

Qualitative Assessment of a Customized Anti-hepatic Herbal Formulation in Bangladesh

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ABSTRACT: Qualitative assessment of herbal formulations is used in pre-health care of many diseases. The potency, composition and safety of herbal formulation are serious concerns in Bangladesh. Therefore, the aim of the present study was to evaluate the qualitative assessment of a customized anti-hepatic herbal formulation in Bangladesh. Isolation of compounds, toxic metals analysis, phytochemical screening, microbial test and determination of SGPT and SGOT level of hepatic model rats were focused in this study. The phytochemical screening revealed the presence of phenol, saponins and tannins. In chemical study, four known compounds were isolated from the formulation (Sodium benzoate, methyl-4-hydroxybenzoate, 4-hydroxybenzaldehyde and 4-hydroxy-3-methoxybenzaldehyde). The results of microbial purity and toxic metals analysis were in favorable limit although the levels of SGOT and SGPT were raised non-significantly. So, based on the study it has been concluded that quality assessment is urgently required for this type of customized herbal formulations.

Key words: Antihepatic herbal formulation, hepatic model rats, heavy metals, microbial impurity, chemical proficiency.

INTRODUCTION

Liver is one of the key organs, considered to have a vital role in metabolism and excretion of xenobiotics from the body.^{1,2} Currently, there is no any alternative way to reimburse for the absence of liver function. The flow of nutrients and control of carbohydrate, protein and fats metabolism are mainly influenced by this major organ³. The dysfunction or injury of liver which is due to the misuse of various drugs and different chemical exposition such as certain anti-biotic, chemotherapeutic agent, carbon tetrachloride, thioacetamide, excessive alcohol consumption and other agents is called hepatotoxicity.⁴ Presently, hepatic disorder becomes a major health problem and has been a great challenge for health professionals, pharmaceutical

and drug regulatory agencies.¹ Since the pre-historic time, the uses of herbal formulations in the treatment of liver injury have a long tradition.^{5,6} Many developing countries continued to get benefit from the rich knowledge of medical herbalism. About 70-80% of the world population relies on non-conventional medicines mainly of herbal origins for their pre-health care particularly in the developing countries, because herbal medicines are relatively accessible, cheaper and less side-effect than the synthetic drugs.⁷ Bangladesh has a rich treasure of plants and herbs. Many plants are being used for the management of several diseases by the traditional healers. Recently, many antihepatic herbal formulations are available in local market and usually prescribed by the herbal practitioners. However, the quality, safety and efficacy of these herbal preparations are also of great concern. The National Poison Information Service reported that at least 785 cases of possible or confirmed adverse reactions to

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herbal drugs, among which hepatotoxicity was the most frequent.⁸

A nationwide population-based study was conducted by multistage sampling in Bangladesh showed that prevalence of fatty liver in Bangladesh is more than 34%.⁹ A large number of populations rely on these types of herbal formulations. Therefore, our present study has been designed to investigate a customized anti-hepatic herbal formulation in Bangladesh to assess the biological safety, efficacy and chemical proficiency.

MATERIALS AND METHODS

Sample collection and extract preparation

Extracts preparation for chemical analysis. Ten bottles (4.5 l) of same brand antihepatic herbal formulation were collected from local market of Dhaka city, Bangladesh. The liquid drug (2.5 l) was sequentially partitioned with n-hexane (3×4.5 ml), dichloromethane (DCM) (3×4.5 ml), ethyl acetate (3×4.5 ml) and butanol (3×4.5 ml) by using separating funnel. The partition was performed in each solvent until exhaustion. Each separated part was concentrated after completion of the separation process under reduced pressure in a Rota vacuum evaporator (EYELA SB-1100) at 40°C, finally, dried by using freeze drier (iLshin Biobase) at -40°C, yielding 4, 13, 27 and 39 g for the n-Hexane, DCM, ethyl acetate and butanol parts, respectively.

Sample preparation for phytochemical and biological analysis. Two liter (2 l) of liquid drug was concentrated directly under reduced pressure in a Rota (Rotary) vacuum evaporator (EYELA SB-1100) at 40°C finally dried by using a freeze drier (iLshin Biobase) at -40°C, was therefore 110 g crude extract was yielded, respectively.

