Original Article

Management of experimental hypochlorhydria with iron deficiency by the composite extract of *Fumaria vaillantii* L. and *Benincasa hispida* T. in rat

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Abstract

The aim of the present study was to search the effective ratio of whole plant of *Fumaria vaillantii* Loisel (*Fumaria vaillantii* L.) and fruit of *Benincasa hispida* Thunb. (*Benincasa hispida* T.) in composite form, namely "FVBH" for the management of hypochlorhydria along with iron deficiency in male albino rats. Hypochlorhydria refers to suppression of hydrochloric acid secretion by the stomach. Hypochlorhydria was induced by ranitidine in this study. We used four composite extracts of the mentioned plant and fruit with different ratios (1:1, 1:2, 2:1, and 3:2) for searching the most effective composite extract for the correction of hypochlorhydria. Gastric acidity is an important factor for iron absorption. Thus, hypochlorhydria causes iron deficiency in rat and it was prevented significantly by the extract treatment at the ratio of 1:1 of the said plant and fruit. The correction of iron deficiency by the composite extract was compared with iron supplementation to hypochlorhydric rat. It was found that preadministration followed by coadministration of FVBH-1 (1:1) able to prevent the ranitidine-induced hypochlorhydria and iron deficiency. The composite extract, FVBH-1 (1:1) significantly (P<0.05) increased the pepsin concentration, chloride level in gastric juice, iron levels in serum and liver along with blood hemoglobin level than other ratios used here. Hence, it can be concluded that FVBH-1 (1:1) is an effective herbal formulation for the management of hypochlorhydria and related iron deficiency.

Key words: *Benincasa hispida* T., hypochlorhydria, liver iron, ranitidine, serum iron

INTRODUCTION

Frequent use of gastric acid inhibiting drugs (ranitidine, omeprazole, etc.,) suppressed the acid secretion and raised the gastric pH.[1,2] Hypochlorhydria is defined as a fasting gastric pH ≥ 4.[1] Use of ranitidine in healthy rat causes hypochlorhydria and oxidative stress[1] and it became chronic hypochlorhydria if it is not treated. Vitamin C inhibits the formation of carcinogenic N-nitroso compounds within the gastric juice of healthy stomach. In hypochlorhydric condition, vitamin C level is diminished in the gastric juice. It is a risk factor for gastric cancer.[3] Hypochlorhydria has many number of etiologies such as sympathetic dominance, antiseretory drug use, excess intake of sugar and refined foods, chronic over-eating, frequent smoking between meals and nutrient deficiencies especially zinc and thiamin. Gastric acid secretion is decreased with advancement of age and it becomes half of the normal in those aged over 60 years.[4] The incidence of hypochlorhydria in the population has been estimated to about 20-50% (in average 30% of the population) aged above 65 years.[5,6] Generally, hypochlorhydria is considered as an influencing factor on nutrition. It also affects the digestion of food and absorption of nutrients such as vitamins and minerals. People, who suffer from hypochlorhydria, feel hungry all the time due to poor absorption of nutrients. Hypochlorhydria is the most important causative factor of anemia due to

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iron deficiency.[7,8] Gastric HCl is an important luminal factor for non-heme iron absorption as it requires an acidic pH [9,10] and it aids in the liberation of iron from food and facilitates the conversion of ferric form to ferrous form.[11] Vitamin C also facilitates the dietary iron absorption by reducing the ferric iron into soluble ferrous iron.[12,13] Iron deficiency reduces the hemoglobin biosynthesis and leads to iron deficiency anemia.[12,14] Vitamin-C concentration is diminished in the gastric juice and also in plasma in hypochlorhydric condition.[15] It was reported earlier that vitamin-C concentration was diminished in ranitidine-induced hypochlorhydric rat and in aged rat also.[16,17] It is not only a problem of the aged but also a problem of most of the people of different age groups. Initially our efforts were being made to manage the hypochlorhydria in experimental condition in rat by the *Fumaria vaillantii* Loisel and *Benincasa hispida* Thunb.[17] *Fumaria vaillantii* Loisel (*Fumaria vaillantii* L.) belongs to family of 'Fumariaceae' and it was reported as a medicinal plant.[18] The whole plant of *Fumaria vaillantii* L. is used for the treatment of constipation, diarrhea, amlapitta, hypochlohydria, and liver complications.[19-21] *Fumaria vaillantii* L. is widely used in folk medicine as a blood purifier in the treatment of skin diseases.[22]

