INTRODUCTION

Xanthium strumarium is a cocklebur or burweed belonging to family Asteraceae and commonly found as a weed in road sides, rice fields, hedges throughout the tropical parts of India. It is commonly called chotagokhru due to the shape of its fruit which look likes the cow’s toe. The whole plant is used as medicine. According to Ayurveda, the plant has cooling, laxative, fattenine, anthelmintic, tonic, digestive, antipyretic activities and improves appetite, voice, complexion, and memory. It cures leukoderma, biliousness, and poisonous bites of insects, epilepsy, salivation, and fever. It is used by various Native American tribes to relieve constipation, diarrhea, and vomiting. Indigenous Chinese applications are as a headache remedy and to assist with cramping and numbness of the limbs, ulcers, and sinus problems. The plant is considered to be useful in treating long-standing cases of malaria and is used as an adulterant for Datura stramonium [1-5].

In continuation of the investigation of bioactive metabolites from X. strumarium, the present work deals with quantitative simultaneous estimation secondary metabolites stigmasterol and β-sitosterol from the petroleum ether extract of leaf, stem, and root part of the plant. Reverse phase high-performance liquid chromatography (RP-HPLC) is now still a most popular tool for herbal analysis. Hence, an attempt has been made to develop HPLC method for estimation of two phytoconstituents (stigmasterol and β-sitosterol) simultaneously from various parts (leaves, Stems, and roots) of X. strumarium. The developed analytical method was validated in accordance with international conference on harmonization (ICH) guidelines [6-10].

METHODS

Chemicals and reagents

Stigmasterol and β-sitosterol were obtained from Sigma-Aldrich, Germany. Acetonitrile, water, and ethanol (HPLC grade) were purchased from Merck Ltd., India. The other chemicals used were of analytical grade.

Authentication of plant material

Fresh and fully grown plants were collected near the roadways of a college campus in the month of October. The sample was authenticated by comparing the morphological characters as described in the literature. The authentication was further confirmed by Dr. H.B. Singh, Chief Scientist, and Head, Raw Materials Herbarium and Museum, NISCAIR, New Delhi. An herbarium sample of this plant is preserved in the Department, Chandigarh College of Pharmacy, Landran (Mohali) for future reference CCP/HB/PA/01.

Collection and preparation of plant materials

X. strumarium L. were collected in bulk quantity after confirmed authenticity from Landran, Mohali (Punjab) in the month of October. The plant parts such as leaves, stems, and roots were manually separated. The plant material was washed with water to remove soil, mud, debris, and other adhering materials and dried thoroughly in an air under shade at room temperature. Coarse powder of each drug was prepared, passed through sieve # 40 and stored in air tight container.
Extraction
Extraction of sterols from the powder of leaves, stems, and roots was carried out using soxhlet extraction method using petroleum ether (60°-80°) as a solvent. Then, the solvent was evaporated in a rotovapor at 60°C until semi-solid consistency. The extracts were dried to constant solid mass in a desiccator and were preserved in desiccator till further use.

Optimization of chromatographic conditions
The chromatographic conditions were optimized using different columns, flow rates, and mobile phase compositions given in Table 1.2. After optimization final solvent system selected was acetonitrile: ethanol:water (85:14:1 V/V/V) and flow rate were 1 ml/minute as it gives a good resolution of the components.

A solution of 1 mg/ml and Shimadzu LC-2010CHT equipped with a spectrophotometric ultraviolet-visible (UV-VIS) detector were used. Chromatographic separation was carried out using a column of quailsil gold C18, 250×4.6 mm, 5 µm particle size, under isocratic conditions with a mixture of acetonitrile:ethanol:water (85:14:1 V/V/V) as a mobile phase at the flow rate of 1 ml/minute and injection volume was 20 µl. Then, using the areas under the peak, the percentage purity was calculated.

Development and validation of HPLC method [6-10]
Preparation of the reference solution
Stock solution of 1 mg/ml in methanol of standard stigmasterol and β-sitosterol were prepared. Further, different dilutions were prepared from stock solution. The samples were sonicated for 30 minutes, filtered through a membrane filter of 0.22 µm.