Chemical Analysis

Toxic metal analysis. Qualitative and quantitative analysis of the antihepatic herbal drug was done by using Atomic Absorption Spectrophotometer (AAS). Flame Atomic Absorption Spectrophotometer (AA-200 and for Chromium AA-800, Perkinelmer) specific hollow cathode lamp for

Copper (wavelength 324.8 nm), Chromium (wavelength 357.9 nm), Cadmium (wavelength 228.8 nm), Lead (wavelength 283.2 nm), Electro-thermal Atomic Absorption Spectrometer (AA-7000, Shimadzu) specific hollow cathode lamp for Arsenic (wavelength 193.7 nm) and for the Mercury vapor Unit Absorption Spectrometer (AA-7000, Shimadzu) specific hollow cathode lamp (wavelength 253.7 nm) were used to analyze the sample. The instrument has minimum detection limit of 0.03 mg/l (Cu), 0.10 mg/l (Cr), 0.01 mg/l (Cd), 0.20 mg/l (Pb) in the flame method, 0.4 µg/l (As) in the furnace method and 0.03 µg/l (Hg) in the mercury vapor unit. Samples were aspirated through nebulizer and absorbance was measured with a blank as reference.

Phytochemical screening. The dried crude extract of the drug was taken for the preparation of sample solution for preliminary phytochemical screening. About 3 g of dried crude extract were dissolved with 30 ml distilled water and kept in water bath at 85-90°C for 5 minutes. Then sample solution was cooled down at room temperature and filtered by using filter paper (Whatman No. 1). Then the filtrate was taken for testing the presence of phytochemicals following the standard protocol.^{10, 11}

Test for alkaloids. (i) Hager's test: 1 ml of sample was treated with 2 ml of Hager's reagent and formation of yellow precipitate indicated the presence of Alkaloid. (ii) Mayer's test: 1 ml of Mayer's reagent was added to 1 ml of sample solution and formation of whitish yellow or cream colored precipitate indicated the presence of alkaloids.

Test for flavonoids. (i) Mg ribbon test: 1 ml of the cool filtrate was added along with dilute HCl from sides of the test tubes and few fragments of magnesium ribbons were also added to the test tubes. Presence of slight pink color in the test tubes indicated the presence of flavonoids. (ii) Few drops of NaOH solution were added to 5 ml of sample. Formation of an intense yellow color, which turns to colorless on addition of few drops of dilute H₂SO₄ indicated the presence of flavonoids.

Terpenoids test. Salkowski Test: 2 ml of each extract was mixed in 2 ml of chloroform and concentrated H₂SO₄ acid (2 ml) was carefully added to form a layer. A reddish brown precipitate of the interface indicated the presence of terpenoids.

Steroids test. Lieberman-Burchard's Test: The extracts were dissolved in 2 ml of chloroform to which 10 drops of acetic acid and five drops of concentrated H₂SO₄ acid were added and mixed. The change of red color through blue to green indicated the presence of steroids.

Phenols test. 2 ml of cool filtrate extracts were treated with 1 ml of Potassium-ferro-cyanide and freshly prepared 1% ferric chloride solution, presence of bluish green color indicated the presence of phenols.

Saponins test. Froth test: 5 ml of cool filtrate was taken in a test tube added with 5 ml of distilled H₂O and shaken vigorously result into persistent foam indicated the presence of saponins.

Tannins test. (i) FeCl₃ test: Few drops of 10% FeCl₃ were added to 5 ml of filtrate and the presence of deep blue-black color indicated the presence of tannins. (ii) Lead acetate test: A little quantity of extracts was mixed with basic lead acetate solution. Formation of white precipitates indicated the presence of tannins.

Fractionation and compound isolation. The DCM part (13 g) was subjected to a normal phase column chromatography prepared by silica gel Si-60 (70-230 mesh) packed by eluting n-hexane: dichloromethane (1:1) as packing solvent. The polarity of the elution mixture was increased by sequentially adding dichloromethane to hexane, ethyl acetate to dichloromethane and methanol to ethyl acetate. Several fractions eluted from this column and were collected (50 ml each) and monitored the fractions using Thin Layer Chromatography (TLC) and UV light.^{12,13} One fraction eluted from 10% n-hexane in dichloromethane showed single spot-on TLC plate and consequently was crystallized out as a pure single compound-1.

