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Grewia asiatica Linn. root extract loaded suspension, microparticulate and nanosuspension dosage form: Fabrication, characterization and anthelmintic evaluation

Tarun Kumar[1], Rimpy Pahwa[2], Munish Ahuja[2*]

1 Department of Pharmaceutical Sciences, Central University of Haryana, Mahendergarh 123031 (Haryana), India

2 Drug Delivery Research laboratory, Department of Pharmaceutical Sciences, Guru Jambheshwar University of Science and Technology, Hisar 125001 (Haryana), India

ABSTRACT

The current study was planned to formulate the *Grewia asiatica* Linn. root extract loaded herbal formulation and evaluated its potential for *in vitro* anthelmintic activity. The root extract of *Grewia asiatica* Linn. was standardized by HPTLC using quercetin and naringenin, as a marker compound. The extract was formulated into a novel microparticulate and nanosuspension dosage form based on the Eudragit S100. The prepared dosage forms were characterized by particle size, morphology, and *in vitro* release behavior. Further, the comparative evaluation of dosage form revealed the presence of higher *in vitro* anthelmintic activity in nanosuspension as compared to conventional suspension and microparticulate dosage form.

Keywords: *Grewia asiatica*; nanosuspension; microparticles; suspension; anthelmintic

INTRODUCTION

Helminths i.e. parasitic worms of the gastrointestinal tract are pathogens, present as a constraints in livestock all over the world. Helminths not only tend to increase other bacterial infections in the body but also reduce growth rate and food production (e.g. milk and meat). Approximately, ten billion people are

*Corresponding author: Munish Ahuja
E-mail: munishahuja17@yahoo.co.in
ORCIDs:
Tarun Kumar: 0000-0002-5450-1586
Rimpy Pahwa: 0000-0003-2087-1105
Munish Ahuja: 0000-0001-6723-140X
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affected in remote areas [rural (68%), & urban (50%)] from soil-transmitted helminthiasis diseases like *Ascariasis*, hookworm, *Trichuriasis,* etc. To treat the infections, several synthetic anthelmintic drugs are available in the market with high cost, toxicity, and drug resistance[1,2]. So on reviewing the literature, herbal drugs were chosen to formulate the required anthelmintic formulation to avoid any side effects and to improve the efficacy with synergistic effects.

Herbal drugs, a part of the traditional system of medicine, are widely present all around. The safer pharmacological profile, economical value, and easy availability make herbal tonics a choice of interest for the development of pharmaceutical formulation[3]. *Grewia asiatica* Linn. (belonging to the family – *Tiliaceae*) is commonly known as "Phalsa" and is found in Asian countries. It is comprised of fruits, shrubs, and trees and has a height of up to 6-8 m. Of 150 species of this genus, nearly 40 species are used in the folklore medicine[4,5]. The term "*Grewia*" comes from the 'Nehemiah Grew' i.e. name of plant physiology founder and "*asiatica*" which reflects the Asian origin of species[6]. This seasonal summer plant contains phenols, tannins, saponins, and flavonoid compounds. Fruits of *Grewia asiatica* contain vitamins, proteins, minerals, fibers, and ash. It has also many reported therapeutic properties such as antimicrobial; analgesic; antipyretic; antioxidant; anthelmintic; antimalarial; anti-hyperlipidemic; rheumatism, radioprotective and anti-diarrhoeal etc[7]. It has already been reported that leaves and extract from *Grewia asiatica* Linn. have high anthelmintic activity but due to the lack of scientific data, and its clinical effectiveness, it needs to be explored more efficiently for further evaluation.

Henceforth, the present study was designed to formulate three different pharmaceutical dosage forms such as suspension, microparticulate, and nanosuspension of *Grewia asiatica* Linn. root extract. However, a standardized extract of *Grewia asiatica* Linn. was used to formulate dosage forms which were characterized by using FT-IR, SEM, and TEM and further evaluated for *in-vitro* anthelmintic activity.

METHODOLOGY

Chemicals and reagents

Grewia asiatica Linn. roots were taken and authenticated in CSIR-NISCAIR, New Delhi, India under voucher specimen no. NISCAIR/RHMD/Consult/2013/2631-141-1 dated 07-01-2014. Albendazole was collected from Coax Bioremedies (Hisar, India). Quercetin, toluene, and ethyl acetate were purchased from Hi-Media Laboratories Pvt. Ltd., (Mumbai, India). Naringenin, formic acid, and methanol were procured from Sisco Research Laboratories

Pvt. Ltd., (Mumbai, India). Silica gel F_{254} HPTLC plates were obtained from Machery-Nagel (Germany). Silica gel UV254, Sodium carboxymethyl cellulose, Tween 80, methyl and propylparaben, lemon oil, Eudragit S100, and Poloxamer 188 were collected from S.D. Fine-Chem Ltd. (Mumbai, India). Other analytical grade chemicals and reagents were used as received.

Preparation of *Grewia asiatica* extract

Grewia asiatica Linn. roots were dried under shade and powdered coarsely which was extracted with distilled water and ethanol. Briefly, alcoholic extracts were prepared with 95% ethanol by a continuous soxhlation process and aqueous extracts were prepared by a cold maceration process. Both extracts were concentrated to a semi-solid residue. Aqueous and alcoholic extracts were suspended in saline phosphate buffer for further usage[8].

Evaluation of anthelmintic activity

Adult Indian earthworms of size 9-12 cm long and 0.1-0.2 cm wide (*Pheretima posthuma*) were collected from the moist soil of the farm field (Hisar, India). The anthelmintic activity of the aqueous and ethanolic extracts of *Grewia asiatica* Linn. roots against *Pheretima posthuma* were evaluated comparatively by employing albendazole as a standard drug. The earthworms were divided into 12 groups of six worms each.

Group 1 : Control (treated with 25 ml of phosphate buffer saline)

Group 2 : Standard (treated with 500 mg of albendazole)

Group 3-7 : Treated with ethanolic extracts of *Grewia asiatica* Linn. (GAEE) roots at five levels of concentration i.e. 12.5, 25, 50, 75 and 100 mg/ml respectively

Group 8-12 : Treated with aqueous extracts of *Grewia asiatica* Linn. (GAAE) roots at five levels of concentration i.e. 12.5, 25, 50, 75 and 100 mg/ml respectively

The worms in group 1 (Control) were treated with phosphate buffer saline, while the group 2 (Standard) worms were treated with albendazole suspension (20 mg/ml) in phosphate buffer saline. Further, GAAE and GAEE were screened at five levels of concentration i.e. 12.5, 25, 50, 75, and 100 mg/ml. The earthworms were placed in petridishes (diameter 9cm) containing 25 ml of the aqueous vehicle (control) or albendazole (standard) or extract as per their treatment protocol. The earthworms were observed for paralysis and death over a period of time and the time taken for paralysis and death of worms was noted and the mean was calculated. The paralysis of worms was established

when the worms become immobile even in warm saline phosphate buffer, while the death was confirmed when the worms showed loss of motility followed by fading of body color [9, 10, 11].