Shimadzu LC-2010CHT equipped with a spectrophotometric UV-VIS detector was used. Chromatographic separation was carried out using a column of Qualisil Gold C18, 250×4.6 mm, 5 µm particle size, under isocratic conditions with a mixture of acetonitrile:ethanol:water (85:14:1 V/V/V) as a mobile phase at the flow rate of 1 ml/minute and injection volume was 20 µl. Detection was performed at 202 nm with 30 minutes runtime (Table 1).

Validation of developed method
Linearity and precision
Calibration curves for stigmasterol (10-500 µg/ml) and β-sitosterol (100-500 µg/ml) were obtained from standard solutions at different concentrations. The precision of the system and the precision of the method were determined as recommended by ICH guidelines. For determination of the precision of the system, Stigmastanol, and β-sitosterol were injected in six replicates on the same day. The acceptance criterion was ±2% for the percentage relative standard deviation (RSD) of the peak area and retention time (RT). For the precision of the method, samples were prepared 6 times, and each of these was injected in duplicate. The precision was determined from the percentage RSD [6-10].

Stability study
The stability of phytosterols in standard stock solutions was investigated at different time intervals at room temperature. The experiment was also carried out to demonstrate the specificity of the developed method for the determination of stigmasterol and β-sitosterol under controlled conditions. The sample of specific concentration was drawn at 3, 6, 9, and 24 hrs, and the peak area responses were recorded under the optimized chromatographic condition.

Robustness and ruggedness
The robustness and ruggedness of the method were investigated by varying the chromatographic conditions, such as the flow rate (±10%), the wavelength of detection (±10 nm) and varying the analytes. The results were indicated by the percentage RSD between the data at each variable condition.

Limit of detection (LOD) and limit of quantification (LOQ)
LOD and LOQ were determined based on solutions of the standard used to construct the calibration curve. LOD and LOQ were calculated using the formula based on standard deviation of response and the slope of the calibration curve. LOD and LOQ were calculated using equations:

$$\text{LOD} = \frac{3.3 \times \sigma}{S}$$

$$\text{LOQ} = \frac{10 \times \sigma}{S}$$

Where S is the slope of the calibration curve and σ is the standard deviation of regression equation intercept (n=10).

Estimation of phytosterols content from petroleum ether extracts of leaves, stems, and roots
The optimized HPLC method was used to estimate the phytosterols in petroleum ether extracts of leaves, stems, and roots of X. strumarium. The solutions of each extract were prepared separately by dissolving (10 mg/ml) in chloroform, and a sample of 20 µl was subjected to HPLC analysis, and the peak area responses were recorded under the optimized chromatographic condition. From the linear regression equation percentage content was determined. The identification of stigmasterol and β-sitosterol in extracts was preferred by comparison of RT. The samples of a standard solution of extracts were stored in the dark to avoid oxidative degradation.

RESULTS AND DISCUSSION
Optimization of chromatographic conditions
A method based on reversed phase HPLC separation combined with UV-VIS detection has been developed for phytosterols analysis in X. strumarium. An isocratic elution was chosen since it is simple, requires only one pump and minimizes the variation of baseline and ghost peaks. For RP-HPLC, various columns are available, but a column: Qualisil Gold C18, 250×4.6 mm, 5 µm Particle size was preferred because its peak shape and resolution were better. During optimization of method conditions, among the different mobile phases employed,

| Table 1: Method parameters of HPLC system |
|------------------------------------------|
| **Column** | Qualisil gold C18, 250×4.6 mm, 5 µm particle size |
| **Flow rate** | 1 ml/minute |
| **Mobile phase** | Acetonitrile: ethanol: water (85:14:1) |
| **Run time** | 30 minutes |
| **Wavelength** | 202 nm |
| **Temperature of column** | 40°C |
| **Injection volume** | 20 µl |
| **Sample solvent** | Methanol |

HPLC: Reverse phase high-performance liquid chromatography

| Table 2: Optimization of chromatographic conditions |
|------------------------------------------|
| **Solvent systems** | **Mode** | **Mobile phase composition** |
| ACN: ethanol | Isocratic | 85:15 |
| ACN: water: ethanol | Isocratic | 85:5:10 |
| ACN: water: ethanol* | Isocratic* | 85:1:14* |
| ACN: water: ethanol | Isocratic | 90:1:09 |

| **Column** | **Flow rate** | **RT (minute)** | **β-sitosterol** |
| C8, 250×4.6 mm | 2 | 25.6 |
| C18, 250×4.6 mm | 1 | 2.8 |
| C18, 250×4.6 mm* | 1* | 8.3* |
| C18, 250×4.6 mm | 1 | 14.5 |

*Most optimized chromatography condition, RT: Retention time
mobile phase: acetonitrile:ethanol:water (85:14:1) was found to be suitable for analysis of stigmasterol and β-sitosterol. Further, a flow rate of 1 ml/minute and an injection volume of 20 µl along with UV detection at 202 nm were found to be optimal conditions for analysis of these phytosterols (Figs. 1-4 and Tables 1 and 2).

