RESEARCH ARTICLE

Novel HYDALJSS08 Polyherbal Formulation Development and Ultra-Performance Liquid Chromatographic Separation, Estimation of Gallic Acid in *Terminalia chebula* Dried Fruits, and a Marketed Siddha-based Polyherbal Formulation "Kabusura Kudineer"

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ABSTRACT

The Siddha-based polyherbal formulation known as "Kabusura Kudineer (Marketed)" contains fifteen plants materials in dried raw form and The Ayush Ministry, Govt. of India highly endorsed for use of "Kabusura kudineer" during the pandemic of COVID-19, due to its immuno-booster properties. The current study aims to develop and document an analytical strategy for the estimation of gallic acid (GA) and methyl, ethyl acetate fraction of *Terminalia chebula* dried fruits and in polyherbal formulations (Marketed, & Developed). One of the active substances of Kabusura Kudineer and developed "HYDALJSS08" Polyherbal formulation is *Myrobalan* (*T. chebula* dried fruit-*Combretaceae*). *Myrobalan* restrains active principal substances are GA. GA is proven for its Anti-viral and immunomodulatory activity. During the pandemic of, COVID-19 ministry of Ayush, Govt of India highly recommended the immuno-booster Siddha-based polyherbal formulation "Kabusura Kudineer" for immune-boosting and treatment of COVID-19. TLC, and FTIR carried out the preliminary identification of GA and the sample. The HPLC method was developed on an Inertsil C_{18} (150*4.6 mm and 5 µm) column. The mobile phase was enhanced for water: Acetonitrile (50:50). The flow rate was 1-mL/min and was GA tracked at 272 nm in a UV detector. The total run time of the chromatogram is 8 minutes and the retention time of GA is observed. The Rf value of GA and sample was found to be 0.27. Validation was carried out according to ICH guidelines. The retention time of GA was 2.2 min, and the Valid parameter of GA is system precision, SD (14247.75), %RSD (0.9), Regression equation y = 25511 x−947505, Correlation coefficient (R2) 0.9999. The adequate Linearity concentration was found to be 50 to 150 µg/mL, LoDs (1.70 µg/mL), LoQs (5.16 µg/mL), Method precision %RSD (0.8), SD (11626.7), Recovery 99.8, and 101.1%. GA content was found in a formulation ("Kabusura Kudineer"- 1.39 µg/mL, developed "HYDALJSS08" Polyherbal formulation-379.4 µg/mL), and ethyl, methyl acetate fraction of *T. chebula* dried fruits was 105.59 and 29.17 µg/mL. The developed (HPLC) UPLC methods have enabled novel, simple, accurate, rapid, easy, reproducible, rugged, and linear analysis in these two fractions, and Siddha-based formulation "Kabusura kudineer" (Marketed), a developed "HYDALJSS08" Polyherbal formulation.

Keywords: Gallic acid, Kabusura kudineer" (Marketed), Ethyl and methyl acetate fraction of *Terminalia chebula* dried fruit, TLC, FTIR, HPLC, Developed "HYDALJSS08" Polyherbal Formulation.

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INTRODUCTION

The Siddha-based polyherbal formulation "Kabusura kudineer (Marketed)" contains fifteen plants materials in dried raw form and The Ayush Ministry, Govt of India also endorsed for use of "Kabusura kudineer" during the pandemic COVID-19, due to its Immuno-booster properties. It is prepared by combining all dried raw materials in equal proportions and pulverizing them. It contains dried raw plants materials of kalmegh-king of bitter (*Andrographis Paniculata* whole plant-*Acanthaceae*), Ginger (*Zingiber officinalis* rhizome-*Zingiberaceae*), Pippali (*Piper longum* dried fruits-*Piperaceae*), Myrobalan (*T. chebula* dried fruit-*Combretaceae*), Clove buds (*Syzygium aromaticum* flower buds-*Myrtaceae*), Indian stinging nettle (*Tragia involucrata* roots-*Euphorbiaceae*), Vasaka (*Adhatoda vasica* leaf-*Acanthaceae*)), Guduchi (*Tinospora cordifolia* stem-*Menispermaceae*), Akarkara (*Anacyclus pyrethrum* roots- *Asteraceae*), Gokanta (*Hygrophila auriculata* whole plant- *Acanthaceae*), Indian borage (*Coleus amboinicus* roots-*Lamiaceae*), Indian costus tree (*Saussurea lappa* roots-*Asteraceae*), Blue flowered glory tree (*Clerodendrum serratum* roots- *Verbenaceae*), Velvet roots (*Cissampelos pareira* roots-*Menispermaceae*), Java grass (*Cyperus rotundus* roots-*Cyperaceae*). The active compounds responsible for Anti-viral and immunomodulatory activity are 6-gingerol, zingerone, eugenol acetate, eugenol, biflorin, eugenin, piperine, gallic acid, pellitorine, ellagic acid, corilagin, quercetin, inulin, chebulinic acid, lupeol, beta-sitosterol, betulin, vasicine, Vasicinone, apigenin, chebulagic acid, luteolin, rutin, costunolide, Dehydrocostus lactone, berberine, cordifolioside, syringin, and andrographolide reported. These compounds are responsible for anti-viral and immunomodulatory activity may be due to their synergistic effects. The present study aimed to standardize and estimate GA in polyherbal formulations and ethyl, methyl acetate fraction of *T. chebula* (*Combretaceae*) dried fruits.[1]