HPTLC Analysis

HPTLC analysis was carried out using aluminum-backed plates pre-coated with silica gel F_{254} (20 x 20 cm, 200 μm layer thickness). The samples were spotted with CAMAG 100 μl syringe using a CAMAG Linomat V automatic sample spotter, starting at the point x = 15 mm and y = 10 mm, in the form of distinct bands of width 4 mm and 10 mm apart. The optimized equipment parameters used were:- application rate 160 nl/sec, slit dimension:- 3 x 0.45 mm and scanning speed of 20 mm/sec. The plates were developed in a linear ascending mode in a twin trough (20 x 10 cm) glass chamber which was previously saturated with the mobile phase for 15 min at a temperature of (25±2)°C and RH of (60±5)%. The chromatogram was developed with a run height of 80 mm and a mobile phase volume of 15 ml (a TLC study was carried out to select the suitable mobile phase for the separation of compounds). The densitometric analysis of plates was performed at 254 nm in absorbance mode employing CAMAG TLC scanner-III having a tungsten lamp as a radiation source and win CATS software (version 1.2.0)[12,13,14].

GAEE-loaded suspension dosage form

The suspension dosage form of GAEE (5%) was formulated using sodium carboxymethyl cellulose as suspending agent (0.5%, 1%, 1.5% and 2%), Tween 80 as wetting agent (0.1%), lemon oil as flavouring agent (0.1%), and methylparaben (0.08%) and propylparaben (0.03%) as preservative.

Evaluation

The prepared GAEE conventional suspensions were evaluated organoleptically for color, odor and taste; pH and viscosity were determined at 25°C; sedimentation volume and ease of redispersibility were measured after 1 day and 1 week respectively; followed by an accelerated stability study at 25°C and 40°C for 3 months.

GAEE-loaded Eudragit S100 microparticles

GAEE-loaded Eudragit S100 microparticles were prepared by spray drying technique. Briefly, an ethanolic solution (250 ml) of Eudragit S100 (1%, w/v) containing GAEE (10%-50%, w/w of Eudragit S100) was sprayed through the spray dryer (LSD-48, JISL, Mumbai, India) under the following conditions:- nozzle: ultrasonic nozzle; inlet air temperature: 85°C; outlet air temperature:

43°C; aspirator: 41%; feed rate: 1%; a vacuum in the system:- 80 mmWC and atomization pressure: 2 kg/cm^2 [15, 16].

Characterization of microparticles

GAEE-loaded Eudragit S100 microparticles were characterized for size by optical microscopy and morphology by scanning electron microscopy.

Microscopical size

Micromeritic analysis of microparticles was carried out using optical microscopy. A pinch of powdered GAEE-loaded Eudragit S100 microparticles was taken on a glass slide and observed under an optical microscope. The eyepiece of the microscope was fitted with an ocular micrometer previously calibrated with a stage micrometer. The photomicrographs were taken using Zeiss Primostar trinocular microscope with a Canon photomicrograph unit.

Morphology

Scanning electron microscope (JEOL, JSM-6100, Tokyo, Japan) was used to observe morphological features and surface topography of microparticles. The gold-coated sample was mounted on a sample holder for capturing the photomicrographs at an accelerating voltage at 10kV at different magnification.

Evaluation of microparticles

Drug content

GAEE-loaded Eudragit S100 microparticles were assayed for the contents of quercetin and naringenin. An accurately weighed 50 mg of the microparticles were dissolved in 10 ml methanol. The contents of quercetin and naringenin were determined by the HPTLC method of analysis as reported above.

In vitro drug release

The *in vitro* release behavior of GAEE-loaded Eudragit S100 microparticles was determined by employing USP type-II dissolution rate test apparatus (TDL-08L, Electrolab, Mumbai, India). GAEE-loaded Eudragit S100 microparticles equivalent to GAEE (50 mg) were placed in muslin cloth, which was tied to the paddle. The paddle was dipped in 500 ml phosphate buffer saline (pH 7.4), maintaining the temperature at (37±0.1)°C, and rotated at speed of 50 rpm. Aliquots of 0.1 ml sample were withdrawn at a specific time interval and substituted with an equivalent volume of release media[17,18]. The withdrawn specimens were investigated for the contents of quercetin and naringenin using the HPTLC method of analysis as mentioned earlier.

GAEE-loaded Eudragit S100 nanosuspension

GAEE-loaded Eudragit S100 nanosuspension was synthesized using the nanoprecipitation method [19]. Briefly, 250 ml solution of GAEE (0.5 %, w/v), Poloxamer 188 (1%, w/v), and Eudragit S100 (1%, w/v) in acetone was prepared. This solution was introduced steadily using a hypodermic glass syringe into distilled water (500 ml), with continuous stirring. A rotary vacuum evaporator (Strike 102, Steroglass, Italy) was used to evaporate the acetone in obtained dispersion. The resulting nanosuspension was dried employing a lyophilizer and spray dryer. For lyophilizer, mannitol (5%, w/v) as cryoprotectant was added in suspension and kept in a deep freezer at -80°C for overnight, followed by lyophilization in a laboratory model freeze dryer (Alpha 2-4 LD Plus, Martin Christ, Germany) at -90°C for 24 h, at 0.0010 mbar. The other batch of nanosuspension was spray-dried using a lab model spray drier (LSD-48, JISL, Mumbai, India) under the following conditions:- nozzle: ultrasonic nozzle; inlet air temperature: 150°C; outlet air temperature: 61°C; aspirator: 42%; feed rate: 4%; a vacuum in the system:- 80 mm WC and atomization pressure: 2 kg/cm^2. The lyophilized powder of GAEE-loaded Eudragit S100 nanosuspension containing equivalent to 1250 mg of GAEE was dispersed in 25 ml Sorenson's phosphate buffer (0.0667M, pH 7.4) to prepare the nanosuspension containing GAEE equivalent of 50 mg/ml [20].

Characterization of nanosuspension

Particle size and zeta potential measurement

The mean particle size and zeta potential of the GAEE-loaded Eudragit S100 nanosuspension were evaluated using Zetasizer Nano ZS90 (Malvern Instruments, UK). The equilibration time and temperature were kept at 120s and 25°C respectively[21].