Benincasa hispida Thunb (*Benincasa hispida* T.) belongs to 'Cucurbitaceae' family and used as a folk medicine.[23] The fruit of *Benincasa hispida* T. has been used in India for centuries in various ailments such as gastrointestinal problems, respiratory diseases, heart diseases, diabetes mellitus, urinary diseases, and Alzheimer's diseases.[1,24,25] In our earlier work it was reported that composite extract of the mentioned plant and fruit is potent antihypochlorhydric agent than individual plant or fruit mentioned here.[17] Therefore, the aim of this study was to search out the effective formulation using the aqueous extract of the said plant and fruit in composite manner for managing the hypochlorhydria and its related iron deficiency.

MATERIALS AND METHODS

Plant materials

The whole plant of *Fumaria vaillantii* L. was collected from sub-Himalayan region (India) in the month of September and the plant was identified in Botany Department, Vidyasagar University, West Bengal, India. The whole plant of *Fumaria vaillantii* L. was air dried and powdered finely by grinding and then stored in air tight vessels as reported previously.[17] The ripe fruits of *Benincasa hispida* T. were collected from the local areas in the month of June and it was also identified in Botany Department, Vidyasagar University, West Bengal, India. The juice of the fruit was collected and stored at 4°C. The herbarium specimens of both the plants were stored in the Botany Department as BMLSM-FV and BH-11, 12-2009.[17]

Extract preparation

The aqueous extracts were prepared as per standard protocol described earlier.[17] The extracts of *Fumaria vaillantii* L. and *Benincasa hispida* T. were used at the ratio (w/w) of 1:1, 1:2, 2:1, and 3:2 to prepare the composite mixture referred to as FVBH-1, FVBH-2, FVBH-3, and FVBH-4, respectively.

Chemicals

All chemicals and reagents used for the study were of analytical grade.

Animals

The young (3-month-old) male albino Wistar rats weighing 100 ± 5 g were used for this experiment. The animals were maintained under standard environmental conditions and were given free access to water and food. The study was approved by Institutional Animal Ethical Committee (IAEC) and IAEC clearance number was "IAEC/BMLSM/2012". National Institute of Health guidelines were followed during the maintenance of the animals and at the time of experimentation.[26]

Experimental design

Animals were divided into seven groups of six rats in each group; duration of the experiment was 30 days (2 days pretreatment and 28 days cotreatment). Hypochlorhydria was induced in healthy rats by orally administrating ranitidine at a dose of 5 mg in 5 ml distilled water/kg body weight on alternative day for 14 days and the dose was selected from trial and error experiments reported previously.[17] All the extracts were administered to the animals through oral route by gavages.

Group I (Control group)

Animals received only distilled water (5 ml/kg) through oral route.

Group II (Hypochlorhydric group or ranitidine treated)

Rats were given distilled water for 2 days in equal volume through oral route and then treated with ranitidine at a dose of 5 mg/kg of body weight on alternative days before meal for 28 days.

Group III (FVBH-1 pre- and cotreated)

Rats of this group were pretreated with FVBH-1 at a dose of 20 mg/kg body weight/day for first 2 days followed by the treatment of ranitidine at the dose of 5 mg/kg body weight on alternative days. Extract cotreatment on each day with the same dose for 28 days.

Group IV (FVBH-2 pre-and cotreated)

Rats of this group were pretreated with FVBH-2 at a dose of 20 mg/kg body weight/day for first 2 days followed by the treatment of ranitidine at the dose of 5 mg/kg body weight on alternative days. Extract cotreatment was continued on each day with the same dose for 28 days.

