INTRODUCTION

Hypochlorhydria is a very common problem caused due to low secretion of hydrochloric acid and increased level of intragastric pH (pH≥4). In hypochlorhydric condition, vitamin-C concentration is diminished in the gastric juice.[1] Vitamin C inhibits the formation of carcinogenic N-nitroso compounds within the gastric juice of healthy stomach and it causes gastric cancer.[2,3] Gastric cancer is the second leading cause of cancer death and the fourth most common cancer in terms of new cases worldwide.[4] The hypochlorhydria is the result of atrophic gastritis and the progression of atrophic gastritis can occur over a period of years. Stress initially increases stomach HCl production and causes indigestion, heart burn, gastritis, and ulcer problems. But chronic stress can lead to hypochlorhydria and reduced function of the pancreas.[5] Hypochlorhydria has so many number of etiologies such as sympathetic dominance, antisecretory drug use, excess sugar and refined foods, chronic over eating, constant smoking between meals, and nutrient deficiencies, especially zinc and thiamin.

In this study, hypochlorhydria in rat was induced by the oral administration of ranitidine, a H₂ receptor antagonist with a standard dose.[6] Our previous investigation reported that Benincasa hispida T has anti-hypochlorhydric activity that prevents the experimental hypochlorhydria and age-induced hypochlorhydria in rat.[6,7] B. hispida belongs to family “Cucurbitaceae.” It is commonly known as “Ash gourd” or “Chalkumra,” or “Kusmanda,” cultivated by villagers (farmers) and available in West Bengal.[8] The fruit of B. hispida has been used in India from ancient times for various ailments such as gastrointestinal problems, respiratory disease, heart diseases, diabetes mellitus,
and urinary diseases. Therefore, the present study was conducted to search out the effective solvent extract of this fruit for the management of hypochlorhydria.

MATERIALS AND METHODS

Plant Material
The ripe fruit of *B. hispida* was collected in the month of June from Midnapur town, Paschim Medinipur district, West Bengal, India. The ripe fruits of *B. hispida* were identified by Prof. R.K. Bhakat in Botany Department, Vidyasagar University, West Bengal, India. The juice of the fruits was collected and stored at 4°C. The concentrated juice was prepared as per standard method and the residue of this juice was used for extract preparation.

Hydro-methanol Extract of *B. hispida*
Five gram residue was macerated with 100 ml of aqueous-methanol (2:3, v:v) mixture at 37°C for 36 hours with intermittent stirring. Then, the extract was filtered and filtrate was dried by low pressure and residue was collected. This residue was suspended in distilled water at a concentration of 4 mg/ml to be used for the experiment.

Ethyl Acetate Extract of *B. hispida*
Using ethyl acetate, 5 g residue was macerated in 100 ml at 37°C for 36 hours with intermittent stirring and the residue was collected and preserved for experimental purpose.

Aqueous Extract of *B. hispida*
Five gram residue was macerated with 100 ml of distilled water at 37°C for 36 hours with intermittent stirring and residue was collected and preserved.

Chemicals
All chemicals were analytical grade and purchased from E. Merck and Loba (India).

Animals
Thirty male Wistar strain young (3 months old) albino rats weighing 100 ± 5 g were selected for this experiment. The rats were acclimatized for a period of 15 days in our well ventilated laboratory prior to the experiment. All studies were conducted in accordance with the National Institute of Health’s Guide for the care and use of Laboratory animals. The work was approved by our Institutional Animal Ethical Committee (IAEC) having Reference No. VU-IAEC/Bio-Med/Exp-16/2011. Animals were housed at an ambient temperature of 25 ± 2°C with 12 hour light: 12 hour dark cycle. Animals were given free access to water and food throughout the experimentation.

Induction of Hypochlorhydria
Hypochlorhydria was induced as per our previously published method in healthy rat by oral administration of ranitidine at the dose of 5 mg/kg body weight in alternative day for 14 days.

Experimental Design
Animals were divided into following five groups containing six rats in each group. All the drugs were administered in oral route by feeding cannula.

Group I (Control Group)
Animals of this group received only distilled water (5 ml/kg) through oral route.

Group II (Hypochlorhydric Group or Ranitidine-treated Group)
Rats of this group were given distilled water for two days in equal volume through oral route and then treated with ranitidine at the dose of 5 mg/kg body weight in alternative day before meal for 7 such doses.

