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PRISMA statement of preferred reporting items for systematic reviews and meta-analyses (Moher D, Liberati A, Tetzlaff J, Altman DG, The PRISMA Group. Preferred Reporting Items for Systematic Reviews and Meta-Analyses: The PRISMA Statement. PLoS Med 2009; 6(7): e1000097.) (http://www.prisma-statement.org/);

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Results: The detailed results of the study should be given and the statistical significance level should be indicated.

Conclusion: Should summarize the results of the study, the clinical applicability of the results should be defined, and the favorable and unfavorable aspects should be declared.

Keywords: A list of minimum , but no more than 5 key words must follow the abstract. Key words in English should be consistent with "Medical Subject Headings (MESH)" (www.nlm.nih.gov/mesh/MBrowser.html). Turkish key words should be direct translations of the terms in MESH.

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ORIGINAL ARTICLE

Thermoreversible Gel Formulation for the Intranasal Delivery of Salmon Calcitonin and Comparison Studies of *In Vivo* Bioavailability

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ABSTRACT

Objectives: We developed original thermoreversible (sol-gel) formulations of salmon calcitonin (sCT) for nasal applications. The sol-gel has been compared with commercial intranasal sprays *in vitro* and *in vivo* studies. The aim of studying sol-gel form is to arrange the viscosity of formulations for a reversible adequate fluidity at different temperatures. This situation may facilitate the use of drugs as sprays and increase the bioadhesive ability to mucosa.

Materials and Methods: Characterization of optimum formulations was studied. Validated analytical assays determined the number of sCT. An approximately equal number of commercial and sol-gel dosages were sprayed into the nostrils of the rabbits. Blood samples were collected from the ear veins of rabbits and determined by enzyme immunoassay plates. These plates were evaluated by Thermo Labsystem Multiscan Spectrum at 450 nm. Thanks to Winnonlin 5.2, pharmacokinetic data were evaluated by a non-compartmental method.

Results: The absolute bioavailability of the formulation at pH 4 and the commercial product (CP) was compared by evaluating the primary pharmacokinetic data area under the curve $0 \rightarrow t_{last}$. The absolute bioavailability of the commercial intranasal spray was measured 1.88 based on maximum concentration (C_{max}) assessment. C_{max} of the sol-gel formulation pH 4 was calculated as 0.99 and the relative bioavailability was obtained 53.3%.

Conclusion: *In vivo* pharmacokinetic data of sol-gel formulation with pH 3 showed significantly higher volume of distribution parameter than the CP (111167>35408). It is thought that the formulation adhered to the nasal mucosa releases sCT slowly and less.

Key words: Salmon calcitonin, thermoreversible gel, sol-gel, bioavailability, nasal spray

Introduction

Human calcitonin hormone (hCT), which consists of 32 amino acids, produced in the thyroid gland regulates calcium levels in the body by increasing the bone calcium level and decreasing the blood calcium level. Salmon calcitonin (sCT) is structurally very similar to human calcitonin, which was synthesized in 1969. It contains the same amino acids as the human calcitonin, however, it differs in terms of the amino acid sequence.[1]

The purity of calcitonin preparations varies based on the type (natural or synthetic) or production process. The hypocalcemic effect of calcitonin was ranked in the order of increasing strength as follows: sCT>eCT>hCT>pCT. sCT is more potent and to last longer than eel, human, and porcine calcitonin.[1,2] sCT inhibits the formation and the interactions of osteoclasts and prevents bone resorption. For this purpose, it is commonly used in formulations for osteoporosis, Paget's disease, and hypercalcemia, especially in the postmenopausal period.[3-5]

The biological activity of 1 mg of sCT is reported to be 6000 international unit (IU).[3] It is rapidly absorbed from nasal mucosa and its bioavailability *via* this route is approximately 3% (0.3-

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30.6%) compared to IM administration.[6] The recommended daily dose of sCT was 200 units with adequate Ca^{++} and vitamin D supplementation. Nasal application is performed with a dosimeter nasal spray pump.[7,8]

Intranasal administration is a suitable route for drugs that require a systemic action and undergo liver first-pass action (by enzymatic and acidic degradation). The molecular weight of most of the existing drugs (peptides or proteins) that penetrate systemic circulation by nasal administration varies between 1000 and 3400. Bioavailability of these drugs (intranasal formulations) is approximately 10% as compared to injection forms.[9,10]

The active substance and excipients used in nasal formulations do not irritate the nasal mucosa. Most of the peptides used in intranasal formulations are used for the treatment of chronic diseases; hence, their reliability is important.[11]

Nasal mucosa is a large capillary-rich area for drug absorption. Absorption and bioavailability values of drugs depend on the molecular structure of the active substance, the drug formulation, the tested species, and, if any, additional delivery devices. Generally, in intranasal formulations containing low molecular weight drugs, the bioavailability is relatively high, while the variability is low. In contrast, the bioavailability for high molecular weight drugs is relatively low, whereas the variability is high.[12,13]

One of the factors affecting drug penetration through nasal mucosa is the viscosity of nasal secretion. Approximately 15-20 mL of mucus is produced daily inside the nose, and the mucus layer is renewed almost every 10-30 minutes. If the mucus layer is thick, contact with the cilia decreases, thereby decreasing the mucociliary clearance. Drug penetration is influenced by the duration of contact of the drug with the mucosa. Mucociliary clearance mechanism, enzymatic degradation, and low permeability of nasal epithelium constitute the most important barriers for peptide/protein structured drugs. Viscous gel formulations that adhere to the mucosa (mucoadhesive) were developed with the aim of increasing the contact duration between the drug and the mucosa. Hence, it is also reported that the releasing of mucoadhesive drugs can be increased to enhance the bioavailability. On the other hand, they may be convenient for drugs even in low doses. It is difficult for high-viscosity gel formulations to provide the correct dosage and proper drug distribution in the nasal cavity. Bioadhesive applications increase the residence time of the drug on the mucosa in terms of patient comfort, compliance, and use. In this regard, alternative formulations such as thermogels may be good choices.[14,15]

Different types of polymers are used in thermogel formulations. Poloxamer 407 (Lutrol F 127) has been used in many thermogel formulations. It is a polymer material with many options for transdermal, ocular, topical, and implant application routes. Polyethylene oxide-polypropylene oxide-polyethylene oxide as triblock polymer can form transparent and temperature-sensitive sol-gels. It is chemically inert and has low toxicity.[16,17] Thermosensitive formulations have the advantage in terms of enhancing, controlling, and sustaining sCT for hypercalcemic effects.[18,19]

In this study, it was aimed that the homogeneous and fluid sol-gel formulation below room temperature should be sufficiently viscous to adhere to mucus at intranasal temperature. Thus, the sprayed drug will be prevented from flowing into the nasal passage or downwards. To research the bioavailability of sCT, thermogel nasal formulations at different pHs have been examined. It is thought that the temperature sensitive pharmaceutical form will allow controlled release of sCT. The formulations are designed to convert from solution to gel form at 34-36 °C, which is the intranasal temperature. The design with active ingredient transformed from solution form into gel form was compared with the commercial product (CP). Determination of the bioavailability and *in vivo* studies of sCT were carried out with enzyme immunoassay-enzyme-linked immunosorbent assay (EIA-ELISA) method.[20-23]

MATERIALS AND METHODS

sCT was purchased from Bachem, Switzerland. Potassium dihydrogen phosphate was purchased from J.T Baker, Netherlands. Sodium dihydrogen phosphate dihydrate, dipotassium hydrogen phosphate, polyethylene glycol (PEG) 1500, sodium dihydrogen phosphate, triethylamine, and *ortho*-phosphoric acid were purchased from Merck, Germany. Acetic acid was bought from Riedel de Haen, Germany. Poloxamer 407 was acquired from BASF, Germany. All other chemicals used were of analytical grade and used without further purification.

Quantification of sCT using high performance liquid chromatography (HPLC)

Quantification of sCT was carried out using HPLC system (HP Agilent 1100, ABD) equipped with injector, quaternary pump, autosampler, column oven and ultraviolet (UV) detector. sCT contains 13 different amino acids, but the determination assay uses a single peak for sCT detection.[24]

For determination of sCT, chromatography parameters such as C18 column (4.6 x 250 mm) (conditioned at ambient temperature), mobile phase composed of phase A (20 mL tetramethylammonium hydroxide (10%), 880 mL distilled water and 100 mL acetonitrile), and phase B (8 mL tetramethylammonium hydroxide in 392 mL distilled water and 600 mL acetonitrile) were used. Each mobile phase was adjusted with *ortho*-phosphoric acid to pH 2.5 (using a pH meter) and degassed for 30 min before use. The mobile phase was pumped at a flow rate of 1 mL/minute, injection volume of 50 µL and UV detector wavelength of 210 nm was used. The quantification method was carried out within the concentration range of 1-75 µg/mL. This analytical method was validated in terms of linearity, specificity, accuracy, precision, robustness, and stability based on International Council on Harmonisation (ICH) guidelines.

Preparation and characterization of thermoreversible formulations

Sol-gels were prepared using mixtures of PEG 1500 and Lutrol F 127 (LF 127) in different proportions with three different pH phosphate buffers (pH 3-5) (Table 1). Magnetic stirrers were placed in the formulations and mixed at a speed of 300 rpm to ensure homogeneity. Gelation temperatures of the formulations were investigated from +4 ˚C to 40 ˚C (with temperature increase interval of 0.5 ˚C) by stirring at 300 rpm in a cooled water bath. For the preparation of sCT loaded formulations, 2.200 IU/mL sCT were added to the formulations. The composition of investigated formulations is presented in Table 1 below.

The amount of PEG 1500 was fixed at 2% in order to determine the gelation temperatures with different poloxamer ratios. Gelling corresponding to the intranasal temperature was followed at intermediate concentrations of the F4 formulation in three pH (Table 2). The formulations were characterized immediately after removal from storage conditions at +4 ˚C.

Density of formulations

This study was conducted using a calibrated pycnometer with a volume of 5.442 mL. The empty weight of the pycnometer was 14.875 g. The weights of the formulations that filled the pycnometer at 25 ˚C were measured. At the end of three replicate studies, the ratio of average weight to volume was evaluated as the density of formulations.

Tonicity studies

Knauer-Semi-Micro Osmometer was first calibrated at "0" miliosmol/kg. For this study, 0.15 mL of pure water was placed in the measuring cup with pipette and placed in the cooling cell. The osmolarities of each formulation were studied in three replicates.

Refractive index measurements

ATAGO RX 7000 a device was first calibrated using pure water. The device was calibrated, when the refractive index reached 1.330 after the chamber of the device was filled up with pure water till the limit. Triplicate measurements were made for each formulation and the average refractive indices were observed.

Conductivity measurements

Mettler Toledo instrument used was first calibrated with conductivity standard solution. The calibration measurement was recorded as 1.413 s/cm ± 2%. The conductivity of the formulations (prepared in 100 mL) was measured in triplicates in a beaker and the average conductivity of each formulation was evaluated.

Viscosity measurements

Viscosity was measured at temperatures between 4 and 40 ± 0.5 ˚C. Briefly, 35 mL of the formulations was placed into the sample measuring cup. Viscosity measurements were taken between 4 ˚C and 40 ˚C. The viscosity measurement (at each temperature) was carried out every 30 seconds in a vibroviscometer to follow the changes related to temperature. The experiment was repeated for each sample in reversely from 40 ˚C to 4 ˚C.

Table 1. Compositions of formulations

Formulation	PEG 1500 % *w/v*	Lutrol F 127 % *w/v*	Phosphate buffer (pH: 3)	Phosphate buffer (pH: 4)	Phosphate buffer (pH: 5)
F1	10	25	+	+	+
F2	7.5	22.5	+	+	+
F3	5	20	+	+	+
F4	2	17.5	+	+	+
F5	1	15	+	+	+

(+) means; all formulations have been prepared according to ratio of excipients and pH
PEG: Polyethylene glycol

Table 2. Composition of optimized formulations

Formulation	LF 127 % *w/v*	PEG 1500 % *w/v*	Phosphate buffer (pH: 3)	Phosphate buffer (pH: 4)	Phosphate buffer (pH: 5)
F4a	18	2	+	+	+
F4b	17	2	+	+	+
F4c	16.8	2	+	+	+
F4d	16.5	2	+	+	+
F4e	16.2	2	+	+	+

(+) means; all formulations have been prepared according to ratio of excipients and pH
PEG: Polyethylene glycol

Evaluation of chromatogram

The formulations were placed in the donor phase as 1 mL (2200 IU/mL) for the transition study. Optimized formulations containing sCT were diluted with 5 mL of the receiving phase. The possible maximum amount for determination would be 2200 IU/5 mL (=440 IU/mL= 73.3 µg/mL). Therefore, formulations containing 75 g/mL sCT were loaded and analyzed separately in triplicates. Evaluation was made to examine, whether the peaks of the excipients in the formulation interfere with the peak of the active substance.

Stability studies

Short-term stability assessment of sCT was carried out based on the repeatability parameters included in the validation studies. The sCT (25 µg/mL in pH 7.4 phosphate buffer) inside the same vial was analyzed 10 times. Monthly stability assessment was performed for formulations containing 11,000 IU sCT in 5 mL. The formulations were kept in capped vials for 3 months at +4 ˚C. The samples were also examined for physicochemical properties at predetermined time intervals (1st day, 1st week, 1st month, and 3rd month). The concentrations of formulations were determined by HPLC. Samples to be examined for stability at +4 ˚C was stored in the refrigerator. Samples to be examined at 25 ± 0.5 ˚C (60 ± 5% RH) and 40 ± 0.5 ˚C (75 ± 5% RH) were stored in climate cabinets.

In vitro release studies

In vitro release studies were carried out using a cellulose membrane (MW: 25.000 Da) using a diffusion tube method specifically designed for our study. The system consists of two parts, *e.g.* transmitter and receiver compartment, and the membranes are left in contact with the receiver compartment. The membranes (3.81 cm^2) are placed tightly between the donor and receiver compartments.

5 mL of pH 7.4 phosphate buffer was receiver phase and tool placed in the lower beaker. Formulations (containing approximately 2200 IU/mL sCT) and CP was placed over the membrane as donor phase. The system was placed in a water bath at 37 ± 0.5 ˚C with magnetic stirrer. The receiving phase was mixed with help of magnetic stirrer at a speed of 300 rpm during the operation. The amount of active substance from the samples was determined at regular intervals by HPLC. The fresh phosphate buffer (pH 7.4) was added to the medium as much as the sample taken. The transition studies were carried out in triplicates.

In vivo studies

CP and the developed formulations must be in the same package to compare their applications to nostrils of rabbits. For this purpose, pediatric packaging of another CP was used. Nasal spray dosimeter control was performed before drug administration.

Nasal spray dosimeter evaluation

Metered dose spray systems of the developed formulations were gravimetrically compared with commercial nasal preparations in terms of spraying amounts. The sprays were weighed on a scale with a precision of 0.0001 g and, then, the amount of each puff was determined gravimetrically by pressing ten times in succession after each puff. The standard deviations and relative standard deviations of the weights were calculated and compared statistically.

The same spray was used for both the developed formulations and the CP to examine, whether there was a difference in the dose of the nasal spray used. Their performance was examined gravimetrically and evaluated statistically in a similar way.

Content uniformity

Since the sensitivity of the kit to be used in the method is between 0 and 100 ng, the amount of sCT to be determined by EIA in rabbit plasma should be within this range. In a similar study, when 2000 IU sCT was applied *IN* to rabbits, sCT was determined between 0 and 100 ng/mL in RIA analysis of serum samples.[25] With this goal in mind, before administering to the rabbits, 5 mL of our formulations were weighed on a balance with 1 g precision to obtain 1000 IU/puff at each spraying with 11.917 g sCT. That is, the formulations were designed to contain 14.300 IU/mL sCT. 100.84 g sCT was weighed and loaded into 5 mL of the CP bearing in mind 5 mL of CP contains 110.00 IU. The formulations prepared were analyzed by HPLC with an injection volume of 10 L using the same analytical method, and their concentrations were checked before application.

Drug administration to rabbits

Drugs were administered to 24 New Zealand-type white rabbits of both sexes (weighing between 2.5 and 3 kg). Rabbits were provided by Faculty of Medicine, Experimental Animal Production Center. Before the studies, ethic approval was obtained from the Ege University, Faculty of Medicine Animal Ethics Committee (2009-95).

The rabbits were divided into four groups consisting of three females and three males in each group. The rabbits were fasted for 24 h before drug administration. During the studies, anesthesia was administered by giving *IM* 50 mg/kg ketamine and 5 mg/kg xylazine.[21]

1 mL of blood samples was taken from six rabbits *per* group at 0 min before even the first administration. The sprays were applied to the nose after being sprayed into the air once. The application consisted of two squeezes and one puff in each nostril of the rabbits. *IV* administration was carried out into the venous vein of rabbit ear using insulin needle.

After the applications, 1 mL of blood samples was taken at 15, 30, 45, 60, 75, 90, 105, 120, and 180 minutes. Blood samples were taken with 22 G1 cannulas and put into EDTA tubes. These samples were then centrifuged at 4 ˚C for 15 min (at 1600 rpm) and the plasma was separated. The plasma samples were stored at -80 ˚C for evaluation by EIA.

Quantification of sCT in rabbit plasma

EIA kit was used for the assay of sCT. The immunoplate in the ready kit was pre-coated with secondary antibody and non-specific binding sites were blocked. The log/logit curve of the optical density *versus* the concentration is determined using standards at known concentrations. Unknown concentrations are estimated by extrapolation.

The kits were maintained at room temperature before use. Assay buffer included in the kit was diluted with 950 mL of distilled water. Five concentrations were obtained by diluting with assay buffer from standard solution at a concentration of 1000 ng/mL (Table 3 below). The primary antibody was rehydrated by mixing with 5 mL assay buffer. Likewise, the biotinylated peptide was also rehydrated by mixing with 5 mL of assay buffer. The positive control was rehydrated with 200 µL assay buffer and centrifuged. 50 µL assay buffer (for total binding), 50 µL standard solutions from less diluted to more diluted and 50 µL of positive control were placed in the first row of the well plates from top to bottom. 50 µL of plasma samples were added to the remaining wells of the plates, respectively. 25 µL of rehydrated primary antibody and rehydrated biotinylated peptide were added to all wells except for the blank, respectively. Plates were covered with acetate plate sealer and incubated for 2 h at room temperature with 300 cycles on a shaker. At the end of this period, inside of the plates was poured out and the wells were washed four times with assay buffer. 100 µL of mixture containing 12 µL of SA-HRP and 12 mL of assay buffer was added to each well and the plates were covered again with acetate. The plates were incubated for 1 h at room temperature at 300 rpm while shaking. Then, the wells were emptied by washing the wells four times with assay buffer. 100 µL of TMB substrate solution was placed in each well and the plates were kept in the dark at 300 cycles on a shaker until the wells were discolored. The reaction was terminated by adding 100 L of 2 N HCl to the wells. The plate was analyzed using a Thermo Labsystem Multiscan Spectrum at 450 nm.

Bioavailability assessments

The results of all plasma samples taken from rabbits after nasal administration of the prepared formulations were subjected to pharmacokinetic analysis compared to the CP administered *via IV* and nasal route. For pharmacokinetic analysis, using WinNonlin version 5.2, Pharsight Corporation program, the data of each rabbit, and pharmacokinetic parameters stated below were evaluated.

Absolute bioavailability: It is the ratio of the amount of an active substance that is administered into the blood circulation by any method to the amount of active substance that enters the blood circulation when administered *IV*. The total area below the blood concentration-time curve when the drug is given *IV* (completely) is considered to be 100%.[26,27]

Relative bioavailability: It is the comparison of the rate and degree of absorption of the active ingredient from the test and reference dosage forms applied in the same way. It is obtained by comparing the blood/plasma concentration-time profile [area under the curve (AUC)] of the formulation to that of the reference and multiplying by 100.[26,27]

RESULTS

Quantification of sCT concentration using HPLC

Linear equation of the calibration curve was determined as y= 16.989x + 30.097 with R^2= 0.9946. Sensitivity of the method was evaluated in terms of the limit of detection (0.276 µg/mL) and the limit of quantification (0.836 µg/mL). Coefficients of variation for repeatability and reproducibility were lower than 2%. Relative standard deviations of both accuracy and precision values were less than 8%. The specific peak of sCT was detected clearly in an irreproachable manner. Retention time of the peak of the active substance was 14.2 min, as shown in Figure 1 below.

Preparation and characterization of thermoreversible formulations

Temperature of the nasal mucosa is about 32-34 °C, while temperature of the entrance part of the nose and nasopharynx is stated to be 31 and 36 °C, respectively. The temperature increases from the nasal vesticulate to the back. No temperature difference was reported between the nostrils.[28,29] Taking all this into account, we designed our formulation to transform into sol-gels at 34 °C.

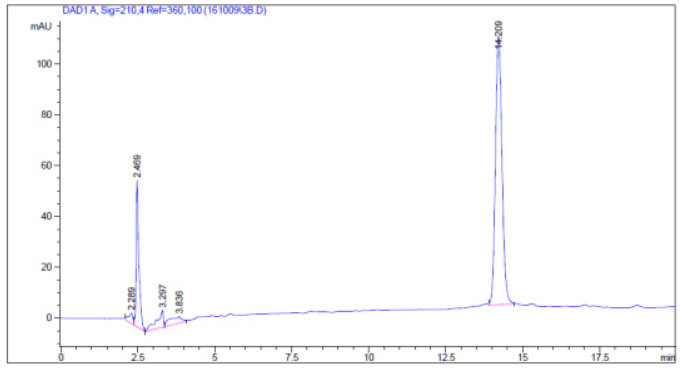

Figure 1. HPLC chromatogram of salmon calcitonin

Table 3. Concentrations of standart solutions for ELISA determination

Standard no	Volume of standard (µL)	Buffer (µL)	Concentration (ng/mL)
Stock	1000 µL	-	1000.0
Std 1	100 µL stock	900	100.0
Std 2	100 µL Std 1	900	10.0
Std 3	100 µL Std 2	900	1.0
Std 4	100 µL Std 3	900	0.1
Std 5	100 µL Std 4	900	0.01

Std: Standard

The density of formulations

The optimal formulation contains approximately 2200 IU/mL sCT. Density, tonicity, refractive indices, and conductivity values of the selected formulations (related with F4e and F4d formulations) are shown in Tables 4-8, respectively. In Table 5, standard deviations between repetitiative measurements are quite small indicating homogeneity in formulations.[30]

Tonicity studies

NaCl solution (0.9%), which has the same isotonia as blood plasma, has an osmolarity of 300 mOsm (less than that of the investigated formulations in Table 6), indicating that all the prepared formulations are hypertonic.

Refractive index measurements

From Table 7, refractive index measurements varied between 1.33 and 1.36. These values suggest the clarity of prepared formulations.[31]

Conductivity measurements

Conductivity values of optimum formulations were determined in the range of 860-1935 S/cm. Because pH of the formulations varied between pH 3-5 and the quantities LF 127 and PEG 1500 are different, the measured values were not the same. As shown in Table 8, conductivity of the researched formulation increases in turn: 4A>4B>3A>3B>5A>5B.

Viscosity measurements

Vibroviscometer used in this study makes measurements using tuning fork vibration method. With the aid of sensor plates vibrating at a frequency of 30 Hz, it is possible to make continuous measurements in a dynamic measuring range with high accuracy/repeatability. Given that measurements can be taken from low to high viscosity, studies were carried out without the need for many spindles compared to conventional rotational viscometer.[32] Viscosity comparison between formulations

Table 4. Gelling temperatures of various gel formulations

Formulations	Phosphate buffer pH: 3	Phosphate buffer pH: 4	Phosphate buffer pH: 5
F4a	25 °C	25 °C	25 °C
F4b	29.5 °C	28 °C	26.5 °C
F4c	30.5 °C	28.5 °C	31 °C
F4d	31 °C (3B)	31 °C (4B)	31 °C (5B)
F4e	36 °C (3A)	35.0 °C (4A)	32 °C (5A)

Table 5. Density of the investigated formulations

Formulations	Weight I (g)	Weight II (g)	Weight III (g)	Average weight (g)	SD	% RSD	Density (g/mL)
3A	5.573	5.578	5.567	5.573	0.006	0.099	1.024
4A	5.596	5.593	5.590	5.593	0.003	0.054	1.028
5A	5.605	5.609	5.598	5.604	0.006	0.099	1.030
3B	5.603	5.595	5.604	5.601	0.005	0.088	1.029
4B	5.615	5.613	5.616	5.615	0.002	0.027	1.032
5B	5.576	5.578	5.583	5.579	0.004	0.065	1.025

RSD: Relative standard deviation, SD: Standard deviation

Table 6. Tonicity measurements of formulations

Formulations	1. Trial (mOsm)	2. Trial (mOsm)	3. Trial (mOsm)	Average (mOsm)	SD	% RSD
3A	360	350	360	356.7	5.8	1.6
3B	360	370	360	363.3	5.8	1.6
4A	380	380	380	380.0	0.0	0.0
4B	380	390	380	383.3	5.8	1.5
5A	390	380	400	390.0	10.0	2.6
5B	420	410	410	413.3	5.8	1.4

RSD: Relative standard deviation, SD: Standard deviation

kept at 34 °C, which is the nasal mucosa temperature, is given in Figure 2. Based on viscosity of the formulations, which increases with temperature, the flow property is observed to be a thixotropic system in non-Newtonian flow. It is suitable for sol-gel form.[33,34]

The evaluation of chromatogram

Number of sCT in the formulations determined using HPLC is given in Table 9. It was observed that the excipients did not interfere with 75 µg/mL of active substance. Highest relative standard deviation (RSD) was found to be 3.31%. Formulations have been under repeatable experimental conditions. The quantities determined during the analysis according to Food and Drug Administration (FDA) and ICH validation criteria are less than 15% repeatability and are appropriate.[35-37]

Stability studies

Stability results of the sCT solution examined at room temperature for 220 min are given in Table 10. Mucociliary clearens occur on average every 20 min due to the nasal application.[38] Ten consecutive analysis stability assessment processes correspond to approximately 11 mucosilyer clearens. Percentage ratios of the samples taken at room temperature based on the value of the first concentration are shown in Table 11.

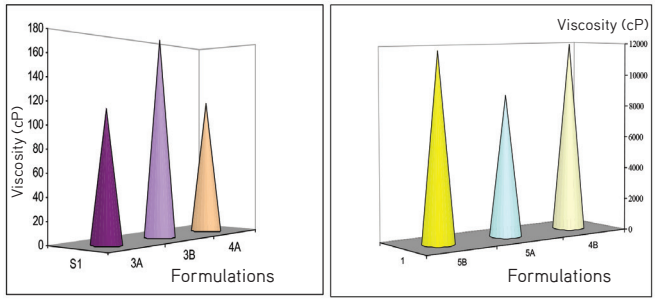

Figure 2. Viscosity measurements of various formulations

Table 7. Measurement of refractive indices

Formulations	Indice I	Indice II	Indice III	Average indice	SD	% RSD
3A	1.3582	1.3582	1.3582	1.3582	0.0	0.0
3B	1.3588	1.3588	1.3588	1.3588	0.0	0.0
4A	1.3302	1.3302	1.3302	1.3302	0.0	0.0
4B	1.3352	1.3352	1.3352	1.3352	0.0	0.0
5A	1.3582	1.3582	1.3582	1.3582	0.0	0.0
5B	1.3587	1.3587	1.3587	1.3587	0.0	0.0

RSD: Relative standard deviation, SD: Standard deviation

Table 8. Conductivity measurements of formulations

Formulations	I (µS/cm)	II (µS/cm)	III (µS/cm)	Average (µS/cm)	SD	% RSD
3A	1620	1620	1620	1620.0	0.0	0.0
3B	1123	1121	1123	1122.3	1.2	0.1
4A	1935	1936	1935	1935.3	0.6	0.0
4B	1850	1850	1852	1850.7	1.2	0.1
5A	881	881	881	881.0	0.0	0.0
5B	864	863	864	863.7	0.6	0.1

RSD: Relative standard deviation, SD: Standard deviation

Table 9. Concentrations of the investigated formulations

Formulations	I. amount µg/mL	II. amount µg/mL	III. amount µg/mL	Average µg/mL	SD	% RSD
3A	86.75	86.36	85.33	86.15	0.73	0.85
3B	86.51	84.33	82.87	84.57	1.83	2.17
4A	73.70	72.47	73.28	73.15	0.63	0.85
4B	67.22	69.31	69.08	68.54	1.15	1.67
5A	80.30	82.17	76.96	79.81	2.64	3.31
5B	85.38	83.97	85.79	85.05	0.95	1.12

RSD: Relative standard deviation, SD: Standard deviation

Table 11 demonstrated that 10% of the initial concentration was lost within 120 min. Based on these results, we limited period of the *in vitro* studies to 120 min.

Short term and monthly stability evaluation of 3A, 3B, and 4A, 4B formulations and CP was carried out at 4 ± 0.5 ˚C and 25 ± 0.5 ˚C, respectively. Figures 3 and 4 compared stability of various concentrations (with respect to time) at 4 ± 0.5 ˚C and 25 ± 0.5 ˚C, respectively.

In Figure 3, it was observed that CP and the 3A formulation showed similar stability at 4 ± 0.5 ˚C at the end of 30 days.

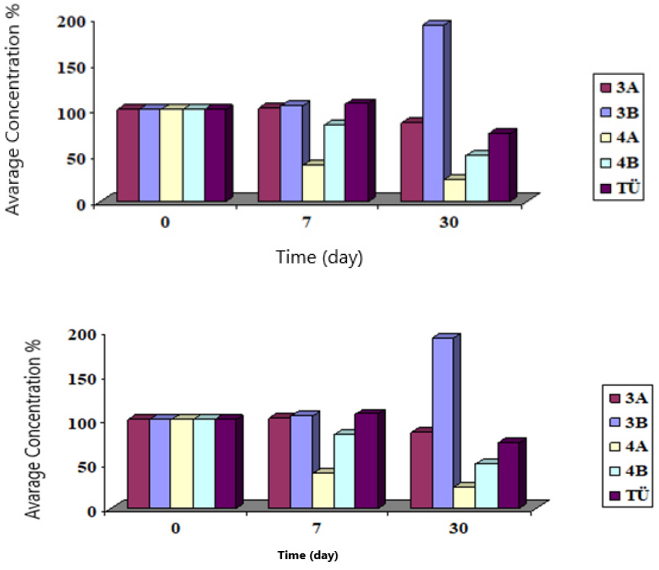

Figure 3. Stability results in terms of mean % concentration *vs.* time (day) at 4 ± 0.5 ˚C

Meanwhile, both CP and formulations could not maintain their stability within the 30 day period.

sCT was not detected in formulations and CP stored at 40 ˚C and 75% humidity on the 7th, 15th, and 30th days.

In vitro release study results

A graph of the cumulative amount of active substance in the receiving phase of the diffusion experiment setup *vs* time is shown in Figure 5. sCT amounts were determined by HPLC. The active ingredient could not be determined in 5A and 5B formulations during diffusion studies. Stability problem was encountered due to the degradation of sCT in a short time at pH 5.[39,40]

The prepared formulations and CP are known to be stable for at least 90 min. Hence, sCT amount expressed as percentage release ± SD in the formulations, and CP was investigated for a period of 90 min (Table 12). CP was noted to be superior in terms of transition as compared to the prepared formulations: 3A>4A>3B>4B.

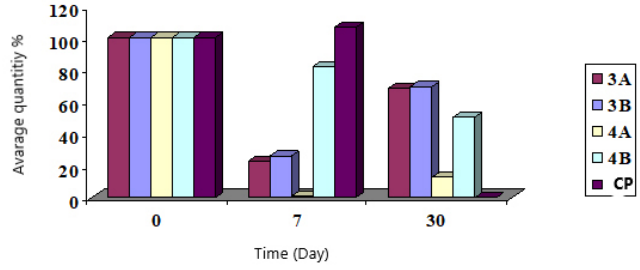

Figure 4. Stability results in terms of mean % concentration *vs.* time (day) at 25 ± 0.5 ˚C

Table 10. Results of short-term stability studies (n: 10 time points) of sCT in HPLC

% Concentration	22 min	44 min	66 min	88 min	110 min	132 min	154 min	176 min	198 min	220 min	Average	SD	% RSD
100	117.3	115.1	113.2	111.8	109.8	108.2	105.0	102.3	98.3	93.3	107.4	7.69	7.16

sCT: Salmon calcitonin, HPLC: High performance liquid chromatography, RSD: Relative standard deviation, SD: Standard deviation

Table 11. % Ratios of samples during a short-term stability studies

Time (min)	0	20	40	60	80	100	120	140	160	180
Ratio (%)	100	98	96.6	95.6	93.9	92.5	89.8	87.4	84.1	79.5

Table 12. % *In vitro* release of sCT with respect to time (min)

Time (min)	3A	3B	4A	4B	CP
0	0.0	0.0	0.0	0.0	0
10	34.4 ± 39.9	1.0 ± 1.2	9.8 ± 17.1	ND	4.5 ± 1.5
30	72.7 ± 24.0	2.7 ± 3.0	18.8 ± 21.1	ND	74.2 ± 16.8
60	62.3 ± 16.8	5.6 ± 6.0	22.5 ± 36.7	ND	78.8 ± 25.2
90	50.7 ± 29.5	4.6 ± 1.5	13.7 ± 10.4	0.2 ± 0.3	73.3 ± 16.9

sCT: Salmon calcitonin, ND: Not determined, CP: Commercial product

Formulations 5A and 5B in pH 7.4 buffer could not be analyzed in any way. This situation was evaluated as stability problem.

In vivo studies

According to *in vitro* studies, 3A and 4A formulations were used considering that they have better performance in terms of membrane permeation as compared to other formulations. 5A and 5B formulations were excluded from the studies due to stability problems, while 3B and 4B formulations were excluded due to gelation at lower temperatures. Furthermore, sCT diffusion from 3B and 4B formulations was limited. Numerous trials were conducted using diffusion tube method and high variations were observed in the results of transition studies.

Nasal spray dosimeter evaluation

The average gravimetric assessments of original drug, 3A and 4A formulations were determined as 0.0739, 0.0720, and 0.0701, respectively. SD values were less than 0.0015 and RSD values were less than 2. Based on the results, it could be said that dose uniformity were achieved in liquid form at the time of pressing. The average gravimetric assessments of original drug after spraying from its original packaging was defined as 0.0927. SD and RSD values were determined as 0.0026 and 2.8416, respectively.

Content uniformity of the investigated formulations

F-test was used to determine, whether there was a general difference between the applications. After F-test, groups with differences were determined using Tukey Honest Significant Difference (HSD) method. Tukey HSD method was also used to compare the formulations in pairs. Significant differences between formulations are shown with asterisks "***" in Table 13 below.[41]

Spr: The original spray of CP

CP: Spray device, in which CP is placed to be compared in the same apparatus

3A: The spray device, in which the 3A formulation is placed to be compared in the same apparatus

4A: The spray device, in which the 4A formulation is placed to be compared in the same apparatus

The concentration of administered formulations

After 2000 IU intranasal administration in an immunoassay study, highest concentration of sCT detected in rabbit plasma was 40 ng/mL.[25] Since sCT detection range of our EIA kit was 0-100 ng/mL, the formulations were designed to administer 1000 IU/puff to the rabbits. The formulations were prepared by loading sCT in 14.300 IU/mL and 71.500 IU/5 mL. In order to eliminate any other factors for the sake of accuracy, 11.917 g sCT was weighed and loaded into 5 mL formulation and the amount of loaded sCT was determined using HPLC before each *in vivo* study. By the way, dose content of the formulations was supported by an analytical method. Dosage of formulations was selected based on the analytical method.

Results of analytical assay of the formulations loaded with sCT before applying to rabbits are given in Table 14. Volume of the spray released from each puff was found to be approximately 0.07 mL.

Quantification of sCT in rabbit plasma

The method used for quantification of sCT in rabbit plasma is ELISA. ELISA is a bioanalytical method, in which substance to be analyzed is measured depending on the antigen-antibody relationship. Sometimes, it is used to quantitatively detect the presence of antigen/antibody in a matrix and occasionally, it is used to detect the amount of analyte in samples of unknown concentration based on calibration curve.

Bioavailability assessments

Before starting the bioavailability studies, the rabbits to be treated were allowed to fast for 24 h. Anesthesia was administered before the application and was continued based on the motility of the rabbits to avoid deviations in blood draws.

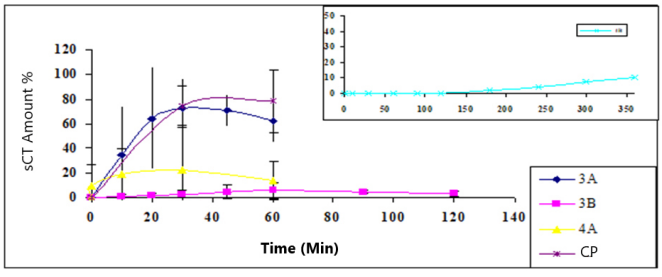

Figure 5. % Salmon calcitonin, release *versus* time (min)

Table 13. Statistical evaluation of dosage differences in sprays

Evaluation of gravimetric dosage differences of sprays at 0.05 level				
Compared formulations	**Differences of averages**	**95% confidence limit**		**Evaluation**
spr - CP	0.019	0.017	0.021	***
spr - 3A	0.021	0.019	0.023	***
spr - 4A	0.023	0.021	0.025	***
CP - 3A	0.002	0.0001	0.004	
CP - 4A	0.004	0.002	0.006	
3A - 4A	0.002	0.0001	0.004	

CP: Commercial product

A catheter was inserted in the rabbits so that blood samples could be collected conveniently, accurately, and on time after injection. In cases where blood could not be obtained from the ear veins during frequent blood withdrawals, blood was taken from the ear arteries. For some of the rabbits, blood was withdrawn from the heart, instead of the veins, due to their complex anatomical structure (*e.g.* no prominent ear veins located) or difficulty in obtaining blood.

The formulations were applied to right and left nostrils of rabbits and blood samples were taken at the stated time periods. The plasma was separated from the blood and stored at -80 °C until quantification using EIA.

Pharmacokinetic constants of *IV* and intranasal applications of the investigated formulations (3A and 4A) and CP were obtained using a program called WinNonlin version 5.2 (Pharsight Corporation). Findings of the pharmacokinetic study using this program are shown in Table 15. Standard dose application was not applied in practice.

