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Characterization and Estimation of Harpagoside in Dried Root Extract and Oral Powder Formulations of *Harpagophytum Procumbens* by Validated RP-HPLC-PDA Method

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ABSTRACT

The present investigation was undertaken to perform structural, physicochemical characterization and to estimate the harpagoside in dried root extract and flavoured powder formulations of *harpagophytum procumbens* (devils claw). The extraction of harpagoside from the root extract was carried out using different solvents and the harpagoside content was estimated using an RP-HPLC-PDA method. The HPLC analysis was carried out using methanol: 0.02% formic acid (60:40 v/v) as the mobile phase, pumped at flow rate of 1mL/min through C_{18} column (Kinetex XB, 150x4.6mm, 5µ). The structural and physicochemical characterization of the root extract was performed using FT-IR, DSC and SEM analysis. Flavoured powder formulations (effervescent and non-effervescent) of the root extract were prepared and the harpagoside content was estimated by HPLC method. Higher percentage of harpagoside (1.6 %) was extracted with water as extraction solvent when compared to methanol and methanol: water (50:50 v/v). The developed RP-HPLC-PDA method resulted in shorter elution time (harpagoside was eluted at 5.3min) and the method was linear with good regression coefficient (R^2>0.998) within the concentration range tested (0.2-4µg/mL). The FT-IR spectra of root extract showed the presence of characteristic peaks corresponding to the harpagoside and the results from photomicrographs and SEM analysis revealed spherical morphology of the particles in the extract. The prepared powdered formulations readily dispersed and dissolved in water and showed more than 98% of harpagoside content even after 6months time period indicating the stability of the formulations. Overall, the results from the present investigation can form basis to establish standardization parameters for formulations containing root extract of devil's claw.

Keywords: *Harpagophytum procumbens,* Structural and physicochemical characterization, RP-HPLC-PDA method, FT-IR, DSC, SEM.

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INTRODUCTION

The utilization of herbal formulations as a part of health care management was still in practice even with the advancement of modern medicine [1]. Moreover, the use of the herbal formulations had increase rapidly in the recent years and more than 80 percent of people around the world are found to be depend on herbal formulations for their health care [2]. The major reasons that can be attributed for the widespread use of these herbal formulations are the ease of availability through online markets and due to the misconceptions regarding their safety aspects [1].

This increase in utilization of herbal products by people had presented new challenges to the regulatory authorities i.e. the establishment of safety and efficacy parameters for the herbal formulations [3]. The general prevalence of concept that these formulations are safe and devoid of any side effects is untrue [1]. Also, most of the herbal formulations are introduced into the online market by different companies claiming that their products are standardized and safe, but the authenticity of such claims needs to be scrutinized [4]. Several studies reported that the most of the herbal formulations available through the online market contains heavy metals in concentrations more than their permissible levels along with the detectable levels of other synthetic drugs to enhance the products efficacy [5, 6]. These factors presence the need to standardize these herbal formulations in terms of their quality, quantity, safety and efficacy.

Standardization of the herbal formulations involves establishment of a set of parameters that defines the qualitative and quantitative characteristics which can be used to assess the products quality, safety and efficacy

parameters [7]. The process of standardization includes several steps of evaluation like the organoleptic, pharmacognostic, phytochemical evaluations etc. and among them the phytochemical evaluation is regarded as the major step as it defines the activity of the formulation [8].

The major hurdle associated with the standardization is the complexity of the herbal formulations. The herbal formulations usually consists of materials of plant origin either in crude form or extracted or processed forms mixed with various excipients [7]. Often this material of the plant origin is prone to lot of variations like the source of the raw material, its storage conditions, drying rates etc. that had direct influence on the activity of the formulation [9]. Moreover, many of the herbal formulations exist as a complex mixture making the separation and analysis of individual components a challenging task. However, with the development of modern chromatographic techniques like TLC, HPTLC, HPLC, GC, etc. and spectroscopic methods like FT-IR, NMR, Mass spectroscopy etc. it had become possible to separate, analyse and establish the composition of the herbal formulations and to detect the extent of adulterations in the formulations [7].

One of the example of such herbal formulations with multiple constituents is the formulations containing dried root extract of *Harpagophytum procumbenes* commonly known as devil's claw [10]. The root extract is known to possess anti-inflammatory and analgesic properties mainly due to the iridoid glycosides that are present in the secondary roots [11, 12]. Along with the harpagoside, which is the main iridoid glycoside (Figure 1) it also contains harpagide and procumbide the other two iridoid glycosides, some flavonoids, sugars, phenolic acids etc [13]. Although the extract contains different types of constituents, the therapeutic activity is believed to be due to the iridoid glycosides and the harpagoside is considered as the major compound [14].

