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Original Research Article (Experimental)

Phytoconstituents assessment and development of standardization protocol for '*Nayopayam Kwatha*', a polyherbal Ayurvedic formulation

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ABSTRACT

Background: *Nayopayam kwatha* (NK) is a well-known polyherbal formulation widely used to cure respiratory ailments, heart problems, and postnatal difficulties. Literature suggests that so far no standardization protocol was developed for NK to validate its quality and purity.
Objective(s): To develop a standardization protocol for NK based on the marker phytoconstituents present in the individual herbs of the formulation.
Materials and methods: The roots of *bala* [*Sida cordifolia* (B1) and *Sida retusa* (B2)], seeds of *jeeraka* (*Cuminum cyminum*), and rhizomes of *nagara* (*Zingiber officinale*) were the ingredients of NK. Since there were two source plants for *bala*, two sets of NK (NKB1 and NKB2) were prepared in the ratio 3:2:1 as per *Vaidya Manorama* and 10:1:1 as per *Arogyaraksha Kalpadruma* along with 1:1:1 as per the general way of Ayurvedic polyherbal decoctions. Both the individual herbs and the *kwatha* (decoction) prepared were analyzed in terms of pharmacognostical, organoleptic, and physcicochemical parameters as per the standard methods. Phytochemical analysis of the individual herbs resulted in the isolation of major phytoconstituents and the *kwatha* was quantified in terms of marker compounds with the aid of HPLC.
Results: HPLC quantification suggests that appreciable amount of marker phytoconstituents of individual herbs are present in the *kwatha*. Thus, the isolated compounds luteolin (*C. cyminum*), 6-gingerol (*Z. officinale*), β-sitosterol (*S. retusa*), and ecdysterone (*S. cordifolia*) can be used as markers to standardize NK.
Conclusion: Characteristics of NK, as well as its individual drugs, were well-established. The present study of NK with respect to its phytochemistry revealed that the classical drug ratios of the polyherbal formulation are of utmost importance rather than the ingredients in equal proportion. The characterization parameters of individual herbs and *kwatha* described in this study may serve as a standard reference for quality control analysis of NK and the method developed in this study can be used as a reliable technique for standardization of NK to ensure the purity and quality of raw drugs used.
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1. Introduction

The science of Ayurveda has a rich traditional knowledge of drug combinations for almost all ailments [1–3]. Ayurvedic dosage forms are mostly polyherbal, which is more effective as compared to the single herb therapies. It is a therapeutic strategy to achieve the augmented therapeutic efficacy at a lower dosage [4,5]. Drug ratios of the individual herbs in an Ayurvedic formulation are of utmost importance in order to attain the desired curative effect [6]. Every formulation has its unique indications and contraindications which help to ensure its quality and safety and make them an ideal treatment of choice for excellent therapeutic effect. Most of these formulations exert their increased efficacy through the phytoconstituents present in the individual herbs. The synergistic (*sarvakarmaja*) activity of individual herbs in such formulations contributes much towards its efficacy [7]. It is amply documented

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that Ayurvedic medicines have adverse effects when formulated or used inappropriately. Hence, it is important to follow the standard protocols regarding the formulation and its uses. As a general principle, most dosage forms are polyherbal combinations of ingredients in equal proportions. However, there are only a few combinations in which the ingredients are mixed in particular proportions to achieve the specific curative effect.

Nayopayam kwatha (NK) is a renowned polyherbal formulation extensively used for all respiratory ailments (*swasa vikara*) especially for bronchial asthma (*thamaka swasa*), cardiac diseases, gas trouble, and postnatal care. It is a very good bronchodilator and carminative in actions. Several Ayurvedic textbooks describe the importance of NK including *Vaidya Manorama* [8] in the chapter *Kasa chiktsa* (treatment of cough) and *Arogyaraksha Kalpadruma* [9] in the chapter *Vata roga chiktsa* (diseases of *vata* origin). *Bala* (*Sida cordifolia*), *jeeraka* (*Cuminum cyminum*) and *nagara* (*Zingiber officinale*) constitute the ingredients of this *kwatha* (decoction). The roots of *S. cordifolia* Linn (B1) are taken as *bala* in Ayurveda Formulary of India (AFI) [10] as are those of *S. rhombifolia* ssp., and *S. retusa* Linn (B2) in Kerala [11]. Ancient Ayurvedic scripts explains different drug ratios of the individual herbs for this formulation to obtain specific therapeutic effects.

For the diseases related to '*vata*' the ingredients *bala* (*S. cordifolia*), *jeeraka* (*C. cyminum*), and *nagara* (*Z. officinale*) are in the ratio 3:2:1 as per *Vaidya Manorama,* and for the treatment of cough the ingredients are in the ratio 10:1:1 as per *Arogyaraksha Kalpadrum*. The reference *slokas* (verses) are cited as; "*Nayopaayayana mithe balajeerakanagarei kwatha peetha pramadhnaty sameerana balam balath*" (*Vaidya Manorama*); and "*Balayaam dasabhir bhage dwabhyaam jeeraka vishwayoo siddhakwatho nayopaya swasa hidma haram param*" (*Arogyaraksha Kalpadruma*). NK is indicated as *vatasamana* (pacification of *vata*) as per *Vaidya Manorama*, and for *swasa* (breathlessness) and *hikka* (hiccough) as per *Arogyaraksha Kalpadruma*.

It is important to ensure the efficacy, stability, and safety of a polyherbal formulation, standardization in terms of physicochemical properties, phytochemical screening of the individual herbs, and physical properties of the final formulation [12,13]. So far, no scientific validation protocol has been developed for NK-based on the marker compound present in the ingredient herbs. Hence, the present study was planned to assess the same. We attempted to develop separate profiles of these *kwathas* with standard markers isolated from the individual plant ingredients to provide leads for clinical research using ingredients in the needed ratios i.e., 3:2:1, 10:1:1, and 1:1:1.

2. Materials and methods

2.1. Collection and identification of plant materials

The ingredients of NK viz., *bala* (B1 - *S. cordifolia* and B2 - *S. retusa*), *jeeraka* (*C. cyminum*), and *nagara* (*Z. officinale*) were procured and authenticated for the study purpose (See Fig. 1). B1 was collected from Kanyakumari region during January-February and its root was cut off, cleaned, washed, and shade-dried. B2 was collected from Tamil Nadu region in the month of December and January, its root was cut off, cleaned, washed, and shade-dried. *Nagara* rhizomes (cultivated locally), were collected in the month of January, peeled, and dried in the sun for a period of 2 weeks. *Jeeraka* seeds were directly purchased from a cultivator in Gujarat state in the month of January. The source plants were authenticated by a botanist from Pharmacognosy unit, Govt. Ayurveda College, Poojappura and a voucher specimen was deposited in the herbarium of Govt. Ayurveda College, Poojappura, Thiruvananthapuram.

2.2. Methods

2.2.1. Macroscopic evaluation

The roots of B1 and B2, fruit of *jeeraka*, and rhizomes of *nagara* were subjected to organoleptic (including sensory) evaluation. The characters evaluated were dimensions, shape of pieces, outer surface, fracture, and odour.

