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RESEARCH ARTICLE

Analytical Method Development, Validation, and Estimation of Lupeol, Quercetin, Vasicine in Polyherbal Formulations and Selected Plant Species by using UFLC-MS

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Abstract

Background: *Kabusura kudineer* (KSK) is a marketed *Siddha*-based polyherbal formulation, and on the authority of the Govt of India, the Ministry of Ayush recommended *Kabusura kudineer* in the therapy of *COVID*-19, and due to its immuno-booster effects. Based on the "*Kabusura kudineer*" (KSK) formulations, we developed a "*Hydaljss*08" polyherbal formulation composed of 16 dried crude materials and 03 fresh forms. Both formulations contain active phytopharmaceuticals such as vasicine, quercetin, and lupeol and these are responsible for anti-viral and immunomodulatory effects, which may be due to their synergistic and additive effects. The chemical nature of vasicine is an alkaloid, quercetin is a flavonoid, and lupeol is a pentacyclic triterpenoid. **Aim:** The current study aims to develop and validate the analytical process for assessing vasicine, quercetin, and lupeol in both dosage forms and in a selected plant species by UFLC-MS. **Methods:** The ultrafast liquid chromatography study was designed by the columns of Inertsil C_8, and Inertsil C_{18}, individually, for vasicine, quercetin, and lupeol respectively. The columns and mobile phase were used as a water C_{18}, 20 mM phosphate buffer pH 2.5: acetonitrile in a combined UFLC method development of vasicine, quercetin, and lupeol. **Results:** A calibration curve and adequate linearity were recorded for vasicine, quercetin, and lupeol by injecting 20.0-60.0 µg/ml, 50.0-150.0 µg/ml, and 25.0-75.0 µg/ml of marker substances. The LOD, and LOQ of the vasicine, quercetin, and lupeol were found to be 1.19, 3.60, 3.80, 11.51, 1.79, and 5.41 µg/ml, and the Ruggedness value of vasicine, lupeol, and quercetin was found in % RSD 0.4%, 0.1%, and 0.1%. **Conclusion:** The developed and validated method showed good linearity with a range of correlation coefficients, new, simple, novel, accurate, specificity, precision, robustness, and ruggedness are within the limits.

Keywords: *Hydaljss*08 Polyherbal Dosage Form, *Kabusura Kudineer* Marketed Polyherbal Formulation, Lupeol, Quercetin, Vasicine

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Abbreviation: ACV: Acyclovir, BALF: Bronchoalveolar Lavage Fluid, COVID-19: Coronavirus disease, DNA: Deoxyribonucleic Acid, EMCV: Encephalomyocarditis Virus, FT-IR: Fourier Transform Infrared Spectroscopy, HPLC: High-Performance Liquid Chromatography, *Hydaljss*08: Hydroalcohalic Polyherbal formulation, HSV-I: Herpes simplex virus, ICH: International Council for Harmonisation, JEV: Japanese Encephalitis Virus, KSK/KKR: *Kabusura Kudineer* Marketed polyherbal formulation, KBR: Potassium bromide, LOD: Limit of Detection, LOQ: Limit of Quantification, LC-MS: Liquid Chromatography and Mass Spectrometry, PDA: Photodiode Array Detector, PEDV: Anti-Porcine Epidemic Diarrhea Virus, RNA: Ribonucleic acid, R2: Correlation coefficient, RSD: Relative Standard Deviation, SARS-COV-2: Severe Acute Respiratory Syndrome Coronavirus 2, SD: Standard Deviation, TLC: Thin Layer Chromatography, TNF: Tumor Necrosis Factor, UPLC/UFLC: Ultra-Performance Liquid Chromatography, UV: Ultra-Violet Spectroscopy, UFLC-MS: Ultra-Performance Liquid Chromatography and Mass Spectrometry, VSV: Vesicular Stomatitis Virus, WHO: World Health Organization.

1. Introduction

The medicinal significance and possible activity of *Siddha*-based formulations have been proven over several centuries against various causative agents such as Chikungunya, influenza, tuberculosis, dengue, and COVID-19. One of the Indian traditional systems of medicine, also known as *Siddha*, is a profitable remedial approach for treating viral respiratory infections like COVID-19 and can *efficiently work* to attack the host response and preventive care to boost the immune system. *Kabusura kudineer* (KSK), an official *Siddha*-based polyherbal formulation, has been used in the Indian traditional system for centuries. The clinical studies proved that the KSK used with vitamin C supplementation and zinc significantly reduces severe acute respiratory syndrome coronavirus two viral loads in coronavirus disease patients. The Government of India, Ministry of Ayush guidelines reported that KSK is prescribing an excellent immune booster for preventing and treating COVID-19[1]. Human coronaviruses were initially identified in mid-1960. It is a group of single-stranded (ss) Ribonucleic acid viruses that cause disease in mammals and birds. It is named due to crown-like spikes proteins on the surface of viruses—coronaviruses cause respiratory tract infections[2]. Severe acute respiratory syndrome coronavirus-2 is spread from person to person. It is an infectious disorder believed to be raised from bats and

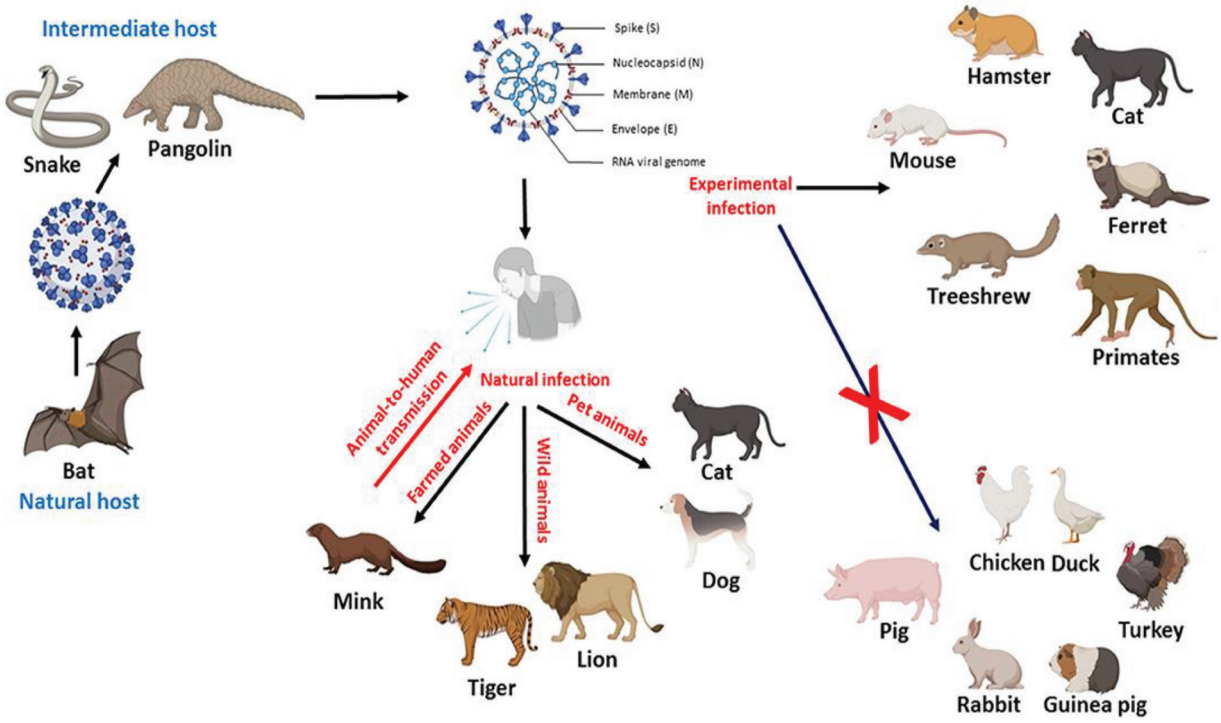


Figure 1. Typical figure of COVID-19 viruses and transmission status.

was communicated to human beings, data illustrated in Figure 1[3]. Statistical data on COVID-19 infection and vaccine administrated in India and globally is described in Table 1.