Coronavirus spread from person to person in several ways, known as SARS-*CoV*-2, believed to be originated from bats and was transmitted to human beings.[2,3] The preliminary recorded case of *Coronavirus* was in Hubei Province, Wuhan city, China.[4-7] The infection has been transmitted globally, notorious for the current pandemic. Globally, as of 12:23 pm CEST, 7 June 2023, there have been 76,77,50,853 confirmed cases of COVID-19, including 69,41,095 deaths, reported to the World Health Organization. As of 10 June 2023, a total of 13,39,67,01,847 vaccine doses have been administered.[8]

Myrobalan is the fully-fledged dried fruit of *T. chebula*, belonging to the family *Combretaceae*.[21] Myrobalan is called the " King of Medicine" in the Tibetan plateau due to its excellent remedial capacity.[9] It is a rich source of about 32 to 34% of tannins, and their composition is distinct with geographical areas. They are ellagic acid, chebulagic acid, chebulinic acid, gallic acid, terchebulin, 1,6-di-o-galloyl-D-glucose, punicalagin, chebulanin, corilagin, neochebulinic acid, and casuarinin. Other components include phenolics and polyphenols such as anthraquinones, terflavin-A, galloyl glucose, corilagin, maslinic acid, and punicalagin. Besides, amino acids, resin, fructose, succinic acid, and beta-sitosterol are also present. Fatty acids are secluded from the myrobalan fruits which are linoleic, palmitic acid, and oleic acid the main substances.[10] The major intention of this study was to develop and document an analytical strategy for gallic acid by using TLC, FTIR, and HPLC methods in the Siddha-based polyherbal formulations "Kabusura kudineer (Marketed)" newly developed "HYDALJSS08" Polyherbal formulation (Asava/Arishta) and ethyl, methyl acetate fraction of *T. chebula* dried fruits.

MATERIALS AND METHODS

Raw Materials

T. chebula (Combretaceae) 's authenticated dried fruits were procured from the KRC crude drugs Chennai (13.0827° N, 80.2707° E). Purchased materials dried at 43°C in a hot air oven until completely dried. Dried crude materials were pulverized by using a grinder as a fine powder (sieve no 10) and packed in a dry airtight container till further use.[11]

Extraction and Fractionation of Gallic Acid

Weigh about 500 gm of *T. chebula* dried fruits fine powder was transferred in a 2000 mL round bottom flask and extracted with 500 mL of methanol and 500 mL of distilled water (1:1) for one week. The extract was drained and collected in the filtrate, and from the filtrate, solvents were evaporated with a rota evaporator under reduced pressures to get viscous mass (188 gm). The dried viscous mass was suspended in 400 mL of purified water and then fractionated with hexane, ethyl acetate, and methyl acetate. The fractions were collected and solvents were evaporated. Ethyl acetate (10 g) and methyl acetate (167 mg) fractions were crystallized with methanol, ethanol, and distilled water. Finally, it will get a gallic acid mixture (both fractions). Preliminary phytochemical analysis of both fractions was identified by TLC (precoated) mobile phase was used as Formic acid: Toluene: Ethyl acetate (1:6:6). Eluted spot detected by iodine as detecting agent.[12] FTIR data was taken and functional groups were identified for both fractions of *T. chebula* (Dried fruits).

Chemical Marker and Chemicals

Gallic acid reference standards were purchased from "Yucca Enterprises, Mumbai" (19.0206° N, 72.8679° E). Required 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, 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- 17.4760781.