2.2.2. Microscopic evaluation

The histological features of roots of B1 and B2, seeds of *jeeraka,* and rhizomes of *nagara* were analyzed in detail.

2.2.2.1. Microscopy of whole drug. Enough number of sections (T.S) of pre-soaked drug were taken and carefully transferred to a petri dish containing water using a fine paint brush. A few thin sections that floated in water were selected and transferred to a watch glass containing safranin stain. After 1 min, the sections were immersed in pure water to remove the excess satin and were thus ready, for mounting on a slide. A stained section was carefully transferred on a clean glass slide using thin paint brush. Two drops of glycerin was added on the section using a dropper and a clean coverslip was placed gently over the section. This slide was then placed on a microscope for examination and direct images were taken at 4x, 10x, and 40x magnifications.

2.2.2.2. Powder microscopy. Sufficient amount of coarsely powdered drug was mounted on a glass slide after mixing with glycerin. Ocular 10x and 40x objectives were used for all observations and diagnostic features were photographed.

2.2.3. Preliminary physicochemical evaluation

Preliminary physicochemical parameters included foreign matter, moisture content, ash value, volatile oil content, different extractive values, fiber content, and sugar content of roots of B1 and B2, *jeeraka* seeds, and *nagara* rhizomes were evaluated according to standard procedures in Ayurvedic Pharmacopoeia of India (API) [14].

2.2.4. Preliminary phytochemical evaluation

The alcoholic (100% ethanol) extract of the individual herbs (B1 and B2, *jeeraka, and nagara*) were subjected to qualitative analysis for the identification of various phytoconstituents including phenols, steroids, flavonoids, alkaloids, etc.

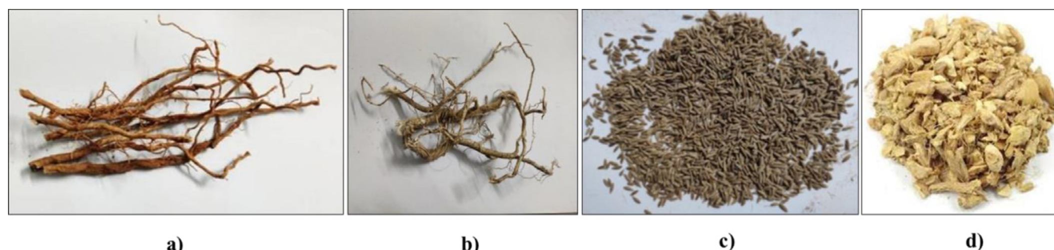

Fig. 1. Collected samples of **a)** *Sida cordifolia*, **b)** *Sida retusa*, **c)** *Cuminum cyminum* and **d)** *Zingiber officinale.*

2.2.5. Isolation and characterisation of major phytoconstituents

Major phytochemicals were isolated from the roots of B1 and B2, *jeeraka* seeds, and *nagara* rhizomes with the aid of gravitational column chromatography. Initially the coarsely powdered materials were subjected to extraction using ethanol (2.5 L x 3days). The extract was then filtered and concentrated under reduced pressure in a rotary evaporator to afford the crude ethanol extract. The crude ethanol extract was then subjected to repeated column chromatographic separation using silica gel (100–200 mesh) with hexane, hexane-ethyl acetate mixtures of varying polarity as eluent in order to obtain the compounds (Supporting information Figure S1, S6, S11 & S16). The isolated compounds were characterised on the basis of various spectroscopic technique.

2.2.6. Preparation of NK

Kwatha was prepared according to the standard procedures as per *Sarngadhara samhita* [15]. The *sloka* (verse) is sited below.

"*Paneeyam shodasa gunam kshunne dravyapalekshipeth*

Mrut patre kwathayeth grahyam astamamsha avasheshitam"

"One pala of coarsely powdered drug is boiled with 16 parts of water in an earthen pot, on mild fire till the required liquid is reduced to 1/8 of the original quantity. This liquid is known as shrta, qwatha, kasaya or niryuha (decoction)".

Crushed root of B1, coarsely powdered seeds of *jeeraka*, and rhizomes of *nagara* were mixed together to achieve a total of 48 g and 768 ml (16 times) of water was added to it. Temperature was maintained at 80–90 °C with the aid of a thermometer, on a gas stove and the total volume was reduced to 96 ml (1/8th). Finally, the decoction obtained was filtered through a 4-layered clean cloth. The same procedure was repeated with B2 to form two sets (NKB1 and NKB2) of *kwatha* in 3:2:1 (24g: 16g: 8g), 10:1:1 (40g: 4g: 4g), and 1:1:1 (16g: 16g: 16g) drug ratios.

The prepared sets of *kwatha* samples were analyzed with respect to the major compounds (β-sitosterol, ecdysterone, luteolin, and 6-gingerol) by analytical technique using HPLC [16–18].

2.2.7. Physicochemical analysis of NK

Different physical parameters of *kwatha* such as total solids, specific gravity, and pH were evaluated using standard pharmacopoeial methods.

2.2.8. Quantitative analysis using HPLC

HPLC analysis was carried out to quantify the major compounds β-sitosterol, ecdysterone, luteolin, and 6-gingerol in the *kwatha*. The analysis was carried out with Agilent 1260 series HPLC system (Agilent Technologies, USA) comprising a quaternary pump, a vacuum degasser, a variable wavelength detector, a 20 μl sample injector, and a column thermostat. The data were analyzed using open lab software.

2.2.8.1. Estimation of β-sitosterol. Ten millilitres each decoction prepared as per the drug ratio was taken in a beaker and kept on water bath maintained at 80 °C for 1.5 − 2.5 hours until a dry residue was obtained. The residue was dissolved in 10 ml methanol by continuously stirring with a glass rod for 20 min. The undissolved portion was filtered through Whatman filter paper no 1 and the clear solution was used for the analysis. A stock solution of β-sitosterol was prepared by dissolving 10 mg of standard β-sitosterol (isolated from the plant extract) in 1 ml methanol and further making it up to a 0.1 mg/mL solution. The detection wavelength was set at 202 nm. The mobile phase consisted of acetonitrile water (in a ratio of 95:5v/v) at a flow rate of 2.0 mL/min. The column thermostat was maintained at 25±1 °C. A stock solution of 10 μl was made up to 1 mL using mobile phase and 20 μl was injected using sampler injector. In case of sample solution of decoction, 20 μl was directly injected after filtration through 0.2 μm millipore membrane filter. The graph obtained with each of the sample was compared with that of the standard and the peak area was measured, which was plotted against concentration. The concentration of β-sitosterol in samples was estimated based on this.

The same procedures were followed for the estimation of ecdysterone, luteolin and 6-gingerol. Different chromatographic conditions of each are given in Table 1.

2.2.9. GC Chromatography-Mass Spectroscopy (GC-MS) analysis of kwatha

Ten millilitres of each *kwatha* were taken in a beaker and kept on a water bath maintained at 80 °C for 1.5–2.5 hours until a dry residue was obtained. The residue was dissolved in 10 ml methanol by continuously stirring with a glass rod for 20 min. The undissolved portion was filtered off through Whatman no.1 filter paper, and the clear solution was used for analysis.