Group III (Hydro-methanol Extract of *B. hispida Pretreatment cum Co-treatment Group*)
Rats of this group received Hydro-methanol extract of *B. hispida* (HMBH) at the dose of 20 mg/kg body weight/day in 5 ml distilled water for two days followed by the treatment of ranitidine at the dose of 5 mg/kg body weight in alternative day (7 such doses) along with treatment of the same extract once a day before meal as above mentioned dose for 14 days.

Group IV (Ethyl Acetate Extract of *B. hispida Pretreatment cum Co-treatment Group*)
Rats of this group received Ethyl acetate extract of *B. hispida* (EABH) at the dose of 20 mg/kg body weight/day in equal volume of distilled water for two days followed by the treatment of ranitidine at the dose of 5 mg/kg body weight in alternative day (7 such doses) along with treatment of the same extract once a day before meal as above mentioned dose for 14 days.

Group V (Aqueous Extract of *B. hispida Pretreatment cum Co-treatment Group*)
Rats of this group received Aqueous extract of *B. hispida* (ABH) at the dose of 20 mg/kg body weight/day in equal volume of distilled water for two days followed by the treatment of ranitidine at the dose of 5 mg/kg body weight in alternative day (7 such doses) along with treatment of the same extract once a day before meal as above mentioned dose for 14 days.

Pyloric Ligation
Animals of all the groups were fasted for 24 hours after completion of above treatment schedule. Under light ether anesthesia, the abdomen of the animals was opened through midline incision and the pylorus was ligated as per standard method. The stomach was placed in the usual position and then abdomen was sutured. Four hours later, pylorus-ligated rats were sacrificed with ether anesthesia. After opening the abdomen, the esophagus was clamped and then stomach was removed. The gastric juice was collected.
and its volume was measured followed by centrifugation. Liver and kidney of each animal were collected and their wet weights were recorded and stomach also collected for the study of biochemical parameters.

**Measurement of pH in Gastric Juice**
The pH of the gastric juice was measured by pH meter. Hypochlorhydria was defined as a fasting gastric pH>4.0.

**Estimation of Free Acidity and Total Acidity**
The free acidity (free HCl) of the gastric juice was measured by titration method with N/10 NaOH solution using Topfer’s reagent (0.5 g diethyl amino azobenzenes/100 ml ethanol) as an indicator and the total acidity was estimated by titration with N/10 NaOH solution using phenolphthalein as an indicator.[12]

**Quantification of Chloride Level in Gastric Juice**
At first, protein free filtrate of gastric juice was prepared. In brief, chloride level was measured in gastric juice by diluting with water followed by mixing with sodium tungstate and H₂SO₄. The mixture was centrifuged and protein free filtrate was collected. This filtrate was titrated against mercuric nitrate solution using diphenyl carbazone as an indicator.[13]

**Assessment of Pepsin Concentration in Gastric Juice**
The pepsin in gastric juice was estimated by the method of Smuual Natelson.[14] In brief, gastric juice was incubated with pepsin substrate (0.5% bovine hemoglobin) followed by centrifugation. Then, supernatant was treated with Folin phenol reagent and absorbance was measured spectrophotometrically at 540 nm.

**Measurement of Vitamin-C Level in Gastric Juice**
Vitamin-C level was measured using the 2, 4-dinitrophenyl hydrazine method.[15,16] Two ml of 10% metaphosphoric acid was added to 0.5 ml of plasma or gastric juice to precipitate protein. After vortex mixing, samples were centrifuged at 900×g for 10 minutes followed by filtration using 0.45-ﬁlter paper. Next, 1.2 ml of the filtrate was mixed with 0.4 ml reaction buffer (5 ml 27 µmol/l copper sulfate, 5 ml 660 µmol/l thiourea, and 10 µmol/l 2, 4 dinitrophenylhydrazine). The mixture was then vortexed and stored in water bath at 37°C for 3 hours. Then, the samples were placed in ice for 10 minutes followed by addition of 2 ml of 12 mol/l H₂SO₄ carefully to the sample. The absorbance of the sample was measured spectrophotometrically at 520 nm. Ten percent metaphosphoric acid was used as blank and 1 mg/dl ascorbic acid was used as a standard.

**Estimation of Lipid Peroxidation in Stomach**
Lipid peroxidation in stomach was assessed by measuring the concentration of thiobarbituric acid-reactive substances. The gastric tissue was homogenized at the tissue concentration of 50 mg/ml in 0.1M of ice-cold phosphate buffer (pH 7.4).