Table 15 compares AUC 0 → last values (the primary pharmacokinetic parameter) of the investigated formulations. Considering that initial sCT amount in the CP is approximately 1.5 times that of 4A formulation, then AUC will increase in the order: 4A>CP>3A. Critical parameters were found to be similar and comparable between formulations 4A and CP.

As seen in Table 16, absolute bioavailability of the intranasal application of CP is 0.856, which is within the range of 0.3-3, stated in the literature.[28,42] Absolute bioavailability of formulation 4A was 0.743, which is very close to CP, even though the initial concentration of formulation 4A is 1.5 times less than CP. For 3A formulation, despite the positive results obtained during *in vitro* studies, *in vivo* results (*i.e.* bioavailability result) were less significant.

Bioavailability of 3A formulation is not at the same level as 4A and CP. Table 16 contains data and bioavailability results of the applications in terms of primary pharmacokinetic parameters (AUC 0 → last value). In Table 17, the relative and absolute bioavailability was evaluated based on the secondary pharmacokinetic parameter (AUC 0 → ∞). The data from Table 17 revealed that absolute bioavailability of CP and the 4A formulations are similar: AUC 0 → ∞ value is 0.254 and 0.234, respectively. On the other hand, bioavailability of 3A formulation is only about 35% of the mentioned bioavailability values (*i.e.,* AUC 0 → ∞ value is 0.157).

Table 14. Quantity of active ingredient in formulations prior to application

Formulations	Rabbit no	Amount (µg/mL)	Application dose (µg)	Application dose (IU)
3A	R1, R2	3270.1	457.8	2746.8
	R3	3401.7	476.2	2857.2
	R13, R14, R15	3078.5	431	2586
4A	R4	2133	298.6	1791.6
	R5, R6	2090.6	292.7	1756.2
	R16, R17, R18	2597.3	363.6	2181.6
CP *IN*	R19, R20, R21	3237.8	453.3	2719.8
	R22, R23, R24	3169.6	443.7	2662.2
CP *IV*	R7, R8, R9, R10, R11, R12	-	1.66	10

CP: Commercial product, *IV*: Intravenously, *IN*: Intranasal, IU: International unit

Table 15. The obtained pharmacokinetic data of sCT administered *via* different routes

Parameters	CP (*IV*)	CP (*IN*)	3A	4A
$t_{1/2}$ (min)	308.9 ± 399.8	84.3 ± 121.5	227.4 ± 148	75.7 ± 49.6
t_{max} (min)	30 ± 30	75 ± 15	105 ± 68.4	57.5 ± 33.4
C_{max} (ng/mL)	17.4 ± 21.5	87.4 ± 95.1	4.8 ± 2.1	37.3 ± 75.6
AUC 0 → t_{last} (min*ng/mL)	786.5 ± 330.9	1838.6 ± 1588.8	466.9 ± 140.2	1231.1 ± 1653.6
AUC 0 → ∞ (min*ng/mL)	3059.5 ± 3221.7	2120.9 ± 1398.9	1279.1 ± 413.5	1477.4 ± 1548.0
Vd (mL)	207.5 ± 96.2	35408.4 ± 53569.3	111167.1 ± 75645.9	40247.5 ± 28583.0
Cl (mL/min)	0.964 ± 0.57	274.2 ± 141.3	395.6 ± 186.6	358.6 ± 187.1
MRT0 → last (min)	73.8 ± 15	61.9 ± 16.7	93.8 ± 6.0	77.1 ± 8.9
MRT 0 → ∞ (min)	474.1 ± 575.8	122.5 ± 197.1	363.2 ± 195.8	146.7 ± 75.8

sCT: Salmon calcitonin, CP: Commercial product, *IV*: Intravenously, *IN*: Intranasal, C_{max}: Maximum concentration, AUC: Area under the curve, V_d: Volume of distribution

Relative and absolute bioavailability assessments based on maximum concentration (C_{max}) were measured in a similar way as AUC. The relative/absolute bioavailability data based on C_{max} is presented in Table 18.

Absolute bioavailability of CP administered *via* nasal route was found to be 1.88 (see Table 18) using C_{max} value as a primary pharmacokinetic parameter.

DISCUSSION

Among the investigated formulations, F4 gelled at a temperature close to the nasal mucosa temperature (*i.e.* 34 ˚C) as desired. For this reason, new formulations based on F4 with modified ratios of Lutrol F-127 as gelling agent along with 2% PEG 1500 were explored. PEG 1500 was used in the formulation due to its water-solubility and amphibian features. It is also non-toxic and non-immunogenic. PEG has been approved by FDA and is used as a carrier in many food, cosmetic, injectable, topical, rectal, and nasal applied pharmaceutical products. It also has penetration enhancing effect. The ratio of PEG 1500 used as 2% is to reduce the variability in the temperature transformation. However, this application is not a PEGylation.[30,31]

According to Table 4, formulations with gelation temperature closer to the nasal mucosa temperature are coded as 3A, 4A, and 5A. Similarly, formulations that tend to have gelation temperature closer to the lower limit of the nasal mucosa temperature are coded as 3B, 4B, and 5B.[13] These coded formulations were selected for further investigations.

Based on viscosity of the formulations, which increases with temperature, the flow property is observed to be a thixotropic system in non-Newtonian flow. It is suitable for sol-gel form.[33,34] Viscosity of formulations with decreasing temperatures from 40˚C to 4˚C was found to be the same as 34 ˚C. Moreover, formulations 3A, 3B, and 4A exhibited a slower gelation transformation.

Nasal formulations should be between 4.5 and 6.5 based on nasal pH.[13] However, pH of commercially available preparations of sCT is around 3. Given that sCT is stable at pH between 3 and 4, pH of the nasal mucosa is around 5. Our formulations were prepared at pH 3, 4, and 5 in order to examine effect of pH on the stability as well as bioavailability.[39-41]

During the diffusion studies, sCT was not detected in the samples or CP after 24 h. Besides, no sCT amount could be determined in the formulation with pH: 5, indicating that the molecule is very unstable in such environments. Stability of commercially available product in transition and storage conditions was also found as very sensitive to be delicate.

When the results of transition studies were evaluated, it is seen that variation between the samples is high. Despite the large

Table 16. Relative and absolute bioavailability data based on AUC 0 → t_{last} values

	AUC 0 → t_{last} (min*ng/mL)	AUC 0 → t_{last}/dose	Absolute bioavailability	Relative bioavailability
CP (*IV*)	786.5	473.8	-	-
CP (*IN*)	1838.6	4.06	0.856	-
3A	466.9	1.05	0.222	25.9
4A	1231.1	3.52	0.743	86.7

AUC: Area under the curve, CP: Commercial product, *IV*: Intravenously, *IN*: Intranasal

Table 17. Relative and absolute bioavailability data based on AUC 0 → ∞ values

	AUC 0 → ∞	AUC 0 → ∞ /dose	Absolute bioavailability	Relative bioavailability
CP (*IV*)	3059.5	1843.1	-	-
CP (*IN*)	2120.9	4.68	0.254	-
3A	1279.1	2.89	0.157	61.8
4A	1477.4	4.31	0.234	92.1

AUC: Area under the curve, CP: Commercial product, *IV*: Intravenously, *IN*: Intranasal

Table 18. Relative and absolute bioavailability data based on C_{max} values

	C_{max}	C_{max}/dose	Absolute bioavailability	Relative bioavailability
CP (*IV*)	17.4	10.5	-	-
CP (*IN*)	87.4	0.195	1.88	-
3A	4.8	0.011	0.11	5.64
4A	37.3	0.104	0.99	53.3

C_{max}: Maximum concentration, CP: Commercial product, *IV*: Intravenously, *IN*: Intranasal

number of trials, consistent values could not be obtained even with the same formulation. It can be said that the release in formulation A is more balanced than in formulation B based on their viscosity. Moreover, release of formulation B was slower than that of formulation A.

Using Tukey HSD method, a difference within 95% confidence interval was observed between the original spray of CP and the investigated spray intended to be used during the application. Using the same method, spray homogeneity was evaluated against our formulations by placing the commercial nasal spray content into the nasal spray to be administered. In spray applications, there was no difference between formulations and commercial nasal product spray homogeneity at 95% confidence interval as seen in Table 13. This indicates reliability of the selected spray device to be used during bioavailability studies.

IU used for sCT herein is defined as the amount of calcitonin that produces an equivalent reduction in blood calcium level in young rats under strictly defined experimental conditions within one hour as the injection of an ampoule or part of an ampoule of international reference preparation of calcitonin.[1] Based on this, spraying volume was taken as 0.14 mL for each nostril to achieve the targeted single dose of 1000 IU. Standardization of sCT dose is very complicated due to precision in the weighing and slight volume differences in the prepared formulations.

ELISA method is generally a heterogeneous non-competitive application. Primary antibody corresponding to the analyte of interest is usually detected on the multiwell plate or solid plastic surface. Biological sample is dispensed onto the multiwell plate and the detected antibody captures the analyte to be measured. Excess analyte is removed by washing. Antigen-antibody complex is determined at the conjugated antibody and its antigen by a two-step retention process. First, the enzyme labeled antibody goes to the analyte and binds to the antibody-antigen complex. The second incubation occurs with a specific substrate solution suitable for enzyme. At this stage, the amount of the colored product is determined spectrophotometrically.[23]

AUC and C_{max} values as main parameters in bioavailability assessment of CPs and the formulations were calculated separately using Winnonlin program after each application to six rabbits. To compensate for differences between the doses administered to rabbits, values were calculated based on the doses administered.

Absolute bioavailability of CP administered *via* nasal route was found to be 1.88 using C_{max} value as a primary pharmacokinetic parameter (Table 18). This finding agrees with the data presented in the literature.[38] Among the developed formulations, 4A formulation has an absolute and relative bioavailability of 0.99 and 53.3%, respectively; the bioavailability value is half that of CP as expected. This could be explained by the fact that the formulation in question is a controlled release hydrogel matrix type preparation designed to release sCT at a much slower rate and in lower quantities than CP.

CONCLUSION

In this study, a sol-gel formulation that is in liquid form at +4 °C as storage condition and gel form at intranasal temperature was developed. When sprayed into nose, the developed formulation adheres to the mucosa. Taken into account the fact that the formulation is in liquid form at the time of spraying, but turns into a gel only after being in contact with the mucosa. Dose uniformity was ensured during the development. Fluidity of the product is reduced, where it is squeezed, allowing the release of the active substance. sCT is stable at pH 3-4 and considering that no sCT was detected in formulations with pH 5. It shows that sCT molecule is very unstable at this pH.

In an *in vitro* release study, formulations 3A and 4A showed faster and more permeation than CP at 10 min. This situation can be attributed to the transition of formulations in the first few minutes. Nevertheless, 3A and the CP showed similar *in vitro* release capability. Overall, formulations A were found to be more stable and have better *in vitro* release capability compared to formulations B based on their viscosity.

CP and developed formulations were further investigated *in vivo* studies. The standard dose was provided with a spray head suitable for rabbit noses. Pharmacokinetic data revealed that CP was able to reach C_{max} value in 30 min. Meanwhile, t_{max} value in EIA analysis was 0 in two of six rabbits administered *IV*, while values such as 15, 45, and 75 min were found in the other rabbits. As this situation could not possibly be explained theoretically, it is likely because of the analysis method used given that the same problem was encountered in another study. Based on AUC $0 \rightarrow t_{last}$ value (the primary pharmacokinetic parameter), the absolute bioavailability of CP administered *via* the intranasal route was found to be 0.856, which is within the range of 0.3-3 stated in the literature.[20,25,42] Absolute bioavailability value for formulation 4A (*i.e.* 0.743) was very close to the value of the CP. As for 3A formulation, absolute and relative bioavailability was found to be 0.222 and 25.9%, respectively, which is below the values of CP and 4A formulation in contrast with the positive results obtained in the *in vitro* studies. A similar situation was observed using the secondary pharmacokinetic parameter, AUC $0 \rightarrow \infty$: absolute bioavailability of CP and 4A formulations were similar (*i.e.,* 0.254 and 0.234, respectively), whereas the 3A formulation has a bioavailability value (*i.e.,* 0.157), which is 35% of the mentioned bioavailability values.

Nasal administration of CP showed an absolute bioavailability of 1.88 based on C_{max} value as the primary pharmacokinetic parameter, which is in accordance with the literature. Among the developed formulations, formulation 4A has an absolute bioavailability of 0.99 and relative bioavailability of 53.3%, half as much as CP. Absolute bioavailability of 0.11 and relative bioavailability of 5.64% for 3A formulation is less than bioavailability values obtained using AUC parameters. This is thought to be due to slow release of sCT from gel form. The obtained virtual volume of distribution (V_d) value from pharmacokinetic data indicates greater plasma protein binding of the sol-gel formulations. Post *IV* and *IN* administration, V_d

values for CP were much smaller than the values of the developed formulations. Based on these results, it was suggested that bioavailability assessment using pharmacodynamic parameters other than EIA and the RIA method would be more meaningful during data evaluation.

Ethics

Ethics Committee Approval: Ege University Faculty of Medicine Animal Ethics Committee (2009-95).

Informed Consent: Not necessary.

Peer-review: Externally peer-reviewed.

Authorship Contributions

Surgical and Medical Practices: A.L.A., E.K., Concept: A.L.A., Design: A.L.A., Data Collection or Processing: A.L.A., G.Y.T., Analysis or Interpretation: A.L.A., E.K., L.D.K., Literature Search: A.L.A., G.Y.T., Writing: A.L.A.

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ORIGINAL ARTICLE

Trachystemon orientalis (L.) G. Don as a Valuable Source of Rosmarinic Acid: Biological Activities and HPLC Profiles

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ABSTRACT

Objectives: *Trachystemon orientalis* (L.) G. Don, colloquially known in Türkiye as "kaldırık", is an edible plant belonging to the Boraginaceae. This plant has been practiced in traditional medicine for many years for its various therapeutic benefits. The effectiveness and chemical composition of plants can vary depending on their parts, age, and extraction solvent. Therefore, the current study aimed to define the biological activities of various parts and extracts of *T. orientalis*, which were collected in distinct seasons as young and mature, and investigate the main component responsible for these biological effects.

Material and Methods: Plant material was collected in different seasons from the northwest of Türkiye. 2,2'-Azinobis-(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) and 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radical scavenging activities were investigated to assess antiradical and antioxidant potential of the extracts. Anti-inflammatory activity of the extracts was also tested using human red blood cell membrane stabilizing method. Folin-Ciocalteu test was conducted to determine the total phenolic content. Reverse phase-high performance liquid chromatography-photodiode array detector (RP-HPLC-PDA) analysis was performed.

Results: Both methanol and aqueous extracts exhibited significant radical scavenging and anti-inflammatory activities compared with control ($p<0.05$). The highest percentage of inhibition on ABTS and DPPH free radicals was obtained in aqueous extracts of the mature herbs and roots, respectively. Methanol extracts of the mature roots and herbs exhibited the strongest anti-inflammatory capacity. Rosmarinic acid possessed a much higher antioxidant and anti-inflammatory effect than the reference compounds used in each assay in our study. High rosmarinic acid content of the extracts suggests that the compound responsible for the great biological activity potential is rosmarinic acid.

Conclusion: To the best of our knowledge, the presence of rosmarinic acid in herbs and roots of *T. orientalis* was shown for the first time in our present study. Phytochemical composition and effective biological activities of *T. orientalis* explain its traditional use and indicate its significant potential in pharmaceutical industry applications.

Key words: Antioxidant activity, anti-inflammatory activity, rosmarinic acid, RP-HPLC-PDA, *Trachystemon orientalis*

INTRODUCTION

Trachystemon orientalis (L.) G. Don is a plant belonging to the Boraginaceae family that is represented by 34 genera and 325 species in Türkiye.[1] This plant is distributed in nearly all Black Sea regions in Türkiye, East Bulgaria, and West Caucasia. *T. orientalis* is called by different names in Türkiye, such as "kaldırık, tomar, burğı, hodan, and ispit". It is an edible and medicinal plant. The leafy and budding plant body is consumed as vegetable, while the roots and petioles are consumed as pickles in several parts of the Black Sea region.[2] It is used

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as an antipyretic, diaphoretic, diuretic, laxative, anticancer, analgesic, antiflatulent, and antirheumatismal in medicinal treatments.[3-5] Chronic and cumulative oxidative damage leads to various pathologic processes such as stimulating the genes incorporated in the inflammatory phases. The occurrence and progression of inflammation-associated conditions such as arthritis, atherosclerosis, Alzheimer's disease, autoimmune diseases, ocular diseases, diabetes, and cancer may be triggered by enhancing the number of free radicals. There has been considerable interest in the role of free radicals in chronic diseases and the protective effect of antioxidants as scavengers of free radicals.[6-9] Inflammatory diseases are commonly treated with steroidal and non-steroidal anti-inflammatory drugs. Besides the beneficial therapeutic effects, they also have crucial side effects. Much research has been conducted to determine new, safe, pharmacologically active plants, and plant-derived compounds with lower side effects.[10,11] Consequently, target of the current study was to analyse and determine antioxidant potentials and anti-inflammatory activities of aqueous and methanol extracts from herbs and roots of young and mature *T. orientalis*, which is the first report on relevant point for this species. Furthermore, to the best of our knowledge, this study identified rosmarinic acid as the major and possibly the most active component of the herbs and roots of this plant for the first time.

MATERIALS AND METHODS

Chemicals

2,2'-Azinobis-(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS), 2,2-diphenyl-1-picrylhydrazyl (DPPH), acetonitrile, acetylsalicylic acid (ASA), gallic acid, propyl gallate, rosmarinic acid, trifluoroacetic acid, and trolox were bought from Sigma-Aldrich (USA). Sodium carbonate, sodium chloride, potassium persulphate, sodium chloride, dimethyl sulfoxide, methanol, ethanol, and other solvents were purchased from either Merck (Germany) or Sigma-Aldrich (USA).

Plant materials

Plant material was collected in different seasons from Akçakoca town and Tahirli village in Düzce province (Türkiye), and identified by one of us (B.B.). Plant material was firstly dried at room temperature. Afterwards, a laboratory-scale mill was used to powder the material. A voucher specimen was housed in the Herbarium of Ankara University, Faculty of Pharmacy (Türkiye). Collection site, date, and herbarium number of the plant sample were noted [*T. orientalis* (L.) G. Don: A3 Düzce, Akçakoca, Tahirli Köyü, 13/02/2016-25/04/2016, AEF 26813].

Preparation of extracts

Preparation of methanol extract: Each part of plant material (5 g) was powdered and, then, the materials were macerated with 50 mL methanol three times for 8 h at 60 °C. Afterwards, they were filtered and combined. A reduced pressure was applied at 40 °C to the mixtures to concentrate them (0.73 g, 14.60% *w/w* young root yield, and 0.42 g, 8.4% *w/w* young herb yield, and 0.66 g, 13.40% *w/w* mature root yield, and 0.31 g, 6.40% *w/w* mature herb yield).

Preparation of aqueous extract: Powdered plant material (5 g) was added to 50 mL of distilled water and boiled for 30 min. The extracts were filtered and afterwards lyophilized (1.15 g, 23.06% *w/w* young herb yield, and 0.73 g, 14.71% *w/w* young root yield, and 1.18 g, 23.70% *w/w* mature herb yield, and 0.92 g, 18.50% *w/w* mature root yield).

Antioxidant activity

Antioxidant activity of the extracts was determined by investigating their scavenging abilities against ABTS and DPPH free radicals.

ABTS free radical scavenging activity

Antioxidant activity was investigated by measuring the scavenging capacity of the samples against ABTS free radicals.[12] First, 7 mM aqueous solution of $ABTS^{+}$ reacted with 2.45 mM potassium persulfate and then a radical cation solution of $ABTS^{\bullet+}$ was produced. This radical solution was maintained at room temperature overnight in dark. Dilution is applied to this dark radical solution with ethanol until obtaining absorbance of 0.700 ± 0.05 at 734 nm. Finally, a working solution was arranged (pH: 7.4). The ABTS solution was prepared freshly in each study to prevent degradation. The test sample was mixed with radical cation solution (100x). Then the mixtures were held for 6 min at room temp. The reference compound was Trolox in this assay. At the final step of the experiment, the absorbance of the samples was measured at room temperature at 734 nm. The experiments were performed at least three times. The percentage of inhibition was computed for each sample at each concentration. Then, half maximal inhibitory concentration (IC_{50}) values were computed using these values. The final results were presented as IC_{50} ± standard deviation (SD).

DPPH free radical scavenging activity

Both qualitative and quantitative methods were used to determine this activity.

Qualitative DPPH radical scavenging activity

Antioxidant capacity of the samples was assessed using qualitative DPPH radical scavenging assay.[13] 0.1 mM DPPH methanol solution (1 mL) was put into the test tube, then, the extracts were inserted in different concentrations and vortexed. The mix was left to stand at room temperature for 30 min. When this DPPH solution is added to a solution containing antioxidant substance, this dark purple loses color with time.

Quantitative DPPH radical scavenging activity

DPPH free radical scavenging capacity of the extracts was examined to determine their antioxidant activity.[14] DPPH stock solution was prepared first. The stock solution of each test compound in methanol was prepared (0.5 mg/mL) and then serial dilutions in equal amounts of methanol were prepared in wells. DPPH solution was added to each well to initiate the reaction. Then, these mixtures were kept in the dark for 30 min. Propyl gallate served as the reference compound.

Absorbance was measured at room temperature at 517 nm. The experiments were performed at least three times. The percentage of inhibition was computed for each concentration of each sample. IC_{50} values were then computed using these values. The final results were presented as IC_{50} ± SD.

Anti-inflammatory activity

Anti-inflammatory activities of the samples were assessed by human red blood cell membrane stabilizing method.[15,16] The protocol was confirmed by the Human Research Ethics Committees of Ankara University, Faculty of Medicine with 14.05.2020/I5-273-20. Fresh human blood was taken from the healthy volunteers. The volunteers without any chronic disease had not taken any medicine for up to 15 days prior to the test (especially steroidal and anti-inflammatory drugs). Then, the centrifuge process was applied at 3000 rpm for 10 min. The packed cells were isolated and washed with 0.85% isosaline at least three times (pH: 7.2). Then, a 10% *v/v* cell suspension was arranged. An equal volume of this cell suspension was added to the tubes consisting of the test samples, and then incubation was applied at 56 °C for 30 min. Afterwards, the tubes were left to cool and centrifuge process was applied at 2500 rpm for 5 min. Then the absorbance was determined at 560 nm. ASA served as the standard compound. The tests were conducted in triplicates. The percentage of inhibition of hemolysis was computed for each concentration of each sample. Then, IC_{50} values were computed and the outcomes were presented as IC_{50} ± SD.

Phytochemical analysis

Total phenolic content quantification assay

Total phenolic contents of the extracts were examined by the Folin-Ciocalteu method.[17] Folin-Ciocalteu's reagent, 20% (*w/v*) aqueous Na_2CO_3 and samples were mixed and the volume was completed with distilled water. The solution was kept at room temperature for 30 min. The absorbance was then determined at room temperature at 765 nm. The same method was applied for gallic acid with the samples and gallic acid calibration curve was drawn. The results were served as gallic acid equivalent (GAE) (mg GAE/g extract dry weight). All experiments were performed at least three times.

Qualitative and quantitative analysis of rosmarinic acid using reverse phase-high performance liquid chromatography-photodiode array detector (RP-HPLC-PDA)

Qualitative and quantitative analyzes of rosmarinic acid (536954, Sigma-Aldrich) in mature leaf and root methanol extracts were assessed using a previously validated method.[18] HPLC system was Agilent 1260 series. The system is equipped with a quaternary pump, an auto-sampler, a column oven, and a PDA detector. Agilent ChemStation software was used for data analysis. The separation was performed on an ACE 5 C18 (250 × 4.60 mm) column with a mobile phase of a mix of trifluoroacetic acid (HPLC grade, ≥99.0%) 0.1% in water (solution A), trifluoroacetic acid 0.1% in methanol (HPLC grade, ≥99.9%) (solution B), and trifluoroacetic acid 0.1% in acetonitrile (HPLC grade, ≥99.9%) (solution C). Gradient profile was (A: B: C), 80: 12: 8 at 0 min, 75: 15: 10 at 8 min, 70: 18: 12 at 16 min, 65: 20: 15 at 24 min, 50:35:15 at 32 min, 25: 60: 15 at 40 min, and 80:12:8 at 45 min. The period between each run was arranged as 2 min. 10 µL was applied for injection. Detection ultraviolet (UV) wavelength was regulated at 330 nm. Column temperature was controlled and arranged to 30 °C. All quantification and validation parameters for rosmarinic acid were given in our previous study.[18]

Statistical analysis

All experiments were assessed at least three times for all test samples. SPSS 23.0 was used to examine the results. Data were tested for significant differences by One-Way ANOVA. Then, *post-hoc* Tukey test was performed. A p value below 0.05 was set statistically significant.

RESULTS

Antioxidant activity

ABTS free radical scavenging activity

Antioxidant activity of the samples was determined by measuring their radical inhibitory capacity on ABTS. The results were presented in Table 1. All extracts exhibited significant radical scavenging effect in a concentration-dependent manner compared to the control (p<0.05). No significant differences were found according to the age of plant. Both mature and young plants were found to be reactive toward ABTS free radical and had a considerable reducing ability. Generally, aqueous extracts exhibited higher radical scavenging activity than those of methanol extracts. The greatest percentage of inhibition was observed in aqueous extracts of mature herbs among all extracts (IC_{50}: 21.86 ± 1.63 µg/mL). Rosmarinic acid was found to be almost 20 fold more effective than trolox, which was used as a standard compound for this assay. IC_{50} value of rosmarinic acid was 0.57 ± 0.02 µg/mL, while trolox was 9.88 ± 0.02 µg/mL.

DPPH free radical scavenging activity

Qualitative DPPH analysis

The outcomes of qualitative DPPH analysis demonstrated that all extracts showed high antioxidant activities according to inhibition zones (Figure 1).

Quantitative DPPH analysis

DPPH free radical scavenging activity of the extracts was assigned as a mark of antioxidant capacity. DPPH inhibition profiles of the extracts are displayed in Table 2. All extracts exhibited a significant DPPH radical scavenging effect compared to control (p<0.05). No significant differences between young and mature plants were noted regarding this effect. Both groups were found to be effective in inhibiting DPPH. Generally, aqueous extracts exhibited better DPPH free radical scavenging potential than the methanol extracts, correlatively to the outcomes of other radical assay (ABTS). Aqueous extracts of the mature root displayed the highest inhibitory effect on DPPH free radicals, followed by aqueous extracts of the young herbs (IC_{50}: 3.50 ± 0.04 and 3.87 ± 0.04 µg/mL, respectively).

Anti-inflammatory activity

Anti-inflammatory effect of the extracts was determined by measuring their protection capacity on human erythrocyte membrane as shown in Table 3. In general, the methanol extracts showed stronger protective effects than those of aqueous extracts and mature plants were more effective than young ones. Methanol extracts of mature roots and herbs exhibited the highest protective effect (IC_{50}: 0.30 ± 0.01 mg/mL) that was similar to the reference compound. Rosmarinic acid was found almost 4 fold more effective in this assay with IC_{50} value of 0.07 ± 0.02 mg/mL than that of the reference compound (IC_{50}: 0.27 ± 0.05 mg/mL).

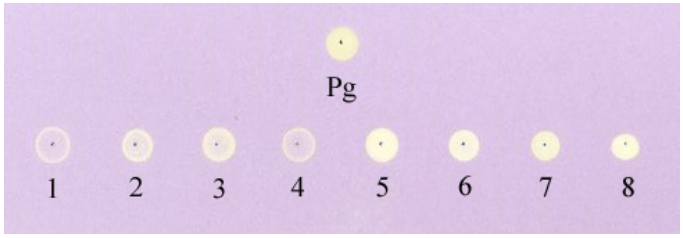

Figure 1. Reactions of extracts with DPPH

1: Young herb methanol, 2: Young root methanol, 3: Mature herb methanol, 4: Mature root methanol,

5: Young herb aqueous, 6: Young root aqueous, 7: Mature herb aqueous, 8: Mature root aqueous, Pg: Propyl gallate, DPPH: 2,2-Diphenyl-1-picrylhydrazyl

Phytochemical analysis

Total phenolic content quantification

Total phenolic content of the extracts was evaluated by the Folin-Ciocalteu method. Gallic acid calibration curves including gallic acid concentration and absorbance values are shown in Figure 2.

All examined extracts had significant amounts of phenolic compounds. Total amount of phenolic compounds of the samples displayed a narrow range from 462.22 ± 8.64 and 578.33 ± 3.14 mg GAE/g extract (dw) (Table 4). In general, aqueous extracts

Table 1. ABTS free radical scavenging effect of various ages, parts and extracts of *Trachystemon orientalis*

Plant/reference	Age	Parts	Extracts	IC_{50} (µg/mL)
T. orientalis	Mature	Herbs	Methanol	30.67 ± 1.51*
			Aqueous	21.86 ± 1.63*
		Root	Methanol	31.22 ± 0.48*
			Aqueous	27.51 ± 0.73*
	Young	Herbs	Methanol	29.84 ± 0.21*
			Aqueous	29.33 ± 0.54*
		Root	Methanol	40.29 ± 0.24*
			Aqueous	25.82 ± 0.61*
Trolox				**9.88 ± 0.02***
Rosmarinic acid				**0.57 ± 0.02***

*$p<0.05$; compared with the control, statistically significant. Each value represents mean ± SD (independently replicated three times)

Table 2. DPPH free radical scavenging effect of various ages, parts and extracts of *Trachystemon orientalis*

Plant/reference	Age	Parts	Extracts	IC_{50} (µg/mL)
T. orientalis	Mature	Herbs	Methanol	4.31 ± 0.04*
			Aqueous	4.33 ± 0.04*
		Root	Methanol	6.12 ± 0.06*
			Aqueous	3.50 ± 0.04*
	Young	Herbs	Methanol	6.24 ± 0.06*
			Aqueous	3.87 ± 0.04*
		Root	Methanol	4.79 ± 0.05*
			Aqueous	4.14 ± 0.04*
Propyl gallate				**1.73 ± 0.02***

*$p<0.05$; compared with the control, statistically significant. Each value represents mean ± SD (independently replicated three times)
SD: Standard deviation, DPPH: 2,2-Diphenyl-1-picrylhydrazyl, IC: Inhibitory concentration

contain higher total phenolic content than methanol extracts. Maximum phenolic content was detected in aqueous extracts of the young herbs followed by methanol extracts of the young roots.

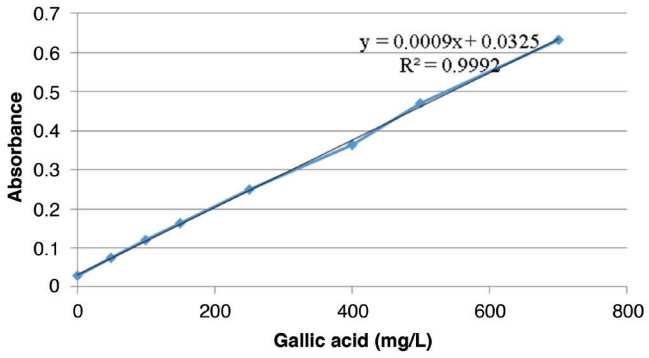

Figure 2. Gallic acid calibration curve

HPLC analysis of rosmarinic acid in T. orientalis extracts

Based on the HPLC analysis results, rosmarinic acid was detected as the major compound in both methanol extracts of mature herbs and roots with contents of 74.56 ± 0.03 mg/g and 77.03 ± 0.01 mg/g, respectively. HPLC chromatograms of *T. orientalis* herb and root extracts were presented in Figure 3. Also, the overlaid UV spectra of standard rosmarinic acid and rosmarinic acid in the extracts were presented in Figure 4.

DISCUSSION

There is a positive correlation between an antioxidant-rich diet and reduction of oxidative damage and inflammation. This antioxidant-rich healthy diet includes a range of plant foods, fruits, and vegetables. In addition, not only for nutrition, but also for oxidative stress and inflammation process, natural compounds and trace elements with antioxidant properties can be a solution in regulating key points.[19-23] Many plants consist of a range of radical scavenging molecules, such as phenolics, flavonoids, anthocyanins, coumarins, alkaloids, and carotenoids. Several studies have indicated that natural phenolic compounds and anthocyanins have antioxidant, anti-inflammatory,

Table 3. Anti-inflammatory activity of various ages, parts and extracts of *Trachystemon orientalis*

Plant/reference	Age	Parts	Extracts	IC_{50} (mg/mL)
T. orientalis	Mature	Herbs	Methanol	0.30 ± 0.01*
			Aqueous	0.45 ± 0.05*
		Root	Methanol	0.30 ± 0.01*
			Aqueous	0.47 ± 0.03*
	Young	Herbs	Methanol	0.34 ± 0.01*
			Aqueous	0.45 ± 0.02*
		Root	Methanol	0.32 ± 0.01*
			Aqueous	0.51 ± 0.02*
ASA				0.27 ± 0.05*
Rosmarinic acid				0.07 ± 0.02*

*$p<0.05$; compared with the control, statistically significant. Each value represents mean ± SD (independently replicated three times)
ASA: Acetylsalicylic acid

Table 4. Total amounts of phenolic compounds of various ages, parts and extracts of *Trachystemon orientalis*

Plant/reference	Age	Parts	Extracts	mg GAE/g extract (dw)
T. orientalis	Mature	Herbs	Methanol	472.22 ± 5.50
			Aqueous	530.56 ± 3.14
		Root	Methanol	462.22 ± 8.64
			Aqueous	559.44 ± 6.29
	Young	Herbs	Methanol	532.22 ± 2.36
			Aqueous	566.11 ± 4.71
		Root	Methanol	570.00 ± 8.64
			Aqueous	578.33 ± 3.14

Each value represents mean ± SD (independently replicated three times)

antidiabetic, and antiproliferative effects.[11,24-27] Rosmarinic acid, one of these phenolic compounds, is an antioxidant found in the structure of many plants from the Boraginaceae family. Some experimental studies have also revealed that rosmarinic acid exhibits inhibitory effects on oxidation and inflammation. Rosmarinic acid represents antioxidant, anti-inflammatory, and hepatoprotective activities and alleviates the impacts of inflammatory diseases including inflammatory bowel syndrome and rheumatoid arthritis.[28,29] Rosmarinic acid has been reported to inhibit colon inflammation *via* binary reduction of NF-κB and STAT3 activation in dextran sulfate sodium-induced mice.[30]

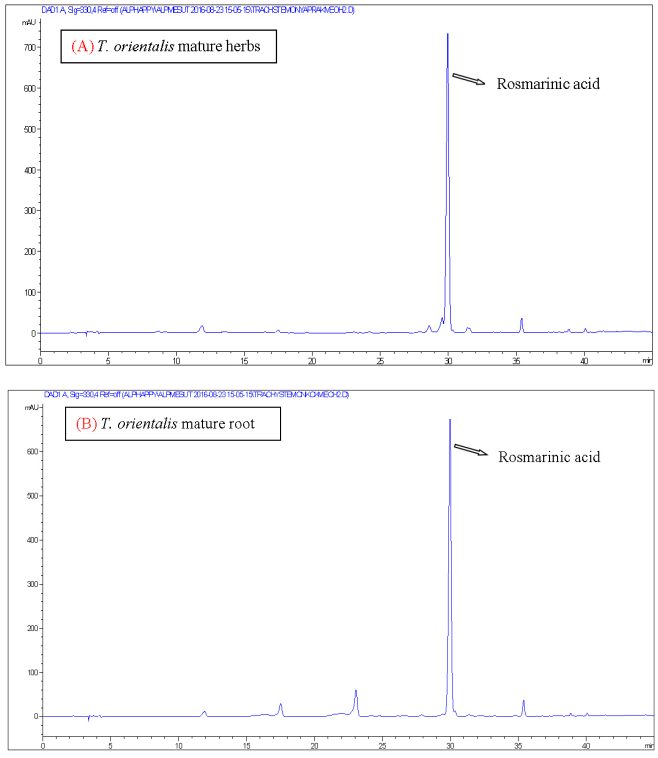

Figure 3. HPLC chromatograms of herbs and roots of *Trachystemon orientalis*. HPLC analysis of rosmarinic acid in *T. orientalis*. (A) *T. orientalis* mature herbs, (B) *T. orientalis* mature roots

HPLC: High performance liquid chromatography

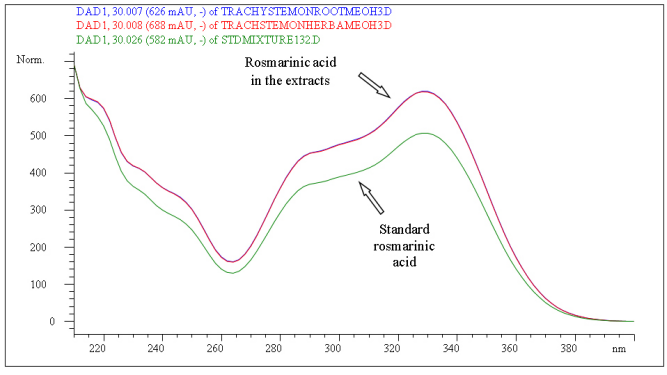

Figure 4. The overlaid ultraviolet spectra of standard rosmarinic acid and rosmarinic acid in the extracts

Anthocyanin-rich extracts and rosmarinic acid, in combination, reduced the symptoms of inflammatory bowel disease.[24] *T. orientalis* has been reported to consist of phenolic compounds, anthocyanins, tannins, essential oils, saponins, and resins and exhibits significant antioxidant activity.[31-33]

Chemical composition of the extracts varies depending on the growing location, environmental conditions, parts, and age of the plant as well as extraction solvent. Thus, different extracts are expected to elicit various clinical responses. For this reason, *in vitro* antioxidant and anti-inflammatory activities as well as phytochemical contents of several parts of *T. orientalis* gathered in different seasons were determined in this study. The results of our study indicated that all parts of *T. orientalis* exhibited significant antioxidant activity regardless of age. Generally, the aqueous extracts exhibited better free radical scavenging activities than the methanol extracts. All extracts offered a substantial anti-inflammatory activity compared with the control. The methanol extracts exhibited better anti-inflammatory activity than aqueous extracts. Both antioxidant and anti-inflammatory potentials of the extracts were increased dose-dependently. The strong antioxidant and anti-inflammatory effects of the extracts are most often correlated with the high content of total phenols. In our study the total phenolics of the extracts was found to be high which supports this information. In addition, rosmarinic acid, a very effective phenolic compound, was found as the main ingredient in our extracts. After obtaining this information, the antioxidant and anti-inflammatory efficacy of not only the extracts but also rosmarinic acid was evaluated. Rosmarinic acid exhibited higher activity than reference compounds tested in all methods. The antioxidant activity of rosmarinic acid was ratified and found to be correlated with the anti-inflammatory effect in our study. Inhibition of free radical production also protects cell membranes against oxidative stress and oxidavite damage.[34,35] This explains the link between rosmarinic acid and *T. orientalis* extracts showing both high free radical scavenging and cell membrane stabilizing effect at the same time. Presence of rosmarinic acid in the herbs and roots of *T. orientalis* was shown for the first time in the current study. Moreover, antioxidant and anti-inflammatory effects of *T. orientalis,* which gathered in both young and mature periods, were presented for the first time as well. Our data supply evidence that *T. orientalis* herbs and roots can be used as potential sources of rosmarinic acid.