The GC-MS phytochemical profiling was performed using GCMS-TQ8030 Shimadzu instrument. One microlitre of sample was injected to a GC, equipped with a MS and a medium polar capillary column Rxi-5Sil MS (30 m × 0.25 mm I. D, 0.25 μm). The oven program had an initial temperature of 60 °C for 2 min, which was then increased to 200 °C for 2 min at the rate of 5 °C per minute, which then increased to 220 °C for 1 min at the rate of 3 °C/min. Finally, the temperature was increased to 250 °C at the rate of 6 °C/min for 7 min. The total run time was 50 min. The detector temperature and the injection temperature were 250 °C, and helium was the carrier gas with purity 99.999% at a flow rate of 1 mL/min. The sample was injected in the split-less mode. The ion energy used for the electron impact ionization (EI) mode was 70 eV. The mass m/z was scanned for a range of 100–1000. The essential chemical constituents were identified by matching mass spectra with spectra of reference compounds in mass spectral library of NIST and WILEY. The relative amounts of individual components were expressed as percentage peak areas relative to total peak area.

3. Results

3.1. Macroscopic evaluation

Macroscopic evaluation of the individual herbs of NK is summarized in Table 2. The data obtained was in good agreement with

Table 1
Different chromatographic conditions of marker compounds.

Chromatographic Conditions	Ecdysterone	β-sitosterol	Luteolin	6- gingerol
Detection Wavelength (nm)	254	202	260	280
Mobile phase	Methanol: Water (1:1 v/v)	Acetonitrile: Water (95:5 v/v)	Water: Methanol: Acetic acid (700:300:10 v/v)	Methanol: 0.05% Ortho phosphoric acid in water (3:2 v/v)
Flow rate (mL/min)	1.2	2	1	1

Table 2
Organoleptic evaluation of the individual herbs.

Characters	*S. cordifolia* (B1) root	*S.retusa* (B2) root	*C. cyminum* seeds	*Z. officinale* rhizome
Dimensions	Length varying from 7 to 10 cm	Length varying from 7 to 14 cm	5 mm long	5–7 cm long
Shape	Long, cylindrical tortuous	Cylindrical, branched tortuous	Elongated, thick in the middle and laterally compressed	Fairly, smooth, laterally compressed bearing many branches
External surface	Smooth, with some thin rootlets	Numerous lateral rootlets are present	5 lines are present	Longitudinally striated
Fracture	Fibrous	Fibrous		Short
Colour	Pale yellow	Pale yellow	Yellowish brown	Yellowish brown to greyish brown colour
Odour	No characteristic odour	No characteristic odour	Aromatic	Characteristic
Taste	Slightly bitter	Slightly bitter	Slightly pungent	Pungent

Table 3
Histological features of the individual herbs.

Plant material	Microscopical characters of transverse section	Characters of powder microscopy
Bala (S. cordifolia) (B1)	Cork-thin walled tangentially elongated cells. Phellogen-a single layer of narrow, thin walled tangentially elongated cells. Cortex-made up of parenchymatous cells. Calcium oxalate crystals and starch grains are present. Secondary phloem-occurs in conical strands. Medullary rays-many, long, uniseriate, extending and reaching up to the cortex in a straight course	Lignified fibers with parenchyma, Calcium oxalate crystals in the parenchyma cells. Starch grains are also seen in the parenchyma
Bala (S. retusa) (B2)	Cork - thin walled, rectangular tangentially elongated cells. Features of phellogen and cortex are similar to *S.cordifolia*. Secondary phloem – appears in cortical strands and are much narrower and linear than in *S. cordifolia*	Similar to *Sida cordifolia*
Jeeraka (C. cyminum)	Epicarp - composed of a layer of colorless cells, with thin walls and a faintly and irregularly striated cuticle. Underlying the epicarp the thin-walled cells of the palisade are seen Endocarp - composed of a layer of fairly large, thin-walled cells. The endosperm - composed of moderately thick-walled cells containing aleuronic microrosette crystals of calcium oxalate	Presence of aleurone grains, surface cells and some calcium oxalate crystals
Nagara (Z.officinale)	Cork-consists of irregularly arranged, tangentially elongated, slightly brown-colored cells. Cortex-contains thin-walled polygonal parenchymatous tissue. Contains suberised oil cells, which holds yellowish-brown oleoresin. The inner cortex contains closed, collateral vascular bundles. Endodermis - characterized by a single layered pericycle. Vascular bundles of stele resemble that of cortex with the exception of a ring of small scattered bundle within the pericycle. Yellow polygonal oleo-resin cell are also present in the cortical region.	Presence of thin walled non lignified fibres, simple starch grains, oleoresin cells and reticulately thickened vessels

the literature which in turn proves the genuinity of the plant material chosen for the study.

3.2. Microscopic evaluation

Histological features of roots of B1 and B2, seeds of *jeeraka*, and rhizomes of *nagara* and their powder characters are given in Table 3. Trans- section of each herb and its powder microscopy images are given in Figs. 2 and 3.

3.3. Preliminary physicochemical evaluation

Preliminary physicochemical evaluations of the individual plants are tabulated in Table 4.

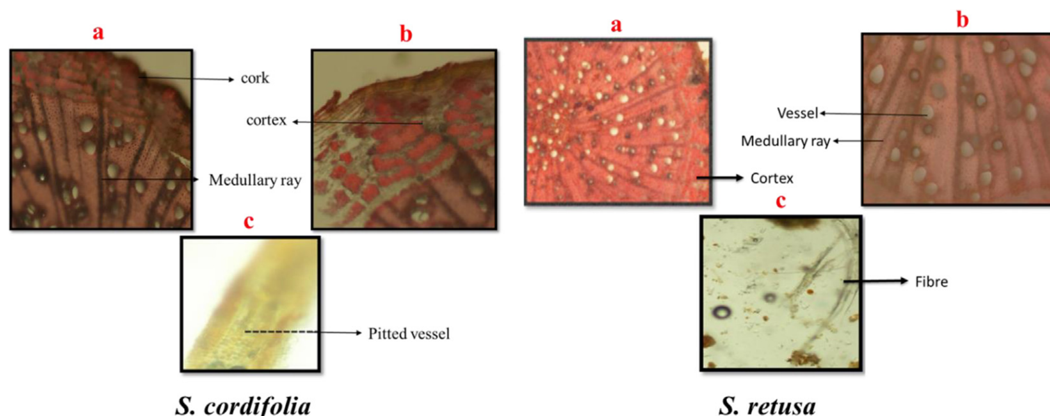

Fig. 2. T.S (a, b) and powder microscopy (c) of *bala*.