Transmission electron microscopy (TEM)

A transmission electron microscope (FEI Tecnai G^2 F20 S-Twin, Bellaterra, Spain) was used to determine the morphology of the prepared nanosuspension at 200 kV[22].

Evaluation of nanosuspension

Drug content

An accurately weighed lyophilized powder equivalent to 50 mg of GAEE was dissolved in methanol with the help of sonication. The contents of quercetin and naringenin were determined by the HPTLC method of analysis.

In vitro release behavior

The *in vitro* drug release of GAEE nanosuspension was studied in USP type-II dissolution rate test apparatus (TDL-08L, Electrolab, Mumbai, India) using the dialysis sac method. The specific amount of nanosuspension (10 ml), placed in a dialysis membrane (cut off: 10,000 Da) was immersed into phosphate buffer solution (500 ml, pH 7.4) at 37°C and stirred at 50 rpm with the help of a sinker. At respective time intervals, aliquots of 0.1 ml sample were removed and replaced with an equal volume of release media[19]. The withdrawn samples were analyzed for the contents of quercetin and naringenin by HPTLC.

Evaluation of GAEE formulations for *in vitro* anthelmintic activity

The conventional suspension, GAEE-loaded Eudragit S100 microparticles, and GAEE-loaded Eudragit S100 nanosuspension, all equivalent to GAEE 50 mg/ml each, were screened for *in vitro* anthelmintic activity using adult Indian earthworm (*Pheretima posthuma*) as mentioned earlier [23]. To rule out the anthelmintic action due to the presence of excipients, the anthelmintic action of excipients was also carried out *i.e.* vehicle control (for suspension), Eudragit S100 (for microparticles and nanosuspension), and Poloxamer-188 (for nanosuspension). In addition, the effect of quercetin, naringenin, and their combination was also comparatively evaluated.

Statistical analysis

All the results are expressed as mean ± standard error of the mean. Analysis of the statistics was determined using one-way ANOVA and Dunnett's *t*-test. A value of $P>0.05$ was considered significant at $P>0.05$.

RESULTS and DISCUSSION

Synthetic anthelmintic drugs have been the mainstay of treatment for the eradication of helminths even though the resistance to them is developing. Their toxicity, increased cost, and inaccessibility in remote areas have added barriers to achieving the goal of deworming. A large proportion of populations in the developing world rely on the traditional system of medicine which use herbs and herbal derivatives to treat several diseases. Several plants have been documented to possess anthelmintic activity in ancient literature and some of them have been tested but still, a large number of plants have not been used clinically[24,25]. Among these plants, *Grewia asiatica* Linn. is one of the active plants which is used traditionally for the treatment of helminthiasis but there is no scientific report to validate their use as anthelmintic. Thus, the roots of *Grewia asiatica* Linn. were used to prepare the aqueous and alcoholic extract.

From both prepared extracts, the alcoholic extract was chosen based on its extractive value (38.6±0.54%,w/w)[8]. Further, the extract was formulated into different pharmaceutical dosage forms and comparatively evaluated for *in vitro* anthelmintic activity.

Selection of extract and its anthelmintic activity

The effect of different concentrations of GAEE and GAAE roots with albendazole on the paralysis and death time of worms were compared in figure 1. It can be observed that the worms treated with vehicle control (saline phosphate buffer) did not show any paralysis or death during the 2 h period of observation. The worms treated with albendazole suspension (20 mg/ml) get paralyzed and died in 29 min and 58 min respectively. It was estimated that GAEE was more efficacious than aqueous extracts. The result of the ANOVA analysis revealed that there was no significant difference in the paralysis and death time of worms treated with albendazole (20 mg/ml) and ethanolic extract (GAEE, 50 mg/ml). However, ethanolic extracts at a concentration of 75 and 100 mg/ml showed better efficacy than albendazole for paralyzing and killing the earthworm. In the previous literature, the anthelmintic activity of methanolic extract of *Grewia asiatica* leaves is already reported with *Ferula assafoetida* Linn. resin, *Ipomoea hederacea* Jacq. seeds, *Lepidium sativum* Linn. seeds, and *Terminalia chebula* Retz. fruits[26].

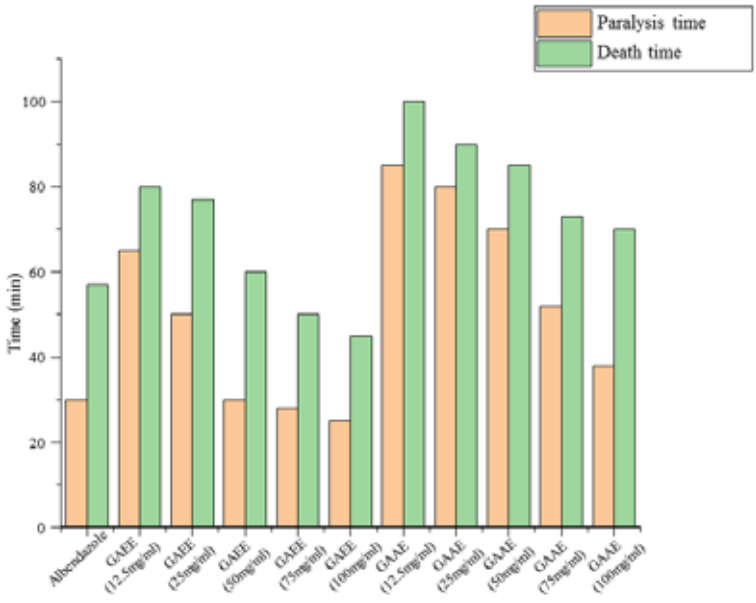


Figure 1. Anthelmintic activity of GAEE and GAAE

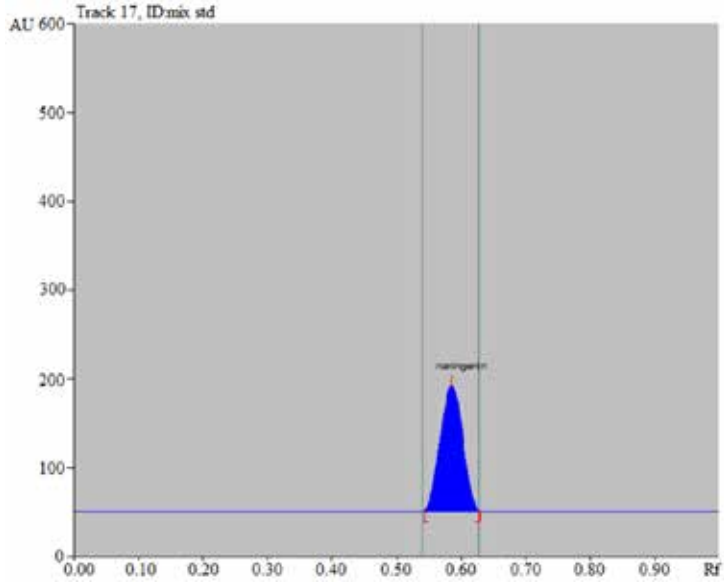
The results of anthelmintic screening of selected plant extracts reveal that the anthelmintic action of extracts followed the order GAEE > GAAE. Thus, GAEE which showed more anthelmintic activity (at a concentration of 50 mg/ml) comparable to the albendazole (at a concentration of 20 mg/ml) was selected for further development of the herbal formulation.