Group V (FVBH-3 pre-and cotreated)

Rats of this group were pretreated with FVBH-3 at a dose of 20 mg/kg body weight/day for first 2 days followed by the treatment of ranitidine at the dose of 5 mg/kg body weight on alternative days. Continuation of the extract cotreatment was performed on each day with the same dose for 28 days.

Group VI (FVBH-4 pre-and cotreated)

Rats of this group were pretreated with FVBH-4 at a dose of 20 mg/kg body weight/day for first 2 days followed by the treatment of ranitidine at the dose of 5 mg/kg body weight on alternative days. Extract cotreatment was continued on each day with the same dose for 28 days.

Group VII (Iron supplemented group)

Rats of this group received ranitidine as hypochlorhydric rat with the same dose from the 1st day of experiment before meal on alternative days for 28 days and supplemented with 0.1 mg iron/kg body weight/day from the 3rd day of experiment for 28 days. Iron supplementation was performed by providing $FeSO_4$ (1 mg iron = 4.97 mg $FeSO_4$) dissolved in distilled water.

Gastric juice collection

The animals of all the groups were fasted for 24 h after completion of mentioned treatment schedule and sacrificed after collecting the gastric juice using pylorus ligation model.[27] Blood was collected from dorsal aorta of each animal by a heparinized syringe. The stomach, liver, and kidney were collected.

Measurement of pH of gastric juice

The pH of the gastric juice was measured by using pH meter on the day of animal sacrifice.

Estimation of free acidity and total acidity

The free acidity (free HCl) of the gastric juice was estimated by titration with N/10 NaOH solution using Topfer's reagent (0.5 g diethyl amino azobenzenes/100 ml ethanol) as an indicator.[28]

Total acidity was estimated by titration with N/10 NaOH solution using phenolphthalein as an indicator.[28]

Quantification of chloride level in gastric juice

Protein-free filtrate of gastric juice was prepared for this purpose. In brief, chloride level was measured in gastric juice by diluting with water followed by mixing with sodium tungstate and H_2SO_4. The mixture was centrifuged and protein-free filtrate was collected. This filtrate was titrated against mercuric nitrate solution using diphenyl-carbazone as an indicator.[29]

Assessment of pepsin concentration in gastric juice

The pepsin in gastric juice was estimated by the method of Smuual Natelson.[30] In brief, gastric juice was incubated with pepsin substrate (0.5% bovine hemoglobin) and centrifuged. Then supernatant was treated with Folin phenol reagent and absorbance was measured spectrophotometrically at 540 nm wave length.

Measurement of vitamin-C level in gastric juice

Vitamin-C level was measured using the 2, 4-dinitrophenyl hydrazine method.[31,32] Two milliliters of 10% metaphosphoric acid was added to 0.5 ml of gastric juice to precipitate protein. After vortex mixing, samples were centrifuged at 900 g for 10 min and filtered through a 0.45 μm filter paper. Next, 1.2 ml of the filtered 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 vortexed and stored in water bath at 37°C for 3 h. The samples were then placed in ice for 10 min and were added to 2 ml of 12 mol/l H_2SO_4 carefully. The absorbencies of samples were measured spectrophotometrically at 520 nm wave length. Ten percent metaphosphoric acid was used as blank and 1 mg/dl ascorbic acid was used as a standard.

Estimation of hemoglobin percentage and hematocrit value

Hemoglobin percentage was determined by the cyanomethemoglobin method[33] and hematocrit value was determined using micro-hematocrit tube.[34]

Estimation of serum iron and liver iron

Serum iron was determined by the method of 'International Committee for Standardization in Hematology.'[35] Serum proteins were precipitated with a reagent containing trichloroacetic acid, hydrochloric acid, and thioglycollic acid. After centrifugation, supernatant was treated with bathophenanthroline reagent to give pink complex, which was measured spectrophotometrically at 535 nm wave length.

Liver tissue was analyzed for nonheme iron by using the bathophenanthroline method.[36] The tissues were dried, weighed, and digested with hydrochloric/trichloroacetic acids at 65°C for 20 h. The resulting mixture was reacted with bathophenanthroline reagent and absorbance was measured spectrophotometrically at 535 nm wave length.