CONCLUSION

To the best of our knowledge, this is the first report to assess the presence of rosmarinic acid in herbs and roots of *T. orientalis.* Moreover, there are no adequate data comparable to the results obtained in our current study. *T. orientalis* has substantial antioxidant and anti-inflammatory characteristics and rosmarinic acid is probably the main responsible compound contributing to these biological activities. Phytochemical composition and biological activities of *T. orientalis* explain its traditional use and indicate its potential applications in pharmaceutical and cosmetic industries.

Ethics

Ethics Committee Approval: The protocol was confirmed by the Human Research Ethics Committees of Ankara University, Faculty of Medicine with 14.05.2020/I5-273-20.

Informed Consent: Informed consent was obtained from all participants.

Peer-review: Externally peer-reviewed.

Authorship Contributions

Concept: B.B., S.Y.S., A.G., T.Ç., M.C., Design: B.B., S.Y.S., A.G., T.Ç., M.C., Data Collection or Processing: B.B., S.Y.S., A.G., T.Ç., M.C., Analysis or Interpretation: B.B., S.Y.S., A.G., Literature Search: B.B., S.Y.S., A.G., T.Ç., M.C., Writing: B.B., S.Y.S., A.G., T.Ç., M.C.

Conflict of Interest: No conflict of interest was declared by the authors.

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ORIGINAL ARTICLE

Preparation and Characterization Studies of Dorzolamide-Loaded Ophthalmic Implants for Treating Glaucoma

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ABSTRACT

Objectives: This study constructed dorzolamide (DRZ)-loaded ophthalmic implants for extended drug delivery and increased drug retention.

Materials and Methods: Carboxymethyl cellulose (CMC) and chitosan (CHI) were used to describe the ophthalmic implants. The implants were prepared by the solvent casting technique in presence of polyethylene glycol 6000 (PEG 6000) as plasticizer. Physicochemical characterization studies including mechanical characteristics [tensile strength (TS), elongation at break, and Young's modulus], bioadhesion studies, and *in vitro* and *ex vivo* drug release studies were conducted.

Results: TS of drug-loaded ophthalmic implants was 10.70 and 11.68 MPa, respectively. Elongation at break of CMC and CHI implants was 62.00% and 59.05%, respectively. The *in vitro* release profiles fit into Higuchi type kinetic model. *Ex vivo* release study results for both implants were correlated with *in vitro* release investigations.

Conclusion: CMC and CHI-based implants provide extended drug delivery. Implants prepared using CMC provided a significantly slower *in vitro* release rate, and drug retention on ocular surfaces increased. Thus, it has been concluded that DRZ-loaded CMC implants could provide effective treatment for glaucoma.

Key words: Dorzolamide, carboxymethyl cellulose, chitosan, ocular implant

INTRODUCTION

Glaucoma is a progressive optic neuropathy resulting from high intraocular pressure (IOP). This condition is stated as the main reason for irreversible blindness after diabetic retinopathy.[1] Purpose of the therapy is to prevent optic nerve damage. Glaucoma is treated with the use of drugs (pharmaceutical therapy). Pharmacologically, beta receptor antagonists, prostaglandin analogs, alpha-2 agonists, and carbonic anhydrase inhibitors are chosen as treatment options.[2] Dorzolamide (DRZ) is one of the carbonic anhydrase inhibitors that decreases the secretion of aqueous humor, thus the IOP is lowered.[3] DRZ eye drop is available on the market under trade name Trusopt® (Merck, N.J., USA). The dosage form contains 2% DRZ aqueous buffered solution at pH 5.6. DRZ 2% eye drops have exhibited the highest and optimal therapeutic effect in clinical trials.[4]

Effective ocular delivery of drug substances is a challenging process. Topical ophthalmic solutions are mostly preferred preparations by clinicians due to their ease of application. However, topical conventional delivery systems remain insufficient due to multifactorial restrictions of the ocular

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anatomy. These can be mainly categorized as intraocular microenvironment, static, dynamic, and metabolic barriers.[5] Intraocular environment includes blood-aqueous and blood-retina barriers. The static barriers include biological structures such as the corneal epithelium, sclera, and conjunctiva. The metabolic barriers contain metabolic enzymes. Dynamic barriers are listed as; blinking, tear turnover, and nasolacrimal drainage, which remarkably decrease the drug bioavailability.[6]

Many sophisticated strategies have been introduced for bypassing the ophthalmic barriers, such as nanoparticles for enhancement of corneal permeation[7-9] and liposomal carriers for enhancement of ocular absorption and precorneal retention.[10,11] Micro or nanoemulsions are used for increasing the precorneal residence time and providing sustained release.[12,13] Hydrogels and ocular inserts are used as primary or secondary delivery systems. The incorporation of nanocarriers into the hydrogels or ocular inserts makes them a secondary delivery system. Hydrogels or ocular inserts can be used as primary drug delivery systems for treating ophthalmic problems.[14,15]

Chitosan (CHI) and carboxymethyl cellulose (CMC) are biodegradable and bioadhesive polymers that are used for the production of hydrogel or ocular implant or insert formulations.[16,17] These polymers are compatible materials with drug substances and biological surfaces. In this study, we aimed to construct an ophthalmic implant for the extended delivery of DRZ to achieve efficient glaucoma treatment. For this purpose, polymeric ophthalmic implants were developed and physiochemically characterized, and then, *in vitro* and *ex vivo* drug release profiles were investigated.

MATERIALS AND METHODS

Materials

CMC and CHI were obtained from Sigma Aldrich (Germany). Polyethylene glycol 6000 (PEG 6000), acetic acid, potassium dihydrogen phosphate (K_2PO_4), and methanol (MeOH) were acquired from Merck-Millipore (USA). DRZ was kindly donated by Deva Pharmaceuticals (Türkiye). The other materials were of analytical quality.

Methods

Preparation of ophthalmic implants

CMC and CHI were used as polymers, PEG 6000 was selected as plasticizer (to provide elasticity) and DRZ was used as active pharmaceutical ingredient, which is dissolved in aqueous polymer-plasticizer dispersion. CMC-based implants include: CHI (1 g) was dispersed in 100 mL aqueous acetic acid solution (1%, *w/w*). CMC-based implants include: CMC (1 g) was dispersed in 100 mL of water. Both of the implant formulations have PEG 6000 (0.1 g) in 100 mL total dispersion volume to improve the mechanic properties.

The dispersions were poured into empty contact lens containers (1-Day Acuvue® moist contact lens container). The lens containers had a diameter of 14.2 mm and a space to hold a gram of mass. Dispersion-loaded containers were left for drying under the fume hood for 24 h at room temperature. According to the placebo weight of containers, the amount of DRZ was adjusted and the strength was obtained as 2 mg DRZ/implant.

Analytical quantification of DRZ

Quantification of DRZ was performed using high performance liquid chromatography (HPLC) (Agilent 1100 series, Germany). The reported analytical method has been slightly changed.[18] In brief, HPLC was equipped with multiple wavelength ultraviolet/visible detectors. Separation was conducted by using a C-18 column (5 µm, 4.6 × 150 mm) (AgilentTech, Germany) at 25 ± 0.5 °C. K_2PO_4 (pH:2.5): MeOH mixture (90:10, v/v) was used as mobile phase. Quantification was achieved at a flow rate of 0.8 mL/min. This analytical method was validated using universal parameters.

Characterization studies of ocular implants

Bioadhesion studies

A previously stated technique was slightly adapted for ocular tissues.[19] Bioadhesive characteristics of implants were detected by applying a texture analyzer (TA-XT Plus Texture Analyzer, Stable Micro System, UK). Swine eyes were obtained from the laboratory animal center and, then, the cornea was isolated from the ocular tissues. The implant was stabilized on a probe of the instrument using adhesive tape and the cornea was placed on the other probe of the instrument. DRZ-loaded implant was contacted to the tissue. Time of contact, rate and applied force were 60 s, 1 mm/s, and 0.2 N, respectively. The work of bioadhesion has been calculated using force-distance graph.

Mechanical characteristics

Mechanical characteristics of ocular implants were investigated using a texture analyzer (TA-XT Plus Texture Analyzer, Stable Micro Systems, UK). Calibration of force was achieved using 2 kg weight and calibration of height was achieved for Tensile Grips (Stable Micro Systems, UK). The samples were arranged by cutting 10 mm x 10 mm were stabilized in grip with primary distance of 50 mm and crosshead speed kept at 2 mm/sec tension mode. Tensile strength (TS) in MPa was measured by dividing the peak load improved during the analysis by the film cross-sectional area. TS, MPa, maximum elongation percentage at break (EAB, %), and Young's modulus (YM, MPa) were computed using equations. 1, 2, and 3, respectively. Tests were performed in triplicate and the outcomes were described as mean values (± standard deviation).

$$\mathrm{TS} = \frac{Fmax}{A} \qquad \text{(equation 1)}$$

$$\mathrm{EAB} = \frac{Lmax}{L0}\ x\ 100 \qquad \text{(equation 2)}$$

$$\mathrm{YM} = S\ x\ \frac{L0}{A} \qquad \text{(equation 3)}$$

F_{max} indicates the maximum force, A is implant cross-sectional area, L_{max} and L0 are the maximum deformation before rupture and primary length, respectively, and S is the slope of force deformation.[20]

Fourier transform infrared spectroscopy (FTIR) analysis

The spectra of ocular implants were obtained in the range of 650-4000 cm^{-1} by using an FTIR spectrometer (NICOLET iS50, Thermo Scientific, USA). Ocular implants were nicely divided and the specimens were directly applied over the crystal of the spectrometer. Scans were performed for each specimen and the force over the specimen was arranged to obtain satisfactory transmittance results.

Thermal analysis

A previous method was applied to detect the thermal behavior of all materials.[21] Specimens were arranged as small parts and (approximately 5 mg) transferred into covered aluminum pans. The temperature was elevated up to 300 °C under a cover of nitrogen gas (50 mL/s) with a heating rate of 10 °C/min using a differential scanning calorimetry (DSC) instrument (Setaram, DSC131, France).

Morphological analysis

Morphological analysis of ocular implants was achieved by using a scanning electron microscope (SEM) (Quattro S, Thermo Scientific, USA). SEM was used with an accelerating voltage of 15.00 kV and surfaces of the isolated specimens were covered with gold and palladium using a sputter (Leica EM ACE200, Leica Microsystems, Germany) at 3 kV for 60 s. SEM micrographs were captured by applying a high vacuum.

Solubility studies

Before the *in vitro* and *ex vivo* studies, solubility of DRZ was investigated using a previously described method.[19] Briefly, an excessive amount of DRZ was added into flasks (10 mL volume of each) that contained distilled water (pH: 7), phosphate buffer (pH: 7.4), and normal saline (NS) solution (0.9% NaCl, pH: 5.5). The flasks were shaken for 48 h and saturated solutions were filtered through 0.22 µm filters and quantified using HPLC.

In vitro drug release

In vitro DRZ-release profile was investigated using a previously reported paddle over disk method.[21] The specimens were placed in vessels that contained a 250 mL phosphate buffer solution (PBS) at pH 7.4. Temperature was kept constant at 37.5 °C. At pre-determined time intervals 2 mL samples were withdrawn from the release medium and completed with the same volume of fresh buffer solution. The samples were analyzed using the validated HPLC method. Release kinetics were also assessed by using zero-order, first-order, Hixson Crowell, Higuchi, and Korsmeyer-Peppas models.

Ex vivo studies

Ex vivo studies were conducted by using removed swine eyes. The eyes were placed into a small beaker (25 mL volume), and 20 mL buffer solution (pH 7.4) was added to cover the surface of the eye. Then, the ophthalmic implant was placed on the eye. At pre-determined time intervals, 1 mL of sample was withdrawn and replenished using fresh buffer.

An eye drop of DRZ was prepared using NS solution-DRZ at 2% (*w/v*) concentration. Similarly, the same *ex vivo* protocol was applied to the eye drops. Briefly, 2 drops (2 mg of DRZ) of preparation were applied to the swine eyes (placed in 20 mL buffer solution at pH 7.4). At pre-determined time intervals, 1 mL of sample was withdrawn and replenished using fresh buffer.

Statistical analysis

The samples were analyzed using the HPLC method. Then, the amount of drug penetrated was calculated retrospectively.

RESULTS AND DISCUSSION

Preparation of ophthalmic implants

Ophthalmic implants in the shape of convex ocular hemispheres were prepared using a previously generated solvent casting method.[21] This technique involves preparing a polymer dispersion, which is poured onto a concave mold (empty contact lens containers). Then, the solvent was removed by evaporation, which caused the reorganization of polymer molecules and engagement with each other. Finally, formation of films was achieved by this phenomenon. After solvent casting, implants had a similar surface with the mold shape. They were dry, elastic, and transparent films. Elastic films were easily removed from the contact lens containers (molds), probably because of the presence of plasticizer.

Mechanical characteristics

Mechanical characteristics of the specimens, including TS, EAB, and YM, are presented in Table 1. EAB is defined as the ability of a film to extend before it breaks. For that reason, if EAB is high, the structure of the implant might be thought to be flexible and soft.[22] TS is described as the maximum load power used to break the film. Rigid and fragile materials exhibit high resistance.[23] EAB and TS of unloaded CMC films were 71.68% and 7.81 MPa, respectively, and the values of loaded CMC films were 62% and 10.70 MPa, respectively. Similar values are also available for CHI films; unloaded CHI films were 69.78% and 8.21 MPa, respectively, and the values of loaded CHI films were

Table 1. Mechanical characteristics of inserts

Samples	The work of bioadhesion (mJ/cm^2)	TS (MPa)	YM (MPa)	EAB (%)
CMC 1% unloaded	0.143 ± 0.046	7.81 ± 0.017	10.77 ± 0.317	71.68 ± 0.049
CMC 1% loaded	0.427 ± 0.163	10.70 ± 0.031	13.80 ± 0.226	62.00 ± 0.03
CHI 1% unloaded	0.255 ± 0.032	8.21 ± 0.02	11.32 ± 0.165	69.78 ± 0.211
CHI 1% loaded	0.434 ± 0.072	11.68 ± 0.012	14.39 ± 0.244	59.05 ± 0.101

CHI: Chitosan, CMC: Carboxymethyl cellulose, TS: Tensile strength, YM: Young's modulus, EAB: Elongation percentage at break

59.05% and 11.68 MPa, respectively. Decline of the EAB and increment of the TS could be explained as the drug molecules interposed the linkages of the polymers.

Similar formulations based on CMC were investigated and it was observed that mechanical properties changed according to the concentration of the active substance. In a study, 1% (*w/w*) of CMC film was developed with different concentrations of active substance and it was found that addition of active substance increases TS of lean film from 17.75 MPa to 58.85 MPa. Also, the amount of casting mass and thickness of the final formulation has a direct effect on the mechanical properties. The increment of mechanical strength is compatible with literature data.[24]

For mechanical assessment of CHI-based formulations, it was observed that polymer concentration and active substance amount had direct impacts on the mechanical properties. In a study, thin film formulations of CHI have been prepared. Then, it was found that mechanical strength has been elevated (7.1 N, 21.6 N, 36.5 N) by increased polymer concentrations (1%, 1.5%, and 2%) and elastic properties were found to be declined. In the same report, it was observed that incorporation of the active substance exhibited similar mechanical behavior.[25]

In the pre-formulation part, it is not possible to remove the lean implants (containing only polymers) from the molds because of their fragility. Thus, plasticizer (PEG 6000) was added onto the lean CMC and CHI dispersions to augment mechanical characteristics. YM is related to film rigidity and ability to undergo elastic deformation under applied stress.[23] Addition of DRZ increased the YM. The data gathered from mechanical experiments (EAB, TS, and YM) has been correlated with each other.

Bioadhesive assessment of the implant formulations is demonstrated in Table 1. Unloaded implants exhibited a lesser work of bioadhesion than loaded formulations. Thus, drug-loaded formulations could be promising delivery systems for eye. Chemical structure of the drug substance in salt form may, thus, affect the bioadhesive properties of implants. Existence of salts has been reported as one of the factors that affect the bioadhesive properties of polymeric drug delivery systems for topical or mucosal administration.[26]

In vivo bioadhesion mechanism of the polymeric films can be explained by the interaction with tear fluid or meibum, which is secreted from holocrine meibomian glands.[27] The early stages of mucosal adhesion include hydration of the polymer *via* normal physiological conditions of the eye surface. The hydrogen bonding capacity of polymers (CMC and CHI) also contributed to mucosal adhesion due to the presence of hydroxyl groups. This functional group has also contributed to the wettability and hydration. Physiologically, the contents of meibum (ester content) could have great potential to increase adhesion properties *in vivo*.

FTIR analysis

FTIR reflects the interactions between the contents of ocular implants and DRZ. These possible interactions will directly affect the characteristics of the ocular implant.[3] FTIR spectra of unloaded CMC and CHI implants treated with DRZ are shown in Figure 1. Four inserts exhibited similar main peaks, but the amplitude varied dramatically with some of them moving. Figure 1 depicts FTIR spectra of the inserts. In DRZ spectrum, the characteristic SO_2 bonds of sulphonamide shifted from 1342 cm^{-1} to 1782 cm^{-1} and 1766 cm^{-1} for 1% CMC-DRZ and 1% CHI, respectively. Other characteristic bands of $-NH_2^+$ stretching at 1281 cm^{-1}, shifted to 1396 cm^{-1} for 1% CHI-DRZ and became widespread for 1% CMC-DRZ.[28,29] 1% CMC-DRZ and 1% CHI-DRZ spectra indicated that the peak intensities of CMC were better than those of CHI formulations. In % CHI spectrum, the characteristic bands of $-CH_2$ stretching at 1083 cm^{-1} became widespread for 1% CHI-DRZ and % CHI spectra, and the characteristic bands of -C=O stretching at 1603 cm^{-1}, intensity decreased with the addition of DRZ for % CHI-DRZ.[30,31] The peak intensity of the films with DRZ was better than those of the films without DRZ. In addition, the double spectra at 2900-2800 cm^{-1} observed in both CMC and CHI formulations are due to the vibrations of -COO group in CMC and CHI.[32] Therefore, we confirmed that addition of DRZ to and use of CMC film can facilitate uniform mixing in the film.

DSC analysis

Determination of solid-state interactions has been performed using DSC. The thermograms are shown in Figure 2 and melting points, enthalpies, and crystallinity indices are presented in Table 2. The enthalpy values of DRZ, CMC 1%, CMC-DRZ 1%, CHI 1%, and CHI-DRZ 1% were 9.455, 29.016, 28.702, 23.892, and 21.233 J/g, respectively, while the melting temperatures of DRZ, CMC 1%, CMC-DRZ 1%, CHI 1%, and CHI-DRZ 1% were 260.41, 219.82, 206.56, 249.14, and 239.53 °C, respectively.

Table 2 displays that results proved the CMC of amorphous structure of polymers and obstructed crystallization.[33,34] Melting points of ocular films have exposed a decline to lower temperatures with larger peaks compared to the bulk polymer by giving variable enthalpy values indicating several thermal transitions as well.[35] A decline in the melting points of the CHI 1% and CHI-DRZ 1% formulations was detected at 10 °C, when CMC was added instead of CHI, causing a greater decrease in CMC 1% and CMC-DRZ 1%. Reduction in CI of CMC 1% and CMC-DRZ

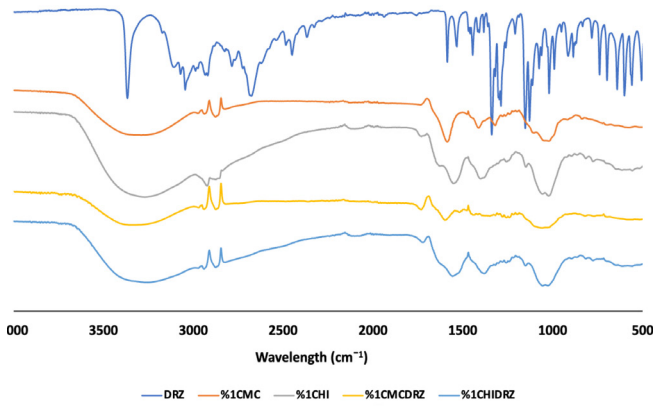

Figure 1. FTIR spectra of active substance and formulations
FTIR: Fourier transform infrared spectroscopy

1% compared to CHI 1% and CHI-DRZ 1% could be attributed to the crystal order in CMC 1% and CMC-DRZ 1% greatly disturbed due to CMC. In a study, salicylic acid was loaded into the CHI-based films and it was reported that the crystallization index of the salicylic acid-loaded formulations increased by about 10% compared to the unloaded films.[36]

SEM analysis of ocular implants

Images of ocular inserts are presented in Figure 3. Obtained data verify that surface of the designed inserts is smooth and plain. Therefore, it could be considered that the implants would not block vision.

In vitro drug release

In vitro drug release profile of a drug delivery system is an important parameter for noticing *in vivo* action of a drug substance. Generally, release experiments are accomplished under sink conditions. European Pharmacopeia describes the sink conditions as a volume of release medium that is at least three to ten times of the active ingredient saturation volume.[37] The solubility of DRZ was found to be 6.65 mg/mL, 6.72 mg/mL, and 38.76 mg/mL in distilled water (pH: 7), phosphate buffer (pH: 7.4), and NS solution (pH: 5.5), respectively. The obtained solubility data agreed with the literature.[38,39] After that, the volume of release medium and content was determined and other parameters were selected by considering normal physiological conditions.

According to the mathematical analysis of *in vitro* release studies (Table 3, Figure 4), the profiles fitted Higuchi-type kinetic model. As indicated in the literature, Higuchi-type release kinetics could express the drug release from polymeric matrices.[40] Moreover, there were some assumptions reported for Higuchi type kinetics, in this case the probable assumptions could be: (i) drug diffusion is one-dimensional, making effects of margins negligible, (ii) the diffusivity of the drug is fixed, (iii) perfect sink conditions are reached.[40,41]

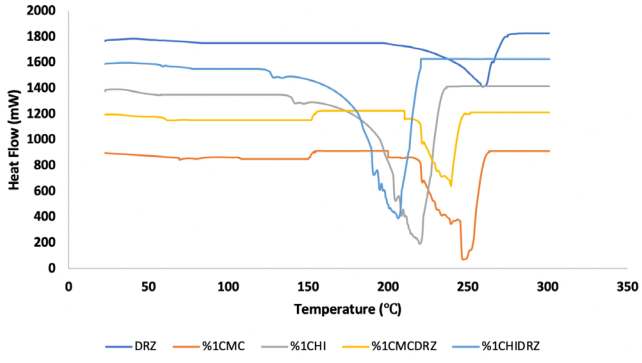

Figure 2. DSC curves of DRZ, CMC 1%, CMC-DRZ 1%, CHI 1%, and CHI-DRZ 1%

DSC: Differential scanning calorimetry, DRZ: Dorzolamide, CHI: Chitosan, CMC: Carboxymethyl cellulose

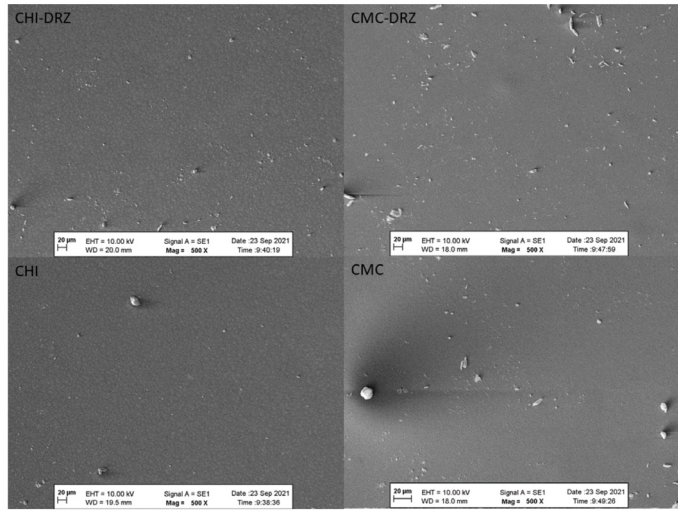

Figure 3. SEM images of the DRZ loaded (CHI-DRZ; CMC-DRZ) and unloaded (CHI; CMC) implants

SEM: Scanning electron microscope, DRZ: Dorzolamide, CHI: Chitosan, CMC: Carboxymethyl cellulose

Table 2. Thermal parameters of active substance and films

Formulations	Melting point (˚C)	Enthalpy ΔH (J/g)	Crystallinity index (%)
DRZ	260.41	9.455	100
CMC 1%	219.82	29.016	32.57
CMC-DRZ 1%	206.56	28.702	32.94
CHI 1%	249.14	23.892	39.58
CHI-DRZ 1%	239.53	21.233	44.529

DRZ: Dorzolamide, CHI: Chitosan, CMC: Carboxymethyl cellulose

Table 3. Kinetic models of formulations

	Zero order	First order	Higuchi	Korsmeyer-Peppas		Hixson-Crowell
	(R^2)	(R^2)	(R^2)	(R^2)	(n)	(R^2)
1.0% CMC-DRZ	0.8156	0.7312	0.9733	0.9313	0.301	0.9461
1.0% CHI-DRZ	0.9352	0.6447	0.9918	0.7818	0.243	0.9172

R^2: Correlation coefficient, n: Release exponent

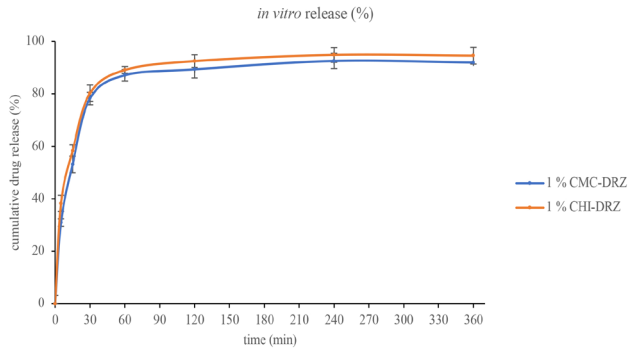

Figure 4. *In vitro* release profile of ocular implants

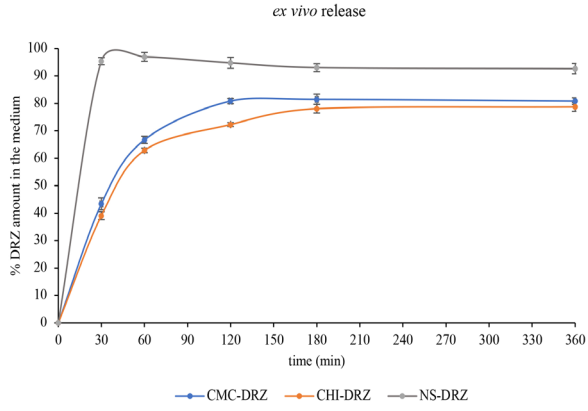

Figure 5. *Ex vivo* release profile of ocular implants

Polymer type and molecular weight directly change release profile of formulations. A similar study investigated the release patterns of sodium alginate, hydroxypropyl methylcellulose (HPMC), and CHI-based ocular inserts loaded with brimonidine.[42] CHI and HPMC based inserts exhibited more than 80% of drug release *in vitro* in the first 30 mins. The sodium alginate based formulations exhibited approximately 80% of drug release *in vitro* in the first 120 mins. The goal of this study was to observe the prolongation of drug contact with the ocular tissue by using biodegradable polymeric systems for daily application. Thus, the amount of DRZ was calculated (2 mg DRZ/implant) according to the dose of the market product's daily application.

Ex vivo release study

Ex vivo drug release profile of dosage form reflects the passage or retention of drug substances throughout the tissue. The applied method detects the residual drug amount on the release medium. Thus, the plateau levels at the 3rd and 6th hours indicate the drug saturation levels (Figure 5). The eye drop (NS-DRZ, 2% DRZ) exhibited instant drug payload in the first 30 min (95.4% ± 1.3), then release percentages of NS-DRZ were found as 93.04% and 92.66% at 3rd and 6th hours, respectively. It was considered that 7.34% of the drug was retained or passed throughout *ex vivo* tissue. The release percentages of CMC-DRZ were 81.5% and 80.9% at 3rd and 6th hours, respectively. The release percentages of CHI-DRZ were 78.1% and 78.7% at 3rd and 6th hours, respectively. Thus, *ex vivo* release outcomes are correlated with *in vitro* release studies. The implants designed with CHI have shown slightly faster *in vitro* release rate, so they could cause faster retention onside *ex vivo* tissues. Between 21.3% and 18.5% of the drug was estimated as retained or passed throughout *ex vivo* tissue.

As previously stated, physiological factors hinder ocular drug absorption and bioavailability.[5,6] It was reported that less than 5% of the applied dose is absorbed into ocular tissues.[39] Therefore, nanoparticulated systems, *in situ* gelling systems, and biodegradable polymeric systems were developed to increase drug permeation, extend the presence of drug substances in ocular tissues, and prolong drug release. NS-DRZ (representing the traditional application) implant exhibits 2.5 and 3.0 fold lower *ex vivo* drug absorption than CMC-DRZ and CHI-DRZ implants.

CONCLUSION

Ophthalmic implants showed remarkable results in both *in vitro* and *ex vivo* investigations for better ophthalmic drug delivery in this study. DRZ-loaded CMC and CHI ocular implants were prepared in the shape of transparent hemispheres; so as not to interfere with the vision. *Ex vivo* release data are correlated with *in vitro* release outcomes. The implant-designed CHI exhibited a slightly faster *in vitro* release rate, so it could cause faster retention in the ocular tissues. DRZ release from implants was biphasic with an initial release lasting about 2 h and then a continuous release lasting up to 6 h. It can be inferred that DRZ-loaded ocular implants can be an effective ocular delivery strategy.

Ethics

Ethics Committee Approval: Not applicable.

Informed Consent: Not applicable.

Peer-review: Externally peer-reviewed.

Authorship Contributions

Surgical and Medical Practices: A.R.C.Ç., Concept: A.R.C.Ç., Design: S.Ö., B.Ü., A.R.C.Ç., Data Collection or Processing: E.Ç., B.S., C.İ.A., Analysis or Interpretation: E.Ç., B.S., C.İ.A., Literature Search: S.Ö., B.Ü., Writing: S.Ö., A.R.C.Ç.

Conflict of Interest: No conflict of interest was declared by the authors.

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ORIGINAL ARTICLE

HERPUD1, a Member of the Endoplasmic Reticulum Protein Quality Control Mechanism, may be a Good Target for Suppressing Tumorigenesis in Breast Cancer Cells

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ABSTRACT

Objectives: Breast cancer is the most frequently diagnosed cancer type and the second leading cause of cancer-related death in women. Recent studies have highlighted the importance of the endoplasmic reticulum (ER) protein quality control mechanism for the survival of many cancers. It has also been recommended as a good target for the treatment of many cancer types. Homocysteine inducible ER protein with ubiquitin-like domain 1 (HERPUD1) functions as one of the main components of ER-associated degradation, which is an ER-resident protein quality mechanism. Today, the association of HERPUD1 with breast carcinogenesis is still not fully understood. Herein, we evaluated the possibility of HERPUD1 as a potential therapeutic target for breast cancer.

Materials and Methods: The effects of HERPUD1 silencing on epithelial-mesenchymal transition (EMT), angiogenesis, and cell cycle proteins were analyzed by immunoblotting studies. To test the role of HERPUD1 on tumorigenic features, WST-1-based cell proliferation assay, wound-healing assay, 2D colony formation assay, and Boyden-Chamber invasion assay were performed in human breast cancer cell line MCF-7. The statistical significance of the differences between the groups was determined by Student's *t*-test.

Results: Our results displayed that suppressing HERPUD1 expression reduced the cell cycle-related protein levels, including cyclin A2, cyclin B1, and cyclin E1 in MCF-7 cells. Also, silencing of HERPUD1 remarkably decreased expression levels of EMT-related *N*-cadherin and angiogenesis marker vascular endothelial growth factor A. Moreover, we determined that cell proliferation, migration, invasion, and colony formation of MCF-7 cells were significantly limited by silencing of HERPUD1.

Conclusion: Present data suggest that HERPUD1 may be an effective target for biotechnological and pharmacological strategies to be developed to treat breast cancer.

Key words: Breast cancer, ERAD, HERPUD1, MCF-7

INTRODUCTION

According to statistical studies conducted in the United States, breast cancer is the most frequently diagnosed cancer type and the second leading cause of cancer-related death in women.[1] It is a hormonal regulation-dependent cancer type; because 70% of breast cancer cells contain estrogen receptors and/or progesterone receptors. Conservative surgery is the most commonly used method in treating breast cancer. Depending on

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the receptor expression profiles, alternative approaches, such as tamoxifen, an estrogen receptor modulator, are used as a treatment option. Today, the hormonal treatment approach can be chosen based on the receptor status of the primary tumor in patients.[2,3] Although alternative treatment approaches and new drug candidates have been introduced through developing technology, there is an ongoing need to better understand the molecular pathogenesis of breast cancer and identify the molecular focuses that support the carcinogenesis process.

Numerous studies have reported that endoplasmic reticulum (ER) stress is highly activated in hematopoietic cancers (leukemia, lymphoma, and myeloma) and solid tumors, including stomach, colon, esophageal, lung, prostate, pancreas, liver, glioblastoma, and breast cancer as well.[4] It has been determined that protein levels are associated with molecular chaperones, which are involved in protein quality control in ER and molecular mechanisms that mediate the removal of misfolded proteins to prevent proteotoxicity highly expressed in many cancer types.[5] This process provides an opportunity for rapidly proliferating cancer cells to adapt against increased proteomic activity. Similarly, it is necessary for providing basal cellular needs and preventing apoptotic cell death signals.[4]

ER is the largest organelle of the cell and functions as a center for crucial biological processes such as protein synthesis, transport, protein folding, lipid and steroid synthesis, carbohydrate metabolism, and Ca^{+2} storage.[6] Protein folding is not a faultless process and it is known that one-third of newly synthesized proteins in cells are misfolded.[7] Although proteins are properly folded, they frequently undergo conformational deterioration inside cells as they are constantly exposed to cellular stresses, such as heat and oxidative stress. This situation leads to rerelease of hydrophobic residues in proteins that results in the formation of protein aggregates and cellular proteotoxicity.[7] Accumulation of misfolded proteins in the ER lumen triggers a process called "ER stress" by lowering free chaperone levels. The only possible way for cells to overcome this stress is by increasing the protein folding capacity of ER and eliminating the misfolded proteins by directing them to the protein degradation process.[8]

Numerous molecular mechanisms are triggered to overcome ER stress in cells and one of the ways to compete with potentially proteotoxic proteins with folding defects is the mechanism known as ER-associated degradation (ERAD).[9] Basically, ERAD involves several steps, including substrate recognition, initiation of retrotranslocation (translocation in the lipid bilayer), ubiquitination, retrotranslocation, targeting to the 26S proteasome, and proteasomal degradation. Targeting for proteasomal degradation is a highly controlled and sophisticated cellular system, in which diverse protein units take part in this process according to the type of targeted protein.[10]

Homocysteine inducible ER protein with ubiquitin-like domain 1 (HERPUD1) is a 54 kDa protein located in the ER membrane. It has been determined to play a role in the retrotranslocation step, which is expressed as the translocation of misfolded proteins from ER to the cytosol to direct 26S proteasome-mediated degradation for the elimination of misfolded proteins.[11-13] To date, there is no identified enzymatic activity of HERPUD1. It has been shown that it regulates ERAD by interacting with proteins involved in the retrotranslocation, ubiquitination and degradation of misfolded proteins.[14-16] HERPUD1 is one of the critical components involved in stabilizing the ERAD multiprotein complex and it effectively directs misfolded proteins to degradation and is thought to be one of the major components of ERAD.[14,17] The interaction analyses showed that HERPUD1 interacted in the ER membrane with Hrd1/SYVN1, an E3 ligase enzyme responsible for protein ubiquitination required to target misfolded proteins to proteasomal degradation, ubiquitin molecule, adaptor subunit SEL1L, OS-9 acting as a lectin and Derlin1, which is proposed as a component of the channel complex.[6,18] Furthermore, HERPUD1 expression is widely distributed in all tissues. In particular, high levels of its expression have been reported in tissues with advanced secretory ability, such as the pancreas.[14] This feature of HERPUD1 suggests that it may play essential roles in tissues with high secretory properties, such as breast and prostate. Today, there is very limited data in the literature associating HERPUD1 with the carcinogenesis process. A study published in 2002 showed that androgens induce HERPUD1 gene expression in prostate cancer cells. Studies conducted with patient samples determined HERPUD1 levels were significantly suppressed in cancer group samples compared to healthy tissues. In these studies, it has been suggested that HERPUD1 may have a function of suppressing tumorigenesis.[19] Also, it was determined that apoptotic cell death was induced in prostate cells overexpressing HERPUD1.[20] Another study exhibited that oxidative stress formation and cell adaptation genes were modulated by metformin administration and HERPUD1 was up-regulated among these genes in doxorubicin-resistant breast cancer cell lines.[21]

In the present study, the effect of the DsiRNA-mediated suppression of HERPUD1 expression was investigated on tumorigenic properties of human breast adenocarcinoma cell line MCF-7. In this context, HERPUD1 expression was silenced by using DsiRNA and then its effects on proliferation, migration, invasion, and colony formation were evaluated in MCF-7 cells. Also, the effect of HERPUD1 on the cell cycle and epithelial-mesenchymal transition (EMT) was evaluated by immunoblotting. Our results showed that the suppression of HERPUD1 expression importantly decreased cell cycle-related protein levels in MCF-7 cells and reduced EMT. In addition, functional assay data indicated that silencing of HERPUD1 strongly limited all tested tumorigenic features of MCF-7 cells, including cell proliferation, migration, invasion, and colony formation abilities. Together, these results suggest that HERPUD1 may be an essential protein in the control of the tumorigenesis of breast cancer cells.

