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Phytoconstituents assessment and development of standardization protocol for ‘Nayopayam Kwatha’, a polyherbal Ayurvedic formulation

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A B S T R A C T

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 (NK1 and NK2) 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 physicochemical 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 ecodyosterone (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 (sarat-karmaja) activity of individual herbs in such formulations contributes much towards its efficacy [7]. It is amply documented...
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 višaka*) especially for bronchial asthma (*thamaka swasa*), cardiac diseases, gas trouble, and postnatal care. It is a very good bronchodilator and particularly for bronchial asthma (*swasa vikara*). Several Ayurvedic textbooks describe the trouble, and postnatal care. It is a very good bronchodilator and particularly for bronchial asthma (*swasa vikara*).

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* (*S. cordifolia* and *B. 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 stain 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*. 

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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 3 days). 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 Sarragadha samhita [15]. The sloka (verse) is sited below.

“Paneeyam shodasa gunam kshunne dravyapalekhipeth
Mrut patre kwhathayeth grahamy astamamsa 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 shrita, qwatha, kasaya or niryuha (decocion)".

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, ecdysonoterone, 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, ecdysonoterone, 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 undisolved 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 95:5 v/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 ecdysonoterone, 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 undisolved 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 x 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

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**Table 1**

| Chromatographic Conditions | Ecdysonoterone | 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         |
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.
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.

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 \(\beta\)-sitosterol-\(\beta\)-\(D\)-glucopyranoside and ecodysterone. 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 \(\beta\)-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, NKB1-1:1:1 and NKB2-1:1:1 respectively. The results are given in Table 6.

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**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%        |

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**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           | -                  | -              | +          | +            |
3.6.1. 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.

Table 6

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

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

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

Fig. 7. Structure of the isolated compound from Z. officinale.
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...
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
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 physicochemical parameters 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
it was found that, altering the ratio of drugs has a slight effect on the total solid contents of NK. These physicochemical 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, NKB1-1:1:1, NKB1-1:1:1) and β-sitosterol (NKB2-3:2:1, NKB2-10:1:1, NKB1-1:1:1). The amount of ecdysterone was found to be more in NKB1-10:1:1. This may be due to the more %w/w of water. Three kwatha samples were taken for the quantification of ecdysterone and β-sitosterol. The amount of ecdysterone (NKB1-3:2:1, NKB1-10:1:1, NKB1-1:1:1) and β-sitosterol was found to be more in NKB1-3:2:1 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, NKB2-10:1:1, 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 analyses 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-sitosterol was observed in the drug ratio 3:2:1, and its active component 6-gingerol is 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 possess potential activity against respiratory diseases and having anti-allergy potential [22]. Luteolin was also found to be present in all drug ratios 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 NKB2-3:2:1, 30 from NKB2-10:1:1, 30 from NKB2-1:1:1. All kwathas contained 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 physicochemical 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. Physicochemical 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

![Fig. 14. GC–MS chromatogram of NKB2-1:1:1.](image-url)
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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Conflicts 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.