The 0.5 ml of supernatant was mixed with 0.5 ml of normal saline and 2 ml of TBA-TCA (0.392 g thiobarbituric acid in 75 ml of 0.25N HCl with 15 g trichloro-acetic acid) mixture. The volume of mixture was made up to 100 ml with 95% ethanol and boiled at 100°C for 10 minutes. This mixture was then cooled to room temperature and centrifuged at 4 000×g for 10 minutes. The whole supernatant was taken into a spectrophotometer cuvette and read at 535 nm.[17]

**Biochemical Assay of Catalase Activity of Gastric Tissue**
The activity of catalase in gastric tissue was measured biochemically.[18] For the evaluation of catalase activity, gastric tissue was homogenized in 0.05M Tris HCl buffer solutions (pH 7.0) at the concentration of 50 mg/ml. Then, the samples were centrifuged at 10 000×g at 4°C for 10 minutes. In spectrophotometer cuvette, 0.5 ml of 0.00035M H₂O₂ and 2.5 ml of distilled water were added and mixed. Absorbance was recorded at 240 nm before the addition of supernatant in the reaction mixture. Then, 40 µl supernatant was added in the cuvette and the subsequent six readings were recorded at 30 seconds interval.

**Statistical Analysis**
Data were expressed as mean±SEM (Standard error of mean). ANOVA (Analysis of variance) followed by a multiple comparison two-tail ‘t’ test was used for statistical analysis of the collected data. Differences were considered significant when P<0.05.

**RESULTS**

**Body Weight and Organo-somatic Indices**
There was no significant difference was observed in body weight as well as in hepato-somatic and reno-somatic indices in ranitidine-treated and extracts of B. hispida pretreatment cum co-treatment groups in comparison with the control group [Table 1].

**Volume of Gastric Juice and Gastric Juice pH**
Volume of basal gastric secretion was decreased and pH increased significantly (P<0.05) in ranitidine-induced hypochlorhydric rat in comparison with the control. ABH showed significant (P<0.05) protective effect in the level of gastric pH and volume of gastric juice compared with other [Figure 1].

**Free Acidity and Total Acidity**
It was found that the level of free acidity was decreased significantly (P<0.05) and total acidity was increased significantly (P<0.05) in ranitidine-induced hypochlorhydric group in comparison with the control group. ABH showed corrective significant difference in the level of free acidity and also total acidity as compare with other solvent extract-treated groups [Figure 2].
Chloride Level and Pepsin Concentration in Gastric Juice
Chloride secretion and pepsin activity were decreased significantly ($P<0.05$) in ranitidine-induced hypochlorhydric rat in comparison with the control group. But, ABH pre-treatment followed by co-treatment recovered the chloride level and pepsin activity in gastric juice significantly ($P<0.05$) in respect to other pre-treated cum co-treated groups [Figure 3].

Vitamin C Concentration in Gastric Juice
A significant ($P<0.05$) depletion was noted in vitamin C concentration in gastric juice in ranitidine-induced hypochlorhydric group in respect to the control group. ABH showed significant protective effect in vitamin C concentration in gastric juice as compare with other extract-treated groups [Figure 4].

Thiobarbituric Acid-reactive Substances Levels and Catalase Activity in Gastric Tissue
Thiobarbituric acid-reactive substances (TBARS) level in gastric tissue was increased and catalase activity was decreased significantly ($P<0.05$) in ranitidine-induced hypochlorhydric group in respect to the control group. Pre-treatment followed by co-treatment of EABH and ABH significantly decreased ($P<0.05$) the gastric TBARS level toward the control. The catalase activity was also recovered significantly ($P<0.05$) by EABH or ABH [Figure 5].

DISCUSSION
The term hypochlorhydria is not common in applied research, though it is a common problem of not only older people but also of any age of people like other gastric disorders. *B. hispida* is used for the treatment of various diseases such as gastrointestinal problems, respiratory disease, heart diseases, diabetes mellitus, and urinary diseases. But there is plethora of scientific explanation about its medicinal properties. Recently, it was revealed that *B. hispida* prevents the Alzheimer’s disease([19]) and we also reported that *B. hispida* has anti-hypochlorhydric activity.[6] In our earlier study, we initially choose the aqueous extract of ripe fruit of *B. hispida* because Indian people are using the fruits as vegetable, cooked with water and water content of the fruit is maximal. Now, this study was conducted to find out the effective extract having anti-hypochlorhydric activity among the different
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Figure 3: Remedial effect of pre-administration followed by co-administration of HMBH or EABH or ABH on chloride and pepsin concentration in ranitidine-induced hypochlorhydric rat. Bars were expressed as mean±SEM (n=6). Bars with different superscripts (a, b, c, d) differ significantly from others (P<0.05). ANOVA followed by multiple comparisons two-tailed “t” test.