Instrumentations

The liquid chromatography was executed on a Shimadzu LC 2010 HPLC (UPLC) instrument consisting of an ultrafast autosampler, UV-VIS detector, and with lab solutions software.

C_{18} Inertsil column (150 × 4.6 mm, 5 µm particle size) was used as the immobile phase. FTIR Model-8400S (Shimadzu), KBR pressing, Software-Shimadzu-IR-Solution.[13]

Preparation of Reference Marker and Test Solutions

Reference marker solution preparation:

Weigh around 50 mg gallic acid was taken and poured into 50 mL of VF, GA dissolved and made up to the mark with the mobile phase. The Final stock solution was prepared according to requirements, maybe 10, 50, 75, or 100 µg/mL with the mobile phase.

Sample solution preparations

Weigh around 50 mg sample was taken and poured into 50 mL of VF. Sample dissolved and made up to the mark with the mobile phase. The Final stock solution was prepared according to requirements, maybe 10, 50,75, or 100 µg/mL with the mobile phase.[19,24]

Developed "HYDALJSS08" polyherbal formulation preparations

Accurately taken 5 mL of liquid sample was transferred into a 50 mL of VF, 30 mL of diluent was sonicated for 10 minutes, made the final volume was with mobile phase, and mixed well. Centrifuged this solution at 5000 rpm for 10 minutes. The supernatant was further diluted 1 to 10 mL with the diluting agent, mixed, well, and injected into the liquid chromatography System.[14] The prepared solution was stored under refrigerator conditions (Figure 1).

ANALYTICAL METHOD DOCUMENTATION

ICH guidelines were followed for the implementation of documentation Parameters. The suggested method is valid for all the important analytical parameters.[20,22]

Accuracy

The accuracy of the proposed work was done for recuperation studies using the method of standard addition. The percentage of recovery was carried out in three different concentrations 50, 100, and 150%, and each concentration was analyzed in triplicate.

Linearity

The linearity of marker solutions was measured by the different concentrations between the ranges of (50–150 µg/mL). The standard curve for the sample was built by plotting the average peak area against the concentration and a regression equation. Pearson's r value was also assessed.

Limit of Detection and Limit of Quantitation Determination

The LoD is the minimum quantity of analyzing substances in a test solution that can be easily notable from zero. The LoQ is the little volume of analyzing materials that can be quantified with allowable accuracy and precision.

System Precision and Method Precision

The precision approach was tested by administering the marker solutions six times with andrographolides. Apex areas were as set on and compared. Precision demonstrated as a %RSD.[17,18]

Specificity

Preparation of placebo solution

Weighed crushed Placebo powder equivalent to 200 mg of gallic acid transferred to 200 mL VF, diffused with 70 mL of mobile phase by 30 minutes of sonication, and made up the volume with the mobile phase. The sample was centrifuged at 5000 rpm for 10 minutes. The above Placebo supernatant solution was diluted up to 5 to 50 mL with the mobile phase. A blank test solution was administered, and the obtained chromatogram was recorded.[19,20]

RESULTS AND DISCUSSION

The recommended technique was initiated to be novel, sensitive, accurate, simple, precise, economical, and quick for the regimen of liquid chromatography development and documentation of Gallic acid in a combined dosage form of polyherbal formulations.[21] The preliminary identification of GA by thin-layer chromatography, mobile phase used as a Formic acid: Toluene: Ethyl acetate (1:6:6) in ethyl, methyl acetate fraction of *T. chebula* dried fruits and marketed Siddha-based polyherbal formulation known as "Kabusura Kudineer," and the Rf value of GA was found to be 0.27. result data summarized in Figures 2 and 3. The FTIR spectral analysis was compassed for ethyl, methyl acetate fraction of *T. chebula* fruits, and standard GA data summarized in Figures 4 and 5. HPLC method expansion and validation of marker GA were executed along with the fraction (Methyl, ethyl acetate) of *T. chebula* fruits and Polyherbal formulations

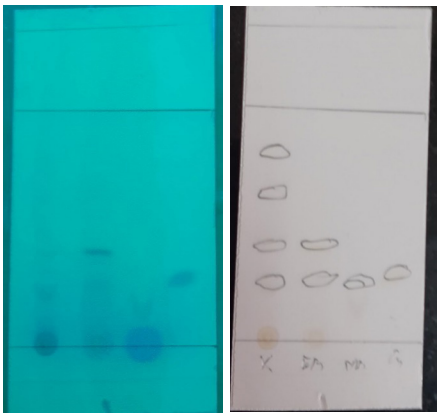


Figure 1: Typical TLC of reference standard GA, and ethyl, methyl acetate fraction of *Terminalia chebula* dried fruits and Polyherbal formulation (Kabusura kudineer)