Phyto molecules' antiviral and immune booster recognition in KSK has been efficiently embedded in molecular docking and various clinical studies[4]. KSK marketed and *Hydaljss*08 polyherbal formulations new are *Siddha*-based polyherbal formulations, contain fifteens similar plant materials in a dried raw form and the Ministry of Ayush, Government of India highly recommended the use of "KSK" during the pandemic of COVID-19, due to its immuno-booster properties, as well as prevents and curable nature of COVID-19. Doctor Thangs Products-Coimbatore manufactures it. Based on the "KSK" formulations, we developed a "*Hydaljss*08" composed of 16 dried materials and 03 fresh forms. Both formulations contain crude dried materials *A. vasica* leaves, *H. auriculata* whole plant, *C. rotundus* roots, *T. involucrata* roots, contains active phytopharmaceuticals are vasicine, quercetin, and lupeol. The presence of the above active compounds in "KSK" may be showing immune-boosting properties. These three compounds are responsible for antiviral and immunomodulatory activity, which may be due to their synergistic and additive effects. Standard literature supports the information having proven antiviral and immunomodulatory activity of vasicine, quercetin, and lupeol[5]. The chemical nature of vasicine is an alkaloid, quercetin is a flavonoid, and lupeol is a pentacyclic triterpenoid, respectively[6]. The analytical method was not reported for the formulations "KSK" as in individual of these compounds and combined. The current study aims to develop and validate the analytical process for the assessment of vasicine, quercetin, and lupeol in both the above dosage forms and in isolated fractions products of *A. vasica* leaves, *H. auriculata* whole plant, *C. rotundus* roots, and *T. involucrata* roots[7]. The preliminary identification of the vasicine, quercetin, and lupeol in the formulations and isolated fractions of *A. vasica* leaves, *H. auriculata* whole plant, *C. rotundus* roots, and *T. involucrata* roots were done by TLC and FT-IR Spectroscopy[8].

1.1 Botanical Description

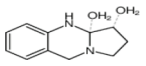
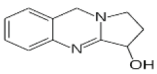
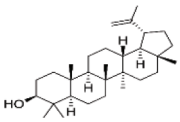
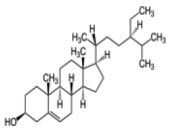
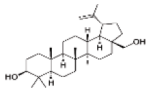
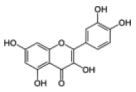
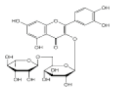
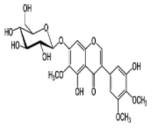
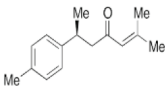
Adhatoda vasica is an effectively recognised plant-based drug in the Indian system of medicine [9]**.** In the Indian indigenous system of medicine for over 2000 years, *Vasaka* plants have been used[10]. The leaves of *Vasaka* contain phytochemical compounds such as glycosides, saponins, alkaloids, tannins, flavonoids, and phenolics[11]**.** The plant contains active quinazoline alkaloids are vasicol, vaccine, azatadine, vaticinate, deoxy vasicinone, Paganini, galactoside, and 9 - acetamido-3, 4 - dihydro pyrido - (3, 4-b)–indole, O–ethyl - A - D galactoside, vasakin, desmethoxyaniflorine, and 7-methoxy vaticinaterespectively[12]. The leaves of *Vasaka* possess a potent immunostimulatory effect and an antioxidant effect[13]**.** The principal active molecule responsible for the immunostimulatory effect of the *Vasaka* extract is yet to be identified[14]. Physico-chemical properties and biological sources of vasicol, vasicine, lupeol, beta-sitosterol, betulin quercetin, rutin, iridin, and ar-turmerone were demonstrated in Table 2.

Hygrophila auriculata Heine plant is found throughout India. Phytochemically screening of *H. auriculata* whole plants contains carbohydrates, glycosides, phytosterols, tannins, flavonoids, terpenoids, sterols, and phenolics. The active chemical constituents present in *H. auriculata* plants are Apigenin-7-O-glucuronide, apigenin-7-o-glucoside lupeol, betulin, stigmasterol, β-sitosterol, and lupeol. Lupeol maximum content was found in the roots (0.25%), and β-sitosterol top content was found in the leaves (0.069%)[15]. The *in-vitro/in-vivo* animal screening proved that lupeol shows potent immunomodulatory activity against the HSV-1 and Acyclovir (ACV) resistant strains[16]**.** Lupeol shows

Table 1. COVID-19 infections statistical data in India and the world

S. No.	COVID-19 Status in India and world					
1	COVID-19 status	No Cases as of12/01/2023 in India	Active Cases as of 12/01/2023 or the Last 24	Discharged as of 12/01/2023	Deceased on 12/01/2023	Total Vaccination as of 12/01/2023
2	India	44681693	2309	44147551	530723 (1.19%)	2201576369
3	World	66,03,78,145	2,30,705	641,224,201	66,91,495	13,10,70,22,929

Table 2. Physico-chemical properties and biological sources of vasicol, vasicine, lupeol, beta-sitosterol, betulin quercetin, rutin, iridin, and ar-turmerone

S. No.	Compound	Molecular formula with mass	Chemical Structure	Biological Source	Nature of compound	Pharmacological effects	References
1	Vasicol	$C_{11}H_{14}N_2O_2$ 206.24 g/mol		*A. vasica* leaves	Quinazoline alkaloid	Antimalarial activity. Immunomodulatory.	12-14
2	Vasicine	$C_{11}H_{12}N_2O$, 188.23		*A. vasica* leaves	Quinazoline alkaloid	Antiviral effects Immunomodulatory Bronchodilator effects	
3	Lupeol	$C_{30}H_{50}O$ 426.7 g/mol		*H. auriculata* whole plant	Pentacyclic triterpenoid	Immunomodulatory antileishmanial	15-18
4	Beta-Sitosterol	$C_{29}H_{50}O$ 414.718 g/mol		*H. auriculata* whole plant	Phytosterol	Immunomodulatory, Antiviral	
5	Betulin	$C_{30}H_{50}O_2$ 442.72 g/mol		*H. auriculata* whole plant	Pentacyclic triterpenoid	Anti-viral, Inhibiting HIV, Antitumor.	
6	Quercetin	$C_{15}H_{10}O_7$ 302.236 g/mol		*C. rotundus roots*	Flavanoids	Immunomodulatory, Antiviral	19-24
7	Rutin	$C_{27}H_{30}O_{16}$ 610.517 g/mol		*C. rotundus roots*	Flavanoids	Immunomodulatory, Antiviral	
8	Iridin	$C_{24}H_{26}O_{13}$ 522.45544 g/mol		*T. involucrata* roots	Isoflavone	Antioxidant activity Neurodegenerative diseases anticancer	25-34
9	Ar-Turmerone	$C_{15}H_{20}O$ 216.32 g/mol		*T. involucrata roots*	Sesquiterpenoid	Antioxidant activity Anticancer Immunomodulatory	

powerful immunomodulatory properties against the antileishmanial parasite[17]. Isolated terpenoid compounds from the *Hylocereus polyrhizus* were identified as lupeol and proved *in-vitro* immunomodulatory activity by increasing macrophage phagocytosis of latex beads[18].