HPTLC analysis

The selection of the mobile phase for running the HPTLC chromatogram was done by a preliminary TLC study. Various composition of mobile phase such as ethyl acetate: methanol: water (60:30:10, 90:20:10, 80:15:05, v/v/v), toluene: ethyl acetate: formic acid (60:40:02, 60:40:04, 60:40:06, 60:40:08, v/v/v) were tried to optimize the resolution of bands. Among these, toluene: ethyl acetate: formic acid (60:40:08, v/v/v) was observed to give the best resolution of quercetin (R_f = 0.44) and naringenin (R_f = 0.62), which act as marker components [27]. Thus, this mobile phase composition was employed further to develop a suitable HPTLC densitometric method of analysis in the GAEE. The identity of bands of quercetin and naringenin in the plant matrix was confirmed by comparing their spectra with the spectra of respective standards.

The HPTLC chromatogram of quercetin, naringenin, a combination of quercetin and naringenin dosage form, and GAEE dosage form is shown in figure 2. The plot between the concentration of naringenin (200-3000 ng/band) and the peak area of naringenin was found to be linear with the equation of line being Y = -159.295 + 5.188X and correlation coefficient (R^2) of 0.99403 [28]. However, the plot between the concentration of quercetin (100-3000 ng/band) and the peak area of quercetin was found to be linear with the equation of line being Y = 99.297 + 1.417X and correlation coefficient (R^2) of 0.99787. The content of quercetin and naringenin in the GAEE were calculated and found to be 2.24 mg and 21.117 mg/gram of the extract respectively. The limit of detection (LOD) & limit of quality (LOQ) was found to be 50 ng & 100 ng respectively for naringenin and 100 ng & 200 ng for quercetin. Also, the 3D densitometric TLC profile of the GAEE suspension dosage form is shown in figure 2[29].

Thus, the developed HPTLC method is suitable for quantification of quercetin and naringenin in *Grewia asiatica* extract and would be useful for standardization of *Grewia asiatica* extract and its formulation.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0.80
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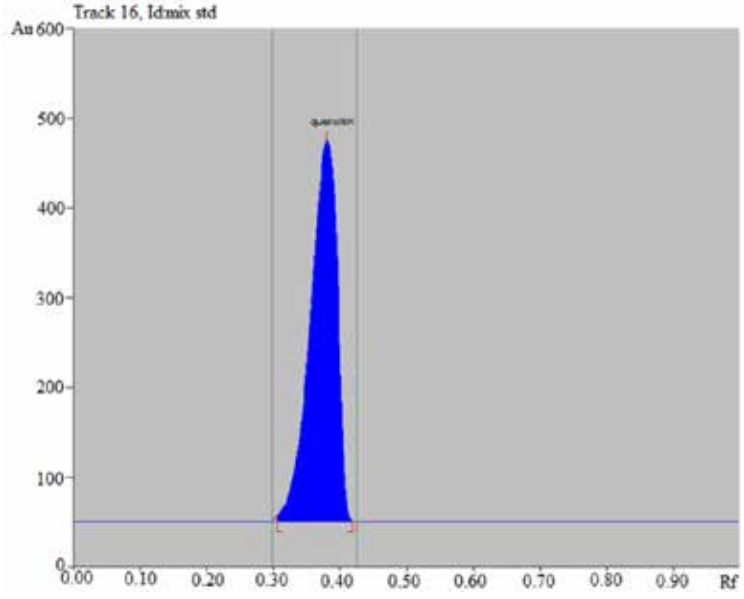
Rf

(a)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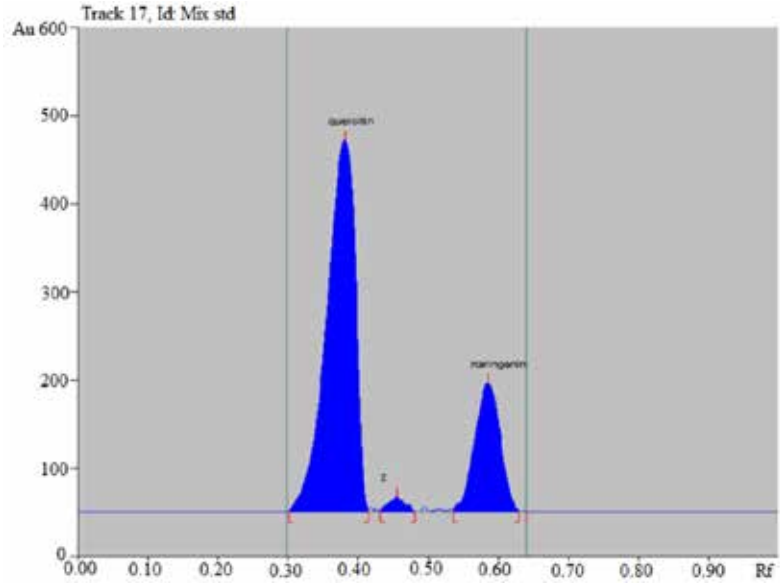
Rf