The ethyl acetate part (27 g) was subjected like previous fractionation technique to a normal phase

column by eluting with using n-hexane: DCM (3:7). The polarity of the elution mixture was increased with DCM, ethyl acetate and methanol sequentially. Several fractions eluted from this column were collected (100 ml each) and monitored the fractions using TLC and UV light. The fraction eluted from the column with 5% ethyl acetate in dichloromethane and 20% ethyl acetate in dichloromethane gave single spot on t.l.c plate and purified as compound-2 and compound-3. Some fractions gave recognizable spots, and similar fractions were gathered and combined according to their number and color of the spot on TLC plate. Depending on recognizable spot of a fraction of DCM: Ethyl acetate (3:7) was subjected to a reversed phase column chromatography prepared by RP-18 silica gel packed with a mixture of acetonitrile and deionized H₂O (1:4). The polarity of the elution mixture was decreased gradually by adding sequentially acetonitrile (ACN). Eluted fractions were collected, concentrated and monitored by TLC and HPLC. The fraction eluted with ACN: H₂O (4:1) was give single spot and isolated in pure form as compound-4.

Spectroscopic characterization. In this study, proton nuclear magnetic resonance (¹H-NMR), carbon nuclear magnetic resonance (¹³C-NMR) and distortionless enhancement by polarization transfer (Dept-135) were used to the structure elucidation of the isolated compounds. The NMR spectra were recorded using CDCl₃ (Compound-1) and CD₃OD (Compound-2, Compound-3 and Compound-4) as the solvent on a Topspin (400 MHz for ¹H and 100 MHz for ¹³C, Bruker, Germany).

Biological analysis

Microbial test. Blood agar, MacConkey agar, Chocolate agar and Saboraud's dextrose agar were used for culturing and observing the growth of bacteria and fungus.¹⁴ Identification of organisms was done as standard ways.^{15,16} All dehydrated media were prepared according to manufacturer's instructions. The sterile media were poured into sterilized petri dishes and allowed to cool. The sterility of the prepared media was checked by incubation of blindly selected plates at 37°C for 24

hours. The method as mentioned by Brown, Poxton and Wilkinson was used; for this liquid drug 1:10 dilution with sterilized distilled water was made. The 100 μ l diluted solutions were spread widely over the surface of medium with sterile inoculation wire. The count was calculated from average colony count/plate.¹⁷

Preparation of hepatic model rats. Adult Long-Evans rats of either sex, bred at Bangladesh University of Health Sciences' Animal House, weighting (220-250) g were used in the study. Some preliminary experiments were done to make hepatotoxic model rats by administering paracetamol at a dose of 3 g/kg body weight^{18,19,20}. Paracetamol was introduced orally at the fasting condition and 7 days later serum SGPT and SGOT levels were determined. The experiments were repeated and finally the hepatotoxic rat model had been developed with a SGPT value of 40 U/l and SGOT value of 232 U/l. All animals had free access of fed and water *ad libitum*.

Formulation of Silymarine and drugs doses. In this study, the standard drug silymarine was used as a positive control. Silymarine was prepared at a dose of 100 mg/kg body weight and the herbal drug was prepared at a dose of 1.25 g/kg body weight of hepatic model rats and orally administrated to the rats for 2 weeks.

Experimental design. Total of 12 rats were used in this experiment by randomly divided into three groups (n=4) normal control group (10 ml/kg), silymarine treated group (100 mg/kg) and drugs treated group (1.25 g/kg). The experimental materials were administered by gastric intubation for 14 consecutive days at doses of 1.25 g/kg body weight. On the 15th day the rats were sacrificed by cervical dislocation and blood was collected for measuring biochemical parameters.

Blood collection. Blood samples were collected from rats kept under fasting conditions (12 hrs) by amputation of the tail tip under diethyl ether anesthesia at 0 day. After cutting the tail tip, about 0.2 ml blood was taken cautiously in micro centrifuge tube to avoid hemolysis. On the 15th day, after the

animals were decapitated, their blood was collected from heart by cardiac puncture. After separating of serum and plasma were stored at 20°C until the estimation of Serum Glutamate Pyruvate Transaminase (SGPT) and Serum Glutamate Oxaloacetate Transaminase (SGOT).

Biochemical parameters. In this study, biochemical parameters as Serum Glutamate Pyruvate Transaminase (SGPT) and Serum Glutamate Oxaloacetate Transaminase (SGOT) were measured by enzymatic method.²¹

Statistical analysis: All analyses were done by using the Statistical Package for Social Sciences (SPSS) 16.0 version for Windows 10. Data are expressed as mean \pm standard deviation (M \pm SD). Statistical comparison between groups was performed using one way ANOVA and paired sample t test.