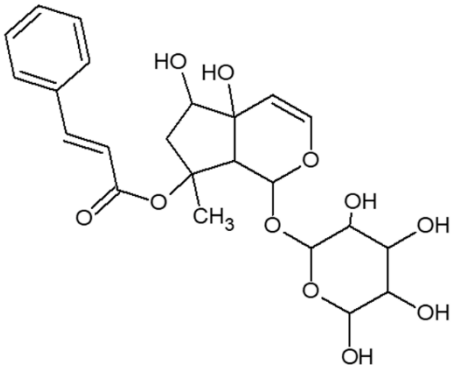

Figure 1: Structure of Harpagoside

Studies on the clinical efficacy of root extract [15, 16] also demonstrated that anti-inflammatory activity of the extract mainly depends on the harpagoside content. The European pharmacopeia also specifies that the dried root extract should contain a minimum 1.5 percent of harpagoside [17]. The root extract is widely available through online markets in form of tablets, capsules, gels and tinctures. However, there is no information regarding the harpagoside content in these formulations along with the data establishing its safety and efficacy. Therefore, with increase in the utilization of root extract as complimentary treatment for painful arthritis, low back pain etc there is a need to standardize the root extract in terms of harpagoside content and physicochemical properties.

Hence, the present investigation was aimed to characterize the commercially available root extract of devils claw for its structural, physicochemical properties and to develop an accurate and precise RP-HPLC-PDA method for the estimation of harpagoside in the root extract. From the literature survey, it was found that only a few methods were reported on the estimation of harpagoside in the root extract [18, 19]. However, these methods may not be applicable for the routine quality control analysis involving large number of samples because of their higher retention times thereby requiring more amounts of organic solvents which is not economical. Hence, there is a need to develop simple and economical RP-HPLC-PDA method with shorter retention time that is suitable for routine quality control analysis. Also, no reports were published till date on evaluating the morphological, structural, physicochemical properties of harpagoside, which are considered as the key parameters for the formulation development.

Therefore, in the present investigation an attempt was made to develop a simple and economical RP-HPLC-PDA method for the estimation of harpagoside in the commercial root extract and in oral powder formulations of devil's claw and to characterize harpagoside for its morphological, structural and physicochemical properties using SEM, FT-IR, and DSC analysis.

MATERIALS AND METHODS

Materials

Harpagoside analytical standard was purchase from Sigma Aldrich (Lot Number: BCBT7346). Commercial root extract of devils claw was purchased for Vigorous tech, China. Formic acid, Methanol and water are of HPLC grade procured from Merck, Mumbai, India.

Instrumentation and Chromatographic Conditions

The analysis was performed on a Shimadzu Prominence HPLC system consisting of DGU-20A3 degasser, SIL-20ACHT auto sampler and a LC-20AD binary pump system interfaced with a PDA detector. Phenomenex Kinetex XB column (150x4.6mm, 5µ) was used as stationary phase with methanol: 0.02% formic acid (60:40 v/v) as mobile phase composition. The mobile phase was sonicated prior to the analysis to remove any entrapped air bubbles and pumped at a flow rate of 1mL/min in isocratic mode. The quantification was performed with an injection volume of 20µL by monitoring the eluents at 280 nm using PDA detector. LC solution software was used for data acquisition and processing.

Extraction and Estimation of Harpagoside in The Dried Root Extract

The extraction of the harpagoside from the root extract was carried out as per the method described in European pharmacopeia [17] using water, methanol and a combination of water and methanol (50:50 v/v) as extracting solvents. Accurately weighed 350mg of extract was transferred to 100mL volumetric flasks each and 50mL of respective solvents were added to them. After vortexing for 5 min the contents were sonicated for about 10 min using ultrasonic bath sonicator at room temperature and the final volume was made up to the mark with the respective solvents. The samples were then centrifuged at 15,000 rpm for 3min and the supernatant was separated and analysed by the RP-HPLC-PDA method for the harpagoside content.

Preparation of Stock and Calibration Standards of Harpagoside

Stock solution of harpagoside was prepared by dissolving accurately weighed 5mg of harpagoside analytical standard in 5mL of methanol to get a stock solution of 1mg/mL. From the stock solution suitable dilutions were made to prepare calibration standards in the concentration range of 0.2 to 4µg/mL.

Method Validation

The chromatographic conditions employed in the present investigation were validated for parameters like specificity, linearity, precision, system suitability etc as per the ICH Q2 (R1) guidelines [20] as follows:

Linearity

Linearity of the method was established by injecting the prepared calibration standards (n=3) over a concentration range of 0.2 to 4.0µg/mL and the obtained peak areas were used for construction of calibration plot. Form the resultant data, parameters such as slopes, intercepts and regression equations were calculated.

Precision

System precision was evaluated by injecting the harpagoside standard (1µg/mL) taken from the same aliquot for six times and percent relative standard deviation (% RSD) for peak areas and retention times were computed. The percent RSD for peak areas and retention times ought to be less than 2.