HPLC chromatogram of standard stigmasterol and β-sitosterol obtained under optimized chromatographic conditions by injecting 20 µl of 10 µg/ml solution. RT for stigmasterol and β-sitosterol was found to be 7.77 and 8.49, respectively which were in good agreement with reported data.

HPLC method development and validation

**Linearity**

Calibration curve for stigmasterol and β-sitosterol were obtained from standard solutions at different concentrations. The linearity of the HPLC method was investigated over range 100-500 µg/ml and 10-500 µg/ml for stigmasterol and β-sitosterol, respectively. The calibration curve for stigmasterol and sitosterol were found to linear, with excellent correlation coefficients \( R^2 = 0.9958 \) and 0.990, respectively. The typical calibration curves of stigmasterol and β-sitosterol have the regression equation, \( Y = 1266.86X + 299118 \), and \( Y = 1056X + 383241 \), respectively (Figs. 3 and 4).

**System and method precision**

The system precision results indicate that the percentage RSD values were 1.33 and 0.027 (RT and peak area, respectively) for stigmasterol and 1.2 and 0.024 (RT and peak area, respectively) for β-sitosterol, which are well within the prescribed limits of the ICH guidelines (percentage RSD <2%). Similarly, the results obtained for the precision of the method are within the prescribed limit (percentage RSD <2%), with the percentage RSD values of the peak area being 0.38 and 0.053 for stigmasterol and β-sitosterol, respectively (Table 3).

**Stock solution stability**

Stability of stock solution was determined for the different time interval. Then the SD and RSD were calculated to check its accuracy. The phytosterols were found to be stable in stock solutions for 24 hrs. The percentage RSD values were 0.061 and 0.021 for stigmasterol and β-sitosterol, respectively (Table 4).

**Robustness and ruggedness**

The robustness and ruggedness of the method were investigated by varying the chromatographic conditions, such as the flow rate (±10%), wavelength of detection (±5 nm), and varying the analysts. The results were indicated by the percentage RSD between the data at each variable condition (Table 5).

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**Fig. 1:** Reverse phase high-performance liquid chromatography chromatogram of standard stigmasterol at 202 nm

**Fig. 2:** Reverse phase high-performance liquid chromatography chromatogram of standard β-sitosterol at 202 nm

**Fig. 3:** Calibration curve of stigmasterol (100-500 µg/ml) by reverse phase high-performance liquid chromatography method. Values are expressed as mean of six determinants

**Fig. 4:** Calibration curve of β-sitosterol (10-500 µg/ml) by reverse phase high-performance liquid chromatography method. Values are expressed as mean of six determinants

**Table 3: System and method precision data**

| Parameter | System precision | Method precision |
|-----------|------------------|------------------|
|           | Stigmasterol     | β-sitosterol     | Stigmasterol     | β-sitosterol     |
|           | (concentration 100 µg/ml) | (concentration 10 µg/ml) | (concentration 100 µg/ml) | (concentration 10 µg/ml) |
| RT        | Mean±SD          | %RSD             | Mean±SD          | %RSD             |
|           | 7.80±0.12        | 1.33             | 8.91±0.109       | 1.2              |
|           | 421806±115.4     | 0.027            | 368819±89.48     | 0.024            |
|           | 422780±121.4     | 0.38             | 378972±98.1      | 0.053            |

Values are expressed as mean of six determinants, RSD: Relative standard deviation, RT: retention time.
The RP-HPLC method is robust and rugged since the percentage RSD values are lower than 2% for all four variables.

**LOD and LOQ**

The results further reveal that the minimum concentration levels at which the analyte can be reliably LOD and LOQ are 3.46 ng/ml and 10.5 ng/ml for stigmasterol and 10.5 ng/ml and 12.2 ng/ml for β-sitosterol, respectively, demonstrating the sensitivity of the method (Table 6).