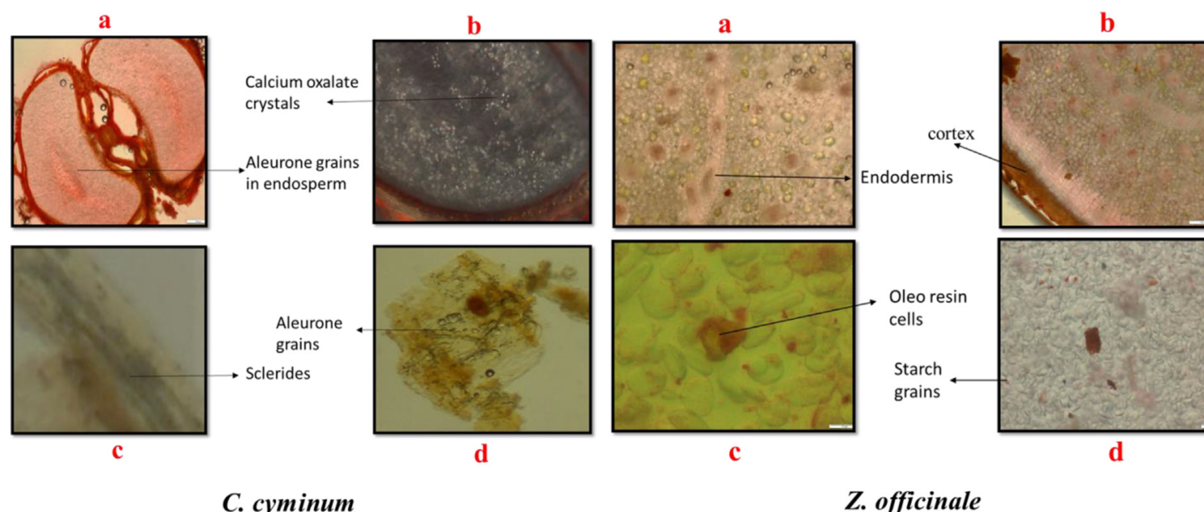

Fig. 3. T.S (a, b) and powder microscopy (c, d) of *jeeraka* and *nagara*.

3.4. Preliminary phytochemical analysis

Preliminary phytochemical analysis of alcoholic extracts of root of B1 and B2, seeds of *jeeraka*, and dried rhizomes of *nagara* revealed the presence of different phytochemicals. The results are given in Table 5.

Table 5
Preliminary phytochemical evaluation of the individual plants.

Experiment	*S. cordifolia (B1)*	*S. retusa (B2)*	*C. cyminum*	*Z. officinale*
Alkaloids	+	+	+	+
Flavonoids	-	-	+	+
Saponins	-	-	+	+
Tannins	+	+	-	+
Phenols	-	-	+	-
Steroids	-	-	+	+

3.5. Isolation of major phytochemicals

3.5.1. *S. cordifolia (B1)*

Ethanol extract of the dried and milled roots of B1 was subjected to separation and purification using column chromatography to obtain β-sitosterol-β-D-*glucopyranoside* and ecdysterone. Structures of the isolated compounds are shown in Fig. 4 and the spectral details of the compounds are depicted in supporting information (S2–S5).

3.5.2. *S. retusa (B2)*

Ethanol extract of the dried and powdered roots of B2 after repeated column chromatographic separation yielded two common phytosterols such as β-sitosterol and stigmasterol. Structures of the isolated compounds are shown in Fig. 5 and the detailed spectral data of the compounds are given in supporting information (S7-S10).

3.5.3. *C. cyminum*

Four compounds were isolated from the ethanol extract of the seeds after repeated column chromatographic separation include cuminaldehyde, leuteolin, 1-(4-(3-methylbut-1-en-1-yl) phenyl) ethan-1-one and apigenin-7-O-glucoside. Structures of the isolated compounds are shown in Fig. 6 and the detailed spectral data of the compounds are given in supporting information (S12-S15).

3.5.4. *Z. officinale*

Acetone extract of the dried and milled rhizomes of *Z. officinale* yielded 8-shogaol and 6-gingerol as the major compounds after column chromatographic purification. Structures of the isolated compounds are shown in Fig. 7 and the detailed spectral data of the compounds are given in supporting information (S17-S20).

3.6. Physicochemical analysis of NK

The specific gravity of the *kwatha* was found to be 1.00 for all the six samples. The pH was found to be around 6–7 and total solids were 0.04, 0.06, 0.09, 0.1, 0.03 and 0.05 for NKB1-3:2:1, NKB2-3:2:1, NKB1-10:1:1, NKB2-10:1:1, NKB1-1:1:1and NKB2-1:1:1 respectively. The results are given in Table 6.

Table 4
Preliminary physicochemical evaluation of the individual plants.

Parameters	*S. cordifolia* (B1)	*S. retusa (B2)*	*C. cyminum*	*Z. officinale*
Foreign matter	0.5%	0.3%	2%	1%
Moisture	10.01%	9.03%	8.4%	9%
Total ash	1.45%	1.34%	8%	6%
Acid insoluble ash	0.645%	0.541%	1%	1.5%
Alcohol soluble extractive	2.012%	1.076%	7%	3%
Water soluble extractive	4.731%	4%	15%	10%
Fiber content	44%	46.02%	5.06%	8%
Total sugar	1.24%	1.78%	0.08%	1.17%
Reducing sugar	0.543%	0.572%	_	0.09%

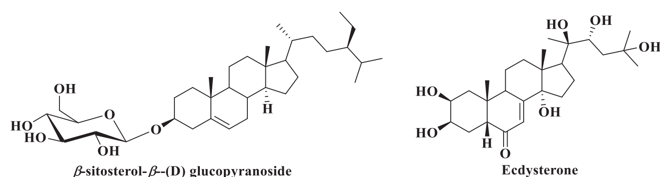

Fig. 4. Structures of the isolated compounds from *S. cordifolia* (B1).

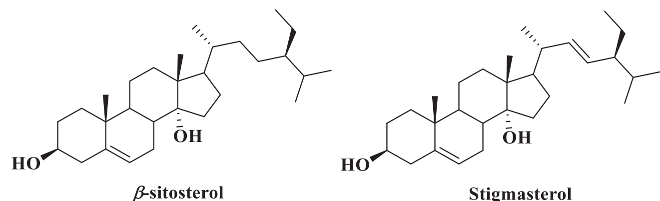

Fig. 5. Structures of the isolated compounds from *S. retusa (B2).*

3.6.1. Quantitative analysis using HPLC

Estimation of the compounds such as β-sitosterol, ecdysterone, luteolin, and 6-gingerol in different ratios of NK were carried out with the aid of HPLC and the results were statistically analyzed and plotted in Fig. 8. Three *kwatha* samples were taken for the quantification of β-sitosterol (NKB2-3:2:1, NKB2-10:1:1 and NKB2-1:1:1) and ecdysterone (NKB1-3:2:1, NKB1-10:1:1 and NKB1-1:1:1). Amount of β-sitosterol was found to be higher in the *kwatha* sample NKB2 in the ratio 3:2:1 and comparatively lower in NKB2-10:1:1 whereas no peaks were detected for NKB2 in the ratio 1:1:1. The *kwatha* sample (NKB1) of the ratio 10:1:1 showed significant amount of ecdysterone when compared to the other two samples. This may be due to the more % w/w of B1 in this proportion. Luteolin was quantified in two sets of three samples in the ratio 3:2:1, 10:1:1 and 1:1:1 with both B1 and B2. Among the *kwathas* made using B2 as the source plant of *bala*, in the ratio 10:1:1 contains significant amount of luteolin while the *kwathas* made using B1 as the source plant of *bala*, the ratio 3:2:1 contains more amount of luteolin. 6-gingerol was also quantified in six samples (three ratios with B1 and B2 are the source plant of *bala*). Amongst the % w/w of 6-gingerol was more in the ratio 1:1:1 both the sample NKB1 and NKB2. Statistical analyses of these four compounds revealed all were found to be significant at a P value < 0.001.