RESULTS AND DISCUSSION

Chemical analysis

Toxic metal analysis. As the part of toxic metal analysis result the correlation coefficient was found for Cu 0.997, for Cr 0.994, for Cd 0.997, for Pb 0.999, for As 0.999 and for Hg 0.996. The values given below are the results (Average of three reading for sample) of the herbal sample for Cu, Cr, Cd, Pb, As and Hg. However, the results showed that the levels of Copper, Chromium, Cadmium, Lead, Arsenic and Mercury were within acceptable limit. (Table 1)

Phytochemical screening. In the present study a preliminary phytochemical analysis was carried out on an antihepatic herbal drug and to identify the phytochemical constituents (Table 2). The phytochemical screening revealed the presence of flavonoids, phenols, saponins were observed and alkaloids, tannins, steroids and terpenoids were not observed in the extract respectively.

Chemical compounds isolation and structure elucidation. About four pure compounds were isolated and characterized from DCM and ethyl acetate extracts of this herbal formulation. Compound-1 (Sodium benzoate) was isolated from

the fraction of the dichloromethane extract and Compound-2 (methyl-4-hydroxybenzoate), Compound-3 (4-hydroxybenzaldehyde), Compound-4 (4-hydroxy-3-methoxybenzaldehyde) were isolated

from the fraction of the ethyl acetate extract of the antihepatic herbal drug. The structures of those compounds were elucidated by extensive NMR (^1H , ^{13}C and Dept-135) techniques (Table 3-6).

Table 1. Effect of toxic metals in antihepatic herbal drug.

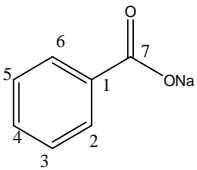
ID	Dilution Factor	Cu-Content (mg/l)	Cr-Content (mg/l)	Cd-Content (mg/l)	Pb-Content (mg/l)	As-Content ($\mu\text{g/l}$)	Hg-Content ($\mu\text{g/l}$)
Blank	1	0.04	BDL	BDL	BDL	BDL	0.45
Herbal Drug	1	0.08	BDL	BDL	BDL	0.57	0.56

Table 2. Preliminary Phytochemical screening of different extracts of herbal drug.

Phytochemicals	Antihepatic herbal drug	Test
Alkaloids	-	Hager's test
Flavonoids	+	i) Mg ribbon test ii) Alkaline reagent test
Phenols	+	Potassium ferrocyanide solution
Tannins	-	i) Lead acetate test ii) Ferric-chloride test
Steroids	-	Salkowaski test
Saponins	+	Froth test
Terpenoids	-	Salkowaski test

Present (+); Absent (-)

Table 3. NMR spectral data of compound 1.

Position	δ_{C} (DEPT) in CDCl_3	δ_{H} (J in Hz) in CD_3OD	Structure
1	129.352 (C)		
2 & 6	128.51 (CH)	7.54 (2H, tt, 8.0 & 1.2)	
3 & 5	130.24 (CH)	8.07 (2H, td, 8.0 & 2.0)	
4	133.82 (CH)	7.563 (1H, tt, 6.8, 1.2)	
7	172.24 (C=O)		

Compound 1: Sodium benzoate

Biological analysis

Microbial test. The result of microbial test is given in Table 7.

Body weight. The body weight in different weeks among all the groups of the rats have not shown any significant changes on one way ANOVA and Pair t-test respectively (Figure 1).

Serum Glutamate Pyruvate Transaminase (SGPT). The SGPT level of drug treated group is shown in Figure 2. The positive control group (Sylimarine treated group) showed non-significant reduction of SGPT level ($M \pm SD$, 0 vs 14, day: 46 ± 17 vs 43 ± 29 , U/l) on 14th day respectively. The SGPT

level of drug treated group unexpectedly increased by 31% ($M \pm SD$, 47 ± 10 vs 62 ± 7 , U/l) on 14th day compared with 0 day, however, the SGPT rise was significantly lower compared to control group.

Serum Glutamate Oxaloacetate Transaminase (SGOT). The SGOT level of drug treated group raised up by 35% compared with baseline value ($M \pm SD$, 167 ± 13 vs 228 ± 33 , U/l) on 14th day. In positive control group SGOT level ($M \pm SD$, 253 ± 10 vs 243 ± 53 , U/l) reduced non-significantly compare to the baseline value at the end of the experiment.