Cyperus rotundus roots, locally called purple nutgrass, is one of the nosy and endemic weeds in intemperate, subtropical, and tropical regions[19]. The active component of *C. rotundus* roots is khellin. Visnagin, ammiol, salicylic acid, caffeic acid, proto-catechetic acid, p-coumaric acid, tricin, isorhamnetin, beta-sitosterol, stigmasterol, chrysoberyl, kaemferol, luteolin, quercetin, rutin, khellol, B-D-Glucopyranoside, and volatile compounds are hyperfine, alpha-copaene, alpha-Erlangen, limonene, P-cymene, beta-Pinene[20,21]. It also contains iridoid glycosides and 3,4-dihydroxy benzoate[22]. The *in-vitro* antiviral activity of baicalein and quercetin was evaluated against the Japanese Encephalitis Virus (JEV) replication *in vero* cells[23]. Quercetin, fisetin, and other bioflavonoids exhibited significant inhibitory activity against the denguevirus[24].

Tragia involucrata Linn. is a medicinal plant used traditionally in Sri Lankan medicine and the Indian system of medicine[25]. The methanolic extracts of Indian stinging nettle roots revealed the presence of flavonoids, alkaloids, carbohydrates, phenolics, tannins, and phytosterols[26]. The principal active compounds present

in Indian stinging nettle roots, such as iridin, quercetin-3-O-rutinoside, tricin 7-O-hexosyl-O-hexoside, and Ar-turmerone, were identified[27,28]. Isolated and characterized colourless compounds from the plants of *T. involucrata* roots are shellsol, 2, 4-dimethyl hexane, 2-methylamine, 2, 6-dimethyl heptane, and vinyl hexyl ether[29]. For the design of new antiviral drugs from plant-based products, quercetin 7-rhamnoside is strongly recommended as a lead compound against the Anti-Porcine *Epidemic Diarrhea Virus* (*PEDV*)[30]. Quercetin, when used along with the Tumor Necrosis Factor (TNF), is recommended against Vesicular Stomatitis Virus (VSV) and Encephalomyocarditis virus[31]. Quercetin is a natural flavonoid compound that has been recently reported to be a potent (Mpro) primary protease inhibitor *in vitro*[32]. Quercetin therapy characteristically reduced interleukin-4and eosinophils while increasing interferon-gamma in blood, Bronchoalveolar Lavage Fluid (BALF), shrinking the allergic airway inflammation by inhibiting mucous cell metaplasia and inflammatory cell infiltration[33]. Quercetin is potentially used in the anti-inflammatory reprogramming of the citric acid cycle and increased antioxidant protection[34].

2. Materials and Methods

2.1 Raw Materials

The authenticated raw materials *A. vasica* leaves, *H. auriculata* whole plant, *C. rotundus* roots, *T. involucrata* roots were purchased from the KRC crude drugs Chennai (13.0827° N, 80.2707° E). Procured materials were dried in a hot air oven until completely dried. Dried crude materials were pulverized using a grinder as a fine powder (Sieve No. 10) and packed in a dry airtight container until further use.

2.1.1 Extraction and Isolation of Vasaka alkaloids (Vasicine and Vasicinone)

Weigh 250 gm of *A. vasica* leaves powder was transferred in a conical flask 2000 ml and macerated with 90% ethanol (1000 ml) for 48 hours. The extract is filtered, and the filtrate is concentrated up to the average value of 90% at 60 °C. The filtrate is mixed with 25 ml of 5% acetic acid solution, warmed at 60°C for 30 mins, and filtered off the Coagulated mass. The acidic filtrate reduced its volume and was shaken with 3 x 25 ml of petroleum ether and 2 x 30 ml of chloroform to remove non-basic red colouring matter. The acidic aqueous extract is adjusted to pH 8.5 with dilute ammonia and extracted successively with 100ml x 2 of chloroform till the aqueous layer gives a negative test with *Dragendorff's* reagent. The chloroform evaporated, and the alkaloidal mixture dried. Finally, it is dissolved in a minimum quantity of chloroform and extracted with acetic acid. The aqueous acidic extract is shaken with activated charcoal for 10 minutes and filtered. The filtrate is adjusted to PH8.5 with dilute ammonia, and the alkaloids are extracted with chloroform. Chloroform is recovered, and the residue thus obtained is dried over calcium chloride and weighed.

2.1.2 Isolation of Vasicinone and Vasicine from Vasaka Alkaloids Mixture

Isolation of vasicinone and vasicine followed by the simple technique from the above mixture of *Vasaka* alkaloids, i.e., TLC. The mobile phase is used as a chloroform: methanol (90:10). The above spot is vasicinone, and the below spot is vasicine. Collect each place in a separate beaker and dissolve the scrapped silica gel mixture in an equal ratio of ethanol and methanol. Stir, warm for 10 min, filter the solution, and evaporate it[35].

2.1.3 Extraction and Fractionation of Lupeol from H. auriculata Whole Plant

Weigh 500 gm of *H. auriculata* whole plant powder materials are extracted with methanol: ethanol (1:1) for one week in a maceration method. The above extract was filtered and dried by the evaporation of the sample. The collected dried extract was suspended in water and fractionated with benzene, n-butanol, chloroform, hexane, ethyl acetate, and pet-ether. Collect the chloroform and hexane fractions, concentrate the mixture until dry, weigh it, and store it in a cool place until further use. It collected bits identified by the thin layer chromatography, and the mobile phase was used as toluene: methanol (9:1)[36,37].

2.1.4 Extraction and Fractionation of Quercetin from C. rotundus Roots

Weighed 250gm of *C. rotundus* roots powder were transferred to a 2000ml Conical flask and extracted with ethanol for one week. The extract was filtered, and the

filtrate was concentrated. The concentrated extract is suspended in water and ethanol; it was fractionated with Pet-ether, n-hexane, butanol, benzene, chloroform, etc. Collect the benzene, chloroform, and hexane fractions, concentrate the solvents with a rota evaporator, dry them, and weigh and store them in a cool place until further use. The preliminary test identified the fractionated products with TLC. The mobile phase was used as butanol, acetic acid, and water (6.25:3.6:0.15). Benzene fraction of *C. rotundus* roots samples small amount is dissolved in ethanol, methanol, and dimethyl sulfoxide while performing TLC, and solutions remain kept aside at room temperature for up to one week. Some parts of the solutions are formed into crystals. Crystals are collected, dried, and weighed (10.5mg). Preliminary analysis of the above crystals was done by thin-layer chromatography, but the clear spots were not eluted. The sample of benzene fraction of *C. rotundus* roots and benzene fraction crystals of *C. rotundus* roots are given for mass spectra analysis. The results obtained from the mass spectra match the molecular weight of quercetin in both samples (Molecular mass: 302.236)[38,39].