Method precision was performed by injecting six replicates of sample solution and the percent RSD for peak area and retention times were calculated.

Accuracy

In the present investigation standard addition method was employed for the determination of accuracy. The method involves the addition of the known concentration of the standard at three levels (80, 100 and 120%) to the known concentration of sample solution. From the data obtained the percent RSD and percent recovery was calculated for each level of addition.

Specificity

Specificity of an analytical method determines its ability to measure accurately and specifically the analyte of interest in presence of other components that are expected to be in the sample matrix. It is determined by comparing the chromatograms of blank, the harpagoside standard and sample and observed for any interference at the retention time of the harpagoside.

Limit of Detection (LOD) and Limit of Quantification (LOQ)

The LOD and LOQ were calculated from the data obtained from the standard calibration curve using the following equation:

$$LOD = 3.3x\sigma/m;\ LOQ = 10x\sigma/m$$

Where 'σ' is the standard deviation of the responses and 'm' is the slope of the calibration curves.

Robustness

Robustness of the analytical method was evaluated by making small deliberate changes (± 2%) to the optimized chromatographic conditions like flow rate, mobile phase composition and wavelength. The harpagoside standard solution (1µg/mL) was injected for each minor change and the system suitability parameters were noted at each change. The parameters should be within the specified limits for the method to be robust.

System Suitability

System suitability tests were performed by injecting the increase volumes (10-50µL) of same concentration of harpagoside standard (1µg/mL) and observed for the changes in the parameters such as retention times, tailing factor and theoretical plate numbers with increase in injection volume.

Stability of the Stock Solution

Stock solution stability in methanol was evaluated by injecting the prepared stock solution at different time intervals over a period of 24hr and observed for the changes in the peak areas and retention times with respect to the 0hr sample. Throughout the study the stock solution was stored at 2-8°C.

FT-IR Analysis

FT-IR analysis was carried out on FT-IR spectrometer (Bruker, Germany) attenuated with ATR fitment. The extract was placed on the ATR crystal (zinc selenium crystal) and the spectra were recorded over a wavenumber range of 4000-500 cm^{-1} at a resolution of 1.0 cm^{-1}.

DSC Analysis

DSC analysis was carried out using Differential scanning calorimeter (Shimadzu DSC-60, Japan). 4mg of the extract was weighed and placed on to the aluminium pan and heated from 30-300°C at a rate of 10°C per minute. An empty aluminium pan was used as reference. Nitrogen gas at flow of 100mL/min was used to maintain the inert atmosphere.

Morphological characterization

Morphological Properties

The morphological properties like colour, appearance of the extract were observed visually. The particle morphology of root extract was assessed by observing the extract under trinocular microscope (Olympus-CH20i) with different magnifications like 10X and 40X and the photomicrographs were taken.

SEM Analysis

The surface morphology of the dried root extract powder was further established using scanning electron microscope (SEM-JEOL, JSM-840A, Japan). The samples was placed on the SEM slab and analysed with a voltage of 25 KV. The images were taken with 100, 250 and 500 fold magnifications.

Preparation of Flavoured Powders of Devils Claw Root Extract

Flavoured powder (both effervescent and non-effervescent) formulations of root extract were prepared as per the formulae given in Table 1 to batch size of 5g per sachet. Extract equivalent to 30mg of harpagoside was accurately weighed and mixed thoroughly with the excipients using spatula in watch glass and stored in aluminium foil pouches until use. The amount of root extract taken for the preparation of powder formulations was selected on the basis of clinical studies reported in the literature [15, 16] stating that the daily administration of more than 50mg of harpagoside is required to achieve the desired therapeutic benefits of the extract. The prepared formulations were then analysed by the RP-HPLC-PDA method for the harpagoside content in the formulations.

Table 1: Composition of flavoured powders of devil's claw root extract

Ingredients (g)	Formulae (5g sachet)		
	F1*	F2	F3
Devils claw root extract	1.88	1.88	1.88
Citric acid	0.5	-	-
Tartaric acid	1.0	-	-
Sodium bicarbonate	1.5	-	-
Microcrystalline cellulose	-	0.75	0.75
Mannitol	-	2.25	-
Lactose	-	-	2.25
Magnesium stearate	-	0.05	-
PEG-4000	-	-	0.05
Colouring agent	0.05	0.05	0.05
Flavouring agent	0.05	0.05	0.05

* Effervescent powder formulation.

Stability Studies

Stability studies of the prepared powder formulations were carried out. The formulations were stored in aluminium foil pouches, sealed and stored 40°C, 75±5% RH for 6 months. During this period the formulations were observed for their morphological properties like texture, colour etc and also analysed for the harpagoside content by RP-HPLC-PDA method.