3.6.2. GC–MS analysis

The methanol extract of all the six *kwatha* samples (NKB1-3:2:1, NKB1-10:1:1, NKB1-1:1:1, NKB2-3:2:1, NKB2-10:1:1, NKB2-1:1:1) were subjected to GC-MS analysis. The essential chemical constituents were identified by matching mass spectra with spectra of reference compounds in mass spectral library of NIST and WILEY. The results are given in the following sections.

3.6.2.1. Kwatha NKB1-3:2:1. The chromatogram corresponds to 29 peaks (Fig. 9). The major compounds identified by the chromatogram include gingerol, benzamide, 4-(1-methylethyl)-, stigmasta-5, 22-dien-3-ol (3.beta. 22E) - etc.

3.6.2.2. Kwatha sample of NKB1-10:1:1. The chromatogram corresponds to 31 peaks (Fig. 10).The major compound identified were benzamide, benzoic acid and gingerol.

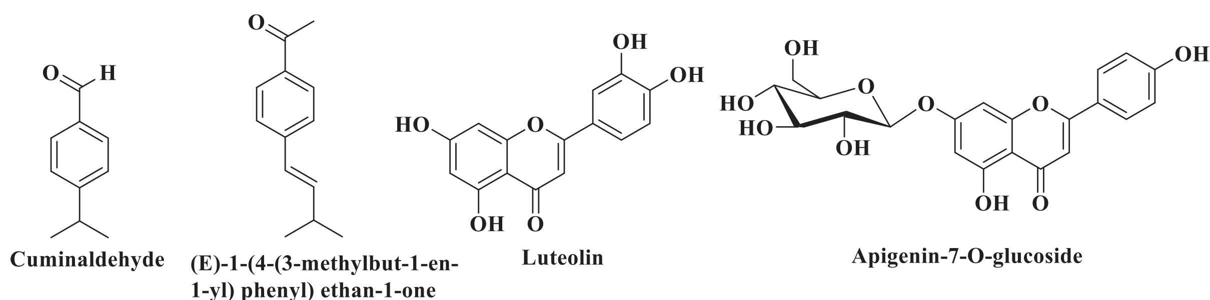

Fig. 6. Structures of the isolated compounds from *C. cyminum*.

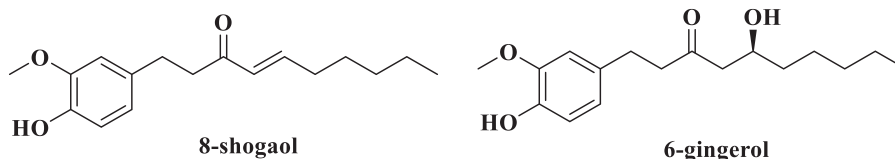

Fig. 7. Structure of the isolated compound from *Z. officinale*.

Table 6
Physicochemical analysis of NK.

Parameter	NKB1-3:2:1	NKB2-3:2:1	NKB1-10:1:1	NKB2-10:1:1	NKB1-1:1:1	NKB2-1:1:1
Specific gravity	1.02	1.05	1.04	1.08	1	1.03
pH	6.56	6.52	6.8	6.65	6.3	6.2
Total solids (g/ml)	0.04	0.06	0.09	0.1	0.03	0.05

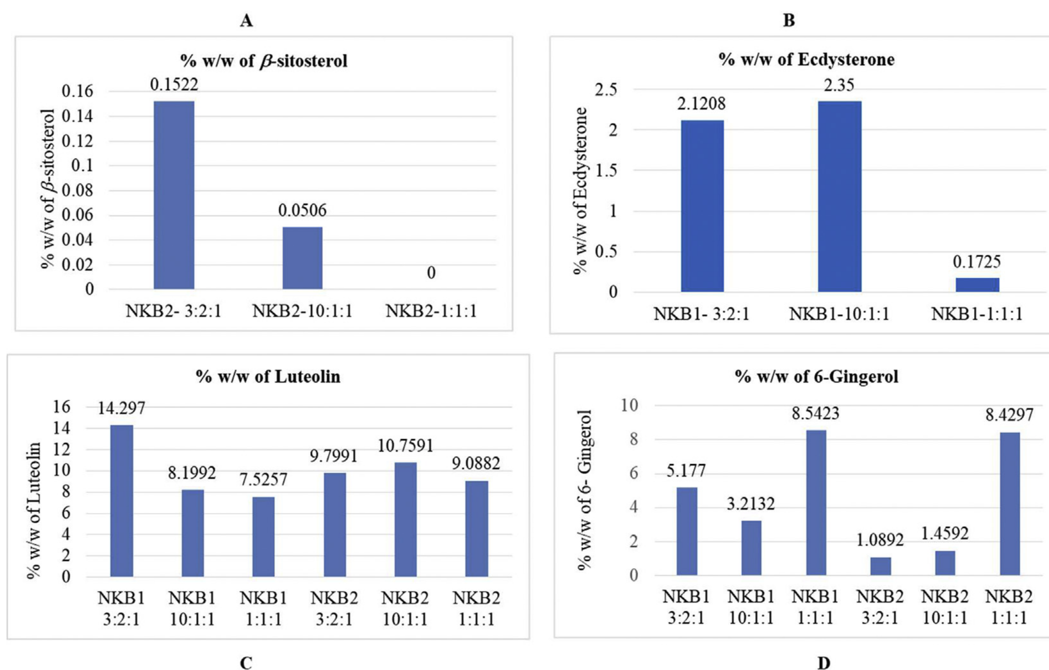

Fig. 8. Mean quantities of the major compounds present in NK.

3.6.2.3. Kwatha NKB1-1:1:1. The chromatogram corresponds to 29 different peaks (Fig. 11). Benzene, gingerol, 2-butanone etc. were identified as the major compounds.

3.6.2.4. Kwatha NKB2-3:2:1. The chromatogram corresponds to 30 different peaks (Fig. 12). 6-Octadecenoic acid and 1, 2-benzenedicarboxylic acid was the major compounds obtained.

3.6.2.5. Kwatha NKB2-10:1:1. The chromatogram corresponded to 30 different peaks (Fig. 13). The major compounds obtained were 9-octadecenoic acid, 12-hydroxy-, methyl ester-2-methoxy-4-vinylphenol, p-cymen-7-ol etc.

3.6.2.6. Kwatha NKB2-1:1:1

The chromatogram corresponds to 30 different peaks (Fig. 14). The major compounds obtained were benzene, benzamide, 2-methoxy-4-vinylphenol, gingerol etc.