2.1.5 Extraction and Fractionation of Quercetin from T. involucrata Roots

It weighed 500gm of *T. involucrata* roots powder in a round bottom flask with solvents such as methanol and ethanol (1:2) for one week. The extract is filtered and collected filtrate; it is concentrated by a rota evaporator at 40°C. The dried extract is dissolved in 250 ml of ethanol and partitioned with chloroform and butanol. Collected the above fraction and evaporate the solvent. The fractionated compound identified as a thin layer chromatography mobile phase is used as a Toluene: Ethyl acetate (8:2)40.

2.1.6 Chemical Marker and Chemicals

Vasicine, quercetin, and lupeol reference standards were purchased from Yucca Enterprises, Mumbai (19.0206° N, 72.8679° E). Required UFLC grade solvents known as acetonitrile, methanol, ethanol, acetone, chloroform, toluene, ethyl acetate, and pet -ether were purchased from the (Central Drug House (CDH) (P) Ltd., Gujarat, India, and Collected from the Dept of Pharmacognosy, and Pharmaceutical Chemistry, JSSAHER-JSS College of Pharmacy, Ooty, Tamil Nadu, India. (Longitude-76°42'25.56"E (76.7071), Latitude -11°24'4.07"N (11.401127), and Chandra Labs, IDA-Prashanth Nagar, Hyderabad, Telangana, India (Longitude-78.4271639, Latitude-17.476078141.

2.1.7. UFLC System and Instruments

The liquid chromatography was carried out on a Shimadzu L.C. 2010 HPLC (UFLC) instrument composed of an ultrafast autosampler and used as a UV-VIS detector. The Lab Solutions software was used as an analysis process. Inertsil C_8 (105 x 4.6mm and 5µm), Inertsil C_{18} (250 x 4.6mm and 5µm), and Inertsil C_{18} (150 x 4.6mm and 5µm) columns were used. FT-IR Model-8400S (Shimadzu), KBR pressing, Software-Shimadzu-IR-Solution, and Shimadzu-LC-MS-8030 were utilized for functional group identifications[42].

2.1.8 Preparation of Standard and Sample Solutions

2.1.8.1 Standard Preparation

50 mg of each weighed vasicine, quercetin, and lupeol were taken and transferred into a 50ml volumetric flask—the sample dissolved in the solvent systems and made up to the final mark with the solvent. The final concentration was prepared at 100 µg /ml with the solvents.

2.1.8.2 Sample Preparations

50mg of each sample was taken and transferred into a 50-Volumetric flask; the piece was dissolved in the solvent systems and made up to the mark with the solvents. The final concentration was prepared up to 100 µg /ml with the solvents.

2.1.8.3 Hydaljss08 Formulation Preparations

Accurately, 5 ml of liquid sample was poured into a 50 Volumetric flask, 30 diluent was added and sonicated for 10 min, and the final volume was made with diluent and mixed well. We centrifuged this solution at 5000rpm for 10 minutes. The supernatant was diluted 1 ml to 10 ml with diluent, mixed well, and injected into an ultra-performance liquid chromatography system. The prepared solution was stored under refrigerator conditions[43].

3. Method Validation

ICH guidelines were followed to carry out the validation parameter study. The proposed analytical methods were validated for precision, linearity, specificity,

robustness, accuracy, repeatability, reproducibility, system suitability, LOQ and LOD.

3.1 Accuracy

The accuracy method was determined for the standard addition and recovery studies. The percentage of recovery carries out three different concentrations: 50%, 100%, and 150% for each reference standard.

3.1.1. Linearity

The linearity was measured by analysing the marker solutions' different concentrations (50–150 µg/ml). The linear curve was formulated for vasicine, quercetin, and lupeol by plotting the average peak area against a regression equation, and concentration was found from the plot. The R^2 was also calibrated[44,45].

3.1.2 Determination of Limit of Detection and Limit of Quantification

The LOD is the small quantity of analysis in a sample that can be calculated but not indeed quantified as an imposed value. The LOQ is the small amount of analyzed substance that can be quantified accurately and precisely for the reference standard vasicine, quercetin, and lupeol.

3.1.3 System Precision and Method Precision

This process was assessed by injecting a reference standard of vasicine, quercetin, and lupeol in different accumulations six times. Peak areas were quantified and computed. Precision was expressed as a % RSD.

3.1.4 Specificity

3.1.4.1 Preparation of Placebo Solution

Crushed placebo powder was weighed equivalent to 200 mg of vasicine, quercetin, and lupeol was poured into a 200ml volumetric flask, dissolved with 70ml of the solvent system, followed by 30 min of sonication, and made up the volume with the solvent system. The sample was centrifuged at 5000rpm for 10 minutes. The above placebo supernatant solution was diluted from 5ml to 50ml with the mobile phase. A placebo solution was injected, and the chromatogram was recorded[46].

4. Results and Discussion

The developed method is a novel, rapid, simple, precise, sensitive, accurate, and economical for the regimen of ultra-fast liquid chromatography development and documentation of vasicine, quercetin, and lupeol in formulations marketed, new, and isolated fractions of *A. vasica* leaves, *C. rotundus*, *T. involucrata* roots, and *H. auriculata* whole plant. The preliminary identification of vasicine, quercetin, and lupeol in formulations and isolated a fraction of plant species was done by the TLC and FT-IR, mobile phase used for separation of these compounds methanol: chloroform (10:90), acetic acid: water: butanol (3.6: 0.15:6.25), toluene: methanol, (9:1) and the R_f value was found to be 0.13 (vasicine), 0.96,0.36 (quercetin) and 0.62 (lupeol) data results are illustrated in Figure 2.