MATERIALS AND METHODS

Materials

All cell culture-grade reagents, including growth media, fetal bovine serum (FBS), L-glutamine, and additional growth requirements were obtained from Biological Industries. Rabbit polyclonal HERP1 (10813-1-AP) (1:3500), *N*-cadherin (22018-1-

AP) (1:1000), and *E*-cadherin (20874-1-AP) (1:1000) antibodies were obtained from Proteintech. Rabbit polyclonal cyclin E1 (#20808) (1:2500), cyclin A1 (#91500) (1:2500), and cyclin B1 (#12231) (1:2500) antibodies were purchased from Cell Signaling Technology. Rabbit polyclonal vascular endothelial growth factor A (VEGF-A) (#E-AB-53277) (1:3000) antibody was provided from Elabscience. Mouse monoclonal *beta*-actin antibody (#A5316) (1:10000) was purchased from Sigma-Aldrich. HRP-conjugated goat anti-mouse (#31430) (1:5000) or goat anti-rabbit (#31460) (1:5000) IgG (H+L) was ordered from Thermo Scientific.

Cell culture

Human breast adenocarcinoma cell line MCF-7 (HTB-22®) was purchased from American Type Culture Collection (ATCC). The cells were propagated in Dulbecco's modified eagle medium (DMEM) supplemented with 10% FBS and 2 mM L-glutamine under a humidified atmosphere of 5% CO_2 and 95% air at a constant temperature of 37 ˚C. The absence of mycoplasma contamination was routinely confirmed using the MycoAlert™ Mycoplasma Detection Kit (Lonza).

siRNA transfection

Negative control DsiRNA and HERP1 DsiRNA were ordered from Integrated DNA Technologies. DsiRNA transfection was performed by Xfect™ RNA transfection agent (Takara #631450) according to the manufacturer's instructions. MCF-7 cells were seeded in 6 well cell culture dishes (3.5 x 10^5 cells/well) and after 24 h, the transfection was applied. Cells were transfected with HERPUD1 DsiRNA or control DsiRNA at the indicated doses.

RNA isolation, cDNA synthesis and quantitative real-time polymerase chain reaction (qRT-PCR)

Total RNA was isolated using the NEB Monoarch™ miniprep total RNA isolation kit according to the manufacturer's instructions (New England Biolabs). Quantification of the isolated RNAs was determined using a micro-volume spectrophotometer (MySPEC, VWR). RNA (1 μg) was reverse transcribed by iScript™ cDNA synthesis kit following the manufacturer's instructions (25 ˚C for 5 min, 46 ˚C for 20 min, and 95 ˚C for 5 min) (Bio-Rad). Gene expression of HERPUD1 was analyzed using iTaq Universal SYBR green reaction mix in a CFX96 RT-PCR instrument (Bio-Rad). The protocol steps were followed according to the manufacturer's instructions. PCR primer sequences are available upon request. Fold change for the transcripts were normalized to the Glyceraldehyde-3-Phosphate Dehydrogenase (GAPDH). Relative quantification was analyzed by using $2^{-\Delta\Delta Ct}$ method. In addition, melting-curve analysis was performed in the last step of qRT-PCR process to test specificity of the PCR reactions.

Protein isolation and western blotting

Protein isolation and immunoblotting studies were carried out as previously reported.[22] MCF-7 cells were lysed with an ice-cold radioimmunoprecipitation assay buffer containing mammalian protease inhibitor cocktail; samples were centrifuged at 14,000 rpm for 30 min at 4 ˚C and the supernatant was stored for further studies. Total protein concentration was measured using bicinchoninic acid assay (Takara). Approximately 30 μg of protein samples was loaded on hand-cast SDS-PAGE gels and electrophoretically separated and then proteins were transferred to the polyvinylidene difluoride membrane. Following the transfer process, blocking, primary antibody incubation, washing, origin-specific horseradish peroxidase (HRP)-conjugated secondary antibody application, washing, and monitoring of the proteins were followed, respectively. Protein bands were monitored using clarity western enhanced chemiluminescence substrate solution in a ChemiDoc XRS+ system (Bio-Rad).

The measurement of cell proliferation

Cell proliferation was analyzed by WST-1-based cell proliferation assay according to the manufacturer's instructions (Takara #MK400). Cells were seeded in a 96 well plate (5000 cells/wells). To measure cell proliferation, 10 μL/well premix WST-1 reactive was applied and cells were incubated for 2 h under conventional cell culture conditions. Absorbance reading at a wavelength of 450 nm was performed on a microplate reader (BioTek, Epoch 2). Measurements were taken at 24 and 48 h. The results were graphed to form proliferation curves.

Colony formation assay

Plate colony formation assay was performed as described before.[22] MCF-7 cells were seeded in 6-well plates with 1000 cells/well and grown under conventional culture conditions for 72 h. At the end of the experiment, cells were washed twice with ice-cold 1X phosphate-buffered saline (PBS). Following this, colonies were fixed with alcohol and stained with 0.05% crystal violet solution (Sigma-Aldrich).

Wound-healing assay

Wound healing assay was achieved as mentioned before.[22] MCF-7 cells were seeded in 12 well cell culture plates with 3.5 x 10^5 cells/well and grown under conventional cell culture conditions. After 24 h, wounds were formed by using a yellow 200 μL tip and cells were washed twice with sterile 1X PBS to remove the cell residues. Following this, cells were grown in a culture medium for 72 h. Percentage of wound closure rates was analyzed with ImageJ software (http://imagej.nih.gov/ij/).

Invasion assay

Boyden-Chamber invasion assay was performed as described before.[22] Transwells with a size of 8 μm pores (Sarstedt) were coated with matrigel. In the upper part of the transwell, 10,000 cells were seeded in serum-free DMEM. In the lower section, a standard 10% FBS containing DMEM medium was added as a chemoattractant. The cells were incubated under conventional culture conditions for 72 h. The cells invaded from permeable Transwell's pores were fixed and stained with a 0.05% crystal violet solution (Sigma-Aldrich). Counting invaded cells was performed by photographing from 5 independent areas using a Sunny SopTop microscope and camera system.

Statistical analysis

The results are presented as mean ± standard deviation. The statistical significance of the differences between the groups

was determined using Student's *t*-test with a minimum confidence interval of 95% using GraphPad Prism 7 software. A value of $p<0.05$ was considered significant.

RESULTS

The determination of the optimum HERPUD1 DsiRNA concentration in MCF-7 cells

To examine the role of HERPUD1 protein in the carcinogenesis process in human breast adenocarcinoma cell line MCF-7, we aimed to suppress HERPUD1 expression using DsiRNA. To determine the optimum HERPUD1 DsiRNA concentration, we transfected the MCF-7 cells with 1, 2 and 5 nM of HERPUD1 DsiRNA. As a control group, cells were transfected with control DsiRNA at 5 nM concentration, which is known not to target any mRNA. The efficiency of silencing HERPUD1 expression was tested at mRNA and protein levels by qRT-PCR and immunoblotting assays, respectively (Figure 1a, b). qRT-PCR results indicated that optimum HERPUD1 DsiRNA concentration was 2 nM, which reduced HERPUD1 mRNA expression by 50% compared to the control DsiRNA administered group (Figure 1a). Consistent with the qRT-PCR results, 2 nM HERPUD1 DsiRNA concentration efficiently suppressed the expression of HERPUD1 protein by 50% compared to control DsiRNA transfected group. These findings showed that the optimum DsiRNA concentration for silencing HERPUD1 expression was 2 nM in MCF-7 cells (Figure 1b).

The investigation of the effects of HERPUD1 suppression on the epithelial-mesenchymal transformation in MCF-7 cells

We analyzed the protein levels of *N*-cadherin and *E*-cadherin, considered markers associated with EMT, in HERPUD1-silenced MCF-7 cells by immunoblotting. Likewise, we tested the expression level of VEGF-A protein, which is associated with the angiogenesis process in cancer cells. Our findings revealed that silencing of HERPUD1 remarkably decreased *N*-cadherin protein levels, whereas *E*-cadherin levels were not affected compared with the control group (Figure 2). Moreover, the silencing of HERPUD1 strongly decreased the expression level of VEGF-A protein compared to the control group (Figure 2).

The evaluation of the effects of silencing HERPUD1 on cell cycle-related proteins in MCF-7 cells

Cyclin proteins are critical regulators for the uninterrupted progression of proteomic and genomic rearrangements throughout the cell cycle. These proteins are a family of proteins that activate specific cyclin-dependent kinases during phase transitions.[23,24] To understand the effect of HERPUD1 protein on the control of the cell cycle in MCF-7 cells, we tested the levels of cyclin A2, cyclin B1 and cyclin E1 proteins in DsiRNA-mediated HERPUD1 suppressed MCF-7 cells by immunoblotting. Our results showed that HERPUD1 suppression remarkably decreased the levels of all tested cyclin proteins compared with the control group (Figure 3).

Determination of the effects of HERPUD1 suppression on MCF-7 cell proliferation

WST-1-based cell proliferation analysis was performed to evaluate the effects of HERPUD1 silencing on the proliferation of MCF-7 cells. Our results indicated that HERPUD1 suppression significantly reduced the proliferation of MCF-7 cells in a time-dependent manner compared with the control group (Figure 4).

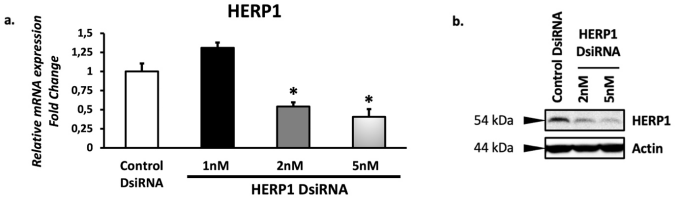

Figure 1. Optimization of effective HERPUD1 DisRNA concentration. (a) MCF-7 cells were transfected with HERPUD1 DsiRNA or control DsiRNA at 1 nM, 2 nM and 5 nM concentrations for 72 h and then the mRNA expression levels of HERPUD1 were examined by qRT-PCR. *GAPDH* was used as a housekeeping gene and normalized relative quantification of the target genes was calculated according to *GAPDH* gene expression. Results are presented in the graph as a fold change compared to the control group (n: 3) (*$p<0.05$). (b) Total protein was isolated from HERPUD1 DsiRNA (2 nM, 5 nM) or control DsiRNA (5 nM) transfected MCF-7 cells. HERPUD1 protein expression was analyzed by immunoblotting studies using HERPUD1-specific primary antibody. Beta-actin was used as a loading control.

qRT-PCR: Quantitative real-time polymerase chain reaction

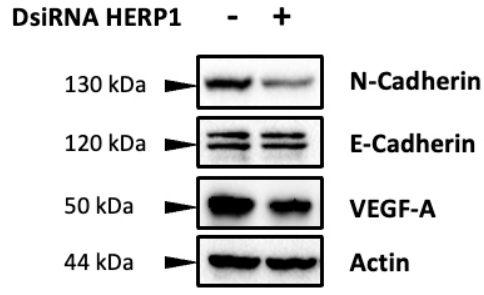

Figure 2. Investigation of the effects of DsiRNA-mediated HERPUD1 suppression on epithelial-mesenchymal transition and angiogenesis-related protein levels. The protein levels of *N*-cadherin, *E*-cadherin, and VEGF-A were examined in 2 nM HERPUD1 DsiRNA and control DsiRNA transfected MCF-7 cells by immunoblotting assay. Beta-actin was used as a loading control.

VEGF-A: Vascular endothelial growth factor A

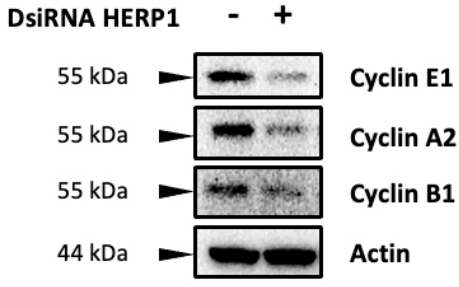

Figure 3. Evaluation of the effects of DsiRNA-mediated HERPUD1 suppression on the protein levels of cell cycle-related cyclins in MCF-7 cells. The protein levels of cyclin A2, cyclin B1 and cyclin E1 were examined in 2 nM HERPUD1 DsiRNA and control DsiRNA transfected MCF-7 cells by immunoblotting assay.

The investigation of the effects of silencing HERPUD1 on the wound healing ability of MCF-7 cells

Wound healing assay model is a relatively easy and cost-effective tool for evaluating the migration ability of cancer cells *in vitro*. Therefore, we used this assay system to test the effect of DsiRNA-mediated HERPUD1 suppression on the migration ability of MCF-7 cells. Our findings displayed that the migration of MCF-7 cells was significantly reduced by HERPUD1 suppression compared to control DsiRNA group (Figure 5a, b).

Testing the effects of silencing HERPUD1 on the colonial growth ability of MCF-7 cells

Acquiring the colonial growth ability of cancer cells is accepted as an important sign of the carcinogenesis process at the cellular level.[25] Thus, to evaluate the relationship of HERPUD1 with tumorigenesis in breast cancer cells, we examined the effects of silencing HERPUD1 expression on colony formation ability in MCF-7 cells by using a 2D colony formation assay. Our results indicated that DsiRNA-mediated silencing of HERPUD1 expression significantly decreased the colony growth ability of MCF-7 cells compared with the control DsiRNA-transfected group (Figure 6a, b).

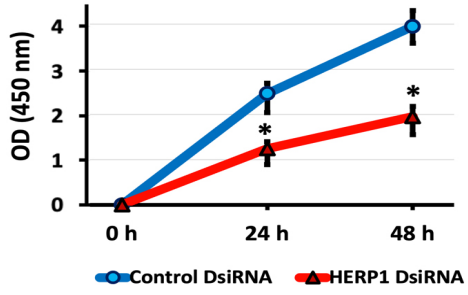

Figure 4. Effects of HERPUD1 suppression on the proliferation of MCF-7 cells. MCF-7 cells were transfected with control DsiRNA or 2 nM HERPUD1 DsiRNA and then proliferation rates were analyzed by WST-1 cell proliferation assay as expressed in the method section. The spectrophotometric results (OD 450 nm) were presented in the graph (n: 3) (*p<0.05).

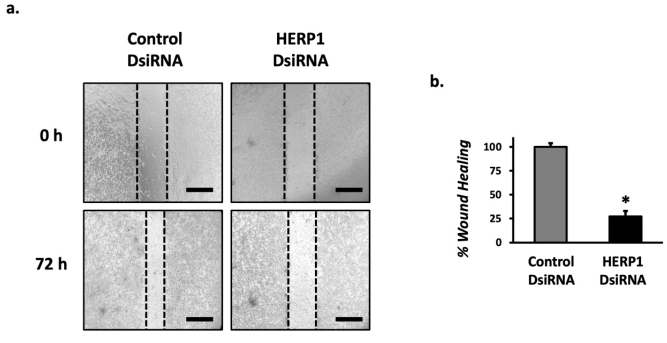

Figure 5. Evaluation of the effects of DsiRNA-mediated HERPUD1 suppression on migration capacity of MCF-7 cells. (a) 0 and 72 h of wound area images (scale: 50 µm). (b) Graphical representation of wound closure rates. Closure rates were analyzed by ImageJ software and the results were presented in % wound-closure (n: 3) (*p<0.05)

The evaluation of the effects of HERPUD1 suppression on the invasion capacity of MCF-7 cells

Invasion is considered one of the distinguishing features of cancer cells and is expressed as the spread of cancerous cells to neighboring or distant tissues and organs. It is an important factor leading to the spread of cancer cells and limiting their treatment efficacy.[26] The effects of silencing HERPUD1 expression on invasion were evaluated by a Matrigel-coated Boyden-Chamber transwell assay in MCF-7 cells. Our data indicated that DsiRNA-mediated suppression of HERPUD1 expression significantly limited the invasion capability of MCF-7 cells compared with the control group (Figure 7a and b).

DISCUSSION

HERPUD1 has N- and C-terminal regions facing the cytoplasm and is localized in the ER membrane. Moreover, it can be induced through ER stress and homocysteine.[14] HERPUD1 has been proposed as one of the significant components of ERAD, which acts as a structure-scaffolding protein in the ER membrane. It acts as a shuttle protein in the retrotranslocation step of ERAD and has no known enzyme activity. On the other hand, it is involved in the regulation of ERAD by interacting with proteins involved in ubiquitination and degradation processes in the retrotranslocation step of ERAD.[16] Moreover, HERPUD1 controls the proteasomal degradation of the inositol 1,4,5-triphosphate receptor and ryanodine receptor proteins, which is a Ca^{+2} channel and has a critical role in Ca^{+2} homeostasis in this

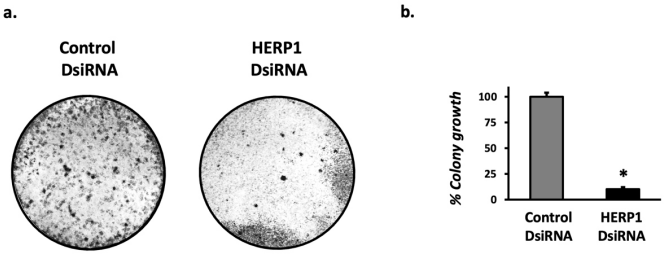

Figure 6. Evaluation of the effects of DsiRNA-mediated HERPUD1 suppression on colonial growth of MCF-7 cells. (a) Colonial growth of MCF-7 cells transfected with control DsiRNA or HERPUD1 DsiRNA. (b) % colonial growth rates were analyzed by ImageJ software and the results were presented in the graph (n: 3) (*p<0.05)

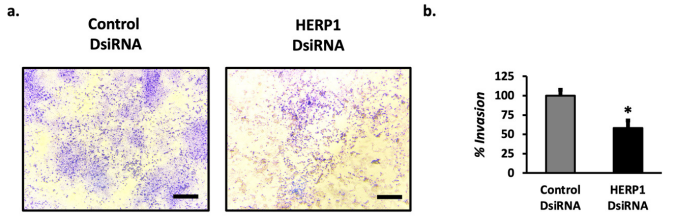

Figure 7. Investigation of the effects of DsiRNA-mediated HERPUD1 suppression on the invasion of MCF-7 cells. MCF-7 cells were transfected with HERPUD1 DsiRNA or control DsiRNA and then invasion capacity was analyzed by matrigel-coated Boyden Chamber transwell assay as described in the method section. (a) Staining of invaded cells on transwell membrane with 0.05% crystal violet solution (scale: 50 µm). (b) Graphical representation of invaded cells as a % invasion (n: 3) (*p<0.05)

way.[27] HERPUD1 exhibits a comprehensive expression profile in all tissues and it has been reported to be expressed at high levels in tissues with high secretory ability, such as pancreas.[14] This finding suggests that members of the ER protein quality control mechanism, an essential mechanism for transition to the secretory pathway of which HERPUD1 is a member, may have important roles in other healthy and cancerous tissues with high secretion ability, such as breast and prostate.

Herein, we evaluate the effects of silencing HERPUD1 on the tumorigenic properties of MCF-7 breast cancer cells. A very limited data is available about the link between HERPUD1 and the carcinogenesis process in the literature. In studies conducted with samples from prostate cancer patients, it was reported that HERPUD1 expression was suppressed to a great extent in cancer group samples compared to healthy group samples.[19] Another study showed that the administration of metformin to doxorubicin-resistant breast cancer cell lines modulates oxidative stress generation and cell adaptation genes. Among these identified genes, HERPUD1 has been reported to be up-regulated.[21] Today, the functional effects of suppressing HERPUD1 expression in breast cancer cells are still unknown. Our data showed that DsiRNA-mediated silencing of HERPUD1 significantly limited the proliferative ability of MCF-7 cells compared with the control DsiRNA-treated group (Figure 4). In our microscopic examinations during this process, we did not observe any characteristics of cell death in HERPUD1-silenced MCF-7 cells.

Since EMT parameters are important in evaluating the tumorigenic capacity of cancer cells, we examined the *E*-cadherin and *N*-cadherin conversion and changes in VEGF-A, which is associated with angiogenesis.[28,29] Our findings showed that the silencing of HERPUD1 markedly reduced *N*-cadherin levels, whereas E-cadherin levels were not affected compared to control DsiRNA-treated MCF-7 cells. Similar to *N*-cadherin results, VEGF-A levels were also importantly decreased in the HERPUD1-silenced group (Figure 2a). ER protein quality control mechanism tightly controls the levels of EMT proteins, tumor suppressors and oncogenic proteins.[30] These results suggest that HERPUD1 is directly or indirectly involved in the regulation of *N*-cadherin and VEGF-A levels in breast cancer cells.

In our trials where we tested the effect of HERPUD1 on the cell cycle proteins, we found that suppression of HERPUD1 caused a decrease in cyclin A2, B1 and E2 levels (Figure 2b). The levels of cyclin proteins are very important for the control of cell cycle phase transitions. Cyclin E1 regulates the progression of G1 phase to S phase.[31] Some retrospective studies have presented an association between high cyclin E levels and an increased risk of death from breast cancer.[32] Cyclin A2 has been proposed as the bridge between cell cycle and invasion in cancer cells and it has been reported to be expressed at high levels in various human tumors.[33,34] However, it does not always show a direct correlation with the degree of tumor aggressiveness.[35] Cyclin B1 plays a regulatory role in the transition from the G2 phase to mitosis.[36] It has been reported that cyclin B1 is overexpressed in primary breast, gastric and colorectal cancer cells.[37-39] HERPUD1 suppression related decreased cyclin levels suggest that HERPUD1 may be directly related to regulating the steady-state levels of cell cycle phase transition proteins.

Advanced colonial growth and increased migration-invasion ability are considered hallmarks of the process of tumorigenesis.[25] To test the possible role of HERPUD1 on the tumorigenic properties of breast cancer cells, we carried out wound healing, 2D colony formation, and matrigel-coated Boyden-Chamber invasion assays. Our data indicated that the colonial growth, migration, and invasion capacity of MCF-7 cells were significantly reduced by HERPUD1 suppression (Figures 4-7). Collectively, these functional data suggested that HERPUD1 has an essential role in regulating the tumorigenic features of MCF-7 cells.

Considering that ERAD mechanism, which functions through large protein complexes, varies according to the characteristics of the substrate molecule to be targeted to the proteasome, it is extremely valuable to investigate the importance of HERPUD1 in breast cancer cells *in vitro* and *in vivo* studies. Together, present data suggested that HERPUD1 has essential regulatory roles on cell proliferation, migration, invasion, and colony formation ability by affecting cell cycle control, EMT, and angiogenesis-related protein levels in MCF-7 breast cancer cells.

Study limitations

In this study, the biological role of HERPUD1 in breast cancer was investigated *in vitro*. In order to better understand the role of HERPUD1 in the molecular pathogenesis of breast cancer, further experimental studies and *in vivo* trials are needed.

CONCLUSION

Collectively, our results suggest that targeting the HERPUD1 protein, an essential component of the ER protein quality control mechanism, may be promising for developing selective treatment approaches for breast cancer.

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Ethics

Ethics Committee Approval: Not applicable.

Informed Consent: Not applicable.

Peer-review: Externally peer-reviewed.

Authorship Contributions

Concept: Y.E., Design: Y.E., Data Collection or Processing: Y.E., Y.D., H.K.D., D.Ç., Analysis or Interpretation: Y.E., Literature Search: Y.E., Writing: Y.E.

Conflict of Interest: No conflict of interest was declared by the authors.

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ORIGINAL ARTICLE

Analytical Quality by Design Driven Development and Validation of UV-Visible Spectrophotometric Method for Quantification of Xanthohumol in Bulk and Solid Lipid Nanoparticles

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ABSTRACT

Objectives: Xanthohumol (XH) is a prenylated chalcone available naturally and has diverse pharmacological activities. It has some limitations in the physiological environment such as biotransformation and less gastrointestinal tract absorption. To overcome the limitations, we prepared nanoformulations [solid lipid nanoparticles (SLNs)] of XH. Therefore, an analytical method is required for the estimation of XH in the bulk nanoformulations, so we developed and validated a quality by design (QbD)-based ultraviolet (UV)-spectrophotometric method as *per* the International Conference of Harmonization (ICH) Q2 (R1) guidelines.

Materials and Methods: The new analytical Qbd based UV-visible spectrophotometric technique is developed and validated for estimation of XH in bulk and SLNs as *per* ICH guidelines Q2 (R1). Critical method variables are selected on the basis of risk assessment studies. Optimization of method variables was performed using the a central composite design (CCD) model.

Results: Multiregression ANOVA analysis showed an R2 value of 0.8698, which is nearer to 1, indicating that the model was best fitted. The optimized method by CCD was validated for its linearity, precision, accuracy, repeatability, limit of detection (LOD), limit of quantification (LOQ), and specificity. All validated parameters were found to be within the acceptable limits [% relative standard deviation (RSD) <2]. The method was linear between 2-12 g/mL concentration with R2 value 0.9981. Method was accurate with percent recovery 99.3-100.1%. LOD and LOQ were found to be 0.77 and 2.36 µg/mL, respectively. The precision investigation confirmed that the method was precise with %RSD <2.

Conclusion: The developed and validated method was applied to estimate XH in bulk and SLNs. The developed method was specific to XH, which was confined by the specificity study.

Key words: AQbD, solid lipid nanoparticles, validation, UV-visible spectrophotometric method

INTRODUCTION

Xanthohumol (XH) is a natural prenylated chalcone obtained from hops. It possesses potential pharmacological applications and is used against inflammation,[1,2] cancer,[3,4] diabetes,[5] melanoma,[6] hyperlipidemia,[7] invasion,[8] angiogenesis,[9] and obesity.[7,10] Due to these excellent therapeutic activities of XH, there is an immediate need to develop a simple, cost effective, rapid, sensitive, and accurate method to quantify XH in several

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matrices.[4] As *per* our knowledge, there are no such ultraviolet (UV)-visible spectrophotometric methods available to quantify XH in bulk and in lipid-based nanocarriers. Therefore, in present research, analytical quality by design (AQbD) has been used to develop a novel simple and cost-effective method for the estimation of XH in bulk and in lipid-based nanocarriers. The structure of XH is represented in Figure 1.

Since the last decade, AQbD approach has gained great importance in method development and validation of various analytical methods. It is generally termed as a systematic approach for developing methods that starts with the already defined objectives and imparts special focus on understanding the process, product, and process control along with quality risk management (QRM).[11] Analytical science is regarded as an important element in the development of pharmaceutical products and thus coincides with the product life cycle. The AQbD approach requires less time and decreases the number of trials of experimentation as compared to conventional types of analytical methods.[12,13] It mainly employs the concept of design of experiments (DOE) and QRM to discover the likely risks as well as interactions associated amongst the method variables, therefore saving a significant amount of time effort and money. DOE is an integral part of AQbD because it provides the best possible method performance. It also permits construction of a statistically significant model that allows different factors and their interactive impact on responses to be estimated. Therefore, adopting principles of AQbD will provide a significant benefit in terms of complete understanding the performance of the method. Application of AQbD in the development of UV-visible spectrophotometric method has utmost priority than other conventional methods.[14] Initially, before proceeding to method development, we have to select the analytical target profile (ATP) or the defined objectives of the study to be selected. Following that, risk assessment studies were used to identify the critical method variables (CMVs) and critical analytical attribute (CAA). CMVs impacting the performance of the method were optimized by central composite design (CCD) to ensure quality within the stated targets.[15,16] The current research work is mainly based on the application of AQbD principles to decrease the variability occurring during measurement of XH to find the best solution. Therefore, a simple, robust, and economic UV-visible spectrophotometric method has been developed and validated as *per* the International Conference of Harmonization (ICH) guidelines Q2 (R1).[17,18]

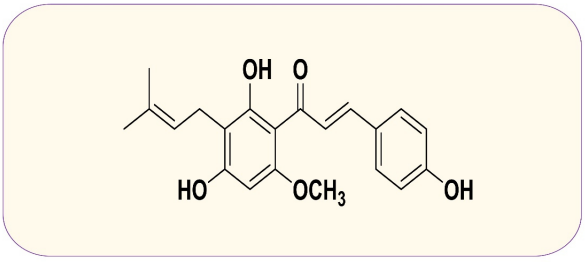

Figure 1. Structure of XH
XH: Xanthohumol

MATERIALS AND METHODS

Experimental

Reagents and chemicals

XanthoFlav (XH) was gifted by Simon H. Steiner, Hopfen GmbH, Mainburg, Germany. Methanol of UV grade was purchased from Loba Chemicals, Mumbai, India. The solid lipid compritol E was gifted by Gattefosse Pvt. Ltd. (Mumbai, India). Lipoid E 80SN was gifted by Lipoid GmbH Germany. Pluronic F-68 was purchased from Loba Chemicals, India. Sephadex-G-25 was purchased from GE Healthcare, Hyderabad, India. All other chemicals used were of analytical grade.

Instrumentation

UV-visible spectrophotometer (1800, Shimadzu) with a set of 1 cm quartz cuvettes was used for the photometric analysis of the sample. Design Expert version 11 Statease software (Minneapolis, USA) is used for optimization. A 0.1 mg sensitive analytical digital balance was used for weighing all the components (Shimadzu).

Analytical method development and optimization

Defining analytical target profile and critical analytical attribute

ATP was established for the systematic development of XH estimation by outlining all of the required quality features of the analytical method using the principles of AQbD strategy. The method objective was defined based on the assessment of the literature and profile of the analyte. Motivation for selecting UV-visible spectrophotometric approach was due to its simplicity and speed of analysis compared to more advanced analytical methods. To satisfy ATP, XH absorbance is selected as CAA.[15,19]

Establishment of cause-effect relationship and risk management

Generation of CAA was made by studying the relationship between variables of the method and this CAA was used for analysis of control noise experiment (C-N-X). Ishikawa fishbone representation is used for depicting the correlation between CAA and method variables (Figure 2). C-N-X strategy used the risk assessment matrix to determine the crucial quality variables that are risky. Rankings were assigned to the recognized risky variables, and the overall score was used to determine the CMVs (Table 1). During the analysis of C-N-X, variables such as detecting wavelength, sampling interval, scanning speed, sample integrity, and solvent variation was investigated. Furthermore, the sampling interval and scanning speed were discovered as CMVs and treated with appropriate experimental design (CCD) for investigative evaluation and optimization.[15,16,20]

Determination of absorption maxima (λ_{max}) for XH analysis

Absorption maxima of XH was determined by scanning solution of 10 mg/mL from 800 to 200 nm by taking methanol as blank. Absorption maxima spectrum of XH is represented in Figure 3. λ_{max} of XH was identified as 369 nm and is used for further analysis.[14]

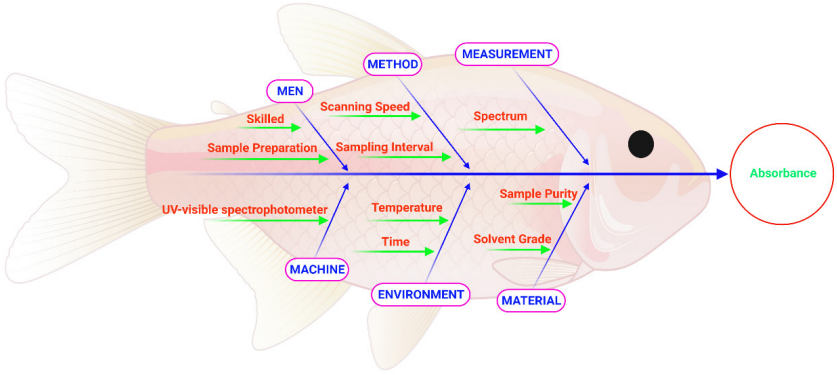

Figure 2. Depicting diagram of Ishikawa fishbone that illustrates the cause-effect correlation between method variables and analytical attributes

Table 1. C-N-X based risk assessment of CMVs for method development

CMVs	Level of risk on absorbance	C-N-X	Strategy used
Sampling speed	1	X	DOE
Sampling interval	1	X	DOE
Solvent	0	C	Controlled
Wavelength	-1	C	369 nm
Purity of sample	-1	N	Quality
Preparation of sample	-1	C	Controlled
Equilibration of detector	-1	C	Controlled

Level: 1- high, 0- medium, -1- low, C/N/X: Control-noise-experimental, CMVs: Critical method variables, DOE: Design of experiment

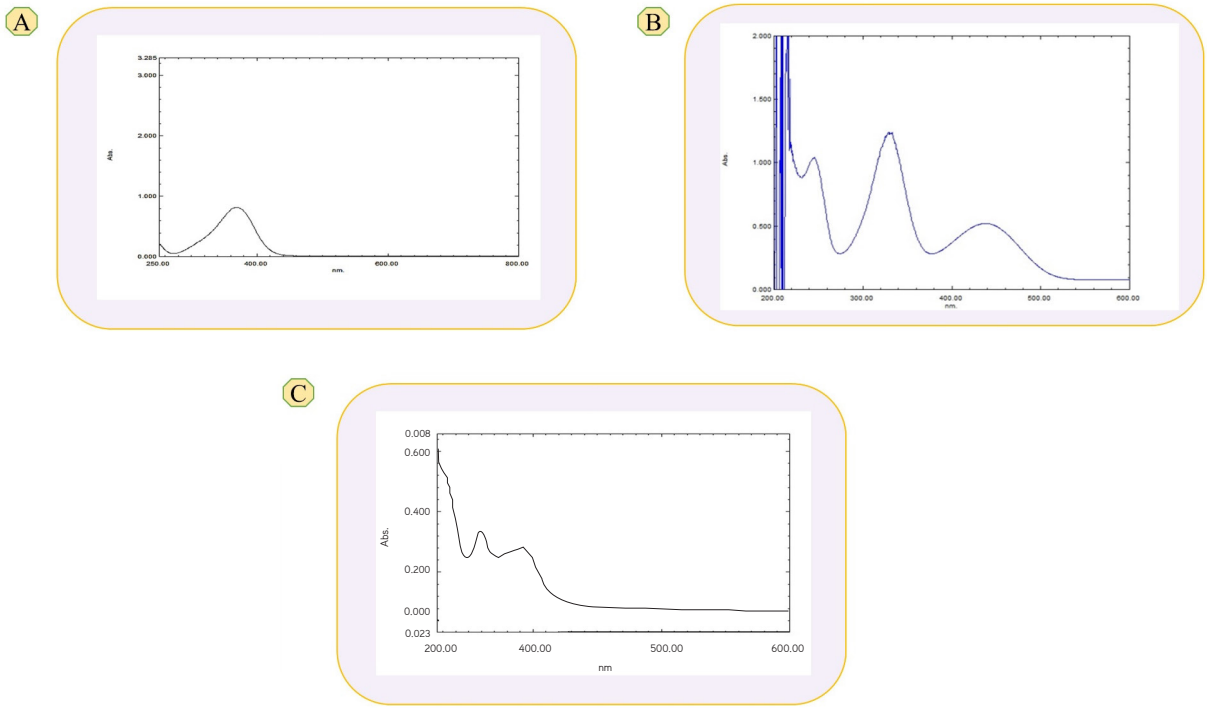

Figure 3. Absorption maxima spectrum of (A) bulk XH, (B) XH-SLNs, (C) blank SLNs (excipients without drug)
XH: Xanthohumol, SLNs: Solid lipid nanoparticles

Optimization of the method by CCD

Identification of optimum conditions of the method and to assure robustness, CCD was used. Based on the risk assessment studies, optimization of the selected CMVs was performed by performing 13 experiments with five center points using CCD. The response variable (absorbance) of XH was evaluated by CCD and measured at 369 nm using 10 µg/mL standard solution. Design expert version 11 software (Stat Ease, Inc., Minneapolis, USA) is used to best fit the obtained data in to suitable mathematical model.[15] Polynomial equations were created as *per* ANOVA for significant model terms with *p* values less than 0.05. A fit summary of the model for the selected CAA suggested a quadratic model as best fit. Correlation coefficient and lack of fit was used to evaluate the suitability of the model. The correlation between CAA and CMVs was investigated using contour plots and response surface plots. Furthermore, numerical and graphical optimization is used to improve the method conditions by software.

The design space created using DOE technique was used to define method control strategies within which minor changes in method performance were tolerated and considered resilient.

Selection of solvent

Solvent for the analysis was selected based on the solubility studies of XH in various solvents such as dimethyl sulfoxide (DMSO), methanol, chloroform, and water. XH has shown highest solubility in methanol and DMSO. Methanol has been selected as a solvent for spectrophotometric method development because DMSO produces toxicity and has stability issues.

Preparation of stock solutions

Primary stock solution was prepared by dissolving accurately weighed 100 mg of XH in 100 mL of methanol, which gives a solution of concentration 1 mg/mL or 1000 g/mL.

Secondary stock solution was prepared from primary stock solution by taking 10 mL of primary standard and volume is made with methanol up to 100 mL, which gives a solution of concentration 100 µg/mL. By using secondary stock, further dilutions were made for analysis.

Analytical method validation

A UV-visible spectrophotometer (Shimadzu, UV-6000) operated with spectral bandwidth of 1 nm was used for analytical method development and validation. Validation parameters including precision, linearity and range, accuracy, repeatability, specificity, limit of detection (LOD), limit of quantification (LOQ), and robustness were evaluated as *per* ICH guidelines Q2 (R1).[18,21,22]

Linearity

Six samples of diverse concentrations (2-12 µg/mL) were prepared from a secondary standard and used for executing the linearity parameter of XH in methanol. It was executed for three days in triplicate (n: 9). Linearity cures was plotted using the obtained data. Correlation coefficient equation and regression equation were determined using the same data.[14,23]

LOD and LOQ

Determination of LOD and LOQ for the method was performed by standard deviation (σ) and the slope of the standard curve.[18] LOD and LOQ were given by the following equations:

LOD= 3.3*σ/S and LOQ= 10*σ/S

Where, σ is standard deviation and S is the slope of the standard curve.