(b)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naringenin
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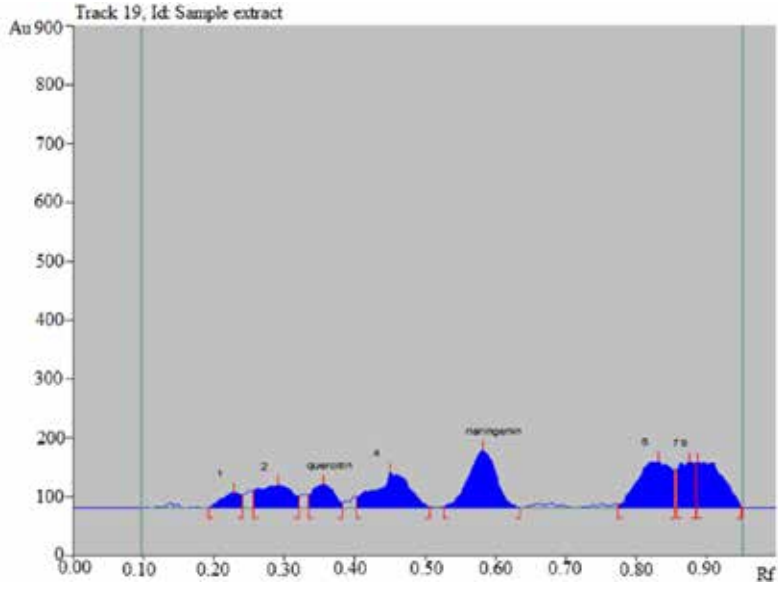
(c)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300
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100
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1
2
quercetin
4
naringenin
5
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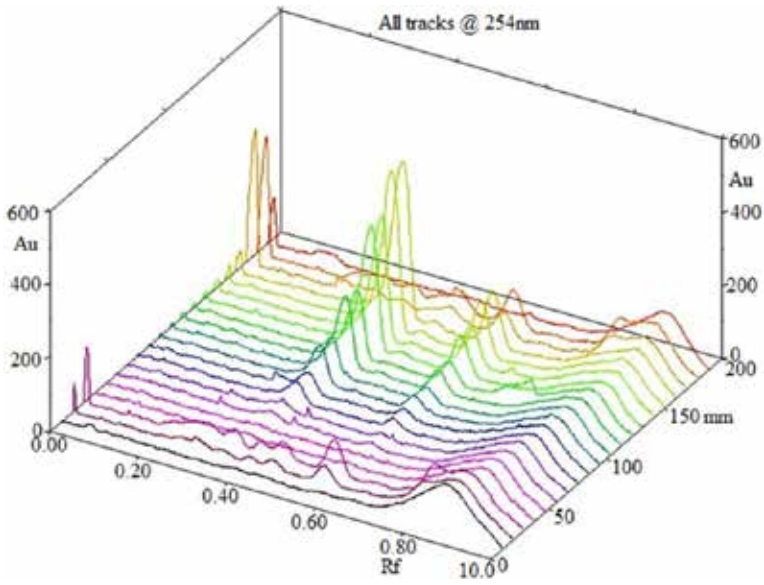
0.00 0.10 0.20 0.30 0.40 0.50 0.60 0.70 0.80 0.90 Rf

(d)



(e)

Figure 2. HPTLC chromatogram of standards naringenin (a), quercetin (b), a combination of quercetin and naringenin (c), and GAEE (d), and densitometric TLC profile showing GAEE (e)

Development of herbal formulation

After confirmation of anthelmintic activity present in standardized *Grewia asiatica* extract, different herbal formulations were developed by using the ethanolic extract for further pharmaceutical applications.

GAEE-loaded Eudragit S100 suspension dosage form

Solution dosage forms, if palatable, are usually chosen, as, these dosage forms are absorbed rapidly with better bioavailability in the human body. Since GAEE was partially soluble in water; so its suspension was selected for developing the dosage form. Sodium carboxymethyl cellulose in concentration of 0.5% (GES_1 and GES_2), 1% (GES_3), 1.5% (GES_4) and 2% (GES_5) was used as suspending agent. As, the suspension without a wetting agent (GES_1) was not dispersible, so Tween-80 was added as a wetting agent at a concentration of 1% (v/v) in formulation GES_2 which was found to adequately disperse the extract. A combination of methyl and propyl paraben was employed as a preservative. To impart a pleasant flavor, lemon oil was incorporated.

Table 1. Physicochemical parameters of GAEE suspension formulations

Physicochemical Parameters	Formulations				
	GES_1	GES_2	GES_3	GES_4	GES_5
Color	Brownish Yellow	Brownish Yellow	Brownish yellow	Brownish yellow	Brownish yellow
Odor	Characteristic	Characteristic	Characteristic	Characteristic	Characteristic
pH	6.51	6.54	6.91	7.12	7.18
Nature	Liquid	Liquid	Liquid	Liquid	Liquid
Texture	Pourable	Pourable	Pourable	Poorly pourable	Poorly pourable
Sedimentation Volume	16.76	16.66	7.69	5.55	5.26
Redispersibility	—	100%	95%	70%	50%
Viscosity (cps)*	300	310	330	370	430

** Viscosity at 100 rpm determined using spindle 3*

Table 1 shows the results of the characterization of various batches of suspension. The pH of the suspension was found to vary from 6.51-7.18, with an increase in the pH of suspension with an increase in the content of sodium CMC. The batch of suspension without Tween-80 was not dispersible while batches prepared with Tween-80 were homogeneously dispersed. Further, it can be observed that the rate of sedimentation decreased with an increase in the concentration of sodium CMC. However, the increase in sodium CMC concentration also affects their redispersibility, which can be attributed to the increase in viscosity. Moreover, suspension prepared using sodium CMC (1.5 %, w/v and 2.0 %, w/v) were poorly pourable. Similar results have been reported in the study evaluating the *Grewia ferruginea* mucilage as a suspending agent in metronidazole suspension[30]. Considering the settling rate, ease of redispersion, and pourability, suspension of batch GES_3 was selected for conducting further studies.

The results (Table 2) of accelerated stability studies done on batch GES_3 of suspension revealed that there was no significant difference in the physicochemical properties of the suspension over the study period. Thus, this batch of *Grewia asiatica* extract suspension (GES_3) appears as the optimal formulation, for further *in-vitro* anthelmintic evaluation.

Table 2. Accelerated stability studies of GES_3 suspension

Physicochemical Parameters	Time Period									
	Initial		15 Days		30 Days		45 Days		90 Days	
	25°C	40°C	25°C	40°C	25°C	40°C	25°C	40°C	25°C	40°C
Color	Brownish Yellow	Brownish Yellow	Brownish Yellow	Brownish Yellow	Brownish Yellow	Brownish Yellow	Brownish Yellow	Brownish Yellow	Brownish Yellow	Brownish Yellow
Odor	Char.	Char.	Char.	Char.	Char.	Char.	Char.	Char.	Char.	Char.
pH	6.91	6.91	6.85	6.95	6.80	6.85	6.78	6.89	6.80	6.85
Nature	Liquid	Liquid	Liquid	Liquid	Liquid	Liquid	Liquid	Liquid	Liquid	Liquid
Texture	Pourable	Pourable	Pourable	Pourable	Pourable	Pourable	Pourable	Pourable	Pourable	Pourable
Sedimentation Volume	7.69	7.69	7.60	7.72	7.62	7.65	7.55	7.60	7.52	7.55
Redispersibility (%)	95	95	95	95	95	90	95	90	95	95
Viscosity (cps)*	330	330	330	330	330	330	330	320	330	320

** Viscosity at 100 rpm determined using spindle 3, Char. = Characteristics*

GAEE-loaded Eudragit S100 microparticles

Three batches of microparticles containing GAEE [10% (GEM1), 20% (GEM2), and 50% (GEM3), w/w of the Eudragit S100] were prepared as shown in Table 3. The average yield of microparticles for the three batches varied from 56% to 67%. The dry product was found to be brownish-yellow in color. The average length–number diameter (*dln*) of three batches was found to be 9.89, 12.31, and 15.99 **μ**m for GEM1, GEM2, and GEM3 respectively, while the respective average volume-surface diameter (*dvs*) was determined to be 15.44, 19.43 and 21.32 **μ**m respectively. Thus, increasing the quantity of *Grewia asiatica* extract from 10%-50% (w/w of Eudragit S100), lead to an increase in the size of microparticles.