The benzene fractions of *C. rotundus* root products were analyzed and quantified by the LC-MS. The interpretations of mass spectra were made,

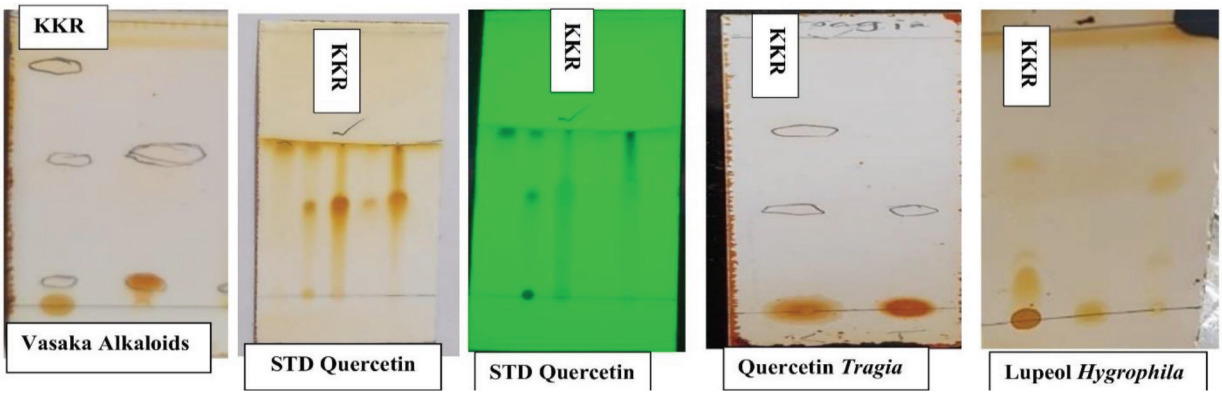


Figure 2. Typical TLC chromatograms of *Kabusura kudineer* (KKR) marketed formulation, isolated *vasaka* alkaloids (vasicine, vasicinone), fraction of *Cyperus rotundus* roots (ref standard quercetin), chloroform fraction ((quercetin) of *Tragia involucrata* roots, and hexane, chloroform fraction (lupeol) of *Hygrophila auriculata* whole plant.

and fragmentation of peaks identified as quercetin content presents in considerable quantities in both samples, resulting in mass spectra summarized in Figure 3.

The ultra-performance liquid chromatography method was carried out of vasicine, quercetin, and lupeol, and in polyherbal formulations marketed and new, and isolated fractions of *A. vasica* leaves, *H. auriculata* whole plant, *C. rotundus*, *T. involucrata* roots. The optimized mobile phase and columns used for liquid chromatography development and validation are 0.1M Sodium pentane sulphonic acid (pH 5.0): methanol, phosphate buffer 4.5: acetonitrile, 0.1% Glacial acetic acid: acetonitrile, and inertsil C8 (105 x 4.6mm and 5µm), inertsil C_{18} (250 x 4.6mm and 5µm), and inertsil C_{18} (150 x 4.6mm and 5µm) column were used as the stationary phase. The retention time of Vasicine, Quercetin, and Lupeol was 1.5, 3.6 and 1.5 minutes, respectively; the data results are summarized in Figure 4.

The ref standard vasicine, quercetin, and lupeol were detected at 295, 371, and 208 nm in the UV detector. While determining the system suitability of vasicine, quercetin, and lupeol were injected six times in the concentrations of 100µg/ml, 100µg/ml, and 50µg/ml to analyze accurate, precise results of the sample, and obtained chromatograms were recorded. Standard deviation (1183.50), (13476.12), (5625.44), and % RSD (0.4), (0.2), (0.1), were found satisfactory. The plate count and tailing factor results were within limits, and the % RSD was 0.266%, so the system is suitable and gives precise results; data are summarized in Table 3.

A calibration curve and adequate linearity were recorded for vasicine, quercetin, and lupeol by injecting 20.0-60.0 µg/ml, 50.0-150.0 µg/ml, and 25.0-75.0 µg/ml of marker solutions. The regression equation was found to be y = 10021x – 46469, y = 65214x + 57224, y = 78293x + 104440, and the correlation coefficient (R2) of vasicine, quercetin, and lupeol was 0.9996, 0.9993,

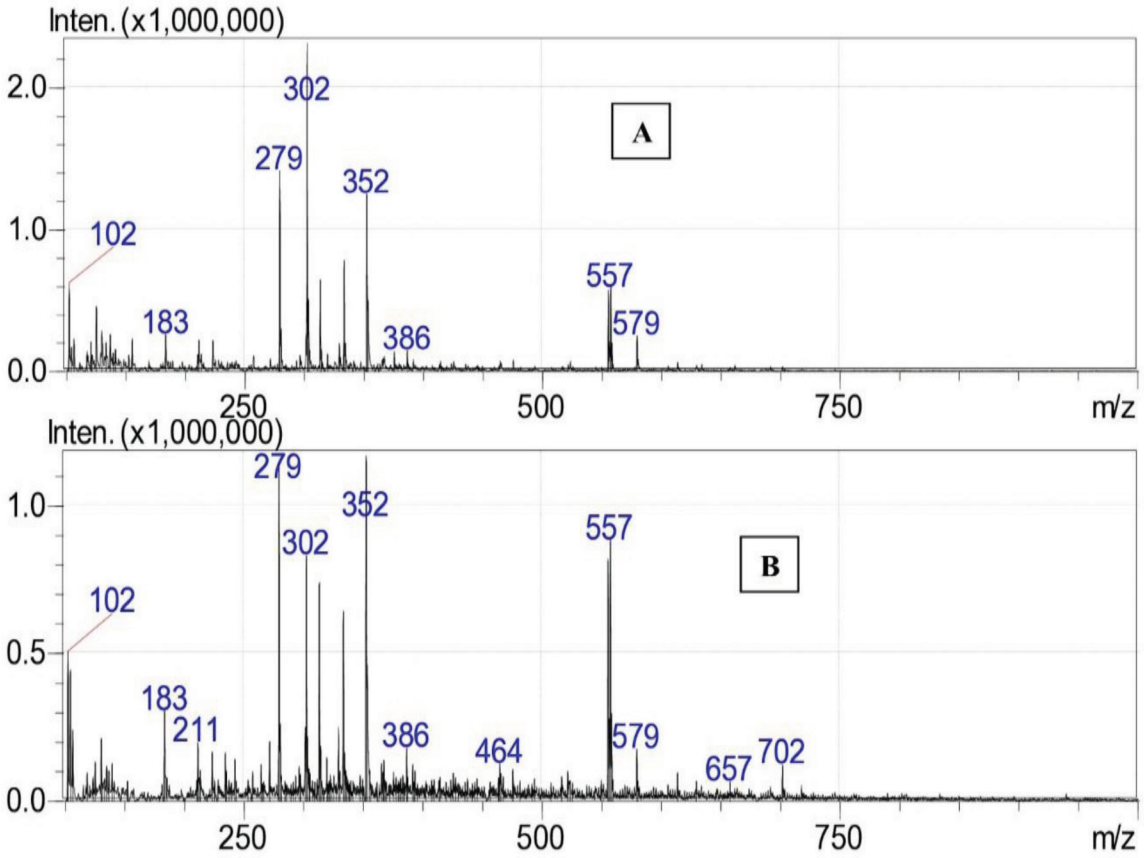


Figure 3. Typical mass spectrum of benzene fractions of *Cyperus rotundus* roots; (**A**)**.** *Cyperus rotundus* roots crystals; (**B**)**.** quercetin-302.236 g/mol.