The use of herbal medicine, an age-old tradition is being revived by practicing day to day for its

affordability, easy availability, less side-effects and natural way of healing.²² However, the lack of standardizations in the herbal-based medicine preparation process is one of the controversial causes about the clinical efficacy of these types of modern formulation.²³ It has been proved that certain herbal formulation contain chemical components that can

protect liver from several sorts of injury. Scientific investigation demonstrates that the presence of alkaloids and flavonoids in herbal formulations have the hepatoprotective effect.²⁴⁻²⁶ The preliminary phytochemical screening of our investigated herbal drug showed the presence of flavonoids, phenols and

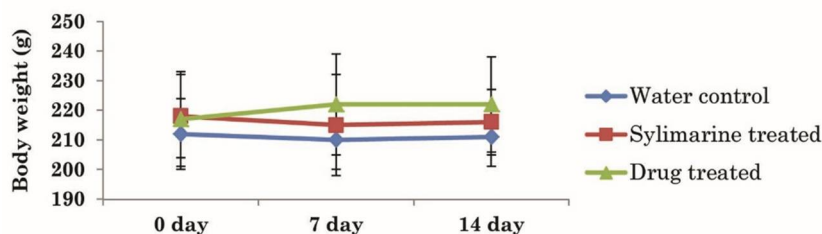


Figure 1. Effect of herbal drugs on the body weight of hepatic model rats

Data presented as mean±standard deviation (M±SD). Statistical comparison between groups was performed using one way ANOVA and paired sample t-test.

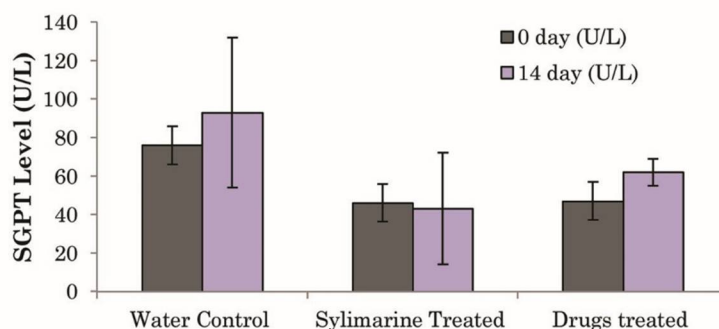


Figure 2. Effect of herbal drugs on the SGPT of hepatic model rats

Data presented as mean±standard deviation (M±SD). Statistical comparison between groups was performed using one way ANOVA and paired sample t test. $p=0.049$; 0 day vs 14th day

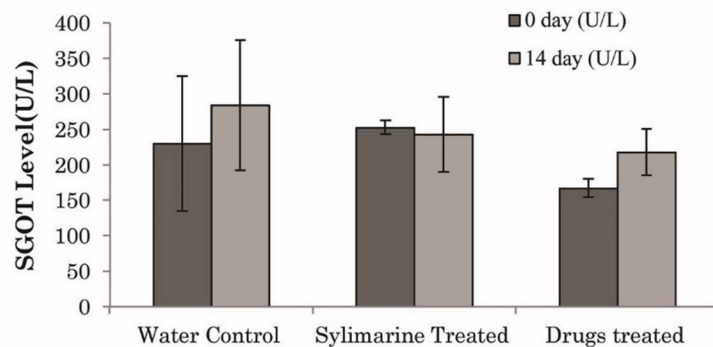
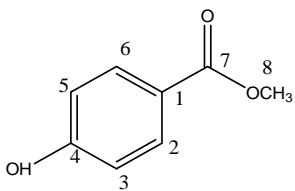


Figure 3. Effect of herbal drugs on the SGOT of hepatic model rats.

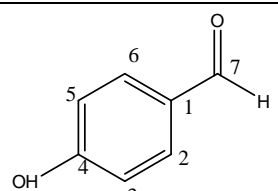
Data presented as mean±standard deviation (M±SD). Statistical comparison between groups was performed using one way ANOVA and paired sample t test. $p=0.049$; 0day vs 14th day.

Table 4. ¹H-NMR spectral data of compound 2.