Biochemical assay of glutamate oxaloacetate transaminase and glutamate pyruvate transaminase

For the assessment of metabolic toxicity, we measured the glutamate oxaloacetate transaminase (GOT) and glutamate pyruvate transaminase (GPT) activities in liver and kidney. Tissues were homogenized separately in ice cold 0.1 M phosphate buffer (pH: 7.4) at the tissue concentration of 50 mg/ml. Then using Kit (Crest Biosystems, Goa, India) enzyme activities were measured and activities were expressed as unit per gram of tissue.[37]

Statistical analysis

Data were reported as means ± SEM. Analysis of variance (ANOVA) followed by a multiple comparison two-tailed '*t*'-test was used for statistical analysis of the collected data. Differences were considered significant when $P < 0.05$.

RESULTS

Volume and pH of gastric juice

Volume of basal gastric secretion was decreased and pH was increased significantly in ranitidine-induced hypochlorhydric rat and iron supplemented rat in comparison to the control. FVBH-1(1:1) extract treatment showed a significant protective difference in the values of gastric pH and volume of gastric juice in comparison to other groups treated with composite extract with different ratio [Figure 1].

Free acidity and total acidity

It was found that the level of free acidity was decreased significantly and total acidity was increased significantly in ranitidine-induced hypochlorhydric group and iron supplemented group in comparison to the control group. FVBH-1(1:1) treatment showed a significant improvement in the values of free acidity and also the total acidity as compared with other composite extracts with different ratio treated groups [Figure 2].

Chloride level and pepsin concentration in gastric juice

Chloride secretion and pepsin activity were decreased significantly in ranitidine-induced hypochlorhydric rat and iron supplemented rat in comparison to the control group. But FVBH-1(1:1) pretreatment followed by cotreatment recovered the chloride level and pepsin activity in gastric juice in respect to other pretreated cum cotreated groups with different formulations of FVBH as well as iron supplemented group [Figure 3].

Vitamin C concentration in gastric juice

A significant depletion was noted in vitamin C concentration in gastric juice in ranitidine-induced hypochlorhydric group and iron supplemented group when compared with the control group. FVBH-1(1:1) or other composite extract treatment significantly increased the vitamin C concentration in gastric juice as compared with ranitidine treated rat and reached to the control level [Figure 4].

Hemoglobin level and hematocrit value

Hemoglobin level and hematocrit value were decreased significantly in ranitidine-induced hypochlorhydric rat in comparison to the control group. But FVBH-1(1:1) pretreatment followed by cotreatment or iron supplementation recovered the hemoglobin level and hematocrit value in respect to other pretreated cum cotreated groups [Table 1].

Iron level in serum and liver

Serum iron and liver iron were significantly depleted in ranitidine-induced hypochlorhydric rat as compared with the control group. FVBH-1(1:1) showed significant elevation in iron level in serum and liver as compared with other groups. This elevation of iron in both serum and liver reached toward the control level and serum iron was comparable to iron supplemented group [Table 1].

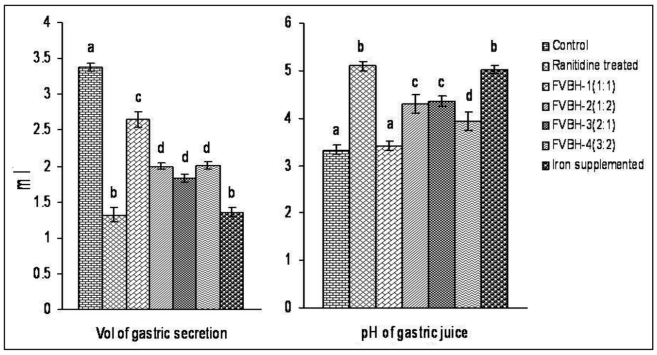

Figure 1: Protective effect of preadministration followed by coadministration of FVBH-1 or FVBH-2 or FVBH-3 or FVBH-4 on volume and pH of gastric secretion in ranitidine-induced hypochlorhydric rat. Data are expressed as mean ± SEM, $n = 6$. Bars with different superscripts (a, b, c, d) significantly differ from each other ($P < 0.05$). ANOVA followed by multiple comparison two-tailed "*t*"-test