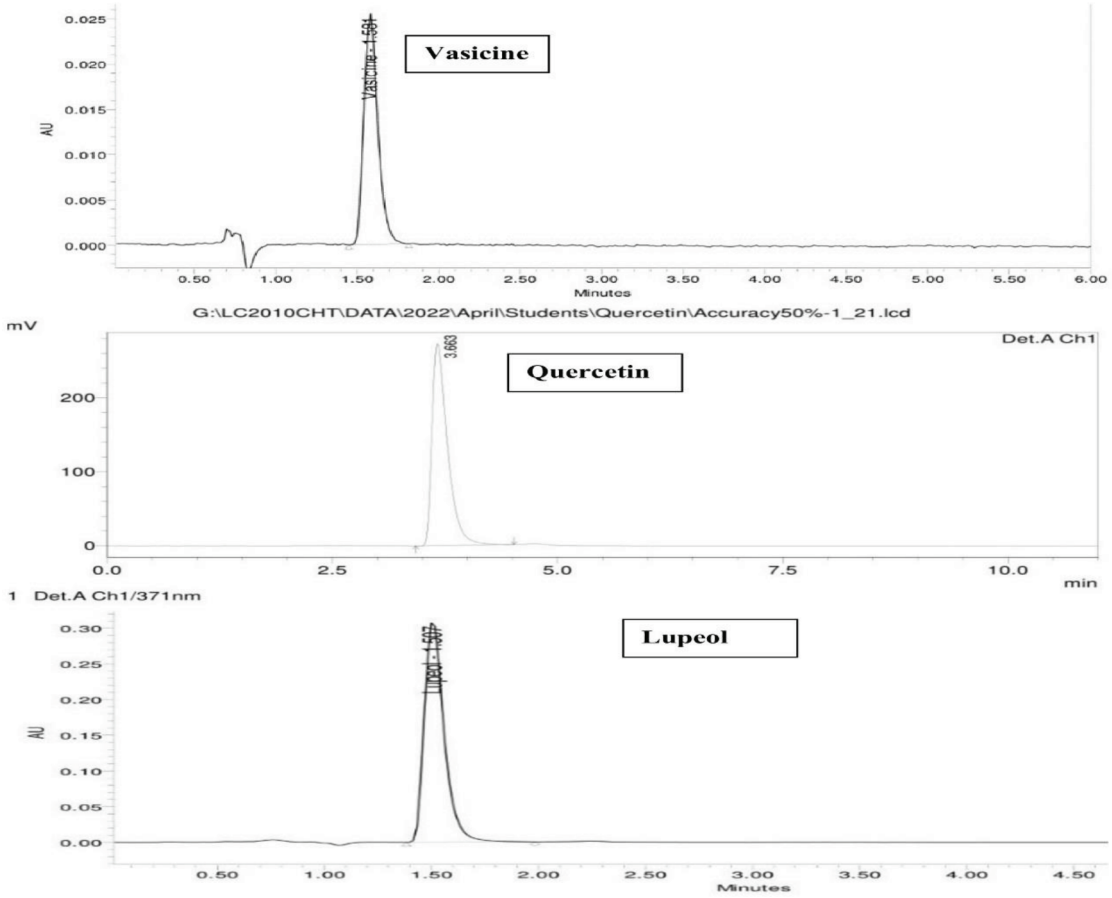


Figure 4. Typical UFLC chromatogram of ref standard vasicine, ref standard quercetin, and ref standard lupeol.

Table 3. System suitability of vasicine, quercetin, and lupeol

S. No.	Parameters	Vasicine	Quercetin	Lupeol
1	Linearity and Range	20-60 µg/ml	50-150 µg/ml	25-75 µg/ml
2	Regression equation	y = 10021 x-46469	y = 65214 x +57224	y = 78293 x + 104440
3	Correlation coefficient	0.9996	0.9993	0.9994
4	Theoretical plates	4668	13450	3327
5	Tailing Factor	1.30	1.45	1.44
6	Detection wavelength (λ_{max})	295 nm	371 nm	208 nm
7	LOD	1.19	3.80	1.79
8	LOQ	3.60	11.51	5.41
9	Slope	10021	65214	78293
10	Intercept	46469	57224	104440
11	Columns	Inertsil C_{18}	Inertsil C_{18}	Inertsil C_{18}
12	Mobile phase	0.1MSodiumpentane sulphonic acid (pH 5.0): Methanol	Phosphate Buffer 4:5Acetonitrile	0.1% Glacial acetic acid: Acetonitrile
13	Mobile Phase and Stationary Phase for the combined method	Waters C_{18} 10 m M Phosphate buffer pH 7.5: Acetonitrile		

0.9994 respectively, the results data demonstrated in Table 4 and Figure 5.

The LODS and LOQ of the vasicine, quercetin, and lupeol were found to be 1.19, 3.60 µg/ml, 3.80, 11.51µg/ml, and 1.79, 5.41 µg/ml, results data illustrated in Table 2. Method precision of the vasicine, quercetin, and lupeol was analyzed by six different concentrations of intraday as well as interday precision was calculated. The standard deviation, % RSD of all reference standards, is within the acceptance criteria limit; data results are illustrated in Table 5.

Table 4. Results of linearity and range for vasicine, quercetin, and lupeol

S. No.	Vasicine		Quercetin		Lupeol	
	Concentration (µg/mL	Area	Concentration (µg/mL	Area	Concentration (µg/mL	Area
1	20.0	152648	50.0	3286132	25.0	2057419
2	30.0	252445	80.0	5238094	38.0	3049248
3	40.0	358154	100.0	6688698	50.0	4082198
4	50.0	457340	120.0	7890281	63.0	5017147
5	60.0	551245	150.0	9790131	75.0	5967802
6	Avg	3612.0	Avg	75089.8	Avg	42369.4

Table 5. Intra- and inter-day results of method precision for vasicine, quercetin, and lupeol

S. No.	Parameters	Vasicine 100µg/ml		Quercetin100µg/ml		Lupeol 50µg/ml	
		Intra-day Precision (n=6)	Inter-day precision (n=6)	Intra-day Precision (n=6)	Inter-day precision (n=6)	Intra-day Precision (n=6)	Inter-day precision (n=6)
		Area	Area	Area	Area	Area	Area
1	Average	315689	322448	6675191	6653331	3935141	4066194
2	Std dev	1183.50	925.2	13476.12	31416.8	5625.44	5919.2
3	% RSD	0.4	0.3	0.2	0.5	0.1	0.1