Precision

UV method precision was determined in terms of variations in intraday and interday (intermediate day) precision. Levels of precision were examined for three diverse known concentrations (4, 6, and 8 µg/mL) of XH prepared from the secondary stock solution for intraday precision of XH three concentrations were determined by taking absorbance of the samples in triplicate three times in a day. Interday precision was executed by measuring the absorbance of selected samples for three days in triplicate. Using linearity curve %RSD was calculated for the samples.[18,24]

Repeatability

Determination of repeatability of the UV method was performed by measuring the absorbance of the XH solution in methanol six times at 4 g/mL concentration.[18]

Accuracy

Accuracy of UV method was estimated by a standard addition method. In this method, a standard stock solution of known amount was added to the test solution (6 µg/mL, prepared from secondary stock solution) of XH at various levels such as 80%, 100%, and 125%. Absorbance for the prepared solutions was determined and concentration was calculated again in triplicate using the linearity curve.[15,18]

Specificity

Specificity test was performed using blank solid lipid nanoparticles (SLNs) (only excipients). Blank SLNs were prepared and a known amount of XH was added to the dispersion. The resulting dispersion was mixed vigorously. 1 mL of sample was taken and subjected to nanoparticle lysis. XH extraction was made by using methanol up to 5 mL. The sample was analyzed by the developed method after filtering through a 0.22 µm filter.[15,18]

Analysis of in-house prepared SLNs of XH

Preparation of solid lipid nanoparticles

XH-loaded SLNs were prepared using homogenization-ultrasonication method. It mainly includes two steps as first the preparation of lipophilic phase and later preparation of aqueous phase. The lipophilic phase is prepared by melting solid lipid (compritol E ATO) 10 °C above its melting point. XH and lipophilic surfactant (lipoid E 80SN) were added to the molten lipid and stirred well. Aqueous phase was prepared by dissolving Pluronic F-68 in water. Both the aqueous and lipid phases heated to the same temperature, *i.e.*, above the melting point of the lipid. Then, under hot conditions, the aqueous phase was added to the lipid phase dropwise with continuous stirring. The

mixture was subjected to high shear homogenizer for 30 min at 6000 rpm followed by probe sonication at 40% amplitude and 35 pulse rate for 10 min. The resultant dispersion was cooled to room temperature for solidification and precipitation of SLNs.[25]

Analysis of solid lipid nanoparticles

The prepared SLNs were analyzed by the developed and validated method for determining percentage-entrapment efficiency (EE) and percentage drug loading (DL).

Entrapment efficiency (%) and drug loading (%)

EE (%) of XH-SLN was determined by separating the entrapped and unentrapped XH using Sephadex G-25 chromatography. The drug in XH-SLN was extracted after the lysis of lipid particles by mixing with methanol followed by filtration through a 0.22 µm filter. Then, both entrapped and unentrapped XH content was determined in triplicate using the above developed and validated UV method. Concentration of XH was calculated using the calibration curve.[21,26]

%EE and %DL were calculated using the following equation:

$$\%EE = \frac{\text{Amount of drug used for formulation-amount of unentrapped drug}}{\text{Total amount of drug in formulation}} \times 100$$

equation (1)

$$\%DL = \frac{\text{Amount of entrapped drug in solid lipid nanoparticles}}{\text{Total weight of solid lipid nanopartcles}} \times 100$$

equation (2)

RESULTS

Analytical method development and optimization

Solubility of XH was found to be freely soluble in methanol and DMSO, based on the toxicity and stability parameters, and methanol was selected as the solvent for UV-visible spectrophotometric method development and validation. The developed and validated method was used for characterization (%EE and %DL) of prepared SLNs. The absorption maxima spectrum was determined using methanol and was found to be 369 nm (Figure 3). To obtain CMVs for developing final spectrophotometric conditions, AQbD approach was used. C-N-X approach was used to identify CMVs using the Ishikawa fishbone diagram and C-E risk assessment matrix. The risk levels of CMVs were identified based on the literature[27] and ranked according to their severity. The parameters with higher severity were selected and optimized using CCD. Total scores for various method variables were calculated and prioritized for DOE investigation. The influence of CMVs on CAA was assessed by CCD. UV-visible spectrophotometer was used to conduct 13 randomized trials in order to get an impartial response with not more than five center points (Table 2A, B) and overlay spectra of all the responses are depicted in Figures 4A and B. CCD has given optimized spectrophotometric conditions with scanning speed -0.147 nm/sec and sampling interval as -0.259 nm with a desirability nearer to 1 *i.e.* 0.903. All important parameters evaluated are found to be present within the specified limits. In addition, numerical and graphical optimizations were performed to identify the best option within the given design space. Furthermore, the impact of variables such as scanning speed and sampling interval on response (absorbance) is evaluated. The responses from all trials were fitted in various kinetic models (linear, 2FI, quadratic, cubic) as it showed best fit to the quadratic model. Investigation of ANOVA for the quadratic model showed a *p* value equal to 0.0053 with R^2 value 0.8698, indicating that the model is significant (Table 3). Effect of CMVs on CAA was studied using response, contour, and 3D plots (Figure 5). The graphical optimization was performed by superimposing the contour of the critical response with contour plots using design expert software that led to an overlay plot with two regions (yellow and gray). The overlay plot of the optimized method is represented in Figure 6A. The design space with yellow shade indicates the area with possible response values, whereas gray area indicates the design space with responses that do not meet the criteria. The optimum conditions were selected on the basis of overlay plot and desirability criteria.

Effect of scanning speed and sampling interval on absorbance

The impact of scanning speed and sampling interval on absorbance was studied from 3D plots, 2D contour plots, and polynomial equation (3) (Figure 5). For interpretation

Table 2A. Design table representing the range of variables used for optimizing the method

Run no	A: Scanning speed	B: Sampling interval
1*	0	0
2	+1	0
3	+1	+1
4	-1	+1
5*	0	0
6	-1	-1
7*	0	0
8	0	+1
9	0	-1
10	-1	0
11	+1	-1
12*	0	0
13*	0	0

*Represents center points of the model

Table 2B. Representing the decoding of DOE codes

Level	A: Scanning speed	B: Sampling interval
-1 (low)	Slow	0.5 nm
0 (medium)	Medium	1.0 nm
+1 (high)	Fast	2.0 nm

DOE: Design of experiment

and optimization purposes, 3D surface plot and 2D contour plot were used. According to the contours produced under optimal circumstances, investigation should proceed with specified center values for both CMVs. In all three 3D response surfaces for the response, similar pattern was seen for both CMVs. At low levels of a sample interval, curvilinear rise in response was seen with progressive increase in scanning speed. Similarly, with low scanning speeds and increasing sample intervals, small increase in responsiveness was seen. However, at low levels of both CMVs, minimal reaction was seen. The actual *vs.* predicted plot illustrated that data obtained from the experiments lie within the specified limits Figure 6B. Different colored points in the figure represent the higher R^2 values implying that the model can explain most variation. Blue, green, and red points represent the lower, middle, and higher values, respectively. Model appropriateness was suggested by satisfactory *p* values in ANOVA and low projected residual sum of squares (PRESS) values (Table 3). Polynomial equation of the quadratic model clearly displays that there is a significant positive impact of scanning speed and the sampling interval individually on absorbance. However, there is a negative effect of scanning speed and the sampling interval combinedly on

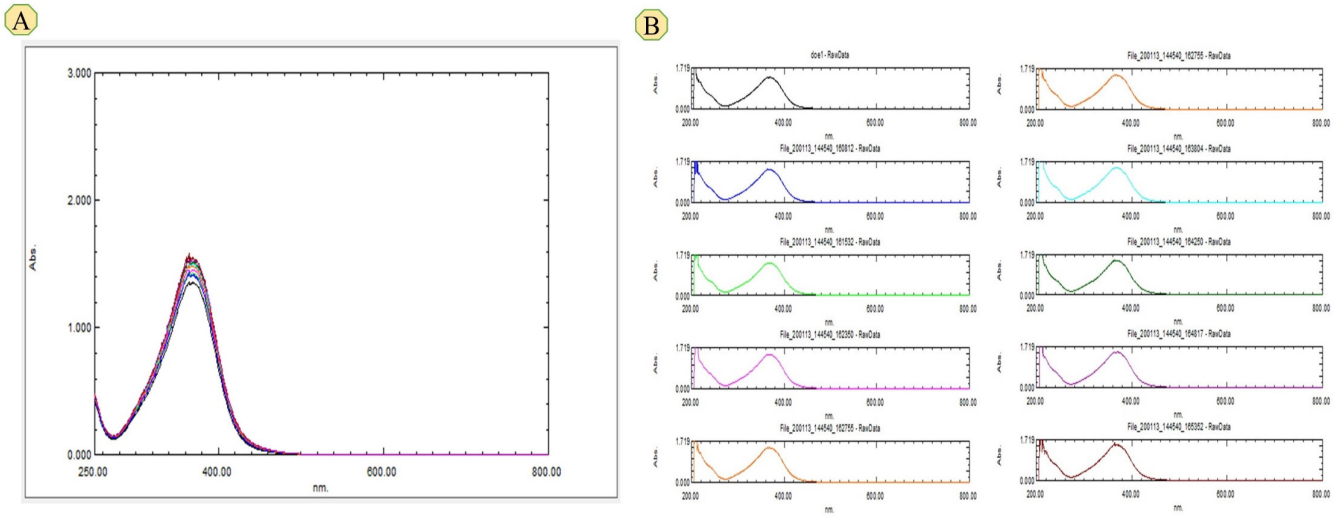

Figure 4. (A) Overlay spectrums of all the experiments given by design, (B) individual spectrums of experiments given by design

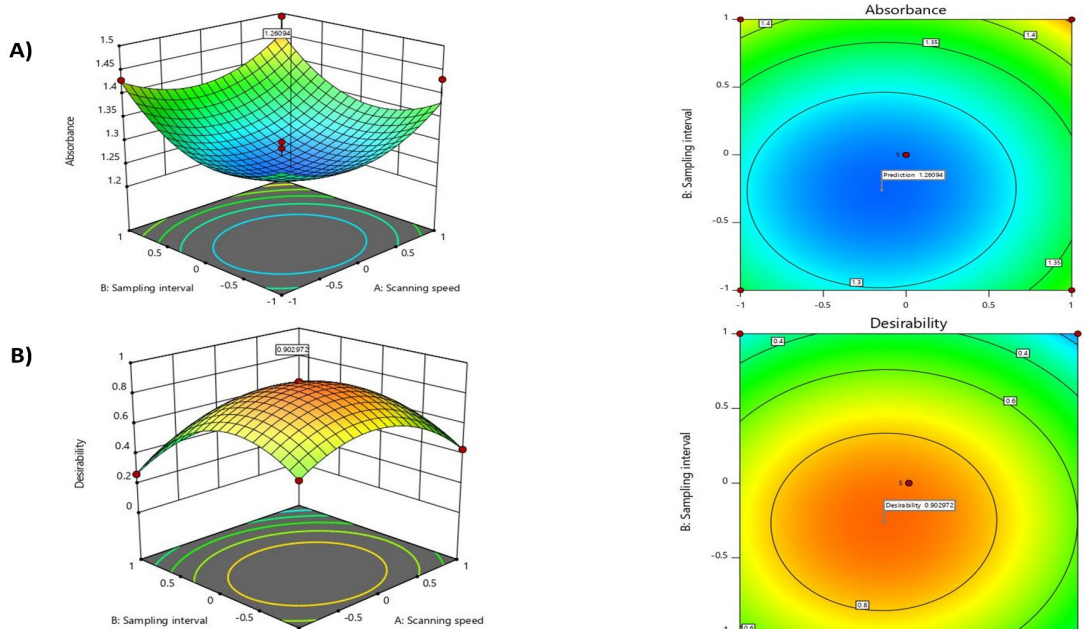

Figure 5. Depicting 3D and contour plots; (A) illustrating the effects of scanning speed and sampling intervals on absorbance. (B) Showing the desirability of the optimized method

Table 3. Representing the quadratic ANOVA model, lack of fit for scanning speed and sampling interval on absorbance

Source	Sum of squares	df	Mean squares	*F* value	*p* value
Model	0.0706	5	0.0141	9.35	0.0053
Scanning speed (A)	0.0023	1	0.00023	1.53	0.2559
Sampling interval (B)	0.0119	1	0.0119	7.89	0.0262
AB	0.0000	1	0.0000	0.0106	0.9209
A^2	0.0244	1	0.0244	16.15	0.0051
B^2	0.0391	1	0.0391	25.88	0.0014
Residual	0.0106	7	0.0015		
Lack of fit	0.0082	3	0.0027		
Pure error	0.0023	4	0.0006		
Cor total	0.0812	12			
Sequential model sum of squares					
Means *vs.* total	23.68	1	23.68		
Linear *vs.* mean	0.0142	2	0.0071	1.06	0.3816
2FI *vs.* linear	0.0000	1	0.0000	0.0022	0.9640
Quadratic *vs.* 2FI	0.0564	2	0.0282	18.67	0.0016
Cubic *vs.* quadratic	0.0026	2	0.0013	0.7998	0.4996
Residual	0.0080	5	0.0016		
Total	23.77	13	1.83		
Lack of fits tests					
Linear	0.0646	6	0.0108	18.42	0.0070
2FI	0.0646	5	0.0129	22.10	0.0052
Quadratic	0.0082	3	0.0027	4.69	0.0847
Cubic	0.0057	1	0.0057	9.70	0.0357
Pure error	0.0023	4	0.0006		
Representing the data of model summary, fit summary and fit statistics					
Model summary statistics					
	Standard deviation	R^2	Adjusted R^2	Predicted R^2	PRESS
Linear	0.0818	0.1752	0.0103	-0.3568	0.1102
2FI	0.0863	0.1754	-0.0994	-1.4285	0.1972
Quadratic	0.0389	0.8698	0.7768	0.2340	0.0622
Fit summary	Sequential *p* value	Lack of fit *p* value	Adjusted R^2	Predicted R^2	
Linear	0.3816	0.0070	0.0103	-0.3568	
2FI	0.9640	0.0052	-0.0994	-1.4285	
Quadratic	0.0016	0.0847	0.7768	0.2340	
Cubic	0.4996	0.0357	0.7633	-35146	
Fit statistics					
Standard deviation	0.0389		R^2	0.8698	
Mean	1.35		Adjusted R^2	0.7768	
CV%	2.88		Predicted R^2	0.2340	
			Adeq precision	7.7458	

PRESS: Projected residual sum of squares, df: Degree of freedom, CV: Coefficient of variation

absorbance. The effects of interaction of CMVs, individually and in combination with CAA and desirability of the method due to these interactions are illustrated in Figure 7.

The polynomial equation for the model is as follows:

$$\text{Absorbance} = +1.27 + 0.0170*A + 0.0386*B - 0.0020*AB + 0.0592*A^2 + 0.0750*B^2 \quad \text{equation (3)}$$

where A is scanning speed and B is sampling interval.

Analytical method validation

Linearity

The obtained linearity chart of XH (Figure 8) was analyzed by its correlation coefficient. The data obtained are represented in Table 4. XH linearity range in methanol was 2-12 µg/mL with a R^2 >0.9981.

LOD and LOQ

Sensitivity of the method was assessed by estimating LOD and LOQ. LOD and LOQ for the developed UV method of XH in methanol were found to be 0.77 and 2.36 µg/mL, respectively.

Precision and repeatability

Determination of precision was performed under prescribed conditions by measuring the absorbance multiple times with homogenous sample. The results for both inter- and intra- day are shown in Table 5A and B. Results of the interday and intra-day precision illustrated that the developed method was stable and precise as %RSD values are <2. Repeatability study was performed by taking absorbance of the XH (4 µg/mL) for six times and the percentage drug recovered was calculated by comparing it with the standard graph. %RSD of drug recovered was found to be less than 2.

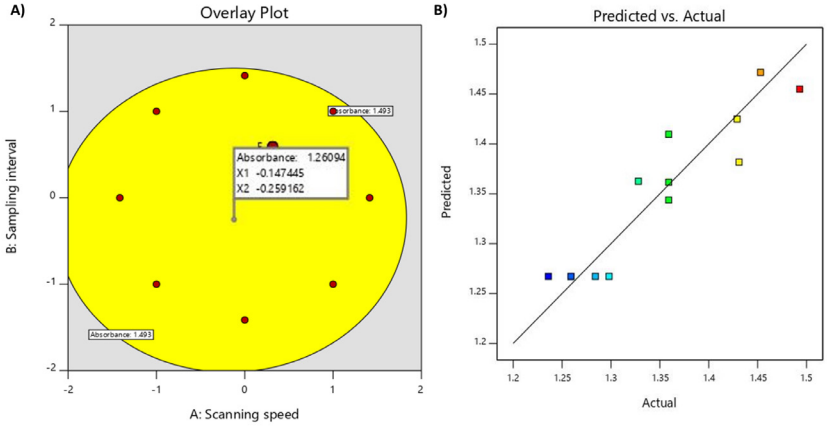

Figure 6. (A) Representing overlay plot of the optimized method, (B) Illustrating the correlation between predicted and actual values

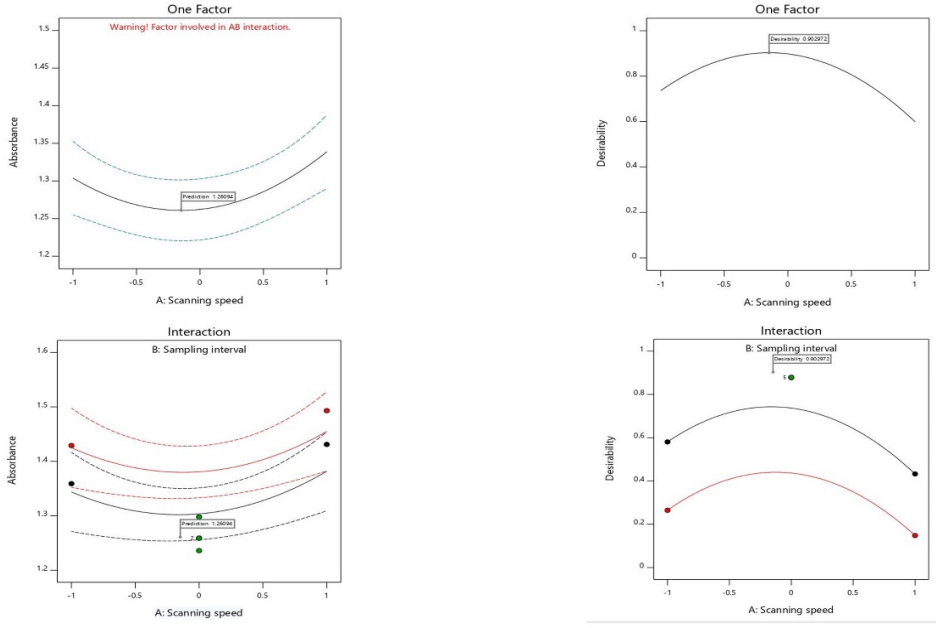

Figure 7. 2D representation of combined and individual effects and desirability of the optimized method

Accuracy

Accuracy study was conducted using the % recovery method. The results are summarized in Table 6. % Recovery was found to be 99.3%-100.1%. %RSD values were found to be within the acceptable limits (%RSD <2). Therefore, it is concluded that the developed method was accurate.

Specificity

Specificity of the developed method was evaluated using blank SLNs. Percentage drug recovery from blank SLNs mixed with known amount of XH was found to be 99.75 ± 0.23%, which indicated the developed method was specific toward XH. There is no interference with the excipients used in the development

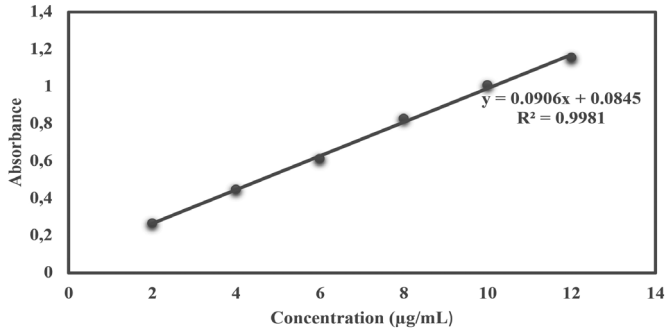

Figure 8. Depicting linearity chart of xanthohumol in methanol

Table 4. Depicting the linearity parameters of XH

Concentration (µg/mL)	Day 1 (Abs)			Day 2 (Abs)			Day 3 (Abs)			Avg
	Trial I	Trail II	Trail III	Trial I	Trail II	Trail III	Trial I	Trail II	Trail III	
2	0.299	0.286	0.292	0.235	0.285	0.268	0.208	0.253	0.271	0.266
4	0.485	0.480	0.487	0.436	0.454	0.475	0.397	0.402	0.399	0.446
6	0.602	0.610	0.608	0.621	0.610	0.622	0.592	0.618	0.626	0.612
8	0.826	0.811	0.825	0.852	0.848	0.844	0.805	0.810	0.815	0.826
10	1.012	1.001	1.010	0.999	1.005	1.012	1.007	1.001	1.020	1.007
12	1.312	1.302	1.310	1.216	1.285	1.295	1.193	1.267	1.222	1.155

Abs: Absorbance, Avg: Average

Table 5A. Inter-day precision data of XH

Concentration (µg/mL)	Absorbance			Mean ± SD	%RSD	% average potency
	Morning	Afternoon	Evening			
4	0.450 0.449 0.448	0.447 0.453 0.455	0.453 0.456 0.454	0.451 ± 0.003335	0.743	102.2
6	0.693 0.692 0.692	0.692 0.693 0.696	0.692 0.692 0.690	0.692 ± 0.001389	0.200	113.0
8	0.897 0.902 0.904	0.898 0.901 0.895	0.898 0.900 0899	0.899 ± 0.002925	0.325	108.8

SD: Standard deviation, RSD: Relative standard deviation

Table 5B. Depicting Intra-day precision data of XH

Concentration (µg/mL)	Absorbance			Mean ± SD	%RSD	% average potency
	Day 1	Day 2	Day 3			
4	0.463 0.464 0.466	0.468 0.468 0.466	0.467 0.469 0.468	0.466 ± 0.002066	0.443	104.4
6	0.684 0.684 0.686	0.686 0.687 0.685	0.687 0.683 0.685	0.685 ± 0.001488	0.217	111.9
8	0.924 0.925 0.922	0.926 0.922 0.925	0.923 0.926 0.925	0.924 ± 0.001642	0.177	111.8

SD: Standard deviation, RSD: Relative standard deviation

of SLNs. Figure 3A-C represents the individual spectrums, which clearly show that there is no peak at the absorption maxima of XH. Therefore, there is no interaction between the excipients used in SLNs with XH.

Analysis of in-house prepared SLNs

The prepared nanoparticles were analyzed for the content of drug encapsulated in the SLNs using equations (1) and (2). Results of %EE and %DL are summarized in Table 7. %RSD <2 is suggesting that the method is efficient for estimation of XH in nanoformulations without any interference.

DISCUSSION

Many UV-spectrophotometric methods have been developed for various drugs for their estimation in bulk and pharmaceutical formulations. In recent years, researchers have also developed UV-spectrophotometric methods for estimation of drugs in nanoformulations, and demonstrated AQbD approach during their developmental process.[15,18] This work has been designed based on the past developed methods for estimation of XH in nanoformulations such as SLNs. Till date, there is no UV-visible spectrophotometric method reported for XH using the AQbD approach. All the results of validation parameters lie within the acceptable limits. The method's specificity and selectivity were accomplished due to the absence of interference from widely used excipients (compritol E ATO, lipoid E80SN, pluronic F-68) in the SLNs. Recovery and accuracy of XH from SLNs were found to be 99.3-100.1% at wavelength 369 nm. LOD and LOQ values were found to be 0.77 and 2.36 µg/mL, respectively. Results of precision studies were found to be %RSD <2, which is an acceptable value. Therefore, the developed method was a reliable and robust method, interference free, which can be used for the estimation of XH in bulk and in nanoformulations.

CONCLUSION

A new robust, simple, and cost-effective UV-visible spectrophotometric method has been developed by using AQbD approach for estimating XH in bulk and nanoformulations. Quality of the analytical method was assured by using AQbD process. The findings point out to the method's originality, simplicity, accuracy, and precision. Statistical analyses of technique validation results support the established methods suitability for application in quality control laboratories. The developed method is more efficient to estimate XH in nanoformulations without interfering with the excipients used in the formulation. Consequently, this developed method can find its applicability in pharmaceutical industries to estimate XH effectively.

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Table 6. Depicting accuracy data of XH

Solvent used	Amount of standard added (%)	Percentage recovery (%)	%RSD
Methanol	80	99.3 ± 0.00215	0.785
	100	100.1 ± 0.00452	0.321
	125	99.54 ± 0.00256	0.685

RSD: Relative standard deviation

Table 7. Results of % of XH entrapped and % drug loading in solid lipid nanoparticles

Formulation	% of XH entrapped ± SD	% of XH unentrapped ± SD	% total amount of XH recovered ± SD	%RSD	%DL
F1	76.64 ± 0.64	22.43 ± 0.22	99.07 ± 0.46	0.469	11.6
F2	63.2 ± 0.35	34.91 ± 0.165	98.11 ± 0.19	0.195	8.34
F3	72.2 ± 0.65	25.93 ± 0.15	98.13 ± 0.75	0.764	12.8

XH: Xanthohumol, SD: Standard deviation, RSD: Relative standard deviation, DL: Drug loading

Ethics

Ethics Committee Approval: Not applicable.

Informed Consent: Not applicable.

Peer-review: Externally peer-reviewed.

Authorship Contributions

Concept: H.V., D.T., R.K., P.G., M.G., Design: H.V., S.K.S., D.T., R.K., P.G., M.G., Data Collection or Processing: H.V., D.T., R.K., S.M., Analysis or Interpretation: H.V., D.T., R.K., S.M., Literature Search: H.V., D.T., R.K., Writing: H.V., D.T., R.K.

Conflict of Interest: No conflict of interest was declared by the authors.

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ORIGINAL ARTICLE

Applications of Dietary Supplements and Aromatherapy for Prophylactic and Treatment Purposes During COVID-19 Pandemic

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ABSTRACT

Objectives: The lack of a specific proven treatment for coronavirus disease-2019 (COVID-19) has led individuals to use different treatment options. Although their effects on COVID-19 have not been proven, interest in dietary supplements and aromatherapy has increased during the pandemic period. In this study, use of dietary supplements and aromatherapy was investigated for COVID-19 among individuals living within the borders of Türkiye.

Materials and Methods: This cross-sectional survey study was conducted among 310 individuals. The questionnaire was prepared using online Google Forms and communicated to the participants *via* social media platforms. The data obtained from the study were analyzed with the statistical program.

Results: The analyzes of the survey revealed that participants increased the usage of supplements mostly prophylactic and for treatment purposes during COVID-19 pandemic, 31.9% individuals declared that they consumed herbal tea/products, 38.1% of them used vitamin/mineral supplements (multivitamin-mineral, vitamins B1, B6, B12, C, D, calcium, coenzyme Q10, iron, magnesium, selenium, and zinc), and 18.4% of the individuals applied aromatherapy (meaning treatment with essential oils). As a result of the study, the most commonly used supplement was vitamin D, the most commonly consumed tea was green tea, the essential oil was thyme oil, and the most eaten vegetable was garlic. Moreover, other frequently used herbal products were found to contain ginger and onion as food and peppermint and eucalyptus oils as aromatherapeutics. Participants often reported that they found it safe to use elevated levels of herbs or herbal products against COVID-19.

Conclusion: Among the individuals participating in this study, it has been observed that the use of dietary supplements has increased during the COVID-19 pandemic period. The study revealed that vitamin D is prominent in self-medication use. Moreover, interest in aromatherapy and dietary supplements has increased. Among aromatherapeutics, thyme stood out over the applied essential oils.

Key words: COVID-19, dietary supplements, Türkiye, herbal, essential oil

INTRODUCTION

New coronavirus disease-2019 (COVID-19), which appeared in December 2019, was declared as a pandemic by World Health Organization (WHO) on March 11, 2020,[1] following days, Türkiye's first cases were detected.[2] With the epidemiological update published by WHO on March 30th, 2021, the weekly number of cases exceeded 3.8 million worldwide. More than 64,000 new deaths have been reported. At the beginning of the pandemic, the number of patients infected with COVID-19 has been more than 126 million, and unfortunately, close to 3 million deaths have been reported. In Türkiye, the number of weekly cases was almost 190 thousand, and the number of weekly deaths was almost 1000. From the beginning of the pandemic in Türkiye, the number of patients infected with COVID-19 virus

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surpassed 3 million and more than 30 thousand deaths were recorded.[3]

Since there are no proven complete treatment options for COVID-19, compliance with wearing masks, hygiene rules, keeping distance, and a balanced food intake is the most effective approach so far. Healthy eating guidelines have been published to strengthen immunity and prevent COVID-19 contamination. Recommendations on optimal nutrition, good hygiene practices, and the use of dietary supplements have been reported.[4,5] The vitamins; A, B6, B12, C, D, and E as well as the minerals, *e.g.* selenium and zinc, have been shown to support immunity by affecting the production and function of antibodies and B and T-cells.[6] Dietary supplements such as cinnamaldehyde, curcumin, lactoferrin, probiotics, quercetin, vitamin D, vitamin C, zinc, and selenium have immune-boosting, antiviral, antioxidant, and anti-inflammatory effects.[7] This has increased the interest in dietary supplements during pandemic. Increasing their advertisements in the media caused unconscious selection and use.[8] High doses of dietary supplements can cause side and even toxic effects.[9,10] Moreover, they may interact with drugs taken together.[10,11]

Based on the scientific evidence, essential oils have antiviral, anti-inflammatory, immunomodulatory, and bronchodilatory activities. Due to their lipophilic nature, it has been suggested that essential oils (*e.g.* eucalyptus, garlic) can easily penetrate viral membranes, inhibit viral replication, and be used for COVID-19[12] for treating respiratory tract infections essential oils are applied topically, orally, and by inhalation. In the topical application, diluted essential oil is applied to the back, chest, and soles of the feet. Application to the chest and back area provides sinus drainage and relaxation of sputum. Because of the application to soles of the feet, essential oils can be absorbed into the bloodstream, followed by a pharmacological effect, only with poor absorption. The oral intake of essential oils is not usually recommended for safety reasons; additionally, if taken orally, they are diluted in water or developed by encapsulation. When essential oils are administered by inhalation, they are distributed to the lungs with 50.0% effective bioavailability and reach the smallest cavities.[13] Aromatherapy has been used in the prevention of plague throughout history. Different forms of aromatherapy application are used in traditional Chinese medicine and have also been used during COVID-19. Given China's position in epidemic prevention, aromatherapy has been suggested to boost immunity, prevent and control COVID-19.[14]

Dietary supplement and aromatherapy applications of individuals against COVID-19 have been investigated in many countries. In Poland, during the COVID-19 pandemic, increased use of immune-related nutrients and foods such as vitamins C and D, zinc, garlic (*Allium sativum* L.- Alliaceae), ginger (*Zingiber officinale* Roscoe- Zingiberaceae) or turmeric (*Curcuma longa* L.- Zingiberaceae) has been reported.[15] In Bangladesh, a study revealed that 57.6% of the individuals used herbal food/products and 11.2% both drugs and herbal food/products as preventive measures against COVID-19. Black seed (*Nigella sativa* L.-Ranunculaceae), clove (*Syzygium aromaticum* L.) (Merr. & L.M.Perry-Myrtaceae), ginger (*Zingiber officinale* Roscoe-Zingiberaceae), and honey are among the herbal food/products used, and *Arsenicum album* (homeopathic drug), vitamins, and zinc supplements were the most used food supplements.[16] Vitamin C was used in Saudi Arabia and Egypt,[17,18] ginger (47.2%) and turmeric (31.6%) were often used in Egypt. The application of these herbs during COVID-19 was associated with age and fear score.[18] In an observational study with participants from three countries, Sweden, UK, and the USA, it was found that the use of probiotics, omega-3 fatty acids, multivitamins, and vitamin D reduced the risk of being infected with COVID-19. On the other hand, it was not associated with the consumption of zinc, vitamin C, and garlic supplements.[19] In West Nusa Tenggara region, use of essential oils, the use of eucalyptus (*Eucalyptus* sp.-Myrtaceae), lemon (*Citrus limonum* Risso-Rutaceae), lemongrass (*Cymbopogon flexuosus* Stapf-Poaceae), lavender (*Lavandula* L. sp.-Lamiaceae) oils were determined during the pandemic period.[20]

The goal of the study was to evaluate the use of dietary supplements and aromatherapeutics used against COVID-19 and the frequency of use. To analyze in more detail the use of dietary supplements, the use of herbals, vitamins, and minerals was questioned separately. The application of essential oils in terms of both oral and other usage methods was also questioned. This study is a comprehensive study that questions both dietary supplements and aromatherapy applications. It is important since it is one of the rare studies, in which the use of essential oils is also questioned.

MATERIALS AND METHODS

The research was conducted as a cross-sectional survey, in which a questionnaire was prepared using online Google Forms, between March 1st and March 10th 2021, and was held in various provinces of Türkiye. Approval was obtained from Gazi University Ethics Committee (22.02.2021-E.33174) for the study. Three hundred ten voluntarily participated individuals aged 18 and older working-living in the borders of Türkiye have been included. It has been communicated to the participants that meet the inclusion criteria through social media platforms such as WhatsApp. The questionnaire form was prepared by scanning the present literature. Overall 26 questions that questioning the descriptive characteristics of individuals, the use of herbs/herbal products, vitamins/minerals, and aromatherapy applications against COVID-19. In the first 14 questions of the survey, the descriptive characteristics of the individuals, whether they were infected with COVID-19 and whether they had influenza and/or pneumonia vaccines during the COVID-19 pandemic were asked. Herbal products, vitamins/minerals, aromatherapy applications, essential oils, and frequency of their use have been researched. Individuals were asked in a separate question, whether there are any herbs/herbal products that they consume as food or not. In this survey, whether individuals found the use of herbs/herbal products safe for COVID-19 was questioned. Scoring was done on a scale of 1 to 5 in which 1 point "I never find it reliable"; 5 points corresponds to "I find it reliable".

Statistical analysis

The data obtained from the study were analyzed with the Statistical Package for the Social Sciences (SPSS) statistical program. The frequency of participant responses is shown in tables and figures. Cross tables were created to correlate the responses and chi-square tests were conducted. The significance for the statistical analysis were set as $p<0.05$. Cramér's V (ϕc) value was calculated to determine the strength of the relationship between variables. It takes on values between 0 and 1 (inclusive), in which 0 corresponds to no relationship between the variables, while 1 corresponds to one variable being completely determined by the other.

RESULTS

Characteristics of participants

Characteristics of the participants are shown in detail in Table 1, in which 310 individuals, 223 (71%) of whom were women, participated in this survey study. The province with the highest participation rate was Ankara followed by Bursa and Hatay, where the participants lived. Majority of the individuals were between the ages of 30 and 44. The most involved occupational groups were healthcare professionals, of whom 61.3% were pharmacists. Majority of the participants (79%) declared that they do not consume alcohol, while 22.6% of the participants still smoke, only 6.5% have smoked and quit in a period of their lives, while 21% of the participants were having chronic illnesses. Among the participants, 48 individuals (15.5%) had COVID-19 previously, and only one of them was hospitalized. Most of the participants did not have influenza or pneumonia vaccines during the pandemic period.

Dietary supplement consumption and aromatherapy applications

A graphic regarding the consumption of herbs is shown in Figure 1. Among the participants, 123 (39.7%) of them reported that they did not increase the consumption of vegetables in their diet during the COVID-19 pandemic. On the other hand, among the rest of the participants, garlic followed by onion was the most consumed food.

The frequency of drinking herbal tea, vitamin-mineral supplements, and aromatherapy for COVID-19 is shown in Table

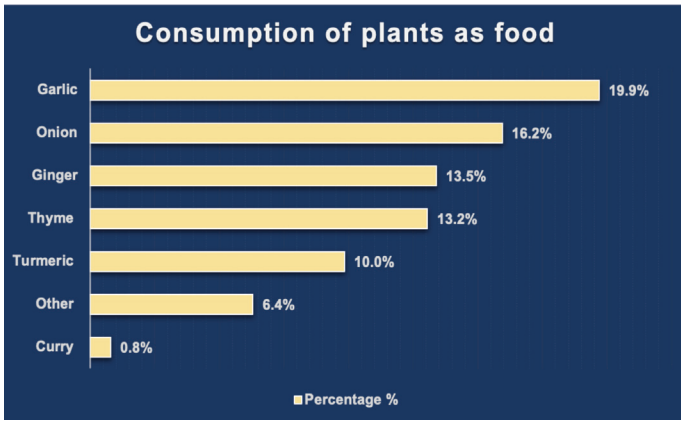

Figure 1. Edible plants and their products that the participants increased their consumption as a food during the pandemic

Table 1. Characteristics of participants (n: 310)

Characteristics	Number of participants (n)	Percentage (%)
Gender		
Female	223	71.9
Male	87	28.1
Age		
18-29	105	33.9
30-44	128	41.3
45-59	61	19.7
≥60	16	5.2
Marital status		
Married	202	65.2
Unmarried	108	34.8
Smoking		
Yes	70	22.6
No	220	71.0
Quit smoking	20	6.5
Alcohol consumption		
Yes	20	6.5
No	245	79.0
Sometimes	45	14.5
Profession		
Healthcare professionals	75	24.2
Civil servant	72	23.2
Housewife	33	10.6
Student	32	10.3
Self-employment	26	8.4
Retired	12	3.9
Other	60	19.4
Healthcare professionals		
Pharmacist	46	61.3
Doctor	15	20.0
Nurse	4	5.3
Dentist	3	4.0
Physiotherapist	2	2.7
Other	5	6.7
Chronic disease		
Yes	65	21.0
No	245	79.0
Infected with COVID-19		
Yes	48	15.5
No	262	84.5
Hospital treatment for COVID-19		
Yes	1	0.3
No	309	99.7
Influenza (flu) vaccine during COVID-19 pandemic process		
Yes	13	4.2
No	297	95.8
Pneumonia vaccine during COVID-19 pandemic process		
Yes	21	6.8
No	289	93.2

COVID-19: Coronavirus disease-2019

2. The vast majority (81.8%) of individuals who used herbal tea or herbal products to protect themselves from COVID-19 during the pandemic period were not infected with COVID-19. Moreover, 81.6% of people who used and stopped consuming herbal tea or herbal products for a while were not infected with COVID-19.