**Table 4: Stock solution stability data**

| Time (h) | Stigmasterol (concentration 100 µg/ml) | β-sitosterol (concentration 10 µg/ml) |
|----------|---------------------------------------|--------------------------------------|
| 3        | 558493                                | 431693                               |
| 6        | 558518                                | 431598                               |
| 9        | 557998                                | 431706                               |
| 24       | 558469                                | 431623                               |
| Mean±SD  | 558344.5±341.4                        | 431655±91.94                         |
| Percentage RSD | 0.061                                  | 0.021                                |

Values are expressed as mean of three determinants, RSD: Relative standard deviation, SD: Standard deviation.

**Table 5: Robustness and ruggedness data**

| Parameter         | percentage RSD of peak area |
|-------------------|----------------------------|
|                   | Stigmasterol | β-sitosterol |
| Flow (2 ml/minute) | 0.65         | 0.39         |
| Flow (0.5 ml/minute) | 0.63         | 0.37         |
| Wavelength (198)   | 0.82         | 0.93         |
| Wavelength (202)   | 0.88         | 1.23         |
| Analyst-I          | 0.48         | 0.54         |
| Analyst-II         | 0.46         | 0.54         |

Values are expressed as mean of three determinants, RSD: Relative standard deviation.

**Table 6: Method validation parameters for the quantization of stigmasterol and β-sitosterol by HPLC method**

| Parameters         | Stigmasterol | β-sitosterol |
|--------------------|--------------|--------------|
| Linear range       | 100-500 µg/ml| 10-500 µg/ml |
| Correlation coefficient | 0.995      | 0.990        |
| Linear regression equation | Y=1266.8X+299118 | Y=1056X+383241 |
| LOD                | 3.46 ng/ml  | 3.99 ng/ml  |
| LOQ                | 10.5 ng/ml  | 12.2 ng/ml  |
| Repeatability (%RSD, n=6) | 1.33       | 1.28        |
| System precision (%RSD)         | 0.027       | 0.024       |
| Method precision (%RSD)          | 0.38        | 0.053       |
| Stability           | 24 hrs      | 24 hrs      |

HPLC: Reverse phase high-performance liquid chromatography, RSD: Relative standard deviation, LOD: Limit of detection, LOQ: Limit of quantification.

**Table 7: Estimation of stigmasterol and β-sitosterol in leaves, stems, and roots of X. strumarium**

| Petroleum ether extracts | Stigmasterol |     | β-sitosterol |     |
|-------------------------|--------------|-----|-------------|-----|
|                         | Peak area mean±SD | Content percentage | Peak area mean±SD | Content percentage |
| Leaves                  | 506362±0.031  | 1.63 | 38836±0.019  | 0.04 |
| Stems                   | 0000         | 0.000 | 427325±0.32  | 0.41 |
| Roots                   | 449816±0.021  | 1.19 | 42339±0.056  | 0.38 |

Values are expressed as mean of three determinants, SD: Standard deviation, X. strumarium: Xanthium strumarium.
linear regression equation and percentage content were determined. Percentage content of Stigmasterol and β-sitosterol were found to be 1.63% and 0.04% w/w respectively in petroleum ether extracts of leaves, 1.19% and 0.38% w/w respectively in roots and percentage content of β-sitosterol was found to be 0.41% w/w in petroleum ether extracts of stems of *X. strumarium* (Figs. 5-7, Table 7).

**CONCLUSION**

A simple, specific, precise, rapid, and reproducible HPLC method has been developed to quantify phytosterols, relevant marker compounds in petroleum ether extracts of leaves, stems, and root part of the *X. strumarium*. The method showed good linear relationship between the peak area and concentrations, acceptable reproducibility and high accuracy. Stigmasterol content was found to be higher in leaves and roots which were 1.63% and 1.19% w/w respectively than stem and β-sitosterol which was 0.04%, 0.41%, and 0.38% w/w respectively in leaves, stems, and roots of *X. strumarium*. The validation procedure confirms that this is an appropriate method for quality control of extracts of *X. strumarium*. Finally, it was concluded that the method is simple, sensitive, and can separate the components from other in plant extracts and estimate them simultaneously.

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