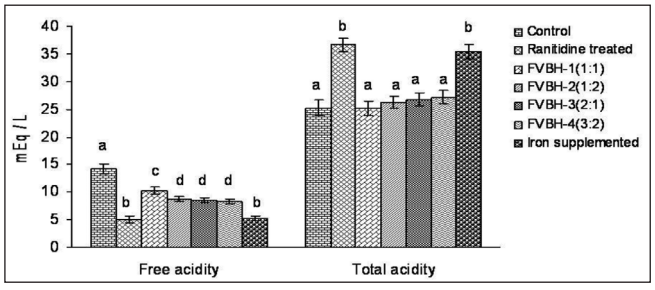

Figure 2: Remedial effect of pretreatment followed by cotreatment of different composite extracts on free acidity and total acidity of gastric secretion in ranitidine induced hypochlorhydric rat. Data are expressed as mean ± SEM, $n = 6$. Bars with different superscripts (a, b, c, d) significantly differ from each other ($P < 0.05$). ANOVA followed by multiple comparison two-tailed "*t*"-test

Biochemical assay of glutamate oxaloacetate transaminase and glutamate pyruvate transaminase activities

For the assessment of metabolic toxicity, we measured the GOT and GPT activities in liver and kidney. No significant alteration was noted in activities of GOT and GPT in liver and kidney in extract treated rats compared with the control group. So the composite extracts with different ratios have no metabolic toxicity [Figure 5].

DISCUSSION

The term hypochlorhydria is not common in applied research. But it is a common problem of not only older people but also at any age of people, like other gastric disorders. Now it is considered as one of the conferring factors influencing nutritional status. Hypochlorhydria is the most important causative factor of anemia due to iron deficiency.[7,9] Gastric HCl is an important luminal factor for nonheme iron absorption as it requires an acidic pH[7,10] and it helps the liberation of iron from food and facilitates the conversion of ferric form to ferrous form.[11] Our initial work showed that composite extract is potent antihypochlorhydric agent than individual plant or fruit extract mentioned here.[17] As iron deficiency is the consequence of hypochlorhydria, so we try to manage the hypochlorhydria along with iron deficiency as well as to find out the effective ratio of plant and fruit in composite extract. In this study, we used the aqueous extracts of whole plant of *Fumaria vaillantii* L. and ripe fruit of *Benincasa hispida* T. at different ratios (w/w) of 1:1, 1:2, 2:1, and 3:2 to prepare the composite mixture referred as FVBH-1, FVBH-2, FVBH-3, and FVBH-4, respectively. The effect of composite extracts with different ratios on iron deficiency in rat compared with the effect of iron supplementation in ranitidine treated rat. From our current studies, we found that gastric pH was increased and volume of gastric secretion was decreased significantly in ranitidine treated rat as compared with control.[1,21] Beside these, significant low levels of chloride, pepsin, and vitamin C concentration in gastric juice were observed and iron deficiency was also noted in ranitidine treated rat as compared with the control. Hypochlorhydria with low vitamin C is the risk factor of gastric cancer and esophageal squamous-cell carcinoma.[3,38] But pretreatment followed by cotreatment of FVBH-1(1:1) has more significantly increased the basal volume of the gastric juice and decreased the pH of gastric juice, increased free acidity, chloride secretion,