Among these applications, mostly green tea and herbal products containing ginger were used to protect against COVID-19; detailed results of using herbal tea or herbal products for protection from COVID-19 are shown in Table 3. Individuals who were infected with COVID-19 consumed mostly thyme hydrosol and herbal products containing ginger during their healing stage at home. However, because of the statistical analysis, no significant relationship was found between the use of herbal tea or herbal products and the state of infection with COVID-19 ($p>0.05$). The herbal tea/product was mostly used by individuals between the ages of 30-44 to protect against COVID-19 (33.3%). A significant relationship was found between the use of herbal tea/product and age ($p<0.05$).

Table 2. Usage frequency of herbal tea/product, vitamin/mineral supplements and aromatherapy

Variables	Number of participants (n)	Percentage (%)
Do you use herbal tea or herbal product to protect against COVID-19?		
Yes	99	31.9
No	162	52.3
I used and stopped	49	15.8
Do you use vitamin/mineral supplements to protect against COVID-19?		
Yes	118	38.1
No	129	41.6
I used and stopped	63	20.3
Do you apply aromatherapy (treatment with essential oils) for COVID-19?		
Yes	57	18.4
No	235	75.8
I applied and stopped	18	5.8

COVID-19: Coronavirus disease-2019

Table 3. Herbal tea or herbal product use among the participants

Herbal tea/ herbal product	I do not use *n (%)	I use it to protect against COVID-19 *n (%)	I only used it during my COVID-19 disease *n (%)	I have used it during my COVID-19 disease and I use it to protect against COVID-19 *n (%)
Herbal product containing ginger	227 (73.2%)	74 (23.9%)	5 (1.6%)	4 (%1.3)
Herbal product containing turmeric	247 (79.7%)	57 (18.4%)	3 (1.0%)	3 (%1.0)
Herbal product containing curcumin	287 (92.6%)	22 (7.1%)	0	1 (0.3%)
Elderberry (Sambucol®)	255 (82.3%)	52 (16.8%)	2 (0.6%)	1 (0.3%)
South African Geranium (Umca®)	288 (92.9%)	20 (6.5%)	2 (0.6%)	0
Green tea	223 (71.9%)	79 (25.5%)	5 (1.6%)	3 (1.0%)
Thyme hydrosol	247 (79.7%)	51 (16.5%)	9 (2.9%)	3 (1.0%)
Thyme oil	275 (88.7%)	28 (9.0%)	6 (1.9%)	1 (0.3%)
Herbal product containing thyme	263 (84.8%)	41 (13.2%)	4 (1.3%)	2 (0.6%)
Herbal product containing carvacrol	291 (93.9%)	16 (5.2%)	2 (0.6%)	1 (0.3%)
Black cumin essential oil	251 (81.0%)	53 (17.1%)	4 (1.3%)	2 (0.6%)
Peppermint essential oil	282 (91.0%)	25 (8.1%)	3 (1.0%)	0
Eucalyptus essential oil	285 (91.9%)	24 (7.7%)	1 (0.3%)	0
Echinacea	295 (95.2%)	13 (4.2%)	2 (0.6%)	0
Ginseng	299 (96.5%)	6 (1.9%)	4 (1.3%)	1 (0.3%)

*n (%): Number (percentage), COVID-19: Coronavirus disease-2019

In order to protect themselves from COVID-19 infection, 33.9% of the participants responded the use of herbs or herbal products to be very reliable, whereas 4.8% never found it reliable. Among the participants, 30% found herbs and herbal products very reliable, whereas 6.8% never found reliable, while having COVID-19 infection (Figure 2).

People who filled the questionnaire (38.1%) reported that they used vitamin/mineral supplements to be protected against COVID-19, and 20.3% used and quit for a period. Vitamins D and C were the most commonly used vitamins by individuals to protect against COVID-19 (Table 4). Individuals infected with COVID-19 also reported that they used vitamins D and C the most, respectively. 80.5% of individuals who used vitamin/mineral supplements to be protected against COVID-19 during the pandemic period were not infected with COVID-19. Also, 85.7% of individuals who used and stopped taking vitamin/mineral supplements for a while were not infected with COVID-19. However, because of the statistical analysis, no significant relationship was found between the use of vitamin/mineral supplements and the state of infection with COVID-19 ($p>0.05$). Similar to the use of herbal tea/product, a significant relationship was found between the use of vitamin/mineral supplements and age, and they were mostly used in individuals between the ages of 30-44 (46.6%, $p= 0.01$).

The essential oils used by entities in aromatherapy applications and their usage rates are shown in Figure 3. Only 24.2% of the individuals participating in the study practice aromatherapy during the pandemic. Individuals who used aromatherapy reported that they mostly consumed thyme (*Thymus* L. sp. or *Origanum* sp., Lamiaceae), peppermint (*Mentha* L. sp., Lamiaceae), and eucalyptus (*Eucalyptus* sp., Myrtaceae) oils; among them, 80.7% of individuals were not infected with COVID-19; moreover, 83.3% of the individuals who applied and stopped the aromatherapy application for a while were not infected with COVID-19. However, no statistically significant relationship was found between aromatherapy applications and COVID-19 infection status ($p>0.05$). The individuals who applied aromatherapy were mostly in the 30-44 age group (36.8%); consequently, a significant relationship between aromatherapy practice and age ($p<0.05$) was determined.

As for the information gathered by the participating individuals,

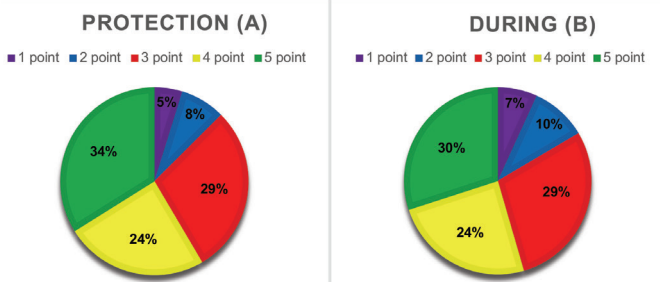

Figure 2. (A) The percentage of participants find the use of herbal products safe for protection COVID-19; (B) The percentage of participants find the use of herbal products safe during COVID-19
COVID-19: Coronavirus disease-2019

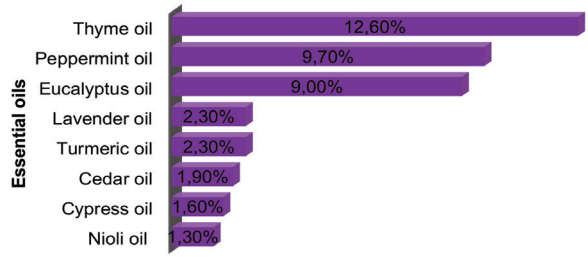

Figure 3. Use of essential oils during pandemic

Table 4. Vitamin/mineral supplements use

Vitamins/minerals	I do not use *n (%)	I use it to protect against COVID-19 *n (%)	I only used it during my COVID-19 disease *n (%)	I have used it during my COVID-19 disease and I use it to protect against COVID-19 *n (%)
Multivitamin-mineral	237 (76.5%)	64 (20.6%)	8 (2.6%)	1 (0.3%)
Vitamin B12	237 (76.5%)	66 (21.3%)	5 (1.6%)	2 (0.6%)
B1 + B6 + B12	260 (83.9%)	43 (13.9%)	5 (1.6%)	2 (0.6%)
Vitamin C	156 (50.3%)	144 (46.5%)	6 (1.9%)	4 (1.3%)
Vitamin D	142 (45.8%)	153 (49.4%)	10 (3.2%)	5 (1.6%)
Iron	265 (85.5%)	41 (13.2%)	3 (1.0%)	1 (0.3%)
Zinc	239 (77.1%)	63 (20.3%)	4 (1.3%)	4 (1.3%)
Magnesium	257 (82.9%)	52 (16.8%)	0	1 (0.3%)
Calcium	274 (88.4%)	33 (10.6%)	3 (1%)	0
Selenium	287 (92.6%)	23 (7.4%)	0	0
Coenzyme-Q10	279 (90.0%)	28 (9.0%)	3 (1.0%)	0

*n (%): Number (percentage), COVID-19: Coronavirus disease-2019

in order to use dietary supplements or aromatherapy, 30% of the individuals responded *via* the internet and television (Figure 4), whereas individuals who stated that they received advice from healthcare professionals who were pharmacists the most.

Cross tabulation was made to determine the relationship between the information sources and the use of herbal teas and products (Table 5). Because of the statistical analysis, a significant relationship was obtained between the individuals using herbal tea/products and the information sources they relied. Further analysis showed that there was a significant correlation between learning the use of herbals from pharmacists, academic articles, social media, neighbors, friends, relatives, and family elders ($p<0.05$).

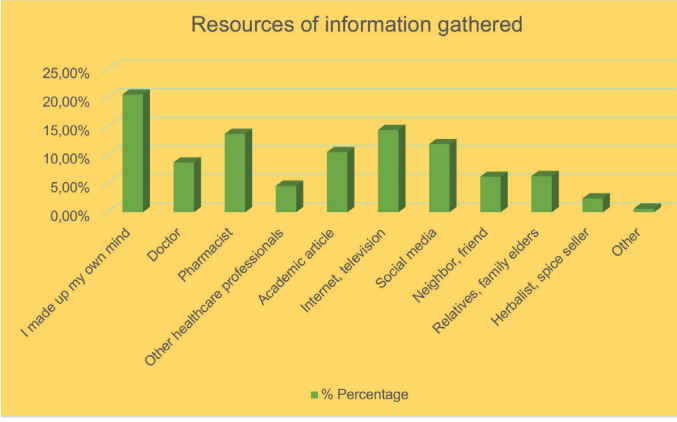

Figure 4. Information resources of participants using herbs

DISCUSSION

At the beginning of the COVID-19 pandemic, studies have accumulated in the literature about investigating the use of dietary supplements. As far as we know, there were no studies questioning the use of aromatherapy and essential oils in addition to dietary supplements in the same questionnaire. In this study, we investigated the use of dietary supplements and aromatherapy in Türkiye for COVID-19. In our study, the province with the highest participation rate was Ankara, the capital of Türkiye, and the highest participation rate of individuals was healthcare workers. Among the participants who smoke and consume alcohol was significantly low, which might suggest that the participants of this survey are particularly conscious about their health. On the other hand, although most of them were healthcare professionals, it is surprising that most participants neither had flu nor pneumonia vaccine during the period that we questioned them. This may be because the Turkish Ministry of Health gives priority to the elderly people over a certain age in vaccination, since most of the participants were between the ages of 30-44 and the number of vaccines was insufficient.

In this study, less than half of the participants who were mostly between the ages of 30-44 used dietary supplements for COVID-19 (31.9% herbal tea/products, 38.1% vitamin/mineral supplements); fewer participants (18.4%) practiced aromatherapy. Additionally, the participants used and quited using herbal tea/products, vitamin/mineral dietary supplements, and aromatherapy applications were 15.8%, 20.3%, 5.8%,

Table 5. The relationship between information sources and use cases of herbal tea/products

	Do you use herbal tea or herbal products to protect yourself from COVID-19 during the pandemic period?			
	Yes *n	No *n	I used and quit *n	Significance
I made up my own mind	43	67	20	$p>0.05$
Pharmacist	39	30	17	$p= 0.001$ $X^2= 14.762$ $\phi c= 0.218$
Academic article	32	23	11	$p<0.01$ $X^2= 12.094$ $\phi c= 0.198$
Internet, television	31	45	15	$p>0.05$
Doctor	21	21	13	$p>0.05$
Social media	21	29	25	$p<0.001$ $X^2= 23.205$ $\phi c= 0.274$
Neighbor, friend	13	14	12	$p<0.05$ $X^2= 8.631$ $\phi c= 0.167$
Other healthcare professionals	12	10	7	$p>0.05$
Relatives, family elders	10	18	12	$p<0.05$ $X^2= 7.008$ $\phi c= 0.150$
Herbalist, spice seller	6	4	5	$p>0.05$

*n: Number, X^2: Chi-square, ϕc: Cramér's V, COVID-19: Coronavirus disease-2019

respectively. Consequently, the most commonly used dietary supplements were vitamins D and C, green tea, and herbal products containing ginger, and zinc have been highlighted for immune support,[6] and our findings are consistent with that reported. Recent studies have suggested that vitamin D is effective in protecting from COVID-19 infection and reducing the severity of existing symptoms.[21] Meta-analysis showed that vitamin D has a slight effect on protection from acute respiratory infections.[22] Findings show garlic and onion to be the most consumed foods. It has been suggested that garlic has antiviral and anti-inflammatory effects, strengthens immunity, and is effective in preventing COVID-19.[23,24] Although some compounds derived from garlic and onions are effective for COVID-19 in *in silico* studies, their usability for COVID-19 has not been proven.[25] Mhatre et al.[26] suggested that green tea polyphenols can be used in the prophylaxis and treatment of COVID-19 due to their antiviral activity. In a previous randomized controlled study, taking capsules containing green tea catechins and theanine prophylactically prevented influenza infection among healthcare professionals.[27] Based on these studies, green tea polyphenols may be an option for protection from COVID-19. However, the amount of catechin in the content of green tea prepared and consumed as tea may not be sufficient for the dose required for COVID-19 prophylaxis. As a matter of fact, according to the results of the research, there was no significant relationship between the consumption of dietary supplements and protection from COVID-19.

This study demonstrated that participants applying aromatherapy mostly used thyme, peppermint, and eucalyptus oils. This result is not surprising as these essential oils are effective against respiratory system pathogens.[13] In addition to all these, essential oils are used in aromatherapy for stress and sleep control.[28] Application of aromatherapy might be useful for controlling stress in individuals caused by COVID-19 pandemics. Interestingly, our findings showed that lavender oil, which is known to have an anxiolytic effect,[29] was used at a low rate by the study participants (2.3%). In our study, we questioned the use of different forms of thyme and its components. Under the title of herbal tea/products, in any period of the pandemic, the rate of use of thyme juice was 20.3%, oregano oil was 11.3%, herbal product containing thyme was 15.2%, and carvacrol-containing herbal product usage rate was 6.1%. The rate of thyme oil usage was determined as 12.6%, which might be the result of previous studies, which demonstrated the antiviral effect of thyme and its components in previous and *in silico* studies against COVID-19.[30,31] It has been shown that different thyme preparations and thymol can be used in respiratory system disorders due to its antispasmodic, antitussive, mucolytic, and expectorant properties.[32] In a randomized clinical study on patients having COVID-19, oral intake of thyme essential oil significantly reduced the severity of symptoms such as fever, cough, shortness of breath, dizziness, muscle pain, anorexia, weakness, lethargy, and fatigue.[33] In a randomized controlled clinical trial, inhaled thyme essential oil improved the respiratory tract condition of patients under mechanical ventilation. Because of the study, thyme essential oil reduced the concentration of airway secretions, facilitated the evacuation of mucous secretions, and had bronchodilator effect.[34] Besides *in vitro* studies, clinical studies also demonstrated that thyme has a potential effect on COVID-19 and its symptoms; however, further studies should focus on choosing appropriate thyme species, the preparation of the product, and the route of administration.

Individuals were asked, if they found the use of herbal tea/products safe for prophylactic usage or for treating COVID-19 infection. More than half of the individuals found the use of the herbal tea/product safe in both situations. Participants marked the option "I made my own decision" (20.6%) as the source from where they obtained information about the usage of the non-pharmaceutical products. Apart from that, they gathered this information mostly through the internet and television (14.4%). Total number of those who acquired information through healthcare professionals such as doctors and pharmacists and academic articles is 37.5%, while the rest gathered the information from unreliable sources. Moreover, the relationship between the source from which the information is learned and the use of herbal teas and products was evaluated. There was a significant relationship between herbal tea/product use and attaining information about the supplements from pharmacists, academic articles, social media, neighbors, friends, relatives, and family elders.

Study limitations

The number of participants was limited to 310 as it was planned as a quick study. The participation rate of the elderly population was low as the research was conducted online.

CONCLUSION

Interest in dietary supplements has increased during the pandemic period, and non-medical information sources have been more effective in deciding on dietary supplements to use. Before the use of the recommended dietary supplements, their protective effects, side effects, and drug interactions should be identified and the public should be informed about these issues. Studies have shown that essential oils have potential effects on COVID-19; therefore, essential oils should be included in published guidelines, and further randomized studies on essential oils should be the focus. Besides dietary supplements and aromatherapy application, the public should be cautious about nutrition, exercise, and hygiene to overcome the COVID-19 pandemic.

Ethics

Ethics Committee Approval: Approval was obtained from Gazi University Ethics Committee (22.02.2021-E.33174) for the study.

Informed Consent: The study was conducted with volunteer individuals who met the inclusion criteria and agreed to participate in the study.

Peer-review: Externally peer-reviewed.

Authorship Contributions

Surgical and Medical Practices: M.M., U.K.Ç., Concept: M.M., U.K.Ç., Design: M.M., U.K.Ç., Data Collection or Processing: M.M., U.K.Ç., Analysis or Interpretation: M.M., U.K.Ç., Literature Search: M.M., U.K.Ç., Writing: M.M., U.K.Ç.

Conflict of Interest: No conflict of interest was declared by the authors.

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ORIGINAL ARTICLE

Development and Evaluation of *In Situ* Gel Formation for Treatment of Mouth Ulcer

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ABSTRACT

Objectives: Mouth ulcers are one of the most prevalent conditions that can be caused by a range of circumstances. Many formulations, such as solutions, suspensions, and ointments are available commercially. However, because there is no long-term effect, no medication can be regarded as totally effective for treating mouth ulcers. The use of bioadhesive methods can boost the therapy efficacy. Because it is easier to administer than prepared gel formulations, the phenomenon of the sol-to-gel conversion can be beneficial. The major goal of this study was to develop and test *in situ* gels for treating mouth ulcers using choline salicylate and borax as model medicines.

Materials and Methods: Because a thermosensitive polymer was employed in this formulation, the sol-to-gel change was thermally reversible, and the frequency of administration was reduced by using the mucoadhesive polymer carbopol. Gelation temperature, pH, gel strength, spreadability, *in vitro* mucoadhesion, and *in vitro* drug release were all measured in the formulations.

Results: The experimental section indicated that viscosity of sols and gel strength increased with increasing temperature, *i.e.*, gel can be created at the site of application owing to body temperature. When poloxamer 407 was used at a concentration of 14 to 16 percent *w/v*, the gelling temperature was close to the body temperature (35-38 °C), but when carbopol 934P was added, the gelling temperature was raised. All formulations had pH between 5.5 and 6.8. All formulations had viscosities of less than 1000 cps, allowing for simple administration of the formulation to a mouth ulcer.

Conclusion: As a result, a correctly developed *in situ* gel for oral ulcers can extend the duration spent at the application site and minimize the frequency of administration. These findings show that the developed technology is a viable alternative to traditional drug delivery systems and can help patients comply.

Key words: *In situ* gel, thermo reversible, mucoadhesive, choline salicylate, mouth ulcer, 2^2 factorial designs

INTRODUCTION

Numerous routes of administration employed so far in new drug delivery systems, localized drug delivery to oral cavity tissues, have been examined for the treatment of periodontal diseases, bacterial and fungal infections, aphthous ulcers, and other disorders.[1] The oral mucosa is the "skin" that covers most of the mouth cavity, besides the teeth. It can be used for multitude of things. Its main purpose is to serve as a deterrence.[2] It protects deeper tissues such as fat, muscle, nerves, and blood vessels from mechanical trauma such as chewing. Oral mucosal disease is the most common disease that affects people. Mouth ulcers are painful round or oval sores that develop in the mouth, usually on the inside of the cheeks or lips.

Mouth ulcers are also called recurrent aphthous stomatitis (RAS), aphthae, aphthosis, and canker sores. The word aphthous is derived from the Greek word "aphtha", which signifies the ulcer. Despite the redundancy, these oral sores are still referred to as aphthous ulcers in medical literature.[3] RAS has an etiology that is either unknown or unclear.[4] Idiopathic RAS, rather than being a singular entity, may be the presentation of several illnesses with quite distinct etiologies. Nutritional deficiencies such as iron and vitamins, especially B12 and C, poor dental hygiene, infections, stress, indigestion,

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mechanical injury, food allergies, hormonal imbalance, and skin illness are all common causes of mouth ulcers. Hematinic deficits and blood disorders, gastrointestinal disorders, immune deficiencies such as in people with human immunodeficiency virus, neutropenia, and other conditions may predispose to RAS, such as microbial illness, chronic prescription of non-steroidal anti-inflammatory drugs, alendronate, nicorandil, and other cytotoxic drugs. In some circumstances, quitting smoking might trigger or worsen RAS.[4,5]

Various topical therapy techniques can be used to effectively treat mouth ulcers. However, there are some problems that emerge from the drug's short retention duration, which could be the cause of limited therapeutic efficacy and should be addressed.[5,6]

Advantages of *in situ* forming polymeric drug delivery systems, such as ease of administration and better patient comfort, have piqued interest. They increase the amount of time spent at the application site. Deformable dosage forms have less adverse effects than other dosage forms because they can conform to the contour of the surface on which they are placed. *In situ* forming polymeric formulations are drug delivery systems that are in sol form before being distributed in the body but gel *in situ* to create gel after being delivered. Recent advances in polymer chemistry and hydrogel engineering have facilitated the development of *in situ* forming hydrogels for drug delivery applications. *In situ* gels have the properties of linear polymer solutions outside of the body, allowing for easy injection/administration. But they gel *in situ* within the body, resulting in prolonged drug release patterns. To accomplish *in situ* gelation, both physical and chemical crosslinking techniques have been used. Hydrogel precursor solutions can be injected and then polymerized *in situ* using intelligent design of monomers/macromers with desired functionalities. The surgery and implantation technique can be completed with minimum of invasiveness thanks to the *in situ* sol-gel transition.[7]

Choline salicylate (ChS), the medication employed in this study, is an analgesic. By acting locally on oral mucosal cells, it reduces pain severity.[8] ChS gel, which is commercially available, gives pain relief but only for a short time since it can be washed away from the site by salivation and tongue movement; accidental engulfing causes adverse effects such as stomach ulcers and increased blood concentration. This is required to examine the formulation that enhances the drug residence time and availability at the application location. Borax is a homeopathic medication with antibacterial properties that has been used to cure mouth ulcers since ancient times. It also keeps the oral mucosa dry, allowing the mouth ulcer to heal more quickly. As a result, it can be used for both to treat mouth ulcers and as a preservative to the formulation.[9]

An attempt was made to develop a thermo-reversible *in situ* gel containing ChS and borax to treat mouth ulcers, to evaluate the formulation for various parameters, and to investigate the effect of the formulation on residence time, gelling temperature, and polymer mucoadhesive properties. Poloxamer 407 and carbopol P 934 were employed as polymers. Poloxamer 407 acts as a gelling agent and is temperature sensitive, while carbopol P 934 is a pH sensitive mucoadhesive polymer.[10]

Objective

The main goal of this research is to develop and evaluate a thermoreversible *in situ* gel for treating mouth ulcers to find the best formula for improving patient compliance.

MATERIALS AND METHODS

This study certify that the project title "Development and evaluation of *in situ* gel formation for treatment of mouth ulcer" has been approved by IAEC at Appasaheb Birnale College of Pharmacy, Sangli (reference no: IAEC/ABCP/13/2015-16) issued on 07-11-2015.

ChS solution BP was obtained from Shreenath Chemicals Bhoisar, Mumbai. Poloxamer 407 (PF127) purchased from Sahyadri chemicals, Islampur, Maharashtra and carbopol 934P was provided as a gift samples by Corel Pharma Chem Ahmadabad. Borax was obtained from Raj Chemicals, Mumbai and sodium hydroxide, methanol, ferric chloride, hydrochloric acid, acetic acid was obtained from S.D Fine-chem limited, Mumbai. All other materials used were of analytical grade.

Instruments required for the work

Franz-diffusion cell (SFDC6 model, manufactured by Logan); ultraviolet (UV)-visible double beam spectrophotometer (manufactured by Jasco, Japan); fourier transform infrared spectroscopy (FTIR-410 model, manufactured by Jasco, Japan), stability chamber (Tempo instruments PVT, Ltd.), and electronic balance, AUX 220 Model (Shimadzu, Japan) were used as the instruments.

Software required for research work

Design Expert Software (Star Ease, Inc.) was used for research work.

Analytical UV-visible method development and validation

A simple UV-visible spectroscopic method was developed for ChS by following the procedure given below.

Preparation of stock solution I

Since the ChS solution BP contains 50% of ChS, 2 mL (1000 mg) of ChS solution BP was mixed in 100 mL phosphate buffered saline (PBS) of pH 6.8 to get 10 mg/mL. Further diluted to get 100 µg/mL concentration of drug.

1 mL, 2 mL, 3 mL, 4 mL, and 5 mL aliquots were withdrawn from stock solution I (100 µg/mL) and diluted up to 10 mL with PBS 0.6 pH in 10 mL volumetric flasks in order to get 10 µg/mL, 20 µg/mL, 30 µg/mL, 40 µg/mL, and 50 µg/mL concentrations of the drug. The absorbance was measured at 238 nm using PBS of pH 6.8 as the blank.

The method was validated using various parameters as *per* International Council for Harmonisation (ICH) guidelines such as accuracy, precision, limit of quantification (LOQ), limit of detection (LOD), and % relative standard deviation (RSD).

Formulation of in situ gel

Preparation and optimization of thermo-reversible PF 127 aqueous solution[11, 12]

The gel was prepared using the cold technique. Poloxamer concentrations ranging from 10% to 20% (*w/v*) were generated by dissolving the polymer in distilled water at temperatures below 5 ˚C in 50 mL. To guarantee complete polymer disintegration, the solutions were stored in refrigerator for 24 h. Temperature of gelation was then determined by visually inspecting each concentration. In a water bath, a beaker holding 20 mL of cold poloxamer solution was stored. A magnetic bead was placed in the beaker and a calibrated thermometer was hung in the beaker so that the tip of the thermometer was in the solution, but it did not touch the beaker's floor and did not disturb the magnetic bead's spin. The system was agitated at 100 rpm with the help of a magnetic stirrer, while temperature was allowed to rise at a rate of 2 ˚C/min. Temperature of gelation was measured, when the magnetic bead stopped rotating due to the production of gel. Concentrations that gelled close to body temperature (35-37 ˚C) were chosen for further optimization with other components.

Optimization of other ingredients with PF 127 concentration

The effect of other ingredients on the gelling temperature of poloxamer solution was studied.

Effect of carbopol 934P on gelling temperature

Carbopol 934P was prepared in various concentrations ranging from 0.1 to 0.5% (*w/v*). For this, a weighed amount of polymer was combined with a little amount of water and allowed to swell overnight. With the use of magnetic stirrer, these concentrations and poloxamer solution were mixed together and the gelation temperature was recorded.

a. Effect of other ingredients on gelation temperature of solution poloxamer 407 and carbopol 934P mixture: The weighed quantity of drug and other ingredients were mixed in the solution containing poloxamer 407 and carbopol 934P. Changes in gelation temperature were noted down.

b. Formulation of batches based on design of experiment: Depending on gelation temperature at or near the body temperature, concentrations were optimized and the experiment was designed by 2^2 factorial design.

Selection of independent variables

Gelation temperature of *in situ* gel at body temperature depends upon concentration of both polymers. Thus, independent variables of both polymers were selected based on gelation temperature and mucoadhesive properties and coded low level as -1 and high level +1 (Table 1).

Experiment design 2^2 full factorial design

Table 1. Coded values for levels of factors

Formulations / Variables	F1	F2	F3	F4
X1	+1	-1	-1	+1
X2	+1	-1	+1	-1

Evaluation of formulation

Prepared batches of formulation were evaluated for the following parameters:

Appearance: The prepared gel was visually inspected under light against white and black background for its clarity.

pH of the gel: Digital glass electrode pHmeter was used to measure pH of the gel by placing the electrode directly into the gel.[13]

Gelation temperature: In a water bath, a beaker holding 20 mL of the formulation's cold solution form was preserved. A magnetic bead was placed in the beaker and a calibrated thermometer was hung in the beaker so that the tip of the thermometer was in the solution, but it did not touch the beaker's floor and did not disturb the magnetic bead's spin. Temperature was allowed to rise at a rate of 2 ˚C/min, while the systems were agitated at 100 rpm. Temperatures of gelation were measured at the point where magnetic bead ceased to rotate due to the formation of gel.[14-18]

Thermoreversible study: Using a constant temperature bath, thermoreversible investigation was conducted. *In situ* gel compositions were kept in a temperature bath at constant temperature. The instrument was adjusted at a temperature of 4-5 ˚C. Temperature was allowed to rise at a rate of 2 ˚C *per* minute and a shift from sol to gel phase was observed as well as changes in viscosity as point rose to the gelling temperature.[16-18]

Similarly, the temperature was allowed to decline until the gel transformed into a sol and the viscosity was recorded as a function of temperature.

Viscosity of all prepared formulations was measured using a Brookfield viscometer (Brookfield viscometer RTV) with spindle no: 62 at the speed of 10 rpm. The rheological properties were also studied by measuring viscosity of all formulations at speeds of 10, 50 and 100 rpm with spindle no: 62.

Shear rate (sec^{-1}) was calculated using the following formula:

Shear rate (sec^{1}) = $2\omega \times R_c^2 R_b^2 \div X^2 \times [Rc^2 - R_b^2]$

Where,

R_c = Radius of the container (in centimeters)

ω = Angular velocity of the spindle (Rad/Sec)

R_b= Radius of the spindle (in centimeters)

ω = $2 \div 60 \times N$

X = Radius at which shear rate is to be calculated (normally the same value as R_b; in centimeters)

N = Spindle speed in RPM

Observed values:

R_c=1.5 cm; R_b = 1.25 cm

Shear stress (dynes/cm²) was calculated using the following formula:

Shear stress = Shear rate (sec^{-1}) ÷ Viscosity (cps)

Drug content

Percentage ChS BP content was determined by dissolving 0.5 g of the gel in 100 mL of pH 6.8 PBS and scanning the resultant solution with UV-visible spectrophotometer set to 238 nm. Calibration curve was used to calculate the drug content.[12,17,18]

Determination of mucoadhesive force

The mucoadhesive force was determined according to Desai and Shirsand[20] description (2018). The assembly, which involved two glass vials, was completed in-house. One is hung in a downward position, while the other is placed on the floor in an upward position. The upper vial is fastened to one end of the thread and a pan is tied to the other end of the thread.[14,18]

A piece of goat buccal tissue was glued to both glass vials with the mucosal side facing out. Before performing the test, these vials were kept at 37 °C for 10-15 min. On the lower vial, around 1 g of gel was applied before the upper vial was inserted and 1 g of weight was added to the pan. The weight was gradually increased until the two vials were still connected. The mucoadhesive force (gm) was calculated using the smallest weights that could separate the two vials. The bioadhesive force was determined using the equation below.

Bioadhesive force = Bioadhesive strength x 9.81/100

In vitro drug release study

Franz diffusion cell was used to conduct an *in vitro* drug (ChS BP) release study of an *in situ* gel. In the donor compartment, 1 mL of formulation (F3) (equal to 1 g of gel) was deposited, and in the receptor compartment, freshly produced PBS (pH 6.8) was poured. A cellophane membrane was fitted between the chambers. One cell as blank was filled with only filled PBS solution. The units were then placed on a magnetic stirrer with thermostat. The medium was maintained at a constant temperature of 37 °C ± 0.5. After each 1 h interval, 1 mL of sample was withdrawn and same amount of PBS solution from blank was transferred into the sample cell for maintaining sink condition. Then, withdrawal amount was diluted to 10 mL in PBS pH 6.8, and concentration of ChS BP was measured using a UV-visible spectrophotometer at 238 nm with PBS pH 6.8 as a blank. The calibration curve was plotted and used to determine the percent cumulative ChS BP release. The best fit model was tested for Korsmeyers, Peppas, and Fickinian diffusion mechanism for their kinetics.[15,18]

Drug diffusion kinetic study

In vitro release data of the formulations was evaluated kinetically to determine drug kinetics. Microsoft Excel 2013 was used to fit the models. The models of zero order, first order, Higuchi, and Korsemeyer Peppas were investigated. Model with best fit was chosen because of its comparatively high correlation coefficient value.[18]

Statistical optimization of in situ gel formulation

Gelatin temperature, viscosity of gel, diffusion of drug at 1 h, and time required for 90% drug diffusion are major variables for performance of the prepared *in situ* gel formulation. Formation of gel at oral temperature is fundamental to the prepared *in situ* gel. Drug release from gel is indirectly proportional to viscosity of the gel. Thus, viscosity of gel is a major variable to consider during design of *in situ* gel formulations. Salivation in the oral cavity restricts sustained release of gel formulations since gel may wash out with saliva. Thus, drug release at 1 h and the time required for 90% drug release must be considered. Both factors help to decide dosing frequency of the formulation. For statistical optimization of *in situ* gel, following criteria for selection of a suitable feasible region were decided (Table 2).

Antimicrobial test

An antimicrobial study was conducted to assess the medication borax antibacterial activity and to determine whether the formulation had enough antimicrobial properties. The test was conducted using the well diffusion method against Gram-positive (*Escherichia coli*) and Gram-negative bacteria (*Staphylococcus aureus*).

5% (*w/v*) of Mac Conkey's agar for *E. coli* and 11.1% (*w/v*) mannitol agar for *S. aureus* was prepared and sterilized. The liquid was then put into sterile glass plate and allowed to set. The bacterial strains were dispersed aseptically over agar after solidification. Each agar plate had three wells; one for the test (F3), one for the standard (ZYTEE), and one for the plane borax solution. The samples were placed in the wells and kept in the refrigerator for 15-20 minutes to allow the materials to diffuse into agar. The plates were then incubated in an incubator at 37 °C for 24 h. Zone of inhibition was assessed after incubation period.[13,15,16]

Animal model study

The study indicated how the produced formulation affected the healing of an oral ulcer in rats. In this study, 15 healthy female Wistar albino rats (weighed 130-150 g) were chosen and separated into three groups, each with five animals. Before anaesthesia, a 5 mm diameter filter paper soaked in 50% acetic acid was placed on the tongue of rats for 60 s to form a circular ulcer. The test group received an optimized formulation (F3), the standard group received ZYTEE gel (a commercialized ChS product), while the control group received no treatment. For 7 to 10 days, the ulcer healing progress was examined.[19-21]

Table 2. Desirable values of dependent variables for optimization

Sr. no.	Response variable	Desired value
1	Gelatin temperature (Y1)	37 °C
2	Viscosity	<1000 cps
3	Diffusion at 1 h (Y3)	40%
4	Time required for 90% drug diffusion	4 hrs

RESULTS

Analytical UV-visible method development and validation

λ_{max} of ChS in PBS 6.8 was found to be 238 nm. The drug follows linearity in the concentration range 10-50 g/mL with a correlation coefficient value of 0.9903. (Table 3). The accuracy of the method was checked by recovery experiments performed at three different levels, *i.e.* 80%, 100%, and 120%. Percentage recovery was found to be in the range of 98.54-99.98%. The low values of %RSD indicate accuracy and reproducibility of the method. Precision of the method was studied as intraday, interday variations, and repeatability. %RSD value <2 indicates that the method is precise (Table 3). Ruggedness of the proposed method was studied with the help of two analysts.

Formulation of in situ gel

Preparation and optimization of thermo-reversible PF 127 aqueous solution: The solution of poloxamer 407 with concentration of 10% *w/v* to 20% *w/v* was prepared in distilled water. Gelation temperatures of the solutions were found as depicted in Table 4.

Concentrations of 15% (*w/v*) to 20% (*w/v*) were considered as optimum for formulation.

Optimization of other ingredients with PF 127 concentration

Effect of carbopol 934P on gelling temperature: The optimum poloxamer concentration solutions were mixed with 0.1% (*w/v*) carbopol solution and gelling temperatures were observed as shown in Table 5.

It was observed that there was an increase in gelling temperature on addition of carbopol 934P. Thus, concentration of poloxamer was increased to form the gel near body temperature. Gelation temperatures were observed as given in Table 6.

Effect of other ingredients gelation temperature of solution poloxamer and carbopol 934P mixture: Other ingredients such as drug ChS (8%), borax (1%), and propylene glycol were added to poloxamer 407 and carbopol 943P solutions and gelling temperature were observed (Table 7), where there was no significant difference upon the addition of other ingredients.

The formulation of batches based on the design of experiment

Different formulation batches F1 to F4 were prepared based on the design of experiment by 2^2 factorial design (Table 7).

Selection of independent variables (Tables 8, 9)

Evaluation of formulation

Appearance: In both solution and gel forms, all the formulations were determined to be clear and transparent. A clear translucent gel created on a mouth ulcer will increase patient compliance because it mimics natural oral mucosa, allowing for daytime application.

Table 3. Results for analytical UV-visible method development and validation

mcg/mL	Observation			Average	SD ±	%RSD	LOD mcg	LOQ mcg
	1	2	3					
10	0.1692	0.1752	0.1632	0.1692	0.006	3.546099	0.112692	0.341491
20	0.3838	0.3888	0.3788	0.3838	0.005	1.302762	0.09391	0.284576
30	0.4951	0.4971	0.4931	0.4951	0.002	0.403959	0.037564	0.11383
40	0.7089	0.7129	0.7049	0.7089	0.004	0.564254	0.075128	0.227661
50	0.8343	0.8457	0.8229	0.8343	0.0114	1.366415	0.214115	0.648833

SD: Standard deviation, LOD: Limit of detection, LOQ: Limit of quantification, UV: Ultraviolet

Table 4. Gelation temperature of poloxamer 407

Concentration of poloxamer 407 (% *w/v*)	Gelation temperature (°C)
11	46
12	42
13	39
14	38
15	37
16	35
17	34
18	30
19	28
20	25

Table 5. Gelation temperature of poloxamer 407 and carbopol 934P mixture

Concentration of poloxamer 407 (% *w/v*)	Concentration of carbopol 934P (% *w/v*)	Gelling temperature (°C)
15	0.1	41.4
16	0.1	41
17	0.1	40.5
18	0.1	39.1
19	0.1	38
20	0.1	37.5

pH of the gel: pH of all formulations was found to be between 5.5 and 6.8 (Table 10). To avoid irritation of the mucosa and further damage to the ulcer, pH of the formulation produced to treat mucus ulcers must be close to neutral. In general, any formulation utilized for the mucosa should have a pH of 4.5 to 7.