4. Discussion

Standardization of herbal formulations is inevitable in order to ensure its purity, quality, safety, and efficacy. Herb-based formulations are widely employed to cure various ailments on account of their higher efficacy, safety, and cost effectiveness. One of the major challenges associated with herb-based formulations is its

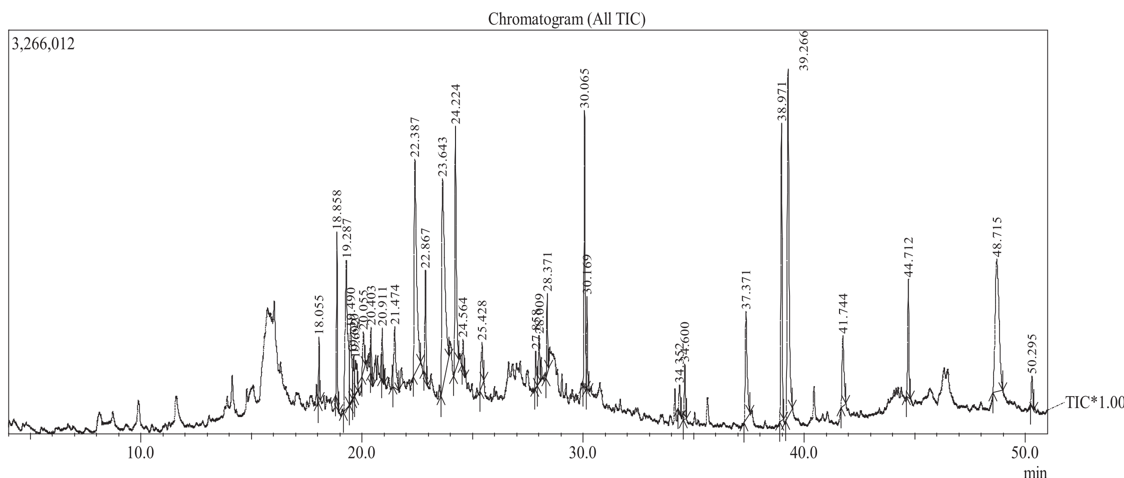

Fig. 9. GC–MS chromatogram of NKB1 3:2:1.

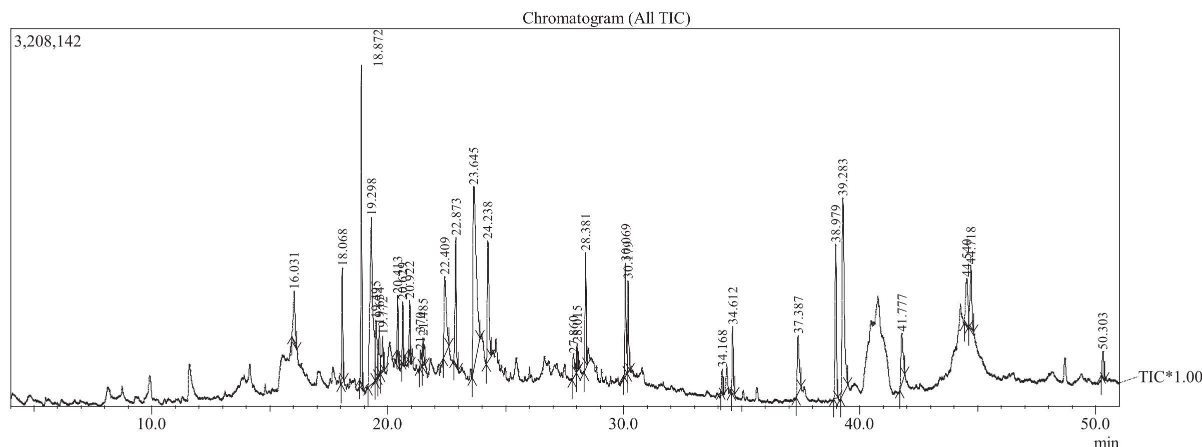

Fig. 10. GC–MS chromatogram of NKB1- 10:1:1.

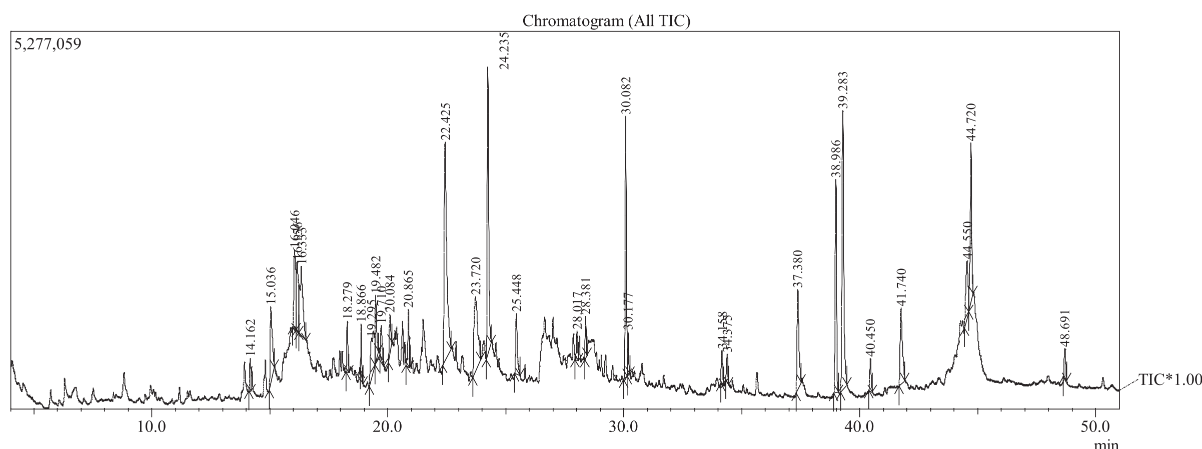

Fig. 11. GC–MS chromatogram of NKB1-1:1:1.

acceptability in modern medicine. Most of the formulations available today in the market have no standardization protocols in terms of marker constituents of the individual herbs used in it. There are some accepted WHO guidelines for the standardization of polyherbal formulations on the basis of several parameters including organoleptic properties, physicochemical analysis, preliminary phytochemical evaluation, and microscopic and macroscopic evaluation of individual plant species used [19]. There is an urgent need to validate basic principles as well as drugs used in the Ayurvedic system of medicine with the help of advanced techniques. In this regard, our efforts are directed towards the development of standardization protocols for the well-known polyherbal formulation NK based on the marker phytoconstituents of the individual herbs.

