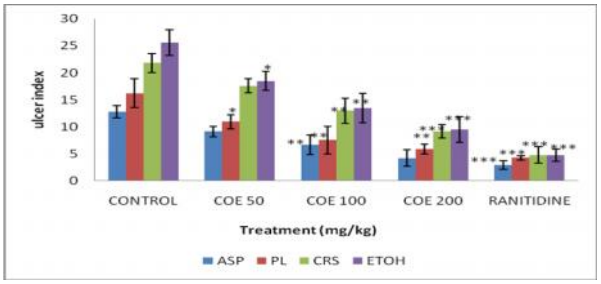

Fig 3: Effect of methanolic extract of *Curculigo orchioides* rhizome (COE) on aspirin (ASA), pylorus ligation (PL) , cold restraint stress (CRS) and ethanol (EtOH),induced gastric ulcers in rats.*P<0.001, as compared to their respective control.**P<0.01,as compared to their respective control.*P<0.05, as compared to their respective control.**

Data are mean±S.E.M. n=6 in each group

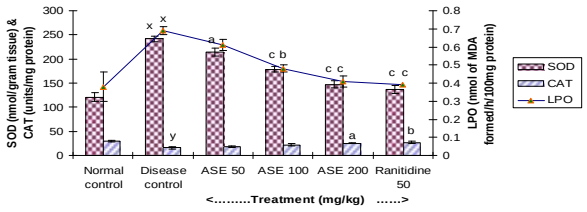

Fig 4: Effect of methanolic extract of *Curculigo orchioides* rhizome (COE) on lipid peroxidation (LPO), superoxidedismutase (SOD) and catalase (CAT) cold restraint stress (CRS) induced gastric ulcers in rats. [a]P <0.001, as compared to their respective control. [b]P <0.01,as compared to their respective control. [c]P <0.05, as compared to their respective control.

Data are mean±S.E.M. n=6 in each group

4. DISCUSSION AND CONCLUSION

The aim of the present study was to assess the role of various mucosal offensive acid-pepsin and defensive mucosal factors. Attempts were made on the necessity of nontoxic, antiulcer compounds preferably from traditional medicinal plants such as *Curculigo orchioides* for their protection against various experimental gastric ulcer models. Attempts were then further made to find out the status of the offensive acid-pepsin and defensive mucosal factors like mucin secretion, mucosal glycoproteins and antioxidant activities in herbal drugs.

Pylorus-ligated ulcers may be due autodigestion of gastric juice, decrease mucosal blood flow and break down of mucosal barrier (Goel and Bhattacharya, 1991). [18] Stress plays an important role in the causation of gastroduodenal ulceration and antistress drugs were found to be effective in stress-induced gastric mucosal damage. Oxidative stress has been proposed to be important etiopathological factor in genesis of peptic ulcer, while enhanced acid-pepsin appears to be directly related to Pylorus-ligated induced gastric ulcers (Senay and Levine, 1967; Guth, 1971) [19, 20]. Disturbance of gastric mucosal microcirculation, alteration in gastric secretion and abdominal gastric motility (Garrick *et al*., 1986; Tariq *et al*., 1986) [21, 22], mast cell degranulation (Cho and Ogle, 1979) [23] and decreased prostaglandin synthesis (Rao et al., 1999) [24] have been considered as pathogenesis mechanisms responsible for the stress-induced gastric mucosal lesions.

Synthetic NSAIDs like aspirin cause mucosal damage by interfering with prostaglandin synthesis, enhance acid secretion, increase back diffusion of H^+ ions, result in leukotrienes production and other product of 5-lipioxygenase pathway and breaking up of the mucosal barrier (Vane, 1971; Brzozowski, 1995; Rao, 2000) [25-27]. Genesis of ethanol induced ulcer due to perturbs superficial mucosal cells, notably mucosal mast cells to release vasoactive mediators notably LTC_4/D_4 , platelet activating factor (PAF) , histamine and reactive oxygen species which damage the gastrointestinal mucosa (Wallace *et al*., 1982; Miller and Henagan, 1984; Peskar et al ., 1986; Boughton Smith and Whittle, 1988) [28-32].

Curculigo orchioides Gaertn. were found to possess ulcer protective effects dose-dependently against Pylorus-ligation-, Aspirin-, CRS-, and ethanol-induced gastric ulcer in rats, as indicated by lower ulcer index. Result of present study on free radical mediated LPO and alteration of circulating enzymatic antioxidant, CAT and SOD , indicate involvement of these enzyme in ulcer.

Gastric ulceration is mainly caused by the alteration of the antioxidant enzymes of the gastric mucosa with concomitant loss of cytoprotection due to decreased activity of the PG synthesis (Das and Banerjee, 1993) [33]. Perhaps development of a novel nontoxic potent antisecretory – antioxidant compound which will prevent oxidative stress will be a suitable way to control this human suffering. Mizui and Doteuchi (1986) [34] showed that the necrotizing substance like ethanol- induced gastric damage could be due to the formation of oxygen derived free radicals resulting in lipid peroxidation and damage of cellular membrane with the release of intracellular component like lysosomal enzymes leading to further damage.

In our present study, the antioxidant property of MeOH extract of *Curculigo orchioides* Gaertn. was found to be changes in SOD, CAT, and LPO levels in rat gastric mucosa. During the ulcer condition there is increase in gastric mucosal SOD and LPO activities. This indicated that the generation of reactive oxygen species during stress might be the causative factor for the inactivation of gastric peroxidase. *Curculigo orchioides* Gaertn. exerts their antioxidant defense mechanism probably by metabolising lipid

peroxides and scavenging endogenous H_2O_2 (Bhattacharya *et al*., 2000; Rao *et al*., 2004) [35, 36]

The present investigation showed that the rhizome of *Curculigo orchioides* Gaertn showed potent antiulcer activity as indicated by low ulcer index or high protection percentage and also antioxidant activity as indicated the SOD, CAT were significantly increased and LPO levels reverted back in rat gastric mucosa.

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