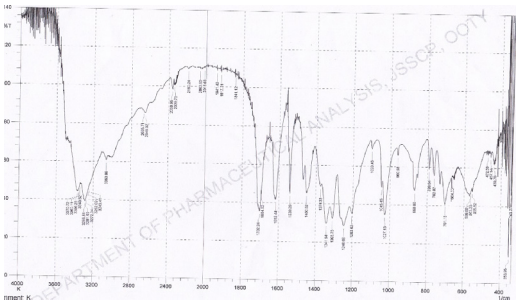


Figure 2: Typical FT-IR spectrum of reference standard GA

"Kabusura Kudineer" (Marketed), "Newly developed "HYDALJSS08" Polyherbal formulation". The optimized mobile phase for liquid chromatography development was used as water: Acetonitrile (50:50) and GA were detected in the UV detector at 272 nm. The retaining time of GA was initiated as 2.2 minute and the full run time of the chromatogram was 8 min data was summarized in Figure 6. To confirm the system's suitability is operating correctly and can give precise and accurate results 100 µg/mL of GA was inserted on six occasions and the chromatograms were put down for the same. Standard deviation (14247.75) and %RSD (0.9) were found to be adequate.[22] The plate count and tailing factor outcomes were initiated to be within the control and the % RSD was reported to be 0.266% so the system is capable and gives pinpoint outcomes. A calibration curve was established for GA by injecting 50, 80, 100, 120, and 150 µg/mL of benchmark solution.[23,24] Linearity was conducted by analyzing the GA average peak area by different injection volumes (concentration), and then, the regression equation of GA was initiate to be y = 25511 x−947505 and the correlation coefficient (R2) for the same was 0.9999 respectively. GA has shown good linearity at a concentration ranging from 50 to 150 µg/ml data outcomes are illustrated in Tables 1 and 2 and Figure 7. The LoDs and LoQs of GA was found to be 1.70, 5.16 µg/mL, data results summarized in Table 3. Method precision was analyzed by six concentrations' % RSD (0.8) and the standard deviation (11626.7) of samples of GA was found to be inside the conformity benchmarks (less than 2.0%). Thus, the method is precise facts outcomes are illustrated in Table 1. The analytical marker of the drugs (50, 100**,** and 150 µg/mL) were added at 50, 100, and 150% levels. The percentage mean recovery of GA was initiate between 99.8 and 101.1%. Information outcomes are demonstrated in Table 3. The outcomes obtained by deliberate variation in method parameters are abstracted. The tailing factor and theoretical plates were found to be within the limits of GA with small variations of flow rate as well as wavelength observed, information outcomes are demonstrated in Table 2. The %RSD of assay values between two analysts should be not more than 2.0. From the outcomes %assay and %RSD earned *obedience* standards 2.0% so the technique is rugged facts outcomes are demonstrated in Table 2.[26,27] Estimation of GA in Ethyl, methyl acetate fraction of *T. chebula* fruits, Kabusura Kudineer polyherbal formulation (Marketed), and developed "HYDALJSS08" Polyherbal formulation was found as satisfactory, Samples results from data as summarized in Table 4 and Figures 8-11.

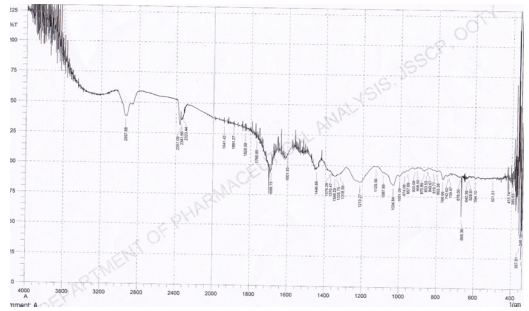
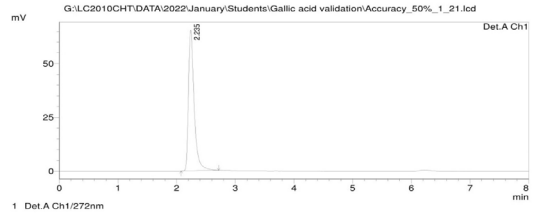


Figure 3: Typical FT-IR Spectrum for Ethyl acetate Fraction of Dried *Terminalia chebula* fruits