Table 3. Characteristics of *GAEE*-loaded Eudragit S100 microparticles

Sr. No.	Batch	Eudragit S100 (mg)	GAEE (%, w/w)	Yield (%, w/w)	Color	dln (µm)	dvs (µm)	Assay
1	GEM1	500	10	56	Creamish-white	9.89	15.44	98%
2	GEM2	500	20	61	Yellowish-white	12.31	19.43	97%
3	GEM3	500	50	67	Brownish-yellow	15.91	21.32	101%

**GAEE = Grewia asiatica ethanolic extract; dln = Average length-number diameter; dvs = Average volume-surface diameter*

Figure 3 (a), (c), and (e) display the particle size distribution curves of three batches of microparticles. The microparticles of GEM1 show a normal distribution curve with a narrow size distribution, while the GEM2 batch shows a bimodal distribution with a broad particle size distribution with two peaks. Microparticles of batch GEM3 also show a very broad distribution of microparticles. The photomicrographs of GAEE-loaded Eudragit S100 spray-dried microparticles of three different batches were observed using an optical microscope. The microparticles appear disc shape to ovoid shape in an optical microscope. However, the exact morphological feature and surface topography can be seen in scanning electron micrographs [figure 3 (b), (d), and (f)]. It can be seen in the micrographs that Eudragit S100 microparticles are disc-shaped with grooved surfaces. It is not unusual to obtain microparticles of irregular shapes, in some earlier studies on microparticles[30,31], microparticles of different shapes such as ribbon, biocone, elongated hexagonal disks, porous, wrinkled, bullets, barrels, pills, and biconvex lens-shaped, etc. were obtained by different methods such as self-assembly, microfluidics, photolithography, and spray drying. The morphology of spray-dried microparticles depends upon a no of factors such as the type of polymer, polymer concentration, solvent composition, feed pump rate, the temperature of drying air, rate of air aspiration, etc. Esposito, et al. 2000 observed that during the spray drying of Eudragit RS 100 microparticles from ethanol/water mixtures, the crust formed during initial drying is impermeable to the solvent which leads to fracture of the crust. The microparticles formed under such conditions are shriveled. Further, it was reported that the collapsed, irregular-shaped microparticles were obtained by spray drying polymer solutions of Eudragit E, Eudragit R, Eudragit S, and Eudragit RL[32]. The results obtained in our study are consistent with the results obtained earlier. In this study, it can be observed that increasing the amount of extract in the microparticles increases the particle size as well as the width of particle size distribution.

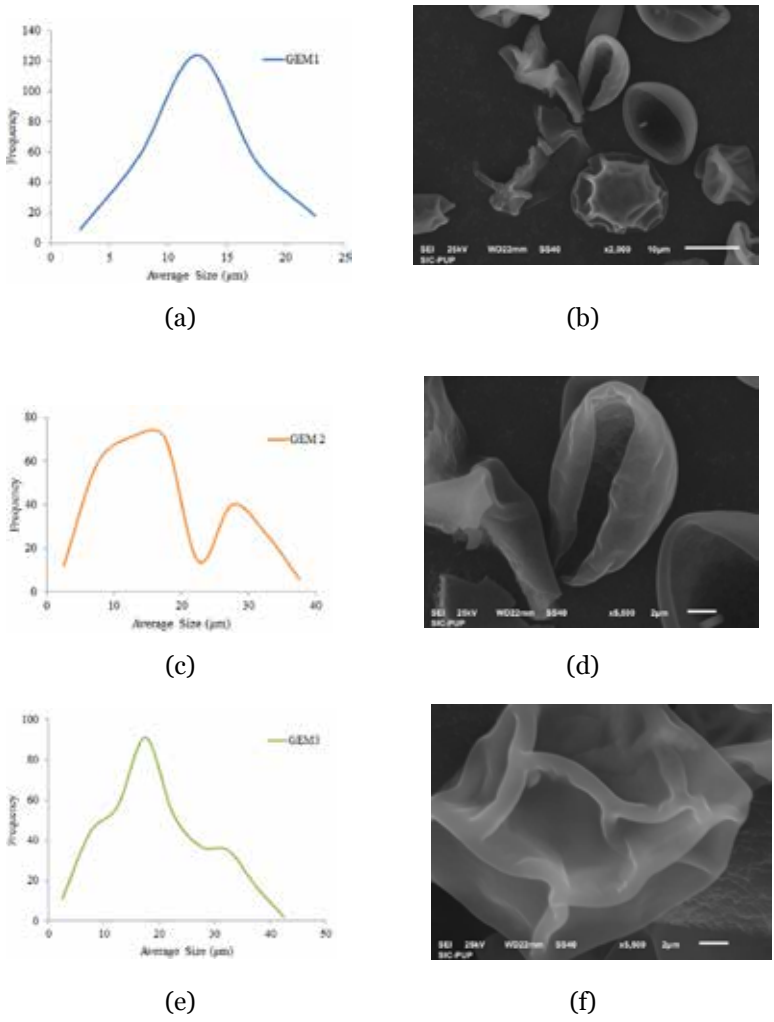
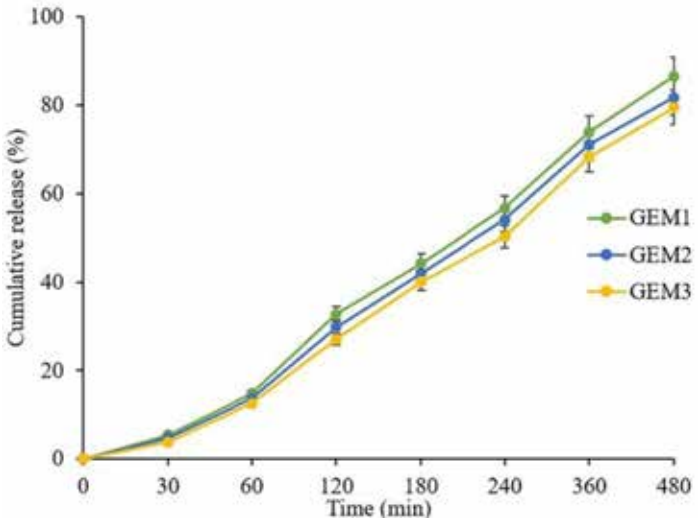
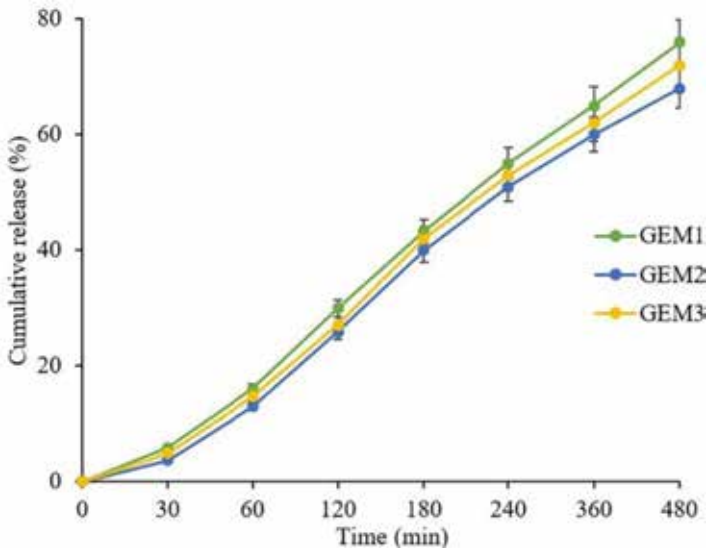