RESULTS AND DISCUSSION

Analytical Method Development

In the initial step of the method development, the chromatographic separations were carried out on Phenomenex Kinetex XB C_{18} column (150x4.6mm, 5µ) using methanol: 0.02% formic acid (50:50 v/v) as mobile phase composition at 1mL/min flow rate. However, under these conditions no peak was eluted even after 30min of run time. Hence, further trails were carried out by altering the mobile phase ratios and keeping the remaining parameters the same. With 70:30 v/v ratio of methanol: 0.02% formic acid, harpagoside was eluted at 3.1 min whereas, with 65:30 v/v and 60:40 v/v ratios, harpagoside was eluted at 4.2 and 5.3 min respectively. Among the three mobile phase ratios, the ratio of 60:40 v/v gave good peak shape, symmetry and theoretical plate number compared to the other two ratios. Hence, the ratio of 60:40 v/v methanol: 0.02% formic acid was selected as mobile phase composition.

Further trails were carried out in order to select the suitable diluent that can maximize the elution capacity of the analyte with minimum or no interferences. Trails were carried out by preparing samples using formic acid (0.02%), water and methanol as diluents. From the results obtained, it was found that sample prepared with water as diluent showed maximum elution strength with higher peak areas and theoretical plate numbers compared to formic acid and methanol. Hence, water was selected as diluent for further analysis.

Overall, Phenomenex Kinetex XB C_{18} column (150x4.6mm, 5µ) as stationary phase, methanol: 0.02% formic acid (60:40 v/v) as mobile phase composition and water as diluent were found to be best suited conditions for the analysis of harpagoside. The chromatogram of harpagoside analytical standard obtained with the optimized conditions was shown in Figure 2A along with the peak purity indices and UV spectra in Figure 2B and 2C respectively.

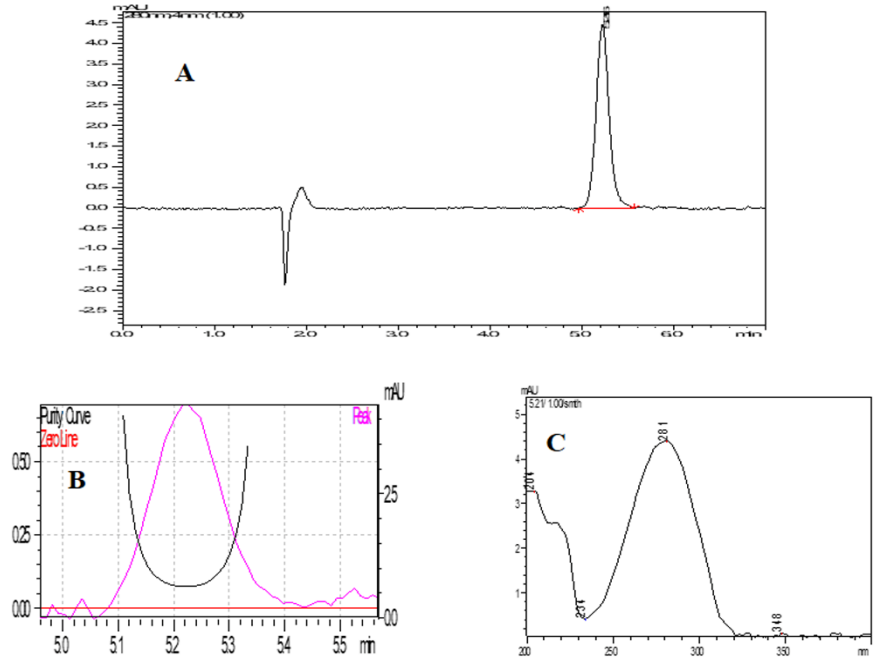

Figure 2: A) Chromatogram of Harpagoside standard (1µg/mL), B) Peak purity index, C) UV spectra of Harpagoside

Estimation of Harpagoside in the Dried Root Extract

The amount of harpagoside in different extraction samples was estimated using the developed RP-HPLC-PDA method. The extraction was carried out in different solvents like water, methanol and combination of water: methanol (50:50 v/v) in order to determine their extraction efficiencies. From the results obtained, it was found that highest percentage of harpagoside (1.6) was extracted with water as extraction solvent compared to water: methanol (50:50 v/v) and methanol. Also, the retention time of the peak obtained with the root extract was matched with the retention time of harpagoside analytical standard, demonstrating that the peak obtained with the extract can be ascertained to the harpagoside. Overall, from the results obtained it can be concluded that the commercial root extract of devil's claw used in the present study contains 1.6% of harpagoside and complies the European pharmacopeia specification [17] (i.e. the dried root extract should contain a minimum of 1.5% of harpagoside).