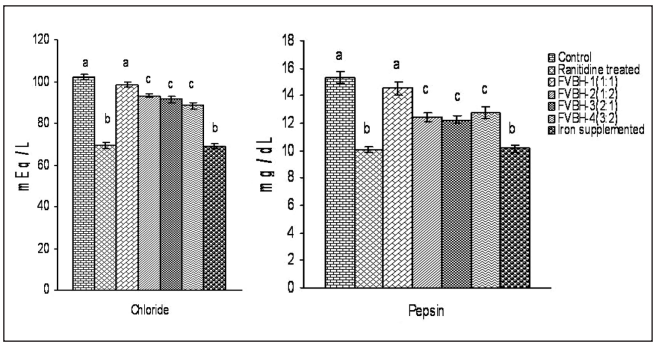

Figure 3: Comparative study on remedial effects of preadministration followed by coadministration of different composite extracts on chloride and pepsin concentrations in ranitidine-induced hypochlorhydric rat. Data are expressed as mean ± SEM, $n = 6$. Bars with different superscripts (a, b, c) significantly differ from each other ($P < 0.05$). ANOVA followed by multiple comparisons two-tailed "*t*"-test

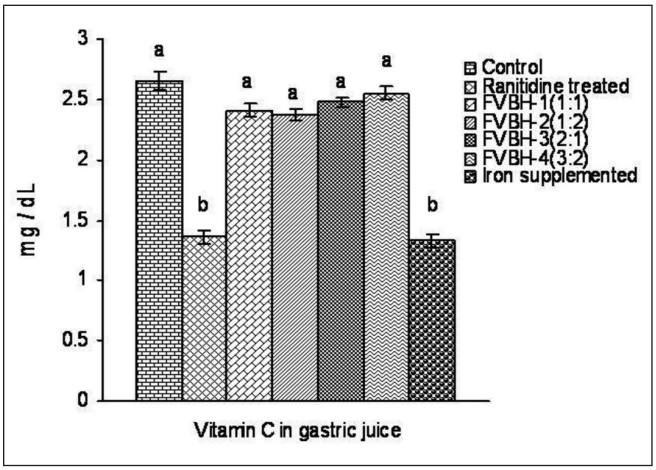

Figure 4: Corrective effect of preadministration cum coadministration of different composite extracts on vitamin C concentration in gastric juice in ranitidine-induced hypochlorhydric rat. Data are expressed as mean ± SEM, $n = 6$. Bars with different superscripts (a, b) significantly differ from each other ($P < 0.05$). ANOVA followed by multiple comparison two-tailed "*t*"-test

Table 1: Corrective effect of different composite extracts of F. vaillantii L. and B. hispida T. on hemoglobin level, hematocrit value and iron content in liver and kidney tissue.

Group	Hemoglobin (g/dL)	Hematocrit value (%)	Serum iron (μmol/l)	Liver iron (μmol/g dry wt)
Control	16.45±0.04^a	52.4±0.28^a	12.35±0.31^a	0.256±0.002^a
Ranitidine treated	12.01±0.03^b	40.2±0.86^b	7.18±0.18^b	0.148±0.001^b
FVBH-1 (1:1)	15.92±0.06^a	51.3±1.1^a	10.24±0.35^c	0.211±0.001^c
FVBH-2 (1:2)	13.02±0.03^b	43.2±0. 37^c	8.25±0.32^d	0.150±0.001^d
FVBH-3 (2:1)	13.05± 0.05^b	43.5±0. 42^c	8.61± 0.33^d	0.151±0.001^d
FVBH-4 (3:2)	14.3±0.06^c	47.4±0.32^d	9.32±0.22^e	0.201±0.001^e
Iron supplemented	16.32±0.12^a	51.8±0.63^a	10.54±0.12^c	0.259 ±0.001^a

Data are expressed as Mean ± SEM, *n:* 6, Values with different superscripts (a, b, c, d, e) in each vertical column differ significantly from other ($P < 0.05$), ANOVA followed by multiple comparison two-tailed "*t*"-test