Figure 4: Effect of pre-administration cum co-administration of HMBH or EABH or ABH on vitamin C concentration of gastric juice in ranitidine-induced hypochlorhydric rat. Bars were expressed as mean±SEM (n=6). Bars with different superscripts (a, b, c, d) differ significantly from others (P<0.05). ANOVA followed by multiple comparison two-tailed “t” test.

Figure 5: Protective effect of pre-administration followed by co-administration of HMBH or EABH or ABH on TBARS level and catalase activity in gastric tissue in ranitidine-induced hypochlorhydric rat. Bars were expressed as mean±SEM (n=6). Bars with different superscripts (a, b, c, d) differ significantly from others (P<0.05). ANOVA followed by multiple comparisons two-tailed “t” test.

solvent extracts of ripe fruit of B. hispida such as HMBH, EABH, and ABH. This study would open the scope of making a standard formulated drug for hypochlorhydria; hence, the composite extract of B. hispida and F. vaillantii was more effective than individual plant extract reported previously. [6] We have found that there were no significant differences in body weight gain among the different groups and no significant difference was observed in food ingestion and water intake behavior throughout the experimental period. Therefore, the above herbal extracts have no general metabolic toxicity. Use of histamine H2 receptor antagonist like ranitidine raises this intra gastric pH. [20] In the present study, we found that ABH increased the basal volume of the gastric juice and decreased the pH of gastric juice by increasing the free acidity and chloride secretion in the gastric juice, supported by our earlier work. [6] Here, we noted that gastric secretory activity was restored after pre-administration as well as co-administration of ABH to ranitidine-treated rat but other two solvent extracts of said fruit did not show satisfactory results for restoring the gastric secretory activity from hypochlorhydric state. This may be due to the prevention of the gastric parietal cell degeneration or by enhancing the secretion of HCl through stimulating cholinergic parietal cell, supported by our earlier work and others. [6,7,21] We also observed that pepsin activity and chloride level were decreased in gastric juice of rats treated with ranitidine in alternate day for 14 days, which was supported by other’s work. [22,23] There are two components of the luminal chloride secreted by the parietal cells, the acidic component of chloride secretion, which is essential for gastric hydrochloric acid secretion, and non-acidic component, which is observed as a transmucosal movement of chloride in excess of hydrogen. [24,25] The pre-treatment followed by co-treatment of ABH increased the chloride level significantly compared with ranitidine-treated groups and other extract-treated group. This may be due to the stimulation of the parietal cell by the active ingredients of ABH. We also found that gastric antioxidant such as vitamin C in gastric juice and catalase activity of gastric tissue were decreased in ranitidine-induced hypochlorhydric rat compared with control. Moreover, products of lipid peroxidation such as TBARS level also increased in hypochlorhydric group in respect to control, which was consistent with other reports. [1,26,27] These findings suggest that hypochlorhydria may cause oxidative stress and produce free radicals; that is why activities of catalase in gastric tissue and vitamin-C level in gastric juice were decreased significantly in hypochlorhydric rats. Results of the present study focused that EABH or ABH extracts increased the gastric secretion along with vitamin C concentration in the gastric juice and they may act as the secretagogues on the stomach, which was supported by our previous investigation. [6] For the recovery of vitamin C level, ABH gave more satisfactory results as compare with other solvent extracts. EABH or ABH extracts also resulted in the significant correction in the level of TBARS in gastric tissue.
toward the control level. Our findings suggest that ethyl acetate extract and ABH having anti-lipid peroxidative and antioxidant property, which was supported by our earlier work and by others [7,19] but aqueous extract of said plant’s fruit (ABH) showed more corrective effect on ranitidine-induced hypochlorhydria in rat.

CONCLUSION

From the results, it may be concluded that aqueous extract of fruit of *B. hispida* has anti-hypochlorhydric activity and it also possesses antioxidant property. These effects may be due to the presence of alkaloids, flavonoids, and other phytochemicals of ripe fruit of *B. hispida*. Further study is required for the isolation, purification, and chemical characterization of active phytomolecule(s).

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