Method Validation

The developed RP-HPLC-PDA method was validated as per the ICH guidelines and the results of the validation were as follows:

Linearity

The calibration curve obtained by plotting concentrations against peak area was evaluated for the correlation coefficient. The response was linear across the selected concentration range of 0.2 to 4.0µg/mL with a correlation coefficient (R^2) greater than 0.998 (Table 2).

Precision

Both system and method precision was evaluated and the results were shown in Table 2. The results indicate acceptable sample stability and method consistency with less than 2% RSD's for peak areas and retention times.

Accuracy

The accuracy was determined by standard addition method and the percent recoveries of the added standard to the sample was calculated and was found to be 98.52-100.4 and the percent RSD's was less than 2 indicating the accuracy of the method to the claimed amount (Table 2).

Specificity

The specificity of the analytical method was determined by comparing the chromatograms of blank, harpagoside standard and sample. For the overlay of chromatograms it can be inferred that there was no interferences of impurities or sample matrix at the retention times of harpagoside indicating that the method was specific for the detection of harpagoside (Figure 3). Also, the peak purity index of the standards and samples was greater than 0.999 further conforming the specificity of the developed method.

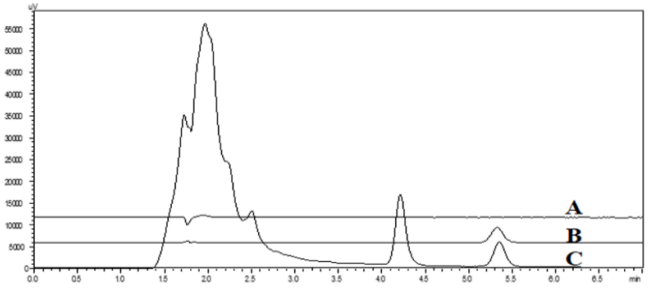

Figure 3: Overlay of Chromatograms of A) Blank, B) Harpagoside standard C) Sample with base shift

Limit of Detection (LOD) and Limit of Quantification (LOQ)

In the present investigation LOD and LOQ was calculated from the standard deviation of the responses and the slop of the regression equation from the calibration curve. The LOD and LOQ were found to be 0.017µg/mL and 0.052µg/mL respectively (Table 2).

Table 2: Validation data of harpagoside

	Parameters	Results
Linearity (n=3)	**Range**	0.2-4µg/Ml
	Regression equation	Y=41931x-1538.13
	Regression coefficient(R^2)	0.9981
	Level of Addition (percent)	**Mean Recovery (% RSD)**
Accuracy (n=3)	80	98.96 (0.15)
	100	98.52 (0.70)
	120	100.4 (0.36)
System Precision (n=6)	**Average Peak area of the standard (%RSD)**	44680.67 (0.93)
Method precision (n=6)	**Average Peak area of the sample (%RSD)**	55287.33 (1.08)
Limit of detection (LOD)	-	0.017µg/Ml
Limit of quantification (LOD)	-	0.052µg/Ml

Robustness

The robustness of the method was determined by making small deliberate changes (±2%) to the optimized chromatographic conditions like mobile phase ratio, flow rate and detector wavelength. Form the results, it was found that altering the mobile phase ratios and flow rate resulted in significant change in retention times whereas, no difference was observed with change in detector wavelength. However, no significant changes in remaining chromatographic parameters were observed indicating that the developed method was robust under these changed conditions.

System Suitability

System suitability tests were performed by injecting increase volumes of same concentration of harpagoside standard (1µg/mL) and the percent RSDs for tailing factor, retention times and theoretical plate number were calculated. From the results it was observed that the percent RSDs for all the parameters were well within the limits and the developed conditions were found to be suitable for analysis of harpagoside.

Stock Solution Stability

The stability of the harpagoside stock solutions was determined by analysing the samples under refrigeration (8±1°C) at different time intervals up to 24 hours. No significant difference was observed in the peak areas, retention times and other chromatographic parameters of the samples compared to the initial sample. Also, no variation in the harpagoside content was observed when compared to the initial sample indicating that the solutions were stable under the employed conditions for 24-hour time period.

FT-IR Analysis

The FT-IR spectra of devil's claw root extract was shown in Figure 4A. The spectra showed characteristic bands at 1145.37 cm^{-1} (-C-O stretching), 1621.00 cm^{-1} (-C=O stretching), 2934.21 cm^{-1} (-C=C stretching), 3253.00 cm^{-1} (-O-H stretching) wavenumbers. Along with these the fingerprint region of the IR spectra also showed prominent band at 969.24 cm^{-1} which can be attributed to the sugar moiety that is attached to the iridoid glycoside ring. Also, the spectra of the root extract was in accordance with the FT-IR spectra of the harpagoside analytical standard (Figure 4B) conforming the presence of harpagoside in the root extract. Together with the molecular structure of the harpagoside (Figure 1) and the results from the FT-IR analysis the characteristic bands observed can be assigned to the ether linkages (1145.37 cm^{-1}), ester linkages (1621.00 cm^{-1}) and phenolic OH groups (3253.00 cm^{-1}) that are present in the harpagoside. (Table 3).