Ayurveda relies on herbal drugs for treatment when compared to other streams of medicine. These herbal products are manufactured primarily from crude drugs. Hence, the quality and purity of crude drugs is an important factor that determines the efficacy of the final product. As a starting point initially the quality of the *kwatha* was analyzed through organoleptic evaluation of individual ingredients used as well as prepared formulations. It revealed that brownish yellow colour, characteristic odour, liquid consistency, and astringent taste were the characteristic features of NK. *Kwatha* samples prepared with different ratios did not show significant differences in organoleptic evaluation apart from the slight colour changes. To ascertain the genuineness of the samples collected, pharmacognostical evaluation was carried out and was compared with the available literature [20]. Organoleptic evaluation of the individual plant species of *kwatha* (*bala*, *jeeraka* and *nagara*) exhibited characteristic features of the plants in terms of dimensions, external surface, fracture, shape, color, odor, and taste. In addition, histological features of the plant parts (roots, seeds and rhizomes) and their powder characteristics were analyzed through compound microscope. Macroscopic evaluation of *bala* revealed that both the roots were cylindrical, tortuous and B2 contains more rootlets than B1. Scent of cumin seeds is the main feature observed and is identical with the features described in pharmacognosy textbook [11,14]. Thus, the collected raw drugs proved to be authentic. In addition, these results can be used to identify the right variety and the adulterants used in formulations. Preliminary physicochemical evaluation based on the parameters such as foreign matter content, moisture content, fiber content, sugar content, total ash, acid insoluble ash, alcohol/water soluble extractive etc. revealed the features of the plant species and are in compliance with the standard values in API. In our analysis, the total fiber content of B1 and B2, *C. cyminum,* and *Z. officinale* was 44%, 46.02%, 5.06%, and 8% respectively. The total sugar content of

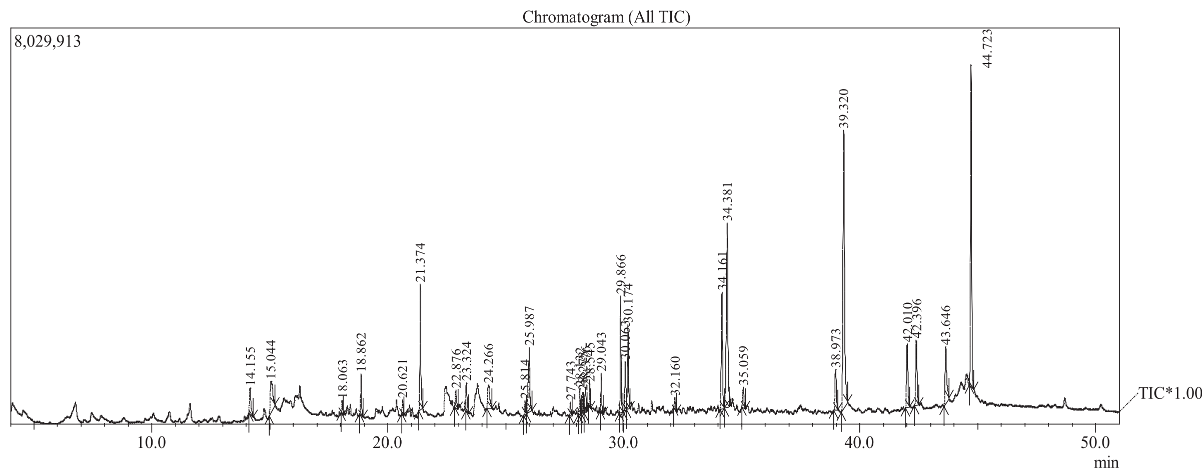

Fig. 12. GC–MS chromatogram of NKB2 - 3:2:1.

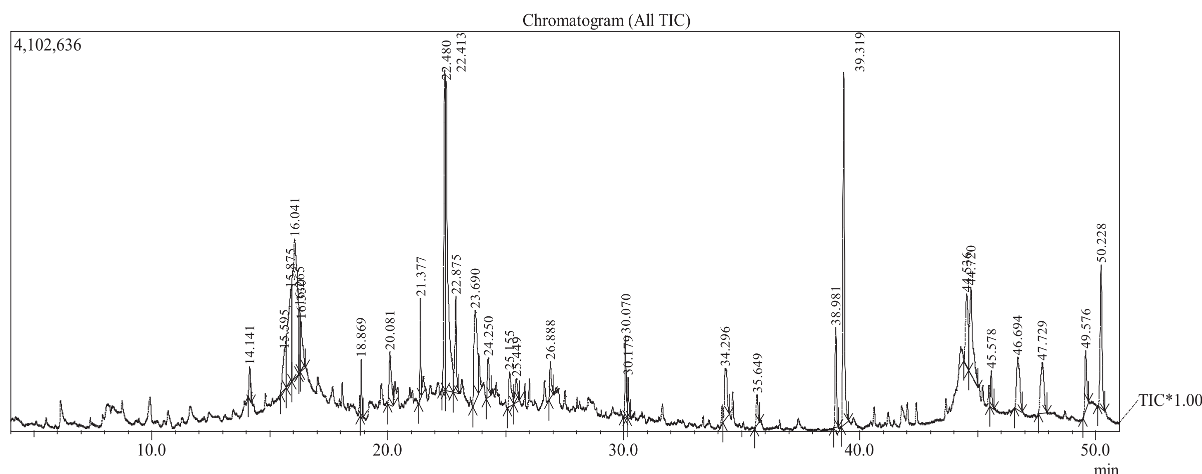

Fig. 13. GC–MS chromatogram of NKB2- 10:1:1.

these drugs was 1.24%, 1.78%, 0.08% and 1.17% respectively. B2 was found to contain maximum fiber and sugar content. The quality and authenticity of the plant species in an Ayurvedic formulation contributes much towards the safety, effectiveness, and acceptability. These basic data obtained can be used for future reference to ensure the authenticity of the plants and to trace out the presence of adulterants. Qualitative analysis of the methanol extract of the ingredients of NK revealed the presence of alkaloid, tannin in *bala;* alkaloid, flavonoid, phenol, steroid, and saponin in *jeeraka*; and steroid, flavonoid, alkaloid, tannin, and saponin in *nagara* and these findings are in accordance with the API standards. Standardization of formulation and its ingredients based on specific marker compounds and its validation is very important. In order to find out the marker compounds of the individual herbs of NK, a detailed phytochemical analysis was carried out. Isolation was carried out with the aid of column chromatography. Alkaloid separation technique was done for getting reported pharmacologically active alkaloids like ephedrine, vasicine, vasicinol etc from B1 and B2 which suggested the presence of these alkaloids in a minimal amount. During the running of column chromatography of cumin, almost all fractions had a smell of cumin. Many of the preliminary fractions contained oil. Gingerol obtained fractions while doing column chromatography created some burning sensation to eyes when kept nearby. Pungency of *Z. officinale* may be due to this compound. The compounds isolated in maximum quantities (major compounds) from each drugs include ecdysterone from B1, β-sitosterol from B2, luteolin from *C. cyminum*, 6-gingerol from *Z. officinale*. Presence of these reported compounds adds to the authenticity of the drugs.