Gelation temperature: Temperature at which the formulation's solution form transforms entirely into semisolid form is known as the gelation temperature. The gelling temperature is the most important requirement for *in situ* gel formulation. At close to body temperature, *in situ* gel formulation for the oral ulcer should quickly change from sol to gel (37 ˚C 5 ˚C), and the resulting gel should not erode or dissolve. The gelling temperature of the produced mixture was determined to be between 34 and 38 ˚C (Table 10).

The gelling temperature and integrity, on the other hand, are mostly determined by the polymer content. At 38 ˚C, formulation F2 formed the weakest gel, whereas formulation F1 generated a strong gel at 35 ˚C. It could be because F2 formulation had lower concentration of both polymers, while the F3 formulation had larger concentration of both polymers.

Because of the observed gelling temperature, it can be concluded that concentration of poloxamer 407 had a proportional effect

Table 6. Gelation temperature of 407 and 934P mixture

Conc. of carbopol 943P (% *w/v*) → / Conc. of poloxamer 407 (% *w/v*) ↓	0.1	0.4	0.6
20	37.2 ˚C	37.8 ˚C	40.3 ˚C
21	35.2 ˚C	36.5 ˚C	39.7 ˚C
22	34.7 ˚C	35.8 ˚C	38.7 ˚C
23	34.1 ˚C	34.9 ˚C	37.8 ˚C
24	32.9 ˚C	32.8 ˚C	33.6 ˚C
25	30 ˚C	31.3 ˚C	32.5 ˚C

Table 7. Gelling temperature of the mixture of ChS, borax, Carbopol 934P, and poloxamer 407 at different concentrations

Ingredients	Concentration (% *w/v*)							
Poloxamer 407	20	21	22	23	20	21	22	23
Carbopol 934P	0.1	0.1	0.1	0.1	0.4	0.4	0.4	0.4
Choline salicylate	8	8	8	8	8	8	8	8
Borax	1	1	1	1	1	1	1	1
Gelation temperature (˚C)	**36.5**	**35.5**	**35**	**33.9**	**37.5**	**37**	**35**	**34.6**

Table 8. Selected independent variables

Level	Variable	X1 (concentration of poloxamer 407)	X2 (concentration of carbopol 934P)
Low	-1	20	0.1
High	+1	23	0.4

Table 9. Composition of *in situ* gel formulation as *per* coded values in experiment design 2^2 full factorial design

Sr. no.	Formulation / Ingredients	F1 % *w/v*	F2 % *w/v*	F3 % *w/v*	F4 % *w/v*
1	Poloxamer 407	23	20	23	20
2	Carbopol 934P	0.1	0.1	0.4	0.4
3	ChS BP	8	8	8	8
4	Borax	1	1	1	1

ChS: Choline salicylate

on gelling temperature, whereas the gelling temperature increased, when the carbopol 943P was added and it is also directly proportional to the carbopol 934P concentration.

Thermoreversible study: In the same way that an increase in temperature causes the sol to gel phase transition in *in situ* gel formulation, a decrease in temperature causes the gel to sol phase transition. The procedure is the polar opposite of sol-gel mechanism. As the temperature rises, the micelles generated at CMC come into touch with one another, resulting in polymerization and thus gel formation. As the temperature drops, micelle pack and micelle entanglement diminish, and the network breaks down. The formulation's gel form begins to transform into a solution form and at a certain point the gel is totally transformed into a solution. Temperature difference between gel and sol is known as gel to sol temperature.

The gelation phenomenon will be aided by a mechanism based on micelle packing and entanglements as well as conformational changes in the orientation of the methyl group in the side chain of the poly (oxy propylene) polymer chain constituting the micelle's core and the expulsion of the hydrating water from the micelle.

It was discovered from the phase diagram in Figure 1 that, when the polymer concentration increased, the gelation temperature decreased, while the sol temperature increased.

In comparison to previous formulations, formulation F1 comprises a larger concentration of polymers resulting in lower gelation and solution temperatures. Similarly, formulation F2 has the lowest polymer concentration, thus it takes more heat to create a gel; but it converts to a sol form fast and at high temperatures, when compared to other formulations.

As can be seen from the phase diagram (Figure 2), the smallest concentration of the polymer has the highest gelation

Table 10. Observations of various evaluation tests

Batch	Appearance	pH	Gelling temperature (°C)	Viscosity (cps)	% drug content	Bioadhesive strength (gm)	Mucoadhesive force (gm)
F1	Clear	5.8 ± 0.05	35 ± 0.2	936.9 ± 7.76	100 ± 1.2	10	0.981
F2	Clear	6.2 ± 0.05	38 ± 0.2	936.9 ± 7.76	99.01 ± 0.9	6	0.588
F3	Clear	5.5 ± 0.05	37 ± 0.1	627.5 ± 6.7	99.86 ± 0.9	18	1.765
F4	Clear	6.8 ± 0.05	36 ± 0.3	443.36 ± 6.84	98.75 ± 0.6	20	1.962

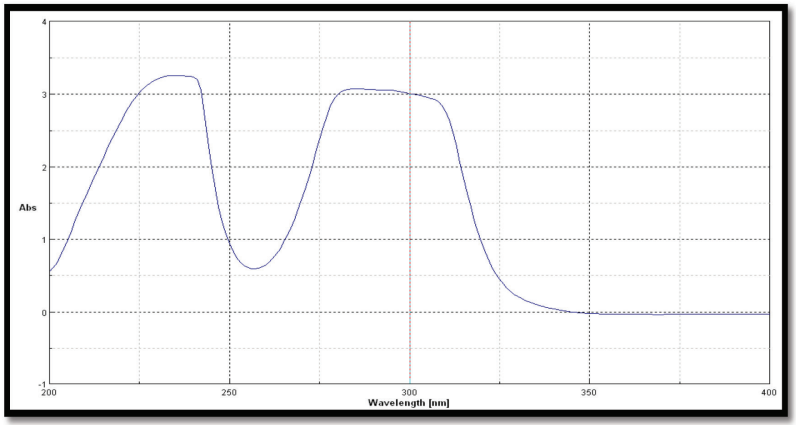

Figure 1. UV spectra of ChS BP
UV: Ultraviolet, ChS: Choline salicylate

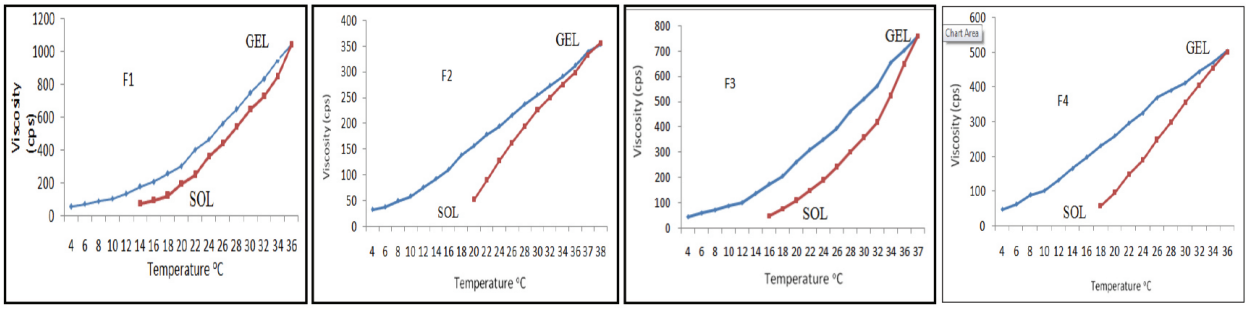

Figure 2. Thermoreversible gel to sol phase diagram of prepared *in situ* gel formulations

temperature and low sol temperature. The micelle created from the smallest amount of polymer was unstable and breaking the hydrogen bond formed during temperature aggregation needed the least amount of energy. The energy required to break the bond is provided by external heat.

Viscosity and rheological properties: This is one of the most significant requirements for *in situ* gel formulation. To remain for a long time at the site of application, *in situ* gel formulation should have a viscosity of more than 100 cps, when it is applied and less than 1000 cps and when it converts to the gel after administration.

Viscosity of all formulations F1, F2, F3, and F4 was found to be polymer concentration dependent. Viscosity increased in the order F1>F3>F4>F2 as the concentrations of polymers poloxamer 407 and carbopol 934P increased. Table 10 provides viscosity (centipoises) of the prepared formulations, and Figures 3a and 3b display the shear rate (sec) and shear stress (dyne/cm^2) of all batches.

It was discovered that viscosity varied depending on the shearing rate. In other words, the ratio of shear stress to the shear rate was not constant, and viscosity dropped as the shear rate increased. As a result, the prepared *in situ* gel was found to be a non-Newtonian fluid. As the shear rate increased, viscosity of the gel dropped. This demonstrated that *in situ* gel was shear thinning pseudoplastic by nature.

Drug content: As stated in Table 10, percent ChS BP of all formulations was determined to be in the range of 98 to 100%. It is possible that discrepancy in medication content is attributable to human mistake during dilution or to production loss during the formulation preparation.

Determination of mucoadhesive force: Mucoadhesion is an interfacial phenomenon that involves two materials, one of which is the mucus layer of mucosal tissue, to which the medication is held together for a long time by interfacial forces. The longer the retention duration, the stronger the mucoadhesive force.

Various studies have shown that the presence of polyoxyethylene groups in poloxamer 407 is responsible for their mucoadhesion *via* H-bonding, but, when it forms gel, the cross linkage between poloxamer 407 increases rendering the polyoxyethylene groups unavailable for mucoadhesion. According to the diffusion interlocking hypothesis, when crosslink density rises, chain mobility falls, and therefore the effective chain length that may penetrate the mucus layer falls, lowering mucoadhesive strength. Thus, addition of carbopol 934P leads to an increase in mucoadhesion. Carbopol is a synthetic mucoadhesive agent. It adheres to the mucosa by a -COOH bond. Formulations F3 and F4 contain higher concentrations of carbopol and indicate strong bioadhesion as compared to other formulations (Table 10).

In vitro diffusion study: An *in vitro* diffusion study was conducted using Franz diffusion cell with pore size of 40 µm and cellophane membrane. In Figure 4, the percentage cumulative ChS BP diffusion obtained from all formulations is displayed. Formulation F2 had the fastest diffusion compared to the other formulations, while formulation FI had the slowest diffusion from the gel. In the case of F2, 90% of the drug was diffused up to 3.5 hours; however, in the case of F1, only 80% of the drug was diffused by 5th hour. It could be because F2 had lower concentration of both polymers, while F1 had higher concentration of both polymers.

In general, the drug diffusion rate reduces as the crosslinking of the polymer in the formulation, such as gel, increases. Based on the findings, it can be concluded that as the polymer concentration grew, the drug diffusion rate decreased. The diffusion of drugs is thus a polymer concentration-dependent process. An *in situ* gel that exhibits 40% drug release after 1 hour and 90% drug release after 4 h was tempted to prepare. F1 formulation was not determined to be optimum (Figure 4).

Diffusion kinetic study: According to data from diffusion studies, the generated *in situ* gel had significant initial drug release (burst effect) and then decreased as gelation progressed. This is a biphasic pattern, which is a common feature of matrix diffusion kinetics. As the concentration of polymer grew, the first burst effect decreased as in the case of F1, which contains high concentrations of both polymers (Table 11).

Korsmeyer-Peppas model is commonly used to confirm the drug release process from the matrix. The "n" value (Korsmeyer-Peppas model release exponential) was used to characterize the various release mechanisms in the following way:

n<0.5 Quasi Fickian diffusion

n-0.5: Diffusion mechanism

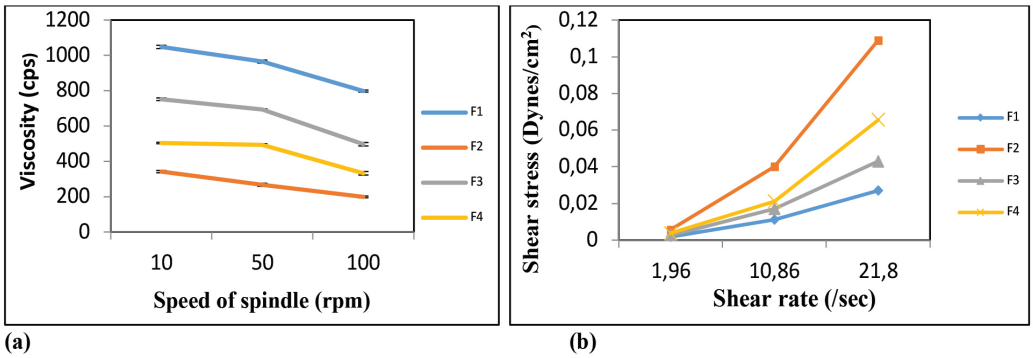

Figure 3. (a) Viscosity (cps) v/s speed of spindle (rpm) graph, (b) shear stress v/s shear rate graph showing non-Newtonian fluid

0.5<n>1 Anomalous non-Fickian diffusion (both diffusion and erosion)

n: 1 case 2 transport (zero order)

n >1 supercase 2 transport relaxation

For each formulation, a graph of log CDR *v/s* log was plotted to determine the diffusion mechanism of the created *in situ* gel according to Korsmeyer-Peppas model. For all formulations, the correlation of co-efficients of all straight lines was determined to be in the range of 0.954 to 0.992.

The n value was recorded for all formulations and utilized to modify the diffusion mechanism from formulations. Since n values of 0.7 and 0.57 were reported, formulations F1 and F4 follow an atypical non-Fickian diffusion mechanism. Due to n: 0.43 and 0.48, respectively, F2 and F3 followed a quasi-Fickian diffusion mechanism (Table 11).

The dissolution data for Higuchi model was investigated to see, if the drug release was diffusion regulated or not. For all formulations, a graph of percentage CDR *vs.* square root of time was drawn. All straight line correlation coefficients were determined in the range of 0.943 to 0.996. As a result, all formulations followed Higuchi's diffusion model (Table 11).

Statistical optimization of in situ gel formulation: Primary process parameter analyses revealed that components such as poloxamer 407 (X1) and carbopol 934P (X2) had a substantial impact on gelation temperature, viscosity, and drug diffusion as well as the time required for 90% drug diffusion. As a result, these two variables were used in subsequent statistical optimization research. For all four formulation batches, all dependent variables revealed several data.

Software stat ease: Design Expert 10 was used to derive conclusions based on the amount of the coefficient and the mathematical sign (positive or negative) they carried.

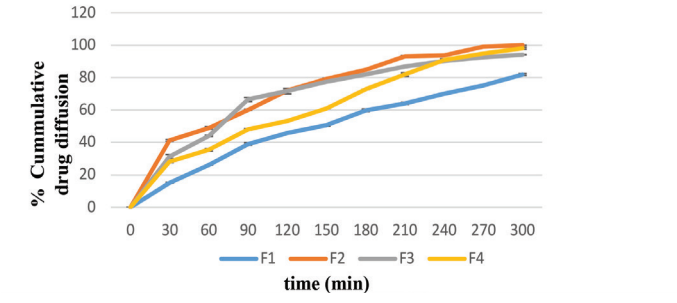

Figure 4. *In vitro* drug diffusion study of prepared *in situ* gel formulations

Optimization of polymer concentrations for gelation temperature: Concerning Y1 (gelation temperature) the data clearly indicated that it is strongly dependent on the selected variables X1 and X2

YI 36.56-0.98 X1-49X2 + 0.042 X1X2

The findings of multiple linear analysis revealed that both coefficients B1 (-0.98) and 3 (-0.49) had a negative sign, indicating that, when individual concentrations of poloxamer 407 or carbopol 934 increase, the gelation temperature decreases. Combination of two polymers, on the other hand, had positive effect on gelation temperature and micellar aggregation. Only when the concentration of poloxamer 407 exceeds the micellar concentration, resulting in the micelle production, gel phase can occur. The hydrophobic sections of the pluronic are kept apart by hydrogen bonding between the POP chains and water, when the material is immersed in cold water. Hydrogen bonding is broken as the temperature is elevated and hydrophobic interactions cause a gel to form. Carbopol 934P was added in escalating quantities to lower the gelation temperature even more. As the concentration of mucoadhesive polymers (carbopol 934P) increased, gelation temperature decreased. It is probable that the ability of mucoadhesive polymers to reduce gelation temperature is linked to increased viscosity the following polymer disintegration and the ability of mucoadhesive polymers to adhere to polyoxyethylene. Chains contained in poloxamer 407 molecules could explain their capacity to lower gelation temperature. This would encourage dehydration resulting in increased entanglement of neighboring molecules and increased intermolecular hydrogen bonding, lowering the gelation temperature. When bioadhesive agents and poloxamer 407 were combined, the effect on gelation temperature revealed that adding carbopol 934P increased micelle packing and tangling, resulting in a drop in gelation temperature. Using a response surface, the relationship between formulation variables (X and X2) and Y1 was further clarified. Figure 5c displays the effects of X1 and X2 on Y. The gelation temperature was reduced as the amount of poloxamer 407 and carbopol 934P was increased (Table 12).

Optimization of polymer concentrations for viscosity: According to the dependent results of multiple linear regression analysis, viscosity is strongly dependent on X_1 and X_2. The fitted equation for the full model relating viscosity to selected factors can be explained by the following polynomial equation:

$Y2\ 661.33 + 114.41X_1 + 238.39X_2 + 33.51\ X_1X_2$

The results revealed that both X_1 and X_2 have positive coefficients. Because of rising X_1 and X_2 values, viscosity is projected to rise. Both elements have favourable effect on

Table 11. Results of drug diffusion a kinetic study

Formulation	Zero order	The first order	Higuchi	Korsmeyer-Peppas	n
F1	0.996	0.966	0.996	0.992	0.7
F2	0.882	0.882	0.989	0.986	0.43
F3	0.83	0.865	0.943	0.943	0.48
F4	0.96	0.96	0.981	0.982	0.57

viscosity, when used separately and in combination. The fact that X_2 has a higher coefficient value than X shows that X_2 is more effective in terms of viscosity than X_1. Surface plot Figure 5d can be used to explain the relationship between selected parameters and response viscosity (Table 12).

Optimization of polymer concentrations for drug diffusion at 1 h: The data clearly indicated that drug diffusion values at 1 h are substantially reliant on the specified independent variables, namely poloxamer 407 concentration and carbopol 934P concentration. Transformed factor is related to the response (release at 1 hour) by the fitted equation (for full model).

$$Y_1 +39.15 - 7.89\ X_1 -3.83\ X_2 -1.12\ X_1X_2$$

Coefficients 1 and 2 for the prediction of release at 1 h were found to be significant at p=0.05. Coefficients 1 (-7.89) and 2 (-3.83) have a negative sign according to the results of multiple linear regression analysis. It appears that increasing the amount of poloxamer 407 or carbopol 934P in the formulation reduces the release levels after one hour. Coefficient of poloxamer 407 is larger than that of carbopol 934P, indicating that poloxamer 407 is more effective than carbopol 934p in terms of 1 h release (Table 12). Using a response surface plot (Figure 5a), the link between formulation variables poloxamer 407 (X_1) and carbopol 934P (X_2) was further explored.

Optimization of polymer concentrations for the time required for 90% drug diffusion: In the case of Y2, the result of multiple regression analysis showed that the coefficient diffusion (+45) and P_2 (+40) bear positive signs. The positive sign of both X_1 and X_2 coefficients indicates that as concentration of both poloxamer 407 and carbopol 934P increased the time required for 90% drug diffusion increased. Summary of regression analysis can be explained by the following polynomial equation:

$$Y4 = 265 +45X_1 +40X_2+10X_1\ X_2$$

Y2 exhibited a good correlation coefficient of 1.000 for all batches F1 to F4. XI had a p value of 0.0001 and X2 had a p

Table 12. Results of experimental design batches of variables

Formulation code	Diffusion at 1 hY1 (%)	Time required for 90% drug diffusion Y2 (hrs)	Gelation temperature Y3 (°C)	Viscosity Y4 (cps)
F1	26.01	6	34	1042
F2	49.01	3.5	38	342.1
F3	44.1	4	37	751.5
F4	35.42	4	35	503.8

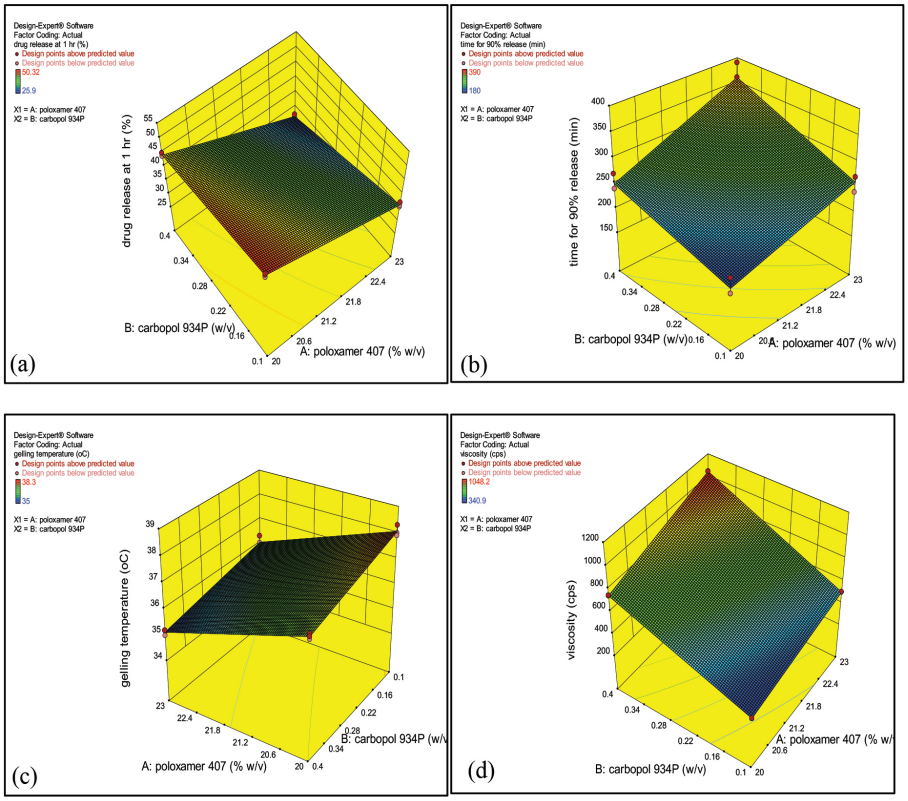

Figure 5. Response surface plot of optimization of polymer concentrations for (a), drug diffusion at 1 hr (b), time required for 90% drug diffusion (c), gelation temperature (°C) (d) viscosity (cps)

value of 0.0001. Both *p* values were less than 0.05, indicating that the independent factors have substantial impact on the time necessary for 90% drug diffusion. The time required for 90 % drug diffusion increased as the concentrations of poloxamer 407 and carbopol 934P rose (Table 12). It could be attributed to an increase in cross-linkage because of higher polymer concentrations resulting in lower drug diffusion from *in situ* gels polymeric network.

The relationship between formulation variables, *i.e.* poloxamer 407 (X_1) and carbopol 934P (X_2), was further elucidated using the response surface plot Figure 5b.

Analysis of variance

The R^2 values for gelation temperature (Y), viscosity (Y2), CPR at 1 h (Y1), and time required for 90% drug release (Y) are 0.9822, 1.000, 0.9959, and 0.9255, respectively, suggesting that dependent and independent variables are well correlated.

Antimicrobial test

Antimicrobial medicines are also used to treat mouth ulcers; these inhibit microbial growth on the ulcer, allowing it to heal more quickly. Borax has antibacterial, antifungal, and antiallergic properties. As a result, borax can be used as both an antiulcer and a preservative. Zone of inhibition obtained by improved formulation (F3) in sol form, conventional ZYTEE gel, and glycerol-borax as shown in Figure 6 and Table 13 can act on both Gram-positive (*E. coli*) and Gram-negative bacteria (*S. aureus*).

There is a negligible difference between zones of inhibition of the standard and the formulation in gel form, which shows that the formulation has preservative properties similar to those of the standard.

Animal model study

In most cases, an oral ulcer heals on its own within 7 to 10 days. The formulations produced to treat mouth ulcers speed up the healing process, requiring less time than natural healing, and reducing the pain associated with ulcers. As a result, the patient's comfort with an oral ulcer will improve.

Wistar albino rats were used as an animal model in this investigation. In comparison to conventional ChS gel (ZYTEE), the effect of a developed formulation (F3) on the healing of an oral ulcer in rats. Ulcer healing properties of the formulation were found to be comparable to those of the reference (Figure 7). The observation was made based on the ulcer's every day ocular observations.

Within 5 days, all animals in the test group that were given the formulation were free of ulcers. Similarly, all animals in the standard-treated group were cured on the fifth day after therapy began. However, on the fifth day, three out of five animals in the control group, *i.e.* those who were not treated, developed an ulcer, and it took them eight days to completely recover. As a result, the developed formulation of *in situ* gel containing ChS is effective for treating mouth ulcers.

CONCLUSION

Using the thermoreversible polymer poloxamer 407 and the mucoadhesive polymer carbopol 934P, a thermoreversible *in situ* gel containing ChS and borax for the treatment of mouth ulcers was successfully created.

It has been determined through compatibility studies that medications and polymers are compatible. When poloxamer 407 was used at a concentration of 14 to 16% (*w/v*), the gelling temperature was close to the body temperature (35-38 °C), however, when carbopol 934P was added, the

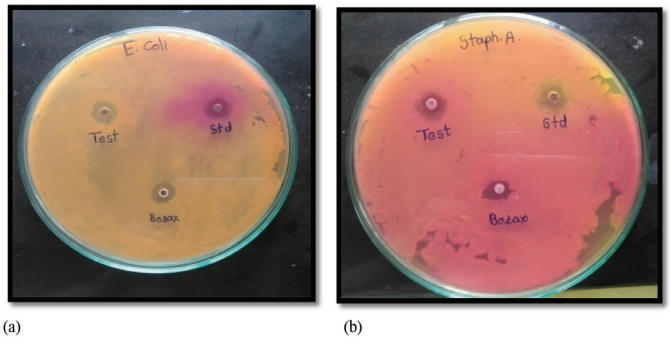

Figure 6. Zones of inhibition of prepared *in situ* gel formation batch F3 (sol form) (a) *Escherichia coli* and (b) *Staphylococcus aureus*

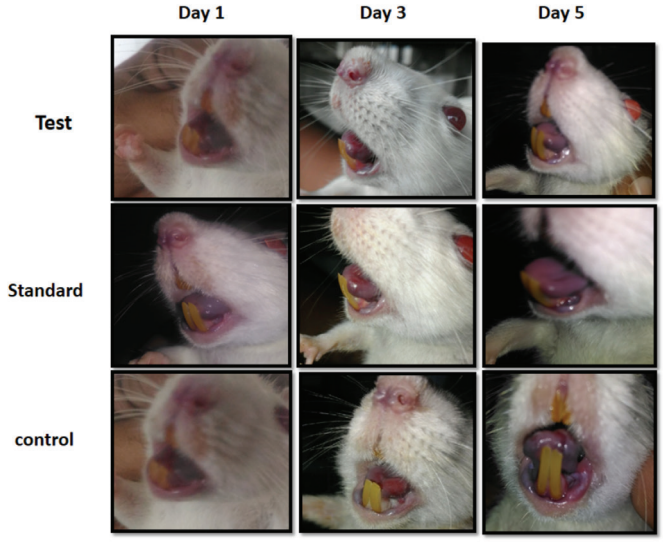

Figure 7. Animal model study

Table 13. Zone of inhibition (mm) shown by prepared formulation

Microorganisms	Formulation	Standard	Glycero-borax
Escherichia coli	22 mm	25 mm	14 mm
Staphylococcus aureus	25 mm	28 mm	17 mm

gelling temperature was raised. Carbopol may cause micelle aggregation, size, and entanglement to decrease, resulting in an increase in gelation temperature. Addition of ChS and borax to the gelation temperature had no effect. The *in situ* gel was thus created based on the gelation temperature, pH, thermoreversibility, viscosity, mucoadhesion study, drug content, *in vitro* drug diffusion, drug diffusion kinetics, statistical formulation optimization, antimicrobial, and animal model study of optimized formulations were all examined.

Thermoreversibility of the formulations was discovered. All formulations had pH between 5.5 and 6.8, which is considered a safe range for mucosal drug delivery. All formulations had viscosities of less than 1000 cps, allowing simple administration of the formulation to a mouth ulcer. Rheological tests revealed that the *in situ* gel had a non-Newtonian flow and was a shear-thinning pseudo-plastic. It is thought to be a beneficial characteristic for *in situ* gel. Content homogeneity of all the formulations was excellent. The insignificant discrepancy between them could be attributable to human error or loss of output. Mucoadhesion was good in all formulations. The formulations F3 and F4 with higher carbopol concentrations have better mucoadhesive properties than the other formulations F1 and F2. According to *in vitro* drug diffusion research, F4 had the lowest diffusion rate and F2 had the greatest. It can be argued that, when viscosity rises, drug diffusion decreases, and the concentration of both polymers is proportional to viscosity. Higuchi model of drug diffusion was seen in all formulations. Formulations Fl and F4 revealed non-Fickian diffusion mechanisms, while F2 and F3 showed quasi-Fickian diffusion mechanisms according to Korsmeyer-Peppas model. The formulation including 0.4% (*w/v*) carbopol 934P and 20% (*w/v*) poloxamer 407 *i.e.* F3 was found to be the most desirable. Antimicrobial testing of the improved sol formulation of F3 revealed a satisfactory zone of inhibition for Gram-negative and Gram-positive microorganisms. As a result, the formulation can be concluded to have good preservation properties. Nevertheless, it revealed a smaller zone of inhibition in gel form, implying that borax diffusion is reduced in gel phase of the formulation. It has antibacterial properties and can be used to treat mouth ulcers. In animal model research, formulation F3 was found to be as effective as standard (ZYTEE) in the healing of mouth ulcers. The formulation was found to be stable under accelerated temperature and humidity conditions in stability investigation.

As a result, a correctly developed *in situ* gel for oral ulcers can extend the duration spent at the application site and minimize the frequency of administration.

Future prospects

In situ gelling systems have garnered a lot of interest in the past decade. *In situ* gel meets the key requirement of a successful controlled release product, increasing patient compliance. The steady and prolonged release of drug from *in situ* gel and its good stability and biocompatibility make it a very reliable dosage form. The use of mucoadhesive compounds and polymers that can both gel *in situ* and interface with mucosa and/or mucus improves formulation performance even more. This system gels at the place of action, when given as a solution. Finally, *in situ* treatments are simple to use and reduce the size, pain, and the colour of lesions. However, more research on its stability and storage conditions statements must be carried out. The above successfully researched formulation looks forward to developing an *in situ* gel spray form for ease of administration in the oral cavity.

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Ethics

Ethics Committee Approval: Ethical approval of this study was obtained from the Institutional Animal Ethics Committee, Appasaheb Birnale College of Pharmacy, Sangli, Maharashtra, India, (reference no: IAEC/ABCP/13/2015-16).

Informed Consent: Animal experiment.

Peer-review: Externally peer-reviewed.

Authorship Contributions

Surgical and Medical Practices: N.H.G., Concept: N.H.G., Design: N.H.G., Data Collection or Processing: N.H.G., Analysis or Interpretation: N.H.G., P.S.H., Literature Search: N.H.G., P.S.H., Writing: N.H.G., P.S.H.

Conflict of Interest: No conflict of interest was declared by the authors.

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REVIEW

A Systematic Review of Healthcare Professionals' Knowledge, Attitudes, and Practices Regarding Adverse Drug Reaction Reporting in Ethiopia

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ABSTRACT

Adverse drug reactions (ADRs) are a prominent cause of morbidity and mortality and higher healthcare expenditures. Healthcare professionals (HCPs) play a crucial role in ADR reporting through spontaneous reporting systems, but under-reporting is their major limitation. The goal of this study is to evaluate HCPs' knowledge, attitude, and practice regarding ADR reporting as well as the factors that influence reporting using research papers that are currently available. A literature search was conducted using sources such as PubMed, Scopus, and Google Scholar to find studies that evaluated HCPs' knowledge, attitudes, and practices regarding ADRs reporting in Ethiopia. A standard procedure of systematic review protocol was used to conduct this review. Demographic factors, sample size, response rate, survey delivery, HCP working setting, and encouraging and discouraging factors of ADR reporting were extracted from articles. A total of 17 articles were included in the systematic review out of 384. The number of HCPs in the included studies ranged from 62 to 708. Response rate ranges from 76.1% to 100%. Most of the research included in this evaluation looked at HCPs, who worked in hospitals. When pharmacists were compared to other HCPs, they were more likely to report ADRs; because they had higher knowledge, attitude, and practice. Lack of understanding, unavailability of reporting forms, uncertainty about the causal relationship between the drug and ADR, and failure to report because the ADR was well known were among the common hurdles to ADR reporting identified in research. To improve reporting, educational initiatives and continued training in pharmacovigilance and ADRs are frequently recommended considerations. In Ethiopia, there is a pressing need to close the gap in HCP knowledge, attitudes, and practice regarding PV and ADR reporting. To address this point, specific educational interventions based on existing gaps in ADR reporting should be developed and integrated into the health education curriculum or provided as in-service training after graduation.

Key words: Adverse drug reactions, knowledge, attitudes, practice, healthcare professionals, reporting, pharmacovigilance

INTRODUCTION

Adverse drug reactions (ADRs) are one of the most common drug-related issues, and they are a considerable cause of illness and death as well as a significant economic burden.[1] ADRs increase the risk of hospitalization, emergency department visits,[2] and length of hospitalization[3] ADRs are monitored using a variety of ways, the most prominent of which is voluntary or spontaneous reporting, which is considered the cornerstone of any pharmacovigilance (PV) system.[3] Reporting of suspected ADRs determines, whether a PV system succeeds or fails.[4]

In the ADR reporting and PV systems, healthcare practitioners play a critical role.[5] ADRs can be reported directly to national PV systems or to pharmaceutical manufacturers by both healthcare professionals (HCPs) and patients.[6] The early detection of signals and dangers related to drug usage is improved by reporting ADRs to the appropriate regulatory body.[7]

Despite widespread worries about drug safety, HCPs still lack understanding of PV and ADR reporting.[8,9] Furthermore, according to recent studies, ADRs are underreported by HCPs, particularly in poorer nations. Only 2-4% of all adverse events and 10% of significant ADRs are reported globally according to reports.[10] Any suspected adverse reaction, specifically those suspected reactions to newly authorized drugs and significant occurrences, should be reported by HCPs such as physicians,

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pharmacists, and nurses.[11] As a result, medicine safety evaluation must be considered an integral element of HCPs' daily clinical practice.[5]

In Ethiopia, a variety of cross-sectional studies have been conducted to assess HCPs' knowledge, attitude, and practice regarding ADR reporting as well as the causes of underreporting by HCPs. To our knowledge, no comprehensive literature review has examined available studies that evaluated HCPs' knowledge, attitude, and practice of ADR reporting. This study evaluates HCPs' knowledge, attitudes, and practices and identifies characteristics that encourage or discourage them from reporting ADRs in Ethiopia.

METHODS

Literature search strategy

To identify published articles that meet the objectives of this systematic review, a literature search was conducted using the databases PubMed, Scopus, and Google Scholar. A review was conducted to verify that the literature was thoroughly covered and that current performance on HCP ADR reporting in Ethiopia was considered. The preferred reporting items for systematic reviews and meta-analyses (PRISMA) procedures were followed for conducting this literature review'.[10] "Adverse reactions" and "drug-related side effects" OR "adverse drug event" OR "adverse drug reaction" OR "drug side effects" OR "drug toxicity" OR "side effects of drugs" OR "toxicity, drug" OR "medication side effect" OR "Ethiopia" were combined search terms to identify eligible articles.

The search was performed on April 2020 with no limitations on study design or publication year (Figure 1). The publication year of the article was not imposed on the search. The articles were chosen based on their titles and abstracts. A manual search was also carried out; significant article reference lists identified throughout the screening process were manually searched to find other qualified studies that had not been discovered previously. To complement the information, an internet search was undertaken using Google Scholar and the generic search engine Google. Through the literature search in electronic scientific databases, the same terms were employed.

Study selection and data abstraction

The literature search comprised all articles were conducted in Ethiopia until February 2020. The authors screened the titles and abstracts of the studies that were identified and evaluated them according to the inclusion and exclusion criteria. The whole text of the selected abstracts was then evaluated. The reviewers revised the all studies that were potentially eligible, and two of the authors agreed on the final inclusion.

Inclusion criteria

Studies were chosen, if they focused on HCPs' knowledge, attitudes, and practices regarding ADR reporting and PV, and they were conducted in Ethiopia. Both electronic grey literature articles searches and published articles in scientific peer-reviewed journal articles were included in systematic review.

Exclusion criteria

Studies on ADR data analysis, patient or consumer reports, medication errors, general adverse drug events, and prevalence and nature of ADRs in hospitals were excluded.

Extraction and assessment of data

A standardized data collection tool was used to perform data abstraction. Author, year of publication, study objective, study period, study population, HCP work setting, number of respondents and percent response rate, survey/study delivery

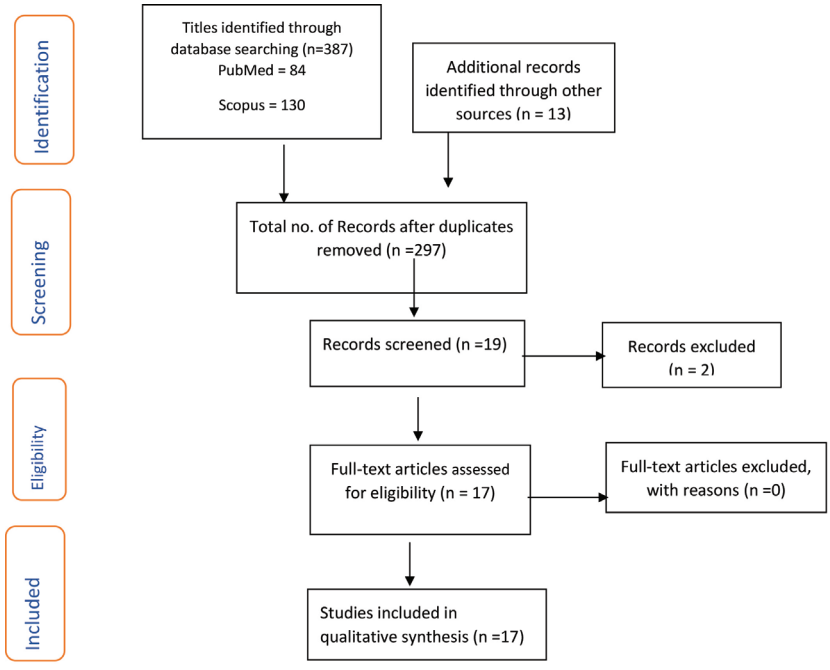

Figure 1. The preferred reporting items for systematic reviews and meta-analyses procedures were followed for conducting this literature review

(mail, face-to-face, self-administrative, e-mail/web), scale or type of questions used (yes or no questions, multiple-choice questions, Likert scale, and open-ended questions), encouraging and discouraging factors of ADR are all characteristics extracted from each eligible study. In the tables, we have included factors that were statistically significant in the research.