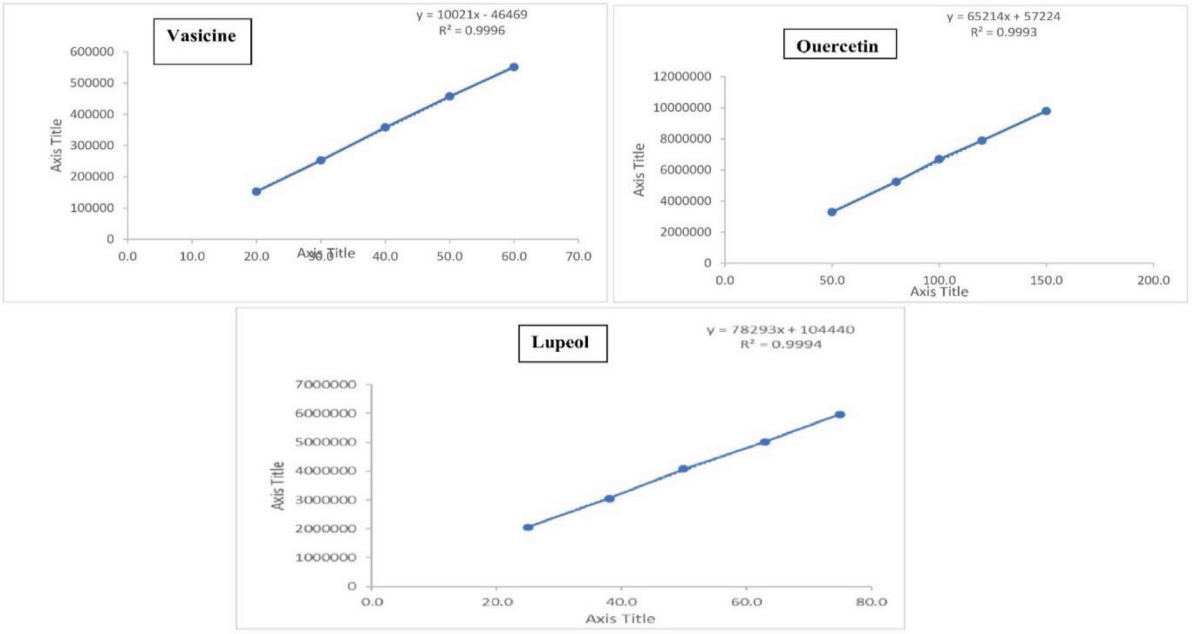


Figure 5. Typical UFLC linearity calibration graph of ref standard vasicine, quercetin, and lupeol.

Table 6. Results of recovery studies for vasicine, quercetin, and lupeol

S. No.	Drugs	Concentration in %	Concentration in (µg/ml)	Nominal Mean % Recovery	Mean % Recovery
		50	50	100.1	
		100	100	101.6	
1	Vasicine	150	150	98.6	98-102
		50	50	98.1	
		100	100	100.2	
2	Quercetin	150	150	148.4	98-102
		50	25	99.8	
		100	50	100.6	
3	Lupeol	150	75	10.9	98-102

Table 7. Results of ruggedness for vasicine, quercetin, and lupeol

S. No.	Vasicine		Quercetin		Lupeol	
1	Analyst 01	100.3%	Analyst 01	99.6%	Analyst 01	99.9%
2	Analyst 02	99.7 %	Analyst 02	99.8 %	Analyst 02	99.7%
3	%RSD	0.4%	%RSD	0.1%	%RSD	0.1%

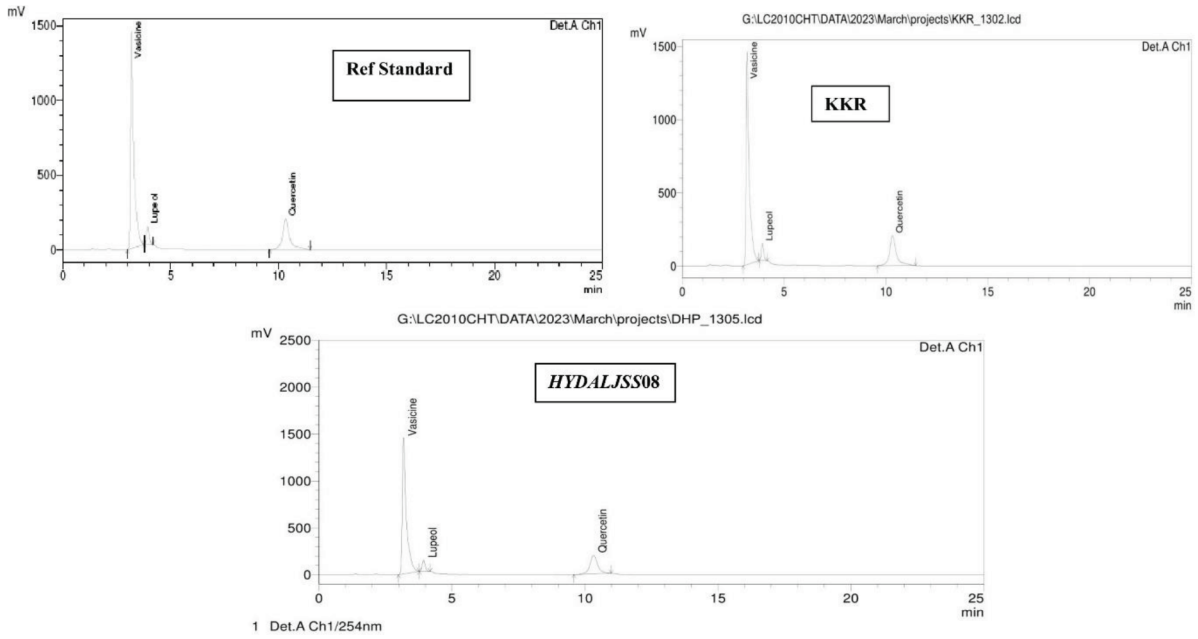


Figure 6. Typical UFLC combined method chromatogram of ref standard vasicine, lupeol, and quercetin, *Kabusura kudineer* (KKR)-marketed, and *Hydaljss*08 polyherbal formulations.

To study the recovery of vasicine, quercetin, and lupeol, accuracy was performed three times to calculate the percentage of healing, and the percentage mean recovery of all markers compounds was found between 98% and 102%; results data were demonstrated in detail in Table 6.

The robustness method of all reference standards was calibrated. The outcomes of the sample obtained by deliberate variation in method parameters, the theoretical plates, and the tailing factor were found to be within the limits of minor deviations of flow rate and wavelength, and the data results are illustrated in Table 3. To determine

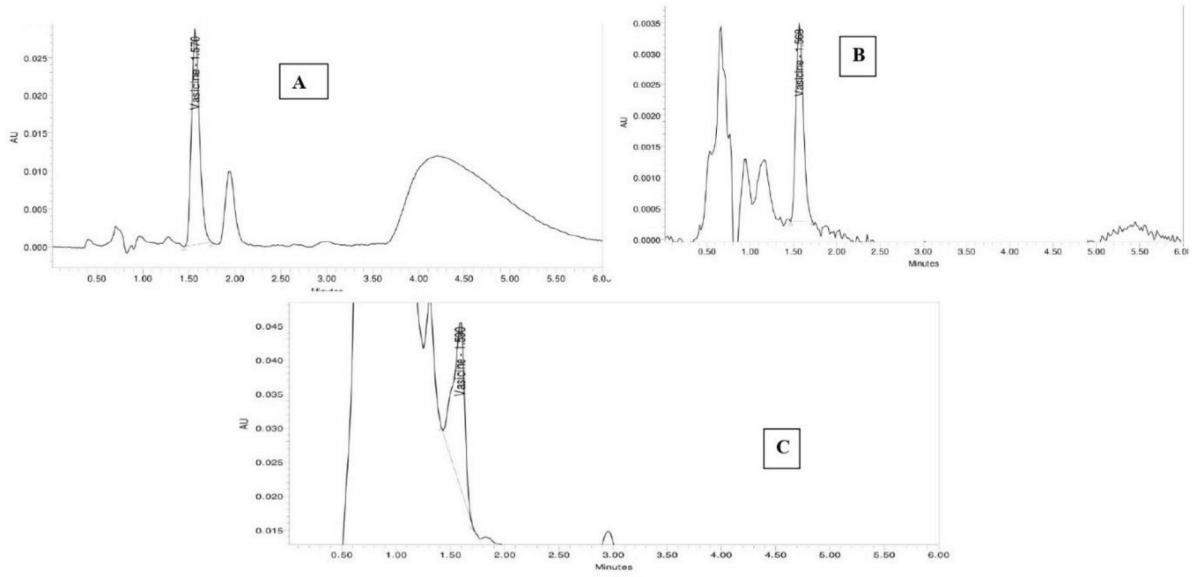


Figure 7. Typical UFLC chromatogram of vasicine isolated from *Adhatoda vasica* leaves; **(A).** *Kabusura kudineer* (KKR)-marketed **(B).** *Hydaljss*08 **(C).** Polyherbal formulations.