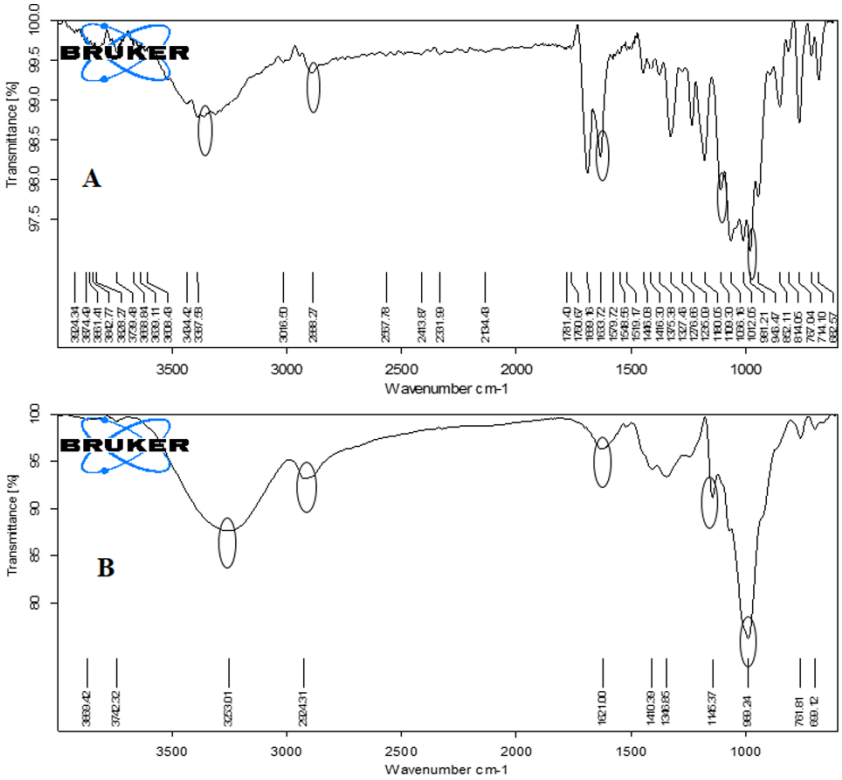

Figure 4: FT-IR spectra of A) Harpagoside analytical standard, B) Devils claw root extract

Table 3: FT-IR Spectral data of Devils claw root extract

Functional group	Vibration	Frequency Range (cm^{-1})	Frequency Obtained (cm^{-1})
Ether (C-O)	Stretching	1300-1000	1145.37
Ester (C=O)	Stretching	1735-1600	1621.00
Phenolic O-H	Stretching	3600-3200	3253.00
Sugar moiety	Stretching	1000-825	969.24

DSC Analysis

The DSC thermogram of the root extract was shown in Figure 5. The thermogram revealed no significant exothermic or endothermic peaks apart from small endothermic peak at 209.9°C. The absence of the significant peaks suggest that the root extract is more of amorphous in nature rather than the crystalline.

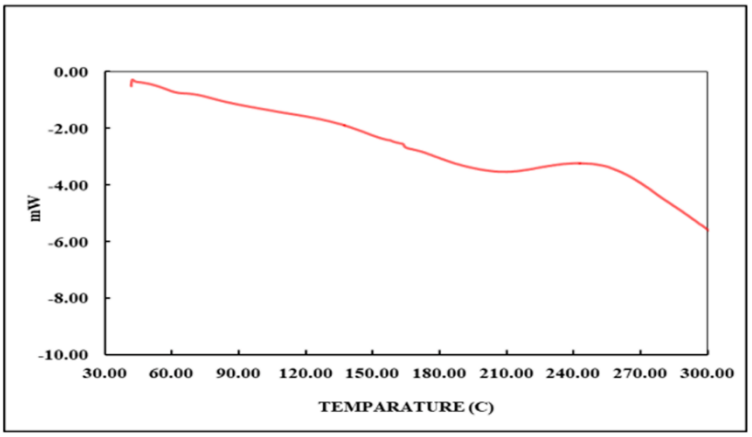

Figure 5: DSC thermogram of root extract

Morphological Characterization

SEM Analysis

The results from the SEM analysis (Figure 6) also revealed the spherical morphology of the particles in the root extract with smooth and wrinkled surfaces. Also, the extract was found to be composed of particles with various sizes ranging from 5µm to 55µm.