RESULTS

Description of eligible study articles included in review

A total of 387 articles was collected from the scientific databases PubMed (n: 84), Scopus (n: 137), and Google Scholar (n: 166) for qualitative analysis. Following the removal of duplicate citations, 297 publications were subjected to title and abstract screening with 17 being chosen for full-text evaluation and eventual inclusion in the systematic review.

A total of 17 articles included in the systematic review was studies using self-administered questionnaires conducted among HCPs in Ethiopia and published between 2012 and 2020 (February). Only one of these was a mixed-method study, in which key informants completed a semi-structured questionnaire and self-administered questionnaires were used.[12] The rest of the studies conducted a cross-sectional design. Six studies were conducted in Amhara Region,[13-19] 4 in the Oromia Region,[20-23] 4 studies in the capital city Addis Ababa,[12,23,24] and one study each was conducted in Tigray Region,[25,26] Harare Region.[27,28] The sample size for the comprised studies ranged from 62[14] to 708[17] HCPs from across databases searched for this study.

Response rate ranges from 76.1%[27] to 100%.[20,21,29-31] Majority of the studies considered in this review surveyed hospital-based HCPs (physicians, pharmacy personnel, nurses, midwives, and health officers) working in public hospital settings. One study involved pharmacists, who worked in community settings.[25] Only nurses working in hospital settings were surveyed in one study.[13] Figure 1 shows a PRISMA flowchart indicating the study selection at each stage. The study characteristics and outcomes are detailed in Tables 1 and 2.

Demographic factors that impact ADR reporting by HCPs

According to one study, gender has a strong relationship with ADR reporting practice, with female physicians are 3.5 times more likely to report ADRs than male physicians.[12] However, another study found no link between different age groups and the likelihood of HCPs reporting ADR.[17] The practice of reporting ADRs is strongly linked to one's educational level. Compared to general practitioners, specialists are 5 times more likely to disclose ADR cases.[12]

In two previous reports, it was discovered that having more job experience as HCP increased the number of reports.[12,27] When compared to physicians with one to three years of experience, those with more than six years of experience were 4.6 times more likely to report ADR instances.[12] HCPs with 10 to 14 years of experience (84.6%) substantially acknowledged that they are aware of the national ADR reporting system and that they are aware of ADR reporting form's accessibility.[27] In addition, one study found that HCPs with less experience were more likely to record ADRs incorrectly.[26] Another research, on the other hand, revealed no statistically significant link between years of service and ADR reporting.[17]

According to studies, having ADR reporting training has a statistically significant relationship with knowledge,[14] and having a high degree of knowledge is associated with ADR reporting.[17] When compared to participants who had received ADR reporting training, professionals who had not received ADR reporting training were 0.722 times (72.2%) less likely to have adequate knowledge. Furthermore, healthcare personnel who had not been trained in ADR reporting were more likely to have poor practice[26] and knowledge.[15]

Comparing ADR reporting among different professions

Several studies indicated that all HCPs have inadequate understanding and habits regarding ADR reporting. Some research, on the other hand, demonstrated statistically significant differences among HCPs.[29] According to studies, physicians have a better likelihood of diagnosing ADRs than other health providers because they either lack confidence in diagnosis or play fewer responsibilities in the ward for intervention.[20,30] Physicians see much more patients with ADR than pharmacists and nurses, according to similar findings.[19]

Gurmesa and Dedefo[23] evaluated the knowledge of HCPs among themselves, finding that physicians (84.2%) and pharmacists (84.2%) were more educated about ADR reporting than health officers (56%) and nurses (25.7%). According to another study, nurses, health officials, and physicians were 93.1% less likely than pharmacy professionals to have an adequate knowledge of ADR reporting.[14] Nurses and health officers had an insufficient degree of understanding of ADR reporting, when compared with pharmacists, according to another finding.[15] According to a survey conducted in Addis Ababa, 72.1% of pharmacists were aware of the yellow card reporting mechanism, while just 40.5% of nurses were.[11] According to a study in South West Ethiopia, pharmacists have significantly more knowledge than other HCPs about the difference between ADRs and side effects, the term PV, the accessibility of a national reporting system, and the availability of an ADR reporting form.[19,27] Two studies comparing the attitudes of HCPs revealed that pharmacists (89.5%) have a positive attitude about ADR reporting, followed by medical doctors (89.5%, 73.6%). Nurses had the worst attitude with only 20% having a positive attitude.[21,27]

However, when compared with physicians and nurses, pharmacists had the least awareness of ADR reporting, according to another study. It also revealed that pharmacists lacked knowledge of how to report ADRs and the types of ADRs that should be reported.[32] Another study, on the other hand, reported no link between respondents' professions and their knowledge and attitudes about ADR reporting.[30]

HCPs knowledge of ADR report

Several studies found that HCPs' awareness of ADR reporting is low, although few respondents were aware of or could define ADR and PV. According to a survey conducted in the

Table 1. Summary of the articles used in this systematic review for data analysis and synthesis

No	Author	Study period	Objective of study	Study population	Study design	HCP work settings	Town/region
1	Adimasu[12]	March-April, 2013	To evaluate indicators of nurse knowledge related to ADR reporting at Felege Hiwot Referral Hospital and University of Gondar Teaching Hospital	Nurses	Cross-sectional	Hospital	Gondar/ Amhara
2	Angamo et al.[14]	January, 2010	To survey the knowledge, attitude and practices of ADR reporting among HCPs in selected health facilities in southwest Ethiopia	Physicians, pharmacy, nurses	Cross-sectional	Hospital and health centers	Jimma zone/ Oromia
3	Kassa Alemu and Biru[19]	May 1-30, 2019	To evaluate knowledge, attitude and practice of HCPs about ADR reporting and the associated factors at selected public hospitals in North East Ethiopia	Nurses, doctors, pharmacy, midwives, and health officers	Cross-sectional	Hospital	Amhara
4	Shanko and Abdela[18]	February-March, 2015	Assessment of baseline knowledge, attitudes and practices of HCPs working in HFSUH	Nurses, physicians, pharmacists	Cross-sectional	Hospital	Harar/Harar Region
5	Teshome et al.[21]	March 3-25, 2016	To analyze knowledge, attitude and practice of HCPs toward ADRs reporting at inpatient wards	HCPs at the inpatient wards of TASH	Cross-sectional	Hospital	Addis Ababa
6	Bule et al.[22]	March-June, 2014	To evaluated the knowledge, attitude and practices of ADR reporting among HCPs in Adama Hospital Medical College	Nurses, doctors, and pharmacists	Cross-sectional	Hospital	Adama/ Oromia
7	Belete et al.[27]	March-June, 2014	To measure the knowledge, attitude and practice of HCPs toward ADR reporting in Boru Meda Hospital	Nurses, doctors, pharmacy, midwifery, and health officers	Cross-sectional	Hospital	North East Ethiopia/ Amhara
8	Seid et al.[26]	March-May, 2017	To evaluate the knowledge, attitudes and practices of HCPs toward ADR reporting	Nurses, health officers, pharmacy	Cross-sectional	Health centers	Gondar/ Amhara
9	Hailu et al.[30]	March-July, 2013	To determine the knowledge, attitude and practices of HCPs regarding (ADR) reporting in Northwest Ethiopia	Doctors, nurses, and pharmacists	Cross-sectional	Hospital	Gondar/ Amhara
10	Nadew et al.[11]	October-December, 2017	To evaluate ADR reporting practices and associated factors among doctors in government hospitals in Addis Ababa	Doctors working in selected governmental hospitals	Cross-sectional mixed-methods study design	Hospitals	Addis Ababa
11	Denekew[16]	September-October, 2013	To evaluate the knowledge, attitude, and practice of ADR reporting and factors affecting reporting among HCPs working in ART clinics of public health facilities of Addis Ababa	Healthcare providers working in ART clinics	Cross-sectional	ART clinics of public health facilities	Addis Ababa

Table 1. Continued

No	Author	Study period	Objective of study	Study population	Study design	HCP work settings	Town/region
12	Gidey et al.[25]	January-March, 2019	To evaluate the knowledge, attitude and practice of ADR reporting and identify associated factors with ADR reporting among HCPs	Nurses, pharmacists, physicians	Cross-sectional	Hospital	Tigray Region
13	Gurmesa and Dedefo[23]	January-June, 2015	To evaluate the knowledge, attitude and practice of HCPs working in Nekemte town toward ADR reporting	Doctors, nurses, health officers, pharmacists	Cross-sectional	Health service centers	Nekemte/ Oromia
14	Mulatu and Worku[20]	May-November, 2012	To evaluate the knowledge, attitude and practice of HCPs toward ADR reporting and associated factors with reporting	Doctors, nurses, pharmacists	Cross-sectional	Hospitals	Amhara
15	Goshime[17]		To evaluate the knowledge, attitude, and practices on ADR reporting among community pharmacists in Addis Ababa	Community pharmacies	Cross-sectional	Community pharmacies	Adds Ababa
16	Tariku and Eshetu Mulisa[15]	January 24-February 7, 2014	To define the status of knowledge, practices, and attitudes toward ADR reporting among HCPs in Nekemte Hospital	Physicians, pharmacists, health officers, nurses, and anesthesiologist	Cross-sectional	Hospital	Nekemte/ Oromia
17	Abay and Dires[13]	May, 2007	To assess the practice of ADR reporting and obstacles of reporting in Gondar University Teaching and Bahirdar Felege Hiwot Referral Hospitals	Physicians, nurses, and pharmacists	Cross-sectional	Hospital	Gondar/ Amhara

ADR: Adverse drug reactions, HCPs: Healthcare professionals, HFSUH: Hiwot Fana Specialized University Hospital, ART: Antiretroviral therapy, TASH: Tikur Anbessa Specilized Hospital

Tigrai Region, 29.3% of respondents did not know the accurate definition of adverse reactions and only 36.8% knew what to report.[26] According to a research conducted in Nekemte, 80%, 66.1%, 45.2%, and 48.7% of health professionals do not understand the difference between ADR and side effects, the word PV, the national ADR reporting system or the existence of an ADR reporting form.[22] In a similar survey conducted in southwest Ethiopia, 79% and 80% of professionals, respectively, did not comprehend the difference between ADR and side effects, nor the phrase PV.[19]

The term PV and its purpose were grasped by 20.2% of HCPs in a survey conducted in North East Ethiopia.[14] Similar findings were that 36.8% knew the term PV.[30] Another survey of health centers in Gondar town found that only 12.7% recognized what the term PV meant and could appropriately define it.[15] In contrast, most HCPs (76.9%,[16] 70.2%[31]) could tell the difference between ADR and side effects.

According to a study conducted on physicians in Addis Ababa, 30.2% had never heard of the ADR reporting system, 49.6% had never heard of national guidelines, and 71.3% had no idea how to submit ADR instances to the appropriate organization.[12]

According to research conducted in Addis Ababa's antiretroviral therapy (ART) clinics, 46.2% were aware of the presence of a national PV center, but only 39.3% knew, where it was located.[25]

According to a research conducted by Teshome et al.,[21] in Addis Ababa, 49.8% of respondents were aware of the responsible entity to whom ADR should be reported. Similar findings were found in Amhara region, 57.1%;[17] in North East Ethiopia, 21.1%;[14] in Gondar, 49%;[15] in West Ethiopia, 24%;[21] in South West Ethiopia, 46.34%;[19] and in Eastern Ethiopia, 61.36%.[27] Regarding the yellow card approach for ADR reporting, 37.4% of HCPs were aware of its existence.[21] 51.8% chose yellow card for ADR reporting, according to similar studies.[14] The yellow card reporting mechanism for reporting ADRs was known to be 57.3%.[23]

Other studies indicated 63.2% and 59.6%,[30] 58.5% and 47.7%[20] recognized the availability of national reporting system and ADR reporting form in Ethiopia, respectively. According to research conducted in the Tigray Region, 39.4% were aware of National Reporting Center and just 31.9% knew where to report.[26]

When it comes to the types of ADRs that should be reported, 69.2% of HCPs believe that all suspected ADRs should be

Table 2. The characteristics of studies that were considered in the systematic review for ADR reporting knowledge, attitude, and practice among Ethiopian HCPs

No	Authors	Study period	Survey delivery method	Sample size (response rate)	Type of questions (scale) used	Measured outcomes (findings)
1	(11)	October-December, 2017	Self-administered questionnaire and key person interview	422 (96%)	MCQ for knowledge and Likert scales for attitude, open-ended interview questionnaire	Knowledge, attitude, practice
2	(17)	April-June, 2014	Self-administered questionnaire	422 (89.9%)	MCQ, likert scale questions	Knowledge, attitude, practice
3	(20)	May-November, 2012	Self-administered questionnaire	708 (88.3%)	Yes/no, Likert scale questions	Knowledge, attitude, practice
4	(23)	January-June, 2015	Self-administered questionnaire	133 (100%)	Yes/no, likert scale questions	Knowledge, attitude, practice
5	(19)	May, 2019	Self-administered questionnaire	120 (95%)	Yes/no, likert scale questions	Knowledge, attitude, practice
6	(19)	March-June, 2014	Self-administered questionnaire	62 (92%)	Yes/no, likert scale questions	Knowledge, attitude, practice
7	(25)	January-March, 2019	Self-administered questionnaire	362 (84.8%)	MCQ, likert scale questions, yes/no	Knowledge, attitude, practice
8	(26)	March-May 2017	Self-administered questionnaires	102 (100%)	Yes/no, MCQs and Likert scale	Knowledge, attitude, practice
9	(14)	January, 2010	Self-administered questionnaires	82 (100%)	Yes/no and Likert scale	Knowledge, attitude, practice
10	(16)	September-October, 2013	Self-administered questionnaires	251 (93.22%)	Yes/no, MCQs and Likert scale	Knowledge, attitude, practice
11	(21)	March, 2016	Self-administered questionnaire	280 (76.1%)	Yes/no and Likert scale	Knowledge, attitude, practice
12	(22)	March-June 2014	Self-administered questionnaire	130 (100%)	Yes/no, MCQs and Likert scale	Knowledge, attitude, practice
13	(18)	February to March, 2015	Self-administered questionnaire	325 (91.4%)	Yes/no, MCQs and Likert scale	Knowledge, attitude, practice
14	(30)	March to July, 2013	Self-administered questionnaire	156(96.1%)	Yes/no, MCQs and Likert scale	Knowledge, attitude, practice
15	(12)	March to April, 2013	Self-administered questionnaire	214 (100%)	Yes/no, MCQs and Likert scale	Knowledge, attitude, practice
16	(15)	January 24-February 7, 2014	Self-administered questionnaire	150 (76.6%)	Yes/no, MCQs and Likert scale	Knowledge, attitude, practice
17	(13)	May, 2007	Self-administered questionnaire	141 (60.8%)	Open and close end questions	Knowledge, attitude, practice

ADR: Adverse drug reactions, HCPs: Healthcare professionals, MCQ: Multiple-choice questions

reported, whereas 15% (12.8%) believe that only major ADRs should be reported.[12] Similar findings were obtained in North East Ethiopia 80.7% life-threatening and 84.2% disability-causing ADRs should be reported.[30]

HCPs' attitudes toward ADR reporting

Studies revealed that the attitude of HCPs toward ADR reporting is positive. The participants agreed that ADR reporting benefits public health, that one report can make a difference and that filling out the ADR yellow form is helpful. They also agreed that ADR reporting should be mandatory. ADR reporting is the responsibility of all health practitioners, according to 95.3% of doctors in Addis Ababa.[11] Other studies in North East Ethiopia 87.7%,[14] 93.0%,[30] in Tigray Region 67.4%,[26] in Southwest Ethiopia 57.31%,[19] in Addis Ababa 84%[21] in East Shoa zone 85.4%,[20] in Harar 60.68%[26] in Addis Ababa 92.7%,[23] and in Nekemte 97.43%.[22]

Based on a survey from North East Ethiopia, majority of the health care professionals strongly agreed that ADR reporting is compulsory (76.3%).[14] Similar findings obtained in Nekemte town (57.9%,[21] 70.1%,[30] 37.8%,[26]) and in Gondar (82%).[32]

According to a survey conducted in the East Shoa zone, 93.8% of HCPs believed that ADRs should be reported on a frequent basis.[20] Similar findings in North East Ethiopia 77.2%,[14] Tikur Anbessa Specialized Hospital 87.3%[23] in Addis Ababa 88.9%,[22] reporting at health center level in Gondar 79.4%,[15] Eastern Ethiopia, Harare 73.9%,[27] Nekemte 78.3%.[22]

Majority of studies also agree that monitoring an ADR is vital for the public (93.6%), the health care system (94.9%), and patient care quality (84.6%).[23] According to a survey conducted in Addis Ababa, 90.1%, 85.5%, and 92.5% of HCPs agree that ADR reporting is beneficial for patients, the public, and the healthcare system, respectively.[25] In a survey in the east Shoa zone 94.7% and 88.6% of respondents agreed that reporting ADR is important for the public and improves the quality of patient care, respectively.[20] A similar result was found in southwest Ethiopia, where 71.95%, 70.73%, and 73.17% agreed that reporting ADR is important for the public, health care system, and patient care, respectively.[19] According to a survey conducted in Harare, 83.4% of HCPs believe that reporting medication safety is critical for the public, and 73.2% believe that reporting ADR is critical for the health care system.[27] According to a survey conducted in Gondar, 96.7% of respondents believe that ADR reporting is beneficial to public health.[32] A similar survey in Nekemte town found that 90.4%, 96.5%, and 98.2% of interviewees stated that ADR monitoring is important for the public, the patient, and the health care system, respectively.[22]

On the other hand, over 77% of HCPs believe that before reporting an ADR, it is necessary to confirm that it is related to the medicine.[27] Also studies with similar findings are conducted in Jimma (85.4%),[19] in Addis Ababa (76.9%),[23] East Shoa zone (76.3%),[20] in Gondar (83.3%),[15] and in Easter Ethiopia (67.8%).[27] 73.7% stated that one ADR report makes a difference.[14] One ADR report can make a difference according to 82.0% of respondents.[32] On the other hand, 57.31% and 56.10% disagreed that one ADR report had no impact and that reporting was irrelevant for the specific patient.[19] About 62.4% disagreed that ADR reporting adds to burdens, while 39.3% were opposed to report only ADR if it causes permanent handicap.[27] ADR reporting is a time-consuming job that produces no results according to 10.5% of health professionals.[30] According to a survey conducted in Tigray, 64.8%[26] believed that reporting adds to their burden, which is more than 32.4% found in the Amhara Region.[15]

The majority of HCPs do not know which form of ADR should be reported regarding the nature of ADR to report, in a survey conducted in Addis Ababa, 35.4% of clinicians disagreed that all suspected ADR instances should be reported.[12] Another survey in Tigray Region found that 51.1% disagreed that only prescribed medications should be reported.[26] Similar results were found in Nekemte by 9.5%.[22] Another survey in West Ethiopia found that 43.6% believe that reporting ADRs is encouraged, when the reaction is serious.[21] Similar findings were obtained in Gondar (44.1%).[15]

HCPs reporting practices for ADRs

According to findings of the studies, HCPs' reporting of ADRs is often poor since many encountered ADRs but did nothing about them. According to a survey conducted in Amhara region, only 38.1% had experience marking ADRs on their clinical records.[17] Similar findings were found in North East Ethiopia, where 29.82% of clinicians experienced at least one patient with ADR in the previous 12 months,[14] only 27% of HCPs in Nekemte town have dealt with ADR patients,[21] only 21.1% of doctors in North East Ethiopia seen patients with ADR in the previous 12 months.[30] Only 15.85% of clinicians in South West Ethiopia had to deal with ADR throughout their work,[19] in Eastern Ethiopia 49.2% encountered ADR in the past 12 months of their clinical practice[27] "in Gondar only 28.6% claim to have reported an ADR to a reporting center at least once".[32] In two studies conducted in Addis Ababa, 43.2%[22] and 38.5%[27] of HCPs said they had seen at least one patient with ADR in the previous year.

In the Tigray Region, however, 74.9% of clinicians experienced ADR in the previous 12 months of practice,[26] survey conducted in Gondar, 55.9% of respondents had encountered at least one patient with ADRs during their job experience,[15] 64.6% of those in the East Shoa zone said they had encountered ADR in their clinical practice.[20] A study of physicians in Addis Ababa found that 84.3% of physicians experienced ADR cases during their professional careers with 87.2% of physicians recording the cases in the patients' medical records.[12]

In most studies on HCPs who have encountered ADRs from their clients, a small number of ADRs have reported. According to a survey conducted in Addis Ababa, only 27.4% of them have reported ADR situations to authorized agencies during their professional careers.[12] Similar results were found in Amhara region 27.7%,[17] in Tigray Region 32.1%,[26] in Gondar 49.1%,[26] a survey in South West Ethiopia among interviewed HCPs none of them reported *via* yellow card to responsible body,[31] in Addis Ababa 10.8%,[27] in East Shoa zone 29.2%,[20] and in North west Ethiopia 28.6%.[32] In contrast, few studies reported that a large number of respondents ever reported ADR. Based on a study in North East Ethiopia, 50% of respondents reported ADRs,[14] another study in North East Ethiopia 83.3%,[30] in Harar 60.68%.[27]

On the other hand, 27.7% of HCPs who reported ADR did so to Food, Medicine and Healthcare Administration and Control Authority, the government agency in charge of monitoring and analyzing ADR in the country.[17] Similar results were found in Addis Ababa 39.36%,[12] in North East Ethiopia 29.41%,[14] and Nekemte 14.3%.[21]

In contrast, a study in East Shoa zone indicated that 67.7% of respondents reported to have never reported ADRs to any of the responsible bodies,[20] similar findings were found in northwest Ethiopia; 46% of respondents who had never reported any ADRs to any reporting centers.[32]

Encouraging and discouraging factors that influence ADR report

Encouraging factors

Accessibility of ADR information sheets at outpatient departments by 80.7%, encouraging all health professionals to report by 75.4%, training to report ADR by 72.8%, encouraging patients to report by 66.7%, drug information center assistance by 66.7%, and easy accessibility to ADR forms by 59.6% were all suggested by Kassa Alemu and Biru[19] as ways to improve ADR reporting. In west Ethiopia, awareness creation on what, when, and to whom to report ADRs accounted for 42.1%, with in-service training accounting for 26.3%.[21]

Discouraging factors

In a survey conducted in North Eastern Ethiopia, respondents agreed that there is a lack of feedback by 58.8%, reporting forms are not available, when needed by 46.4%, not knowing where to report by 46.4%, not knowing how to fill and report the report form by 41.2%, other colleagues are not reporting ADR cases by 37.7%, and it is unclear whether there is a causal link between the drug and ADR by 35.9%.[19] According to a study from eastern Ethiopia, the causes for under-reporting were inaccessibility of the reporting form (53.9%), ambiguity of how to report (51.9%), and lack of feedback from the responsible entity (41%).[26] In a research in West Ethiopia, under-reporting of ADRs was due to a lack of awareness and information about what, when, and to whom to report them (30.8%), and a lack of commitment from HCPs (25.5%).[21,22] Another survey found that the reasons for not reporting were the need to be certain of how to report ADR (52.9%), the unavailability of ADR reporting forms (51%), and the lack of feedback (47.1%) were all factors for not reporting.[15] According to a survey conducted in Gondar, respondents stated that they were unsure about reporting ADRs (23.2%), that they had not received feedback (18%), that they did not have access to reporting forms (15.3%), and that they did not report since the ADR was quite well (17.3%).[32]

DISCUSSION

A spontaneous ADR reporting system is essential for effectively discovering new ADRs, but it has one main drawback: under-reporting.[8] HCPs are accountable for identifying, recording, and reporting ADR. Their assistance in detecting and reporting ADR at an early stage is crucial.[33] Many factors including lack of awareness, uncertainty about who should report, challenges with reporting procedures, lack of feedback on submitted reports, and rapid resolution of adverse occurrences affect ADR reporting.[34,35] ADR reporting is strongly linked to HCPs rs knowledge and attitudes.[36] To improve reporting processes, it is critical to examine healthcare practitioners' knowledge, attitude, and practice in relation to ADR reporting.[37]

This systematic review focused on health care professionals' knowledge, attitude, and practice regarding ADR reporting as well as the many factors affecting ADR reporting in Ethiopia. The findings of this review study revealed that the primary hurdles to reporting by health care personnel were a lack of understanding of basic concepts linked to PV and ADR reporting process. Majority of research found that health professionals lacked knowledge and experience but had a positive attitude toward reporting ADRs. Most health care professionals suggested giving continuous education or special training courses relevant to PV and the ADR reporting process to improve ADR reporting.

According to studies, there is a link between demographic parameters and professional setting characteristics as well as HCPs reporting ADR. Few studies have described that sex and education have significant associations with ADR reporting practice. Female physicians were 3.5 times more likely to report ADRs than male physicians.[12] This could be because females are more likely than their male counterparts to report ADRs.[38] Furthermore, females may be more aware of PV and ADR reporting.[39] With regard to level of education, similar with a study done in Ghana and Egypt.[40] It is indeed possible that this is due to specialists receiving ADR training and having more expertize in the field. In addition, experts knew more about PV and ADR reporting than general practitioners. This put them in a better position to notify the national PV center about any ADRs they faced.[40]

HCPs with more work experience are more likely to practice, have higher expertise, and have a good attitude toward ADR reporting.[12,26] Similarly, HCPs with less working experience have poor ADR reporting practices.[26] Increased years of experience are linked to greater awareness of the national ADR reporting system's existence.[26] This finding is similar to one found in an Ugandan study, in which more experienced HCPs were four times more likely than less experienced professionals to have ever reported.[41] This could be due to increased exposure to numerous classes of drugs and better understanding of their properties as a result of extensive work experience. Furthermore, experienced HCPs have the opportunity to participate in in-service trainings and other scientific conferences. Years of experience among physicians and other healthcare personnel were similarly linked to knowledge and attitudes concerning PV and ADR reporting according to the study.[41] Only 23% of intern pharmacists and physicians in South Africa recognized how to report ADRs according to a recent survey, yet percentage was familiar with the reporting form having seen it before.[38] Van Hunsel et al.[46] discovered that there is no standard for teaching PV at universities, which could be one cause for this.[42-45] Attending ADR reporting training was found to have a strong relationship with knowledge, and the level of knowledge[14] was linked to ADR reporting.[17,24] In comparison to participants who had received ADR reporting training, HCPs who had not received ADR reporting training were less likely to have adequate knowledge. On the other hand, HCPs who had not undergone ADR reporting training were more likely to have poor practice[26] and knowledge.[15] This finding is consistent with the study by Lewis et al., which included providing training to physicians under reporting rate was 36%.[4] Physicians were provided personalized training on how and why to report suspected ADRs in the study's spontaneous component. However, despite most events being mild to moderate in nature, this low underreporting rate may indicate, in addition to the

effect of training, a greater motivation to report ADRs in this patient. This is similar to a study conducted in Spain which found that participation in educational activities related to the detection and resolution of drug-related problems was linked to ADR reporting.[39] This could be due to the impact of training to increase the grasp of health professionals on the reporting process.

In this review, research revealed statistically significant disparities in knowledge, attitude, and practice regarding ADR reporting among healthcare workers. According to studies, physicians have a better likelihood of diagnosing ADRs than other healthcare providers because they either lack confidence in diagnosing or have less duties on the ward to intervene.[20,30] Similar findings were found with physicians seeing more patients with ADR than pharmacy professionals and nurses.[31] This could be because physicians were older and had more years of experience than pharmacists. Pharmacists, on the other hand, claimed to have a better understanding of PV and ADR than physicians.[40]

When compared with other HCPs professionals (physicians, nurses, and health officers), pharmacists have more knowledge.[14,15,21,27,31] This disparity in knowledge could be due to the nature of pharmacist training, which places a strong emphasis on drugs and their safety.[45] Similar findings were found in a study from South Africa on various degrees of knowledge among different occupations with nurses having the least understanding on how to report.[42] According to the study, an alarming 92% of respondents felt that physicians should be held accountable for reporting. "Who is accountable for adverse drug reporting?" is a question that has to be addressed. Nurses are not fully aware of their role in ADR reporting, according to Van Hunsel et al.[46] found a similar result with 89% of nurses preferring to refer the report to the physicians for completion. Workload, inattention, trust in reporting, and fear of litigation are all possible explanations for low nurse reporting rates according to other studies.[46]

The outcomes of this review article revealed an Ethiopian lack of understanding about PV and drug safety. The figs of the several papers revealed that HCPs' awareness of ADR reporting is minimal, even though a small proportion of them were aware of or could define ADR and PV. Insufficient awareness of PV ideas, methods, and functioning was found in most trials and was identified as a major obstacle to reporting ADRs. Numerous studies[14,15,22,26,30,31] showed that a large number of respondents have limited knowledge on the definition of ADR, the difference between ADR and side effects, the term PV, the national ADR reporting system, and the availability of ADR reporting forms. It was clearly indicated that a portion of health care professionals have limited information or have never heard the existence of a national ADR reporting system, national guidelines and do not know about the PV system and how to report ADR cases to the responsible body.[12,24,26,27] Several HCPs are untrained, which can lead to inadequate knowledge of ADR reporting. This represents an important issue that needs to be addressed; the PV center in Ethiopia should provide training for HCPs. This review showed that only a few HCPs were aware of the existence of an ADR system in Ethiopia. This meant that most of the professionals did not have information about the center responsible for monitoring ADRs in Ethiopia. Similarly, a lack of knowledge about the national ADR reporting system was reported in different regions of the country. This is a critical observation, which is undoubtedly related to the current underreporting of ADRs. This finding is similar to a study in Nigeria, where lack of knowledge of the forms and procedures for reporting is cited as a determinant factor for reporting.[43] Moreover, a systematic review on the determinants of ADR reporting conducted in Spain confirmed that knowledge of health professionals appeared to be strongly related with reporting in a high proportion of studies.[44] A similar study in Spain also indicated that having the basic knowledge needed to report ADR was a determinant factor for ADR reporting.[39] This implied that a certain level of knowledge is required for a health professional to report ADR. Those health professionals with adequate knowledge have a higher chance of understanding the key procedures of reporting such as what to report, where to report, and when to report, which in turn encourages reporting.

HCPs' positive attitudes regarding ADR reporting were discovered to be a critical factor in predicting ADR reporting. Positive attitudes such as the perception that reporting will benefit public health, increase patient safety, and contribute to a better understanding of drug risk, are essential characteristics to consider, when designing interventions aimed at increasing HCP reporting rates. Most study participants believed that reporting is vital for the public, improving patient safety and the health-care system; that one report can make a difference; that filling out the ADR yellow form is helpful; and that ADR reporting should be mandatory.[12,14,16,22,24,28,30,31] This is the same as a study in Sweden, where majority (80.9%) of the HCPs were in opinion that ADR reporting is the duty of physicians, nurses, and pharmacists.[46] This implied that health professionals had appropriately recognized ADR reporting as a professional obligation.

Similarly, HCPs have stated that before reporting an ADR it is necessary to confirm that the ADR is linked to the drug.[15,20,23,26,27,31] In research, a bigger number of respondents was concerned about legal liability during reporting. This indicated that the majority of health professionals working in hospitals across the country are unaware that any reported case cannot be utilized as a source document for legal difficulties as stated clearly in the ADR reporting guideline.

Only a few studies found that respondents agreed that reporting increased their workload.[15,26,27,30] Though it may take some time to fill out the report forms, the percentage of respondents who hold this belief, as revealed by this study, may influence motivation to report adverse responses. HCPs should consider ADR reporting a responsibility and be familiar with current PV systems.

Several surveys identified factors that facilitate ADR reporting to improve PV system training to report ADR, encouraging patients to report, availability of ADR information sheets, encouraging all health professionals to report, and drug information center assistance.[14,21] Many HCPs stated that

they have encountered ADR during their practice; however, a sizable percentage do not report it to the appropriate authorities (regulatory authorities, manufactures, *etc.*).

Different articles, on the other hand, have looked into the reasons for underreporting ADRs. Common reasons indicated were lack of knowledge,[14,21,25] and lack of feedback.[14,15,25,32] Besides reporting forms are not available, when needed 46.4%.[14,15,25,32] Other reasons were that other colleagues are not reporting ADR cases,[14] uncertain that causal association between drug and ADR,[14,15,32] did not report because the ADR was well known (17.3%).[32] This is identical to a similar review conducted in Europe.[8] Similar reasons under ADR reporting were also mentioned in the qualitative section of this study. This implied that, if relevant organizations worked to reduce these barriers, the reporting rate might be improved. Lack of effective feedback mechanisms from the concerned organization through various channels may deter health professionals from reporting ADR. In a few of the studies included in this review, feedback from the PV center with information regarding the reported ADR was identified as a positive element that could improve reporting. Receiving personalized feedback from a PV center was thought to be a major motivator to report an ADR in the future in a study done in the Netherlands.[47] Furthermore, this finding suggests that health practitioners across the country have linked ADR reporting to legal and ethical concerns. This indicated that health professionals' perceptions of various impediments are essential in establishing the causes of underreporting and that removing these impediments could lead to an increase in spontaneous reporting. ADR reporting is hampered by the difficulty of reporting mere suspicions, health professionals, who are encouraged by one-sided drug advertising, and the idea that only safe pharmaceuticals are allowed on the market.

Based on the studies included in this review, HCPs' reporting of ADRs is often poor, as many encounter ADRs but do nothing about them. Responses based on encountered *vs.* reported ADRs were used to assess healthcare practitioners' practices. The disparity between the number of respondents, who encountered an ADR in practice and the proportion, and who reported an ADR was alarming.[12,15,17,20,23,26,32] Few of the health professionals who reported ADR reported it to Ethiopia's PV center, which is in charge of monitoring and analyzing ADR.[12,14,17,21] Low reporting is a big concern among health professionals, according to this article. The fact that most health professionals lacked fundamental comprehension of the reporting system could explain the low reporting rate. Poor feedback and limited reporting options may have an additional impact on reporting. Similarly, despite having strong understanding and awareness of ADRs, Toklu and Soyalan[47] observed a low level of practice by healthcare providers. According to Fadare et al.[48] despite the fact that 80% of respondents experienced an ADR, less than half of them (42.7%) chose not to report it.

Health practitioners with a low degree of knowledge were more likely to record adverse events incorrectly. Many previous studies have found a link between low levels of knowledge among health practitioners and bad ADR reporting practices.[8,41,43] Furthermore, health workers who did not obtain ADR reporting training were more likely to practice poorly. A study conducted in Spain backs this up.[39] Similarly, in a study conducted in Uganda, HCPs also showed poor training in areas of ADR and reporting.[37] As a result, more training is required in terms of identifying ADR, the goal of ADR reporting, and the availability of resources for ADR reporting.

Findings from this review have important implications. Different measures should be developed to improve HCPs' limited knowledge of the ADR and its reporting. Multiple interventions appear to have had more impact than single interventions according to systematic evaluation of efforts to enhance ADRs reporting.[44] Several studies have found that educational interventions such as oral workshops, oral presentations, group discussions, developing ADR newsletters in hospitals, and ongoing training in PV and ADR reporting increased knowledge and attitude scores.[49-51] Incorporating PV-related activities into undergraduate and postgraduate training programs could help improve reporting. In a study conducted in Nigeria and Italy, similar strategies were suggested as a solution to enhance reporting.[52,53]

Other studies have found that offering incentives to health practitioners improves ADR reporting.[54,55] In a study conducted in Spanish that included both financial incentives and educational activities, the average number of ADRs reported increased by up to sixfold.[55] Improved reporting rates were achieved by increasing the accessibility of yellow cards on wards and encouraging the use of web-based reporting. As a result, empowering HCPs in detecting and reporting suspicious drug reactions and employing evidence-based tactics is critical to improve Ethiopian PV systems. This is particularly crucial for less experienced health workers and those, who have never had ADR reporting training. Nonetheless, more research is needed to determine the impact of these interventions on ADR reporting knowledge and practice in our setting.

The main limitation of this review was that data were extracted based on self-reported information and the possibility of reporting errors and recall biases could not be ruled out in studies. The cross-sectional design used in these articles may not be able to prove a causal link between ADR reporting and explanatory variables. Inconsistencies in the study were population of interest, data gathering scales, and methodology. Because some of the questions in the eligible studies were closed-ended and others were open-ended, the outcomes of this evaluation could be influenced by differences in how they were asked. The encouraging and discouraging variables that influence reporting are not necessarily worded exactly as they appear in the articles; slight edits were made to fit them into the final list of factors. These modifications are unlikely to influence the review's principal finding.

CONCLUSION

Knowledge, attitudes, and practice of HCPs toward PV were found to be associated with ADR reporting in this systematic

review. Sex, level of education, years of experience, and profession appear to have an impact on reporting among personal and professional factors. When compared to other HCPs, pharmacists had more expertise, good attitudes, and a higher reporting rate. Involvement of HCPs in maintaining drug safety necessitates a thorough understanding of PV ideas, processes, and functions. ADR reporting is a vital component of ensuring drug safety at the individual and population levels, and HCP participation is critical. To improve ADR reporting by HCPs, it is recommended to design customized curricular interventions based on existing gaps in knowledge and attitudes that can be integrated within the health education curriculum or in-service training after graduation.

Ethics

Peer-review: Externally peer-reviewed.

Authorship Contributions

Concept: Z.G.A., N.A., Design: Z.G.A., N.A., Data Collection or Processing: Z.G.A., N.A., Analysis or Interpretation: Z.G.A., N.A., Literature Search: Z.G.A., N.A., Writing: Z.G.A., N.A.

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