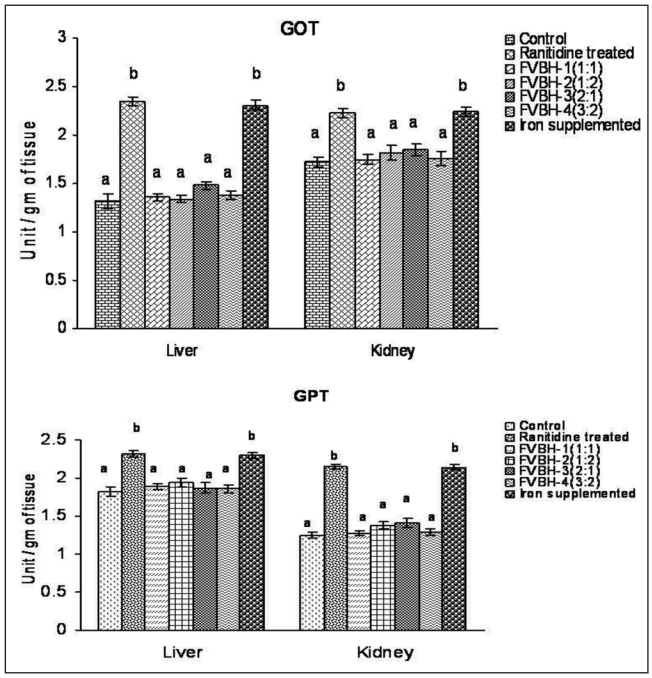

Figure 5: Protective effect of preadministration followed by coadministration of FVBH-1 or FVBH-2 or FVBH-3 or FVBH-4 on GOT and GPT activities in liver and kidney in ranitidine-induced hypochlorhydric rat. Data are expressed as mean ± SEM, $n = 6$. Bars with superscripts (a, b) significantly differ from each other ($P < 0.05$). ANOVA followed by multiple comparison two-tailed "*t*'-test

pepsin concentration, and vitamin C level in the gastric juice, supported by our previous works.[1,17,21] 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 nonacidic component, which is observed as a transmucosal movement of chloride in excess of hydrogen.[39,40] The pretreatment followed by cotreatment of FVBH-1(1:1) increased the chloride level significantly as compared with ranitidine treated group and other groups. This may be due to stimulation of the parietal cells to secrete chloride by the active ingredients of the composite extract supported by our earlier works.[1,21] Pepsin activity and secretion level of both were increased due to increased HCl secretion by the stimulation of composite extract as it acts as secretagogue reported earlier.[13] We also observed that FVBH-1(1:1) significantly increased hemoglobin level, hematocrit value, serum iron, and liver iron as compared with other groups, where hemoglobin level was reset to the control and also recovered hamatocrit value, which comparable with iron supplemented group. This could be done due to preventing the hypochlorhydria and providing the iron absorption factor like gastric juice with low pH and vitamin C. Iron level in serum and liver tissue were also reached toward the control level in FVBH-1(1:1) pretreated cum cotreated group. No significant alteration was noted in GOT and GPT activities in liver and kidney in extract treated rats as compared with the control. So the composite extracts with different ratios have no metabolic toxicity.

We observed here that the iron supplementation has no effect on gastric secretion, which has been reflected here from the values of sensors of hypochlorhydria such as pH of gastric juice, free acidity, chloride level, pepsin concentration, and vitamin C in gastric juice comparable with hypochlorhydric group. It only prevented the development of iron deficiency in ranitidine treated rat reported by other study.[41] Iron is a causative agent for lipid peroxidation.[42] A significant increase in lipid peroxidation in liver has been observed in rats[43] because of increased iron store in the liver due to aging. Excess iron causes stress in body since iron is catalyst for lipid peroxidation and for reactive radicals generation.[44] Iron supplementation amplifies oxidative stress, the inflammatory response, and mucosal damage in the rat[45] and it threats the body specially in the aged because it causes lipid peroxidation, reported by our earlier work,[17] and therefore iron supplementation was not the proper therapy for the iron deficiency due to hypochlorhydria without correcting hypochlorhydric state. In these circumstances, herbal treatment is appropriate therapy for managing the iron deficiency with hypochlorhydria.

CONCLUSION

From the present study, it may be concluded that FVBH-1(1:1) is the effective formulated composite extract not only for the management of hypochlorhydria but also for iron deficiency due to hypochlorhydria.

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