Position	δ_C (DEPT) in CDCl ₃	δ_H (J in Hz) in CD ₃ OD	Structure
1	122.04 (C)		
2, 6	115.38 (CH)	6.803 (2H, d, 8.8)	
4	160.61 (C-OH)	4.859 (1H, s)	
3, 5	131.95 (CH)	7.848 (2H, d, 8.8)	
7	170.04 (C=O)		
8	52.09 (CH ₃)	3.824 (3H, s)	

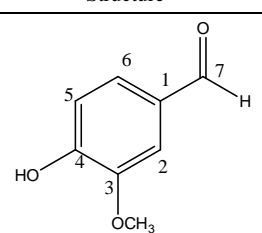
Compound 2: Methyl-4-hydroxybenzoate

Table 5. ¹H-NMR spectral data of compound 3 (δ in ppm and J in Hz).

Position	δ_C (DEPT) in CDCl ₃	δ_H (J in Hz) in CD ₃ OD	Structure
1	133.65 (C)		
2, 6	128.49 (CH) 130.15 (CH)	7.366 (2H, d, 3.6)	
3, 5	111.87 (CH)	6.567 (2H, d, 3.6)	
4	160.50 (C-OH)	4.598 (1H, s)	
7	177.67 (-CHO)	9.532 (1H, s)	

Compound 3: 4-hydroxybenzaldehyde

Table 6. ¹H-NMR spectral data of compound 4 (δ in ppm and J in Hz).

Position	δ_C (DEPT) in CDCl ₃	δ_H (J in Hz) in CD ₃ OD	Structure
1	129.94 (C)		
2	108.78 (CH)	7.40 (1H, s)	
4	147.158 (C-OH)	4.867 (1H, s)	
3	151.67 (C-OCH ₃)		
5	127.54 (CH)	6.90 (1H, d, 8.4)	
6	114.38 (CH)	7.37 (1H, m)	
7	190.87 (HC=O)	9.712 (1H, s)	
8	56.15 (-OCH ₃)	3.905 (3H, s)	

Compound 4: Vaniline (4-hydroxy-3-methoxybenzaldehyde)

Table 7. Effect of herbal drug on microbial culture (Aerobic).

Sample ID	Test	Incubation temperature (°C)	Incubation duration	Results
H1	Bacteria	37 °C	36-48 hours	No growth
H1	Fungi	37 °C	3 weeks	No growth

saponins. No significant improvement was found on hepatic model rats in the present investigation. Additionally, no growth of bacteria and fungi were found in microbial test. In the toxic metal investigations the levels of Copper, Chromium, Cadmium, Lead, Arsenic and Mercury were under

the maximum residual level (MRL). The body weight of different groups of rats was found to be almost unchangeable in different weeks. As expected, the SGPT and SGOT level of water-control group was increasing and silymarin treated group was decreasing. Unexpectedly, the SGPT and SGOT

value of drug treated group was raised up non-significantly by 31% and 35% on 14th day compared to baseline values. Different scientific studies suggested that, due to largely missing of valid scientific data, pesticides, food preservative and flavor agents contaminating herbal preparations may act against the potential hepatoprotective effects of silymarin flavonoids/flavonolignans²⁷. So, the presence of significant level of food preservative, pesticides and flavor agents seems to be another problem which certainly may affect the health of consumers. In the present study, four chemical compounds were isolated and characterized by comparing NMR-data with known compounds as (1) sodium benzoate, (2) methyl-4-hydroxybenzoate, (3) 4-hydroxybenzaldehyde and (4) 4-hydroxy-3-methoxybenzaldehyde from this herbal formulation. Among them sodium benzoate and methyl-4-hydroxybenzoate, i.e. methyl-paraben/nipazin are very popular as pesticides transformation product and commonly used as food preservative in foods and pharmaceuticals industry. 4-hydroxybenzaldehyde is produced by removing of methoxy group in position-3 of 4-hydroxy-3-methoxybenzaldehyde which is vanillin both are the derivatives of benzaldehyde. It was mentioned that benzaldehyde and its derivatives are also commonly used as flavoring agents or food adjuvant in pharmaceuticals, foods and beverages.²⁸ As well as, there are numerous factors like the variety of the plants used, climatic conditions during plant growth, seasonal and geographic conditions and extraction methods are also affecting the composition of herbal preparations. The maintenance of good manufacturing practices including safety, efficacy and quality are other important factors during the production of herbal preparation which often being violated.^{22,29}

CONCLUSION

On the basis of the obtained results it may be concluded that such types of herbal medicine before marketing should undergo rigorous testing regarding safety, efficacy and chemical composition. The

regulatory procedures should also be improved in this regards.

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CONFLICT OF INTEREST

We declare that we have no conflict of interest.

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