These major compounds were used for comparative quantitative analysis of *N. kwatha*. Six different *kwatha* samples was prepared for the study, based on the standard procedures as per *Sushruta samhita* [15] and these *kwatha* (decoction) were subjected to physicochemical analysis based on its physcicochemical parametres such as specific gravity, pH, and total solids. Specific gravity of the *kwatha* prepared in three ratios was found to be around 1. The formulation NKB2 in the ratio 10:1:1 showed highest specific gravity and NKB1-1:1:1 having lowest specific gravity. pH of the *kwatha* was in an acceptable range for all formulations and was found to be around 6.2–6.8. Altering the drug ratio has no effect on its pH and specific gravity. The sample NKB2 in the ratio 10:1:1 was found to contain more amounts of total solids whereas NKB1 in the ratio 1:1:1 having least amount of total

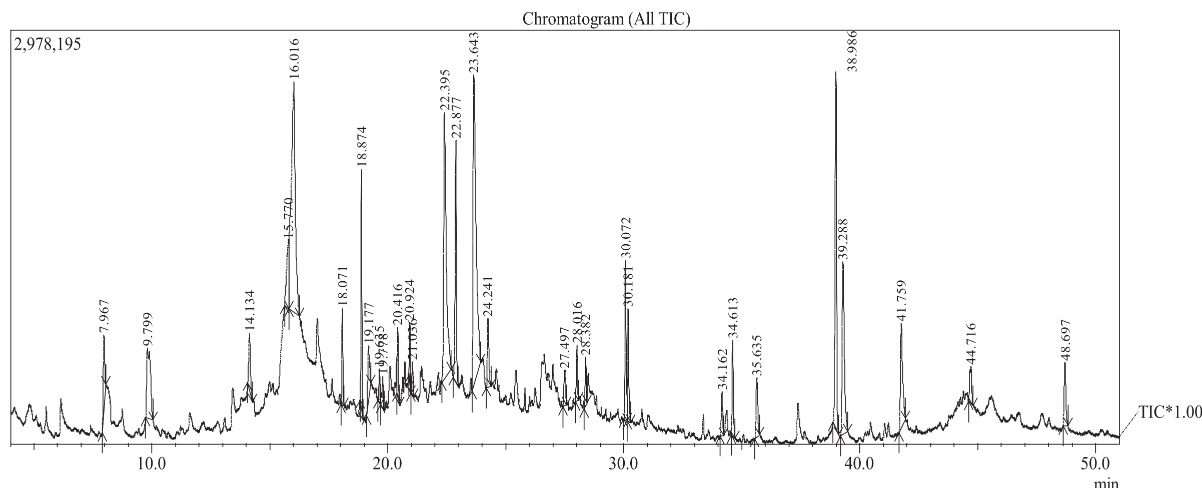

Fig. 14. GC–MS chromatogram of NKB2-1:1:1.

solids. It was found that, altering the ratio of drugs has a slight effect on the total solid contents of NK. These physciochemical parameters will serve as reference for future analysis and furnish information to identify the quality of the formulation.

Quantitative analysis of NK, based on the markers was done using HPLC and identification of volatile components was achieved through GC–MS analysis. Methanol extract of the *kwatha* was chosen for the analysis because it has a polarity similar to that of water. Three *kwatha* samples were taken for the quantification of ecdysterone (NKB1-3:2:1, NKB1-10:1:1, NKB1-1:1:1) and β-sitosterol (NKB2-3:2:1, NKB2-10:1:1, NKB2-1:1:1). The amount of ecdysterone was found to be more in NKB1-10:1:1. This may be due to the more %w/w of *bala* in this proportion. Amount of β-sitosterol was found to be more in NKB2- 3:2:1 which was unexpected. No peaks were detected for NKB2-1:1:1. Among the *kwathas* made using B2, NKB2-10:1:1, contained more amount of luteolin while the *kwathas* made using B1, NKB1-3:2:1, contained more amount of luteolin. Among the six *kwathas*, the % w/w of 6- gingerol was more in NKB1-1:1:1. From the area percentage of standard 6-gingerol was 823525.47 and area percentages of NKB1-1:1:1, NKB2-3:2:1 and NKB2-1:1:1 were more than the standard. This suggests that the amount of 6-gingerol increased in NK than the extract. NKB2-1:1:1 and NKB2- 1:1:1 contained more amount of gingerol than in other samples. Statistical analyes of these four compounds, showed significant at a P value < 0.0001. By analyzing the chromatogram, precise peaks were obtained for luteolin and 6-gingerol.

Pharmacological/therapeutic action of NK can be attributed to the bioactive phytoconstituents present in the individual herbs of the formulation. The constituent *Z. officinale* and its active component 6-gingerol are known to possess potential activity against respiratory disorders and cardiovascular diseases [21]. In our study, it is evident that NK in all drug ratios contains 6-gingerol in appreciable amount and substantially higher in the drug ratio 1:1:1 and there is a notable variation in B1 and B2. Literature suggests that flavonoids including luteolin are beneficial in controlling various respiratory diseases and having anti-allergy potential [22]. Luteolin is also found to present in all drug ratio of NK in significant amount. Thus, these two compounds contribute much towards the therapeutic potential of NK against respiratory problems. In addition, ecdysterone was present in higher amount in the drug ratio of 10:1:1 of NK when B1 was used, in accordance with its weight percent in the ratio. However, in the case of B2, significant amount of β-sitosterol was observed in the drug ratio 3:2:1 and not detected in the ratio 1:1:1 which is unusual. Both ecdysterone and β-sitosterol are pharmacologically important and from this study, we can conclude that B1 can be a better choice when compared to B2 for the NK formulation. Classical drug ratios are very important rather than the ingredients in equal proportion [23,24].

GC–MS analysis of methanol extract of the six *kwathas* was done in qualitative manner in order to find out the volatile components of the formulation. As the technique of GC–MS has limitations in identifying the compound based on the chemical nature, all the compounds were not detected. A total of 29 compounds from NKB1-3:2:1, 31 from NKB1- 10:1:1, 29 from NKB1-1:1:1, 30 from NKB2-3:2:1, 30 from NKB2- 10:1:1, 30 from NKB2-1:1:1. All *kwathas* contain gingerol as a common compound in different percentages. The results will serves as a reference for future studies.

5. Conclusion

NK was analyzed in detail in order to explain the rationale of the particular proportion of ingredients with respect to its phytochemistry. The *kwatha* was characterized in terms of organoleptic evaluation and physcicochemical analysis. Brownish yellow colour, characteristic odour, liquid consistency, and astringent taste were observed as the characteristic features of NK. These preliminary quality parameters can be used for the quality assurance of the formulation as a reference. Physcicochemical parameters of the *kwatha* revealed that altering the drug ratio has no effect on its pH and specific gravity while it will affect the presence of total solids. Authenticity of the individual herbs in the *kwatha* was examined through pharmacognostic, physicochemical, and phytochemical analysis. Ecdysterone from *S. cordifolia*, β-sitosterol from *S. retusa*, luteolin from *C. cyminum*, and 6-gingerol from *Z. officinale* were identified as the marker compounds and were used for HPLC quantification. According to the HPLC analysis, presence of these compounds were identified in the *kwatha* sample in appreciable amount (except the non-detection of β-sitosterol in NKB2-1:1:1), thus contributing to the therapeutic efficacy of the *kwatha*. While analyzing HPLC, luteolin and gingerol were found to be present in more quantities (%w/w) which contribute to its pharmacological action. NK with different drug ratios has different profiles. Also, a notable change was observed in the phytochemical profile of *kwatha* by using two source plants for *bala i.e. S. cordifolia* and *S. retusa*. The present study is the first report of standardization of

NK based on the marker constituents in the individual herbs. This may prove to be a remarkable contribution to the existing knowledge, especially in the field of quality control and standardization. The method developed for HPLC and GC–MS analysis in this study can be used as a reliable technique for standardisation of NK to ensure the purity and quality of raw drugs used.

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Conflict of interest

None.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jaim.2021.05.002.

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