PeakTable

tector A Ch1 272nm

Peak#	Ret. Time	Area	Height	heoretical Plates/met	Area %	Tailing Factor
1	2.235	752141	65435	16490.010	100.000	1.675
Total		752141	65435		100.000	

Figure 4: Typical liquid chromatogram of reference marker GA

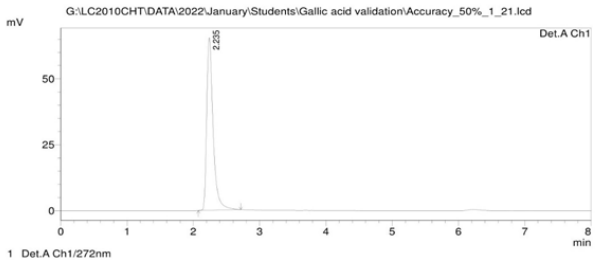


Figure 5: Typical liquid chromatogram of reference marker GA

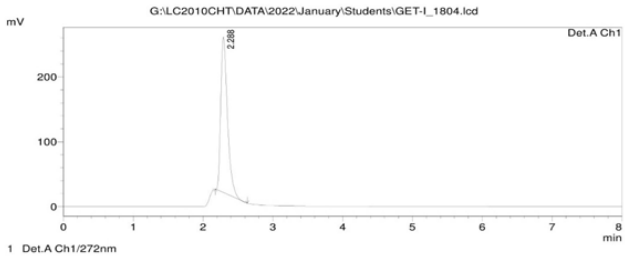


Figure 6: Typical liquid chromatogram of GA in ethyl acetate fraction of terminalia chebula dried fruits.

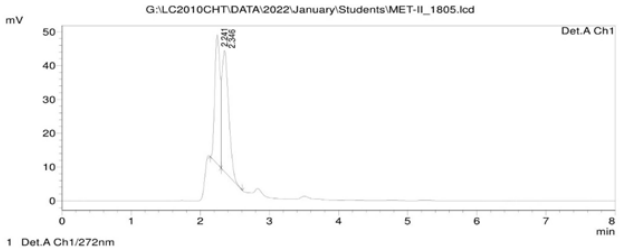


Figure 7: Typical liquid chromatogram of GA in methyl acetate fraction of terminalia chebula dried fruits

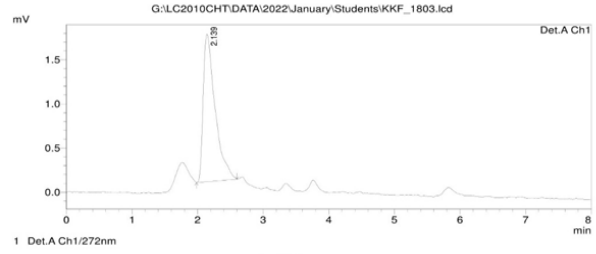


Figure 8: Typical liquid chromatogram of GA in "Kabusura Kudineer" Marketed

Table 1: Intra- and inter-day results of method precision and results of linearity and range of gallic acid

S. No	*Intra-day Precision (n=6)*		*Inter-day precision (n=6)*		*Linearity and Range*	
	Con 100 µg /mL	*Area*	*Con 100 µg /mL*	*Area*	*Concentration (µg/mL)*	*Area*
1	1	1474503	1	1534503	50.0	340164
2	2	1473166	2	1523166	80.0	1075077
3	3	1497609	3	1497609	100.0	1605908
4	4	1474855	4	1534855	120.0	2112083
5	5	1461940	5	1511940	150.0	2884606
6	6	1477221	6	1517221	-	-
7	Average	1476549	Average	1519882	-	-
8	Std dev	11626.7	Std dev	14247.75	-	-
9	% RSD	0.8	% RSD	0.9	-	-

Table 2: System suitability and ruggedness results of GA

S. No	*Concentration (µg/mL)*	*Area*	*Ruggedness Results of GA*	
1	Linearity & Range	50-150 µg/mL	Analyst 01	99.4%
2	Regression equation	y = 25511 x-947505	Analyst 02	99.7 %
3	Correlation coefficient	0.9999	%RSD	0.2%
4	Theoretical plates	17411.12	-	-
5	Tailing Factor	1.518	-	-
6	LOD	1.70	-	-
7	LOQ	5.16	-	-
8	Slope	25511	-	-
9	Intercept	947505	-	-