Figure 3. Size distribution frequency curve (a, c, e) and scanning electron micrograph (b, d, f) of GAEE-loaded microparticles of batch GEM1, GEM2, and GEM3

Figure 4 shows the *in vitro* release behavior of three batches of microparticles. The microparticles were observed to release the drug over 8 h with 73%, 68%, and 70% of quercetin release from GEM1, GEM2, and GEM3 respectively. In terms of naringenin, the percentage release was found to be 85%, 82%, and 79% from GEM1, GEM2, and GEM3 respectively. Since there is no significant difference in the release rate of microparticles, the microparticles of batch GEM3 owing to their higher drug loading were selected for further evaluation of *in vitro* anthelmintic activity.



(a)



(b)

Figure 4. *In-vitro* release profile of quercetin (a) and naringenin (b) from microparticles batches of GEM1, GEM2 and GEM3

GAEE-loaded Eudragit S100 nanosuspension

GAEE-loaded Eudragit S100 nanosuspension (GEN) dried using a spray dryer was found to lose redispersibility after drying while the nanosuspension dried using a lyophilizer was found to be redispersible. Thus, the spray drying method was rejected and lyophilization was adopted. The lyophilized powder of GAEE-loaded Eudragit S100 nanosuspension after redispersion was analyzed for particle size and zeta potential. The Z-average particle size of 224.3 (dnm) and polydispersity index (PDI) of 0.153 indicate that the nanoprecipitation method provides suspension of nanometric size particles while the zeta potential was found to be -13.7 which represents the stability of nanosuspension. Further, the lower PDI values indicate monodisperse nanosuspension.

Figure 5 displays the transmission electron micrographs of the GAEE-loaded Eudragit S100 nanosuspension. The nanoparticles are spherical in shape having size consistent with the results of particle size analysis.

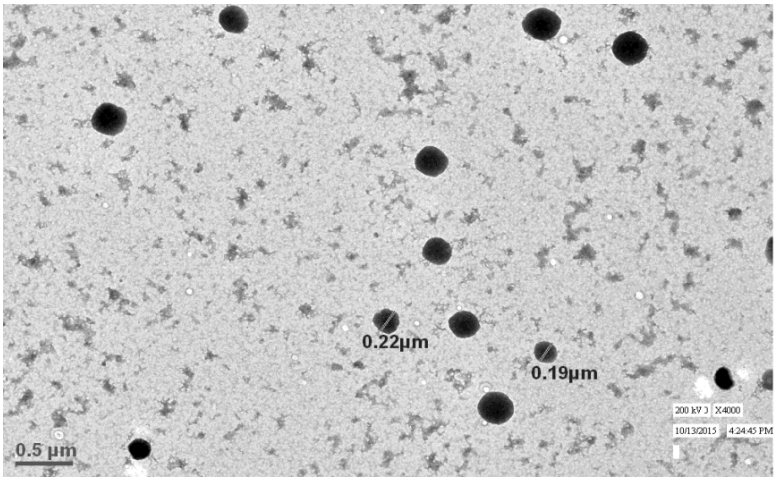
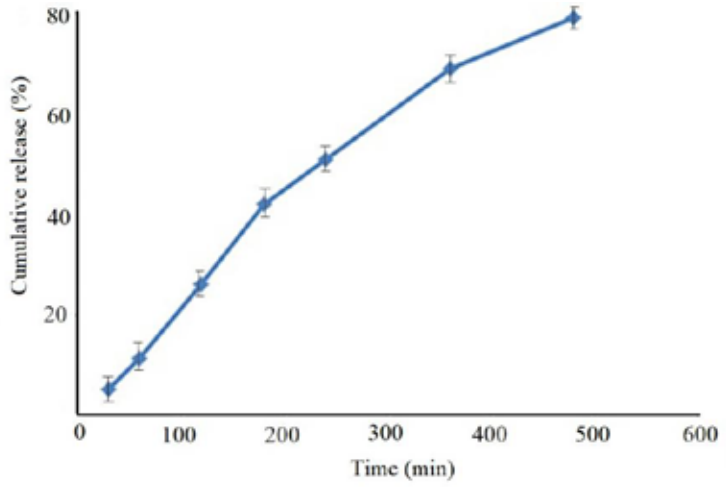