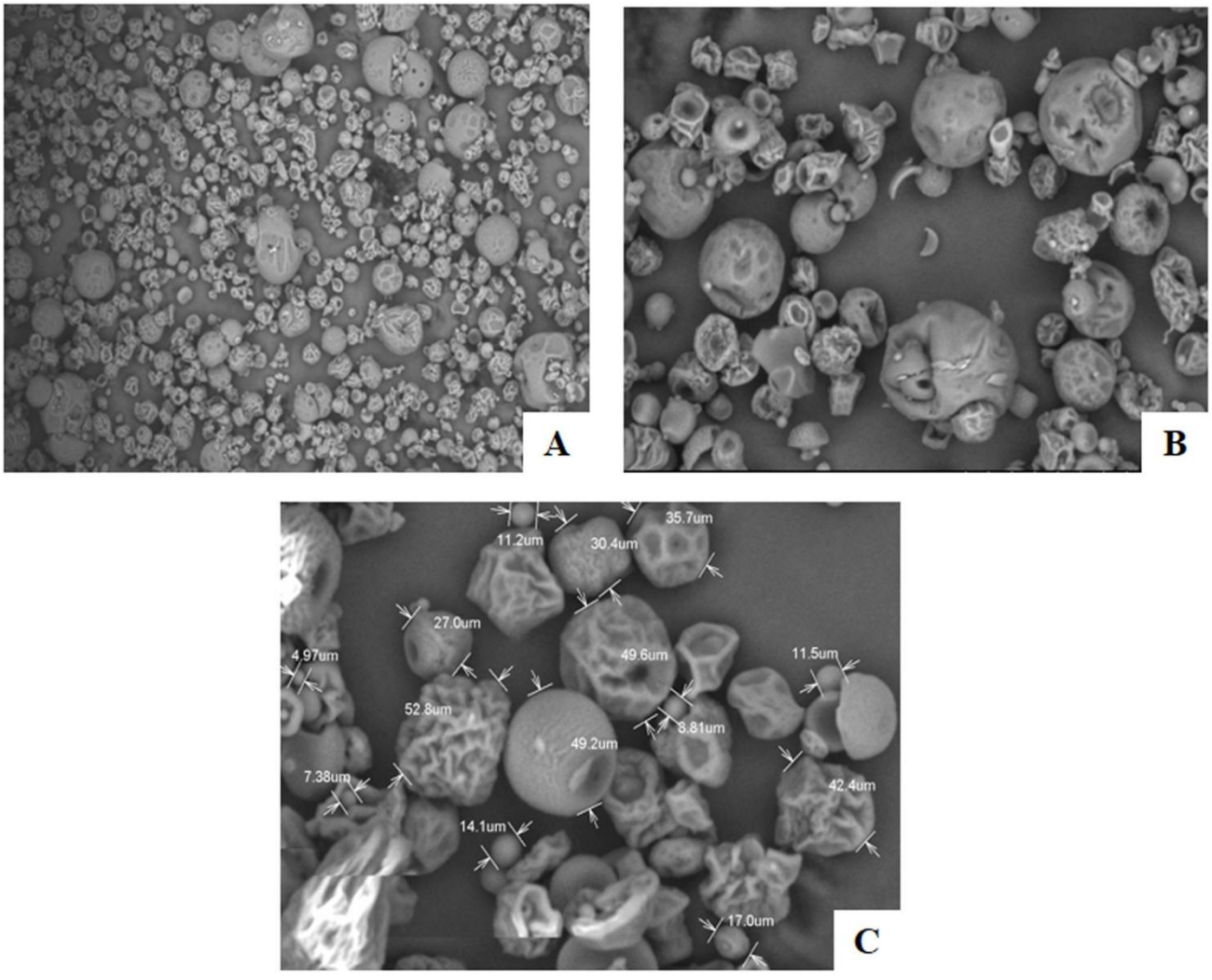

Figure 6: Scanning electron micrographs of root extract taken at 100 fold (A) 250 fold (B) and 500 fold (C) magnifications.

Preparation of Flavoured Powders of Devils Claw Root Extract

The flavoured powders (effervescent and non-effervescent) were prepared as per the formulae given in Table 1 to a batch size of 5g per sachet. The required ingredients were thoroughly mixed using spatula in dry and clean watch glass (Figure 7) until all the ingredients were uniformly mixed. The powder mixture was then packed in aluminium foil pouches and stored in desiccator until use. The prepared powders were intended to be administered thrice a day by dissolving them in full glass of water. The formulations dissolved quickly in water and devoid of any undissolved matter making them easier to administer. The harpagoside content in the powder formulations was analysed by the developed RP-HPLC-PDA method and was found to be 98.7, 98.3 and 99.5 percent respectively for F1, F2 and F3 formulations indicating that the developed analytical method is suitable for estimation of harpagoside in devil's claw formulations.