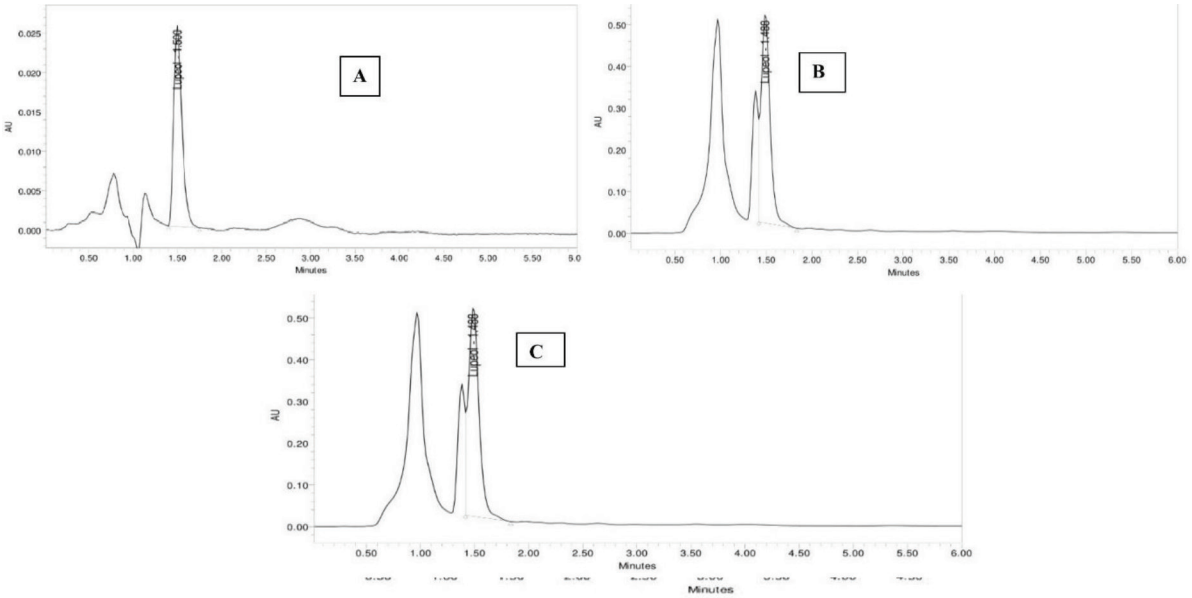


Figure 8. Typical UFLC chromatogram of (lupeol) chloroform; **(A).** hexane; **(B).** Fraction of *Hygrophila auriculata* whole plant, and *Hydaljss*08; **(C).** Polyherbal formulation.

the variation in the study of vasicine, quercetin, and lupeol assay by two different analysts in the method of ruggedness. The % relative standard deviation of assay values between two analysts should be less than 2.0, as demonstrated in the results data detailed in Table 7.

Specificity was determined to all marker compounds to analyze excipients or diluents not detected while performing the assay. The columns and mobile phase were used as a waters C_{18} (250mm x 4.6mm and 5µm), 20 mM Phosphate buffer pH 2.5: acetonitrile in a combined UFLC method development of vasicine, quercetin, and lupeol and compounds were detected at 254 nm in U.V. detectors. The retention time of vasicine, lupeol, and quercetin was found to be 3.178, 3.929, and 10.321 mins, respectively, and the data results are illustrated in Figure 6. Estimation of vasicine, quercetin, and lupeol

Table 8. Percentage purity calculation of vasicine, quercetin, and lupeol in isolated, fractions of *A. vasica* leaf, *C. rotundus* roots, *T. involucrata* roots, and *H. auriculata* and polyherbal formulations

S. No.	Drugs	Sample	Area	% Assay	in µg/mL	in mg/mL
1	Vasicine	Ref standard vasicine	158602	-	-	-
		Isolated vasaka alkaloids from *A. vasica* leaf	240833	30.5	29.281	0.0292
		Isolated vasicine from *vasaka* leaf by TLC	171172	21.7	20.832	0.0208
		Kabusura kudineer – marketed	8898	2.4	2.304	0.0023
		*Hydaljss*08 polyherbal formulations	172703	21.9	21.024	0.0210
2	Quercetin	Ref standard Quercetin	3263899	-	-	-
		Benzene fraction of *C. rotundus* roots	39997	0.6	0.58	0.00058
		Chloroform fraction of *C. rotundus* roots	65089	1.0	0.96	0.00096
		Hexane fraction of *C. rotundus* roots	64685	1.0	0.96	0.00096
		Chloroform fraction of *T. involucrata* roots	26657	0.4	0.39	0.00039
		Kabusura kudineer-marketed	2282	0.03	0.12	0.00012
		*Hydaljss*08 Polyherbal Formulations.	154571	2.3	2.202	0.0022
3	Lupeol	Ref standard Lupeol	1994277	-	-	-
		Chloroform fraction of *H. auriculata* whole plant	3465692	88.1	44.035	0.0440
		Hexane fraction of *H. auriculata* whole plant	163327	4.2	2.0752	0.0020
		Kabusura kudineer-marketed	186863	4.7	2.3742	0.0023
		*Hydaljss*08 polyherbal formulations	5898473	149.9	74.946	0.0749

in polyherbal formulations marketed, and new and isolated fractions of *A. vasica* leaves, *C. rotundus* roots, *T. involucrata* roots, and *H. auriculata* whole plant were found satisfactory[47-49]. Data results are illustrated in Table 8 and Figures 7 and 8.

5. Conclusion

The present developed process is a simple, novel, rapid, accurate, and reliable method that has evolved and estimated for the vasicine, quercetin, and lupeol present in *A. vasica* leaves, *C. rotundus* roots, *T. involucrata* roots, and *H. auriculata* whole plant and marketed *Siddha*-based dosage forms called as "KSK" and a *Hydaljss*08 by using liquid chromatography. The LOD and LOQ of the vasicine, quercetin, and lupeol were found to be 1.19, 3.60 µg/ml, 3.80, 11.51 µg/ml, and 1.79, 5.41 µg/ml, and ruggedness value of vasicine, lupeol, and quercetin were found in % RSD 0.4%, 0.1%, and 0.1% respectively. The results revealed that the method showed better linearity, recovery, reproducibility, and low limits of detection, quantification, ruggedness, and specificity. Thus, it is acceptable to be used in quantifying vasicine, quercetin, and lupeol in *Ayurvedic*-based polyherbal formulations and isolated, fractionated products in plant species.

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