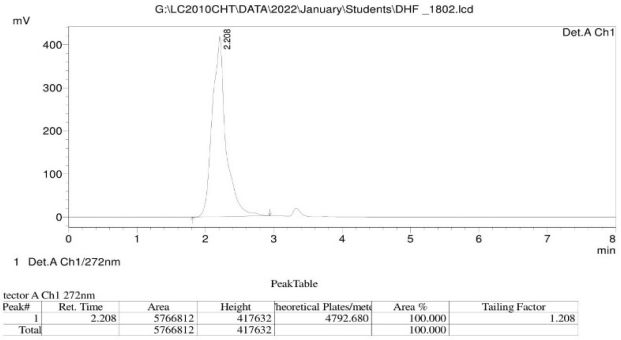


Figure 9: Typical liquid chromatogram of GA in developed "HYDALJSS08" polyherbal formulation (Asavas/Arishtas).

Table 3: Results of recovery studies for GA

S. No	*Concentration in (%)*	*Concentration in (µg/mL)*	*Area*	*% Recovered*	*% Recovery*
		50	752141	99.0	
1	50	50	761276	100.2	99. 8
		50	751136	98.8	
		100	1505219	99.0	
2	100	100	1512494	99.5	99.4
		100	1514387	99.6	
		150	2294031	100.6	
3	150	150	2307185	101.2	101.1
		150	2312963	101.5	

Table 4: Percentage purity calculation of isolated GA in *Terminalia chebula* dried fruits and in polyherbal formulations

S. No	Sample	Area	% Assay	in µg/mL	in mg/mL
1	KKR-Kabusura Kudineer-Marketed.	21147	1.4	1.39	0.00139
2	DHF-Developed "HYDALJSS08" Polyherbal Formulation.	5766812	379.4	379.4	0.379
3	GET-Estimation of GA in Ethyl acetate fraction	1604378	105.6	105.59	0.10559
4	MET-Methyl acetate fraction of Gallic acid.	443286	29.2	29.17	0.02917

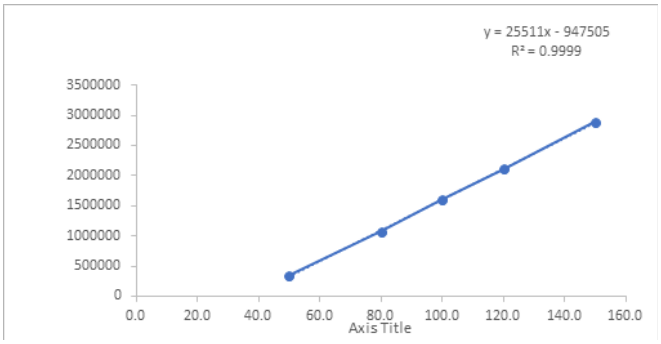


Figure 10: Typical linearity graph for GA

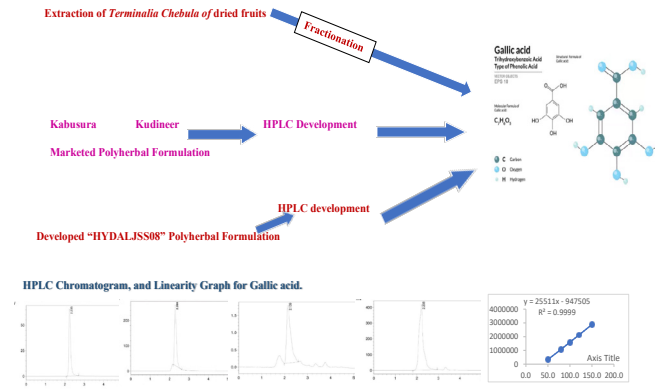


Figure11: Typical Graphical Hypothesis, and Abstract HPLC development, validation of GA, in Fraction of *Terminalia Chebula* dried fruits and polyherbal formulations marketed and developed

CONCLUSION[28,29]

In the present work, a novel, rapid, linear, reliable, accurate, and simple method was developed to estimate GA and chemical compounds present in ethyl, methyl acetate fraction of *T. chebula* fruits along with polyherbal formulations "Kabusura kudineer" (Marketed), Newly developed "HYDALJSS08" Polyherbal formulation by using HPLC. The obtained results of GA give an idea that the method appears to have logical linearity, recovery, reproducibility, and low LoD and LoQ. The developed method is most widely accepted in the identification and quantification of GA in *T. chebula* fruits as well as combined dosage form development in Ayurvedic-based polyherbal formulations.

AVAILABILITY OF DATA AND MATERIALS

The supporting information for this study's findings is available at the corresponding authors upon reasonable request.

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