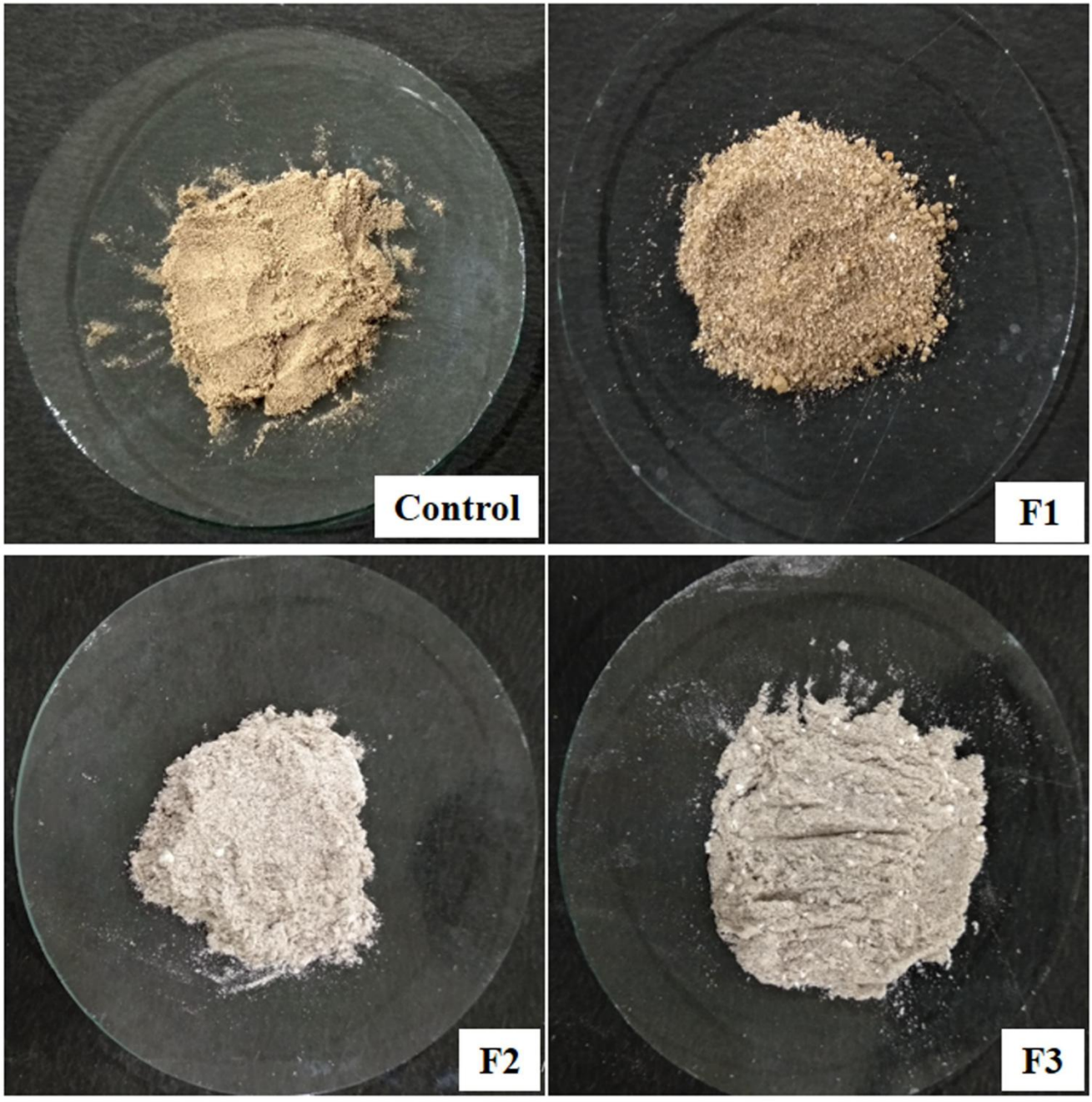

Figure 7: Different formulations of the root extract along with the control (extract alone)

Stability Studies

The prepared formulations were stored in aluminium foil pouches at 40°C with relative humidity of approximately 75±5% for 6 months and observed for the harpagoside content along with the morphological properties. No change in colour and texture of the powder was observed throughout the observation period and especially no formation of powder lumps was observed. All the formulations showed more than 98-101% of harpagoside content even after 6months time period indicating that the formulations were stable throughout the observation period.

CONCLUSION

In consideration of the increase in the commercial use of the devils claw root extract and need for its standardization the results from the present investigation can form basis to establish fundamental characteristics of the root extract that can be used for further establishment of the standardization parameters to ensure quality, safety and reproducibility of the devils claw formulations. The developed RP-HPLC-PDA method provides rapid and precise estimation of harpagoside in the root extract with simple mobile phase compositions and faster elution times. The results from the FT-IR, DSC and SEM analysis can be used for establishing the structural, morphological and physicochemical characteristics of the root extract which are the important factors in the development of devils claw formulations.

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CONFLICT OF INTERESTS

The authors declare that they have no conflict of interests.

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