References

[1] Patwardhan B. Ayurveda: the designer medicine. Indian Drugs 2000;37(5):213–27.
[2] Mukherjee PK, Harwansh RK, Bahadur S, Banerjee S, Kar A, Chanda J, et al. Development of ayurveda-tradition to trend. J Ethnopharmacol 2017;197:10–24.
[3] Kumar S, Dobos GJ, Rampp T. The significance of ayurvedic medicinal plants. J Evid Based Complementary Altern Med 2017;22(3):494–501.
[4] Parasaruman S, Thing GS, Dhanara SA. Polyherbal formulation: concept of ayurveda. Phcog Rev 2014;8(16):73–80.
[5] Chandrasekaran CV, Sundarajan K, David K, Agarwal A. In vitro efficacy and safety of poly-herbal formulations. Toxicol Vitro 2010;24(3):885–97.
[6] Karole S, Shrivastava S, Thomas S, Soni B, Khan S, Dubey J, et al. Polyherbal formulation concept for synergic action: a review. J Drug Deliv Therap 2019;9(1):453–66.
[7] Mukherjee PK, Banerjee S, Kar A. Exploring synergy in ayurveda and traditional Indian systems of medicine. Synergy 2018;7:30–3.
[8] Moos Vayaskara NS. Vaidyamanoraka-Kasa chikitsa. 3rd ed. Kottayam: Vaidya saradhi Press; 1979. p. 81.
[9] Syamala B. Arogyarakshakalpadrumam-Vataroga chikitsa. Thrissur: Samrat Publishers; 2000. p. 64.
[10] List of single drugs of plant origin. The Ayurveda Formulary of India. Part 1. Second revised edition. Delhi: Govt. of India; 2003. p. 309. Published by the controller of publications, Civil lines.
[11] Narayananayeri K, Kolammal K. Pharmacognosy of ayurvedic drugs Kerala. In: Thrivurpathapuram: Pharmacognosy unit. 1st ed. Govt Ayurveda College Thrivurpathapuram; 1993. p. 71.
[12] Crockett SL, Khan IA. Challenges of standardization: marker compounds in plant species related and unrelated to top-selling herbs. J Herbs, Spices, Med Plants 2008;3(10):13–24.
[13] Li Songlin, Han Quanbin, Qiao Chunfeng, Song Jingzheng, Cheng Chuen Lung, Xu Hengxi. Chemical markers for the quality control of herbal medicines: an overview. Chin Med 2008;3:7.
[14] Ayush Do, editor. The Ayurveda pharmacopoeia of India. vol. 1. New Delhi: Ministry of Health and Family welfare; 2007. p. 213–4, part 1.
[15] Panditha Sarngadharacarya, Acharya Sharngadhara, Sharngadhara Samhita, with Dipika commentary of Adhamalla and Gudhartha Dipika commentary of Kasirama. The Sarangadhara samhitha. Pt. Parasuraman Shastri Vidyasagar, editor. Krishnadas Ayurveda series 14. Varanasi: Chowkhamba Krishnadas Academy; 2013.
[16] Muti HY, Olimat S. HPLC method of analysis for determination and standardization of luteolin and vanillic acid in dry extract of paronychia argentea lam. Orient J Chem 2018;34(6).
[17] Shah UM, Patel SM, Patel PH, Hingorani L, Jadhav RB. Development and validation of a simple isocratic HPLC method for simultaneous estimation of phytoestrogens in Cissus quadrangularis. Indian J Pharmaceut Sci 2010;72(6):753–8.
[18] Kamal YKTK, Singh M, Ahmad S, Alam P, Salam S. Stability-indicating RP-HPLC method for the determination of 6-gingerol in polyherbal formulations. J Anal Sci Technol 2015;23(6).
[19] Gavali Jyoti. WHO (world health organization) guidelines for standardization of herbal drugs. Int Ayurvedic Med J 2015;3.
[20] Joshi Devendra, Joshu Geetha. Standardization Of Herbal Medicine. Quality control and standardization of Ayurvedic medicines. Varanasi: Chaukambha orientalia; 2017. p. 113–7.
[21] Townsend Elizabeth A, Siviski Matthew E, Zhang Yi, Xu Carrie, Hoonjan Bhupinder, Emala Charles W. Effects of ginger and its constituents on airway smooth muscle relaxation and calcium regulation. Am J Respir Cell Mol Biol 2013;48(2):157–63.
[22] Lago Joao Henrique G, Toledo-Arruda Alessandra C, Merrak Marcia, Barrosa Kaidu H, Martins Milton A, Tiberio Iolanda FL C, et al. Structure-activity association of flavonoids in lung diseases. Molecules 2014;19(3):3570–95.
[23] Karthika RS, Shahul Hameed A, Meenu MT. HPTLC estimation of gallic acid and ellagic acid in amrtottara kvatha prepared in two ratios. Int. J. Ayur. Pharma Res 2019;7(8):1–9.
[24] Karthika RS, Shahul Hameed A, Meenu MT. Estimation of ellagic acid in ayurvedic polyherbal formulation amrtottara kvatha by HPTLC method. Int J Adv Res 2019;7(7):452–7.