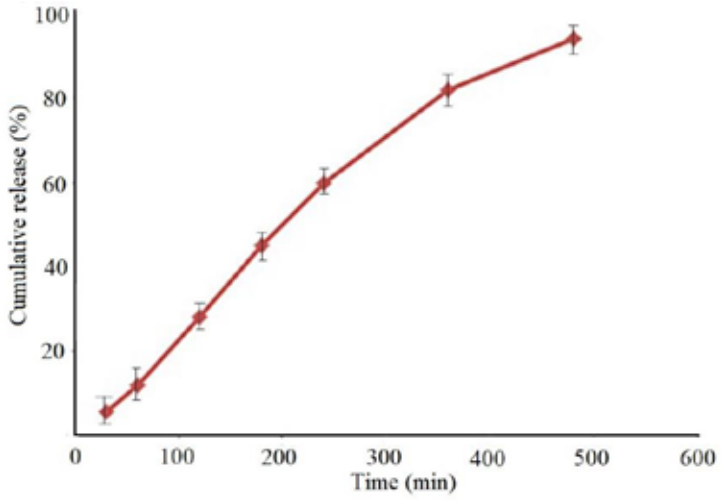
Figure 5. Transmission electron micrographs of GAEE nanosuspension dosage form

Figure 6 shows the *in-vitro* release profile of *Grewia asiatica* extract nanoparticles in terms of quercetin (a) and naringenin (b) respectively. It can be observed that nanosuspension provided 76% release of quercetin and 92% release of naringenin over 8 h period. Quercetin and naringenin have poor oral bioavailability which is attributed to their poor aqueous solubility[33,34]. Nanosuspensions of hydrophobic bioactive have been prepared to improve their solubility and bioavailability. During earlier studies, the nanosuspensions of quercitin[33] and naringenin[34] were observed to show a sustained release profile.

In the present study, microparticulate and nanosuspension dosage forms of GAEE have been prepared, it can be observed that the release of quercetin and naringenin (biomarker components) is relatively faster from the nanosuspension dosage forms as compared to the microparticulate dosage form.



(a)



(b)

Figure 6. In vitro release profile of quercetin (a) and naringenin (b) from the nanosuspension dosage form

Comparative anthelmintic evaluation of suspension, microparticles, and nanosuspension

The results of *in vitro* anthelmintic screening of conventional suspension of GAEE (GES_3), GAEE-loaded Eudragit S100 microparticles (GEM), and GAEE-loaded Eudragit S100 nanosuspension (GEN) with albendazole drug as a standard is shown in figure 7. The formulations of GAEE were tested at a concentration equivalent to 50 mg/ml of GAEE as a drug while the preliminary screening of GAEE at 50 mg/ml was found to be comparable with albendazole (20 mg/ml). It can be observed that the conventional suspension paralyzed and killed the earthworms at a comparable time to that of GAEE. Further, there was no effect of vehicle control (GES) on earthworms. The results of HPTLC studies showed the presence of eight phytoconstituents in GAEE. Among these, quercetin and naringenin were identified to contain 2.24 mg and 21.117 mg/gm of the extract respectively. So, quercetin and naringenin equivalent to that present in GAEE at 50 mg/ml were also tested for *in vitro* anthelmintic activity. The results show that the effect of quercetin is more than naringenin while the combined effect of quercetin and naringenin indicates synergistic action. However, the combined effect of quercetin and naringenin is still less than the effect of GAEE, indicating that other ingredients present in the GAEE are also responsible for its anthelmintic activity.

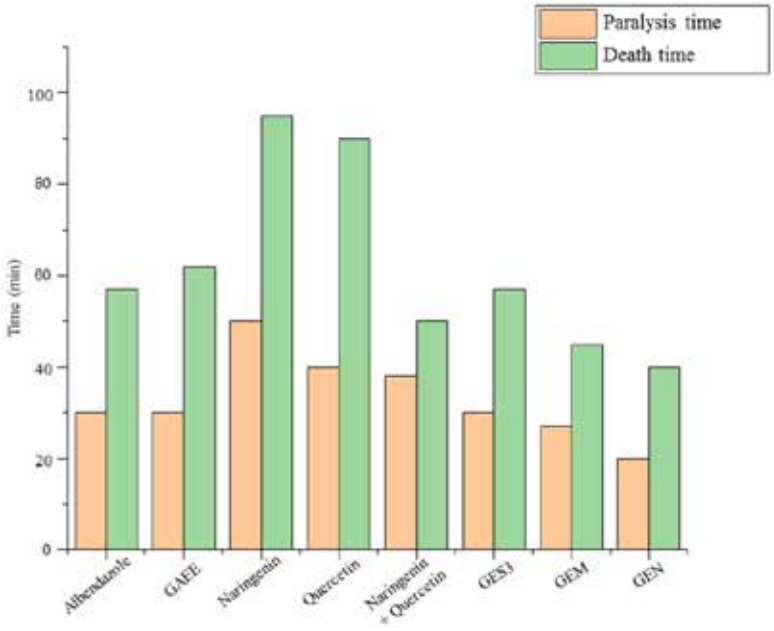


Figure 7. Anthelmintic activity of suspension, microparticulate, and nanosuspension

On comparing the effect of microparticles and nanosuspension dosage forms, it can be observed that both microparticulate and nanoparticulate dosage forms of GAEE showed significantly higher anthelmintic activity than the standard albendazole. Further, the effect of nanosuspension is more pronounced than the microparticulate dosage form. Also, there was no effect of the blank microparticles and nanosuspension which confirms that the effect is due to GAEE.

Herbal drugs i.e. *Grewia asiatica* Linn roots have numerous therapeutic and curative properties. So, the extract of the plant was employed to develop a new and low-cost herbal formulation with better activity and efficacy. For qualitative fingerprinting, the HPTLC study was carried out by using quercetin and naringenin as marker compounds. Then the conventional suspension dosage form of ethanolic extract of *Grewia asiatica* was prepared and evaluated. Microparticles and nanosuspension dosage forms were also prepared simultaneously with the help of spray drying and freeze-drying process respectively. Further, the comparative anthelmintic evaluation of all the selected dosage forms was done against the Indian earthworm *Pheretima posthuma*. The results of the study revealed that in nanosuspension dosage form, significant anthelmintic activity is present as compared to conventional suspension and microparticulate dosage form. However, the excipients exert no anthelmintic action. So, it can be concluded that a stable and non-toxic herbal anthelmintic formulation can be prepared with improved stability, convenience, and compliance.

STATEMENT OF ETHICS

All the necessary ethical rules were followed while performing research.

CONFLICTS OF INTEREST

The authors declare no conflict of interest.

AUTHORS CONTRIBUTION

Conceptualization	:	Tarun Kumar
Methodology	:	Tarun Kumar
Validation	:	Tarun Kumar, Rimpy
Formal Analysis	:	Rimpy, Munish Ahuja
Investigation	:	Tarun Kumar
Resources	:	Tarun Kumar, Munish Ahuja
Writing - Original draft	:	Tarun Kumar
Writing - Review and editing	:	Rimpy, Munish Ahuja
Supervision and Project administration	:	Munish Ahuja
Funding acquisition	:	Munish Ahuja

FUNDING

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