Abstract

Hedera helix L. is traditional herbal medicine used in the treatment of asthma. The main objective of this study was to formulate a microemulsion, a gel, and an ointment containing the ethanol extracts of H. helix L. and to evaluate in vitro and ex vivo permeation. Thin-layer chromatography (TLC) and high-performance liquid chromatography (HPLC) were used to identify and quantify hederacoside C at 210 nm. Evaluation of release and permeation was done by Franz diffusion cells, dialysis cellulose membrane, and natural rabbit skin. Using dialysis cellulose membrane and rabbit skin, various parameters such as permeation coefficient, flux (J), and release kinetics were analyzed with different kinetics models. In the three formulations, release behavior of the microemulsion and gel were similar, but the ointment showed slow release. Maximum amount of drug released from the microemulsion, gel, and ointment formulations through the dialysis membrane was 70.239%, 63.9157%, and 40.9154%, respectively. Release via rabbit skin was 66.051%, 59.5%, and 37.64%, respectively. According to the Korsmeyer-Peppas kinetic model, the microemulsion, gel, and ointment formulations contained 99.10%, 98.23%, and 97.45% of the medicine, respectively. This study demonstrates that in vitro and ex vivo diffusion cell experiments can be utilized to develop formulations of herbal medicines.

Keywords: Hedera helix, hederacoside C, topical formulations, dialysis membrane, rabbit skin, release kinetics
methanol, acetonitrile, disodium hydrogen phosphate, triethanolamine, potassium dihydrogen phosphate, and ethanol (95%) were also purchased from Sigma-Aldrich. Polysorbate 80 and glacial acetic acid were purchased from Fisher Scientific (Hampton, NH, USA). Cuprophan dialysis cellulose membrane (hydrophilic 128 x 345 mm) was obtained from Medicell Membranes Ltd. (London, England). All other chemicals used were of high purity grade.

Plant Collection and Extraction from *Hedera Helix* L.
The leaves of *H. helix* were collected from Quetta, Balochistan between August and September 2015. The plant was identified by a taxonomist (Dr. Rasool Bakhsh Tareen, University of Balochistan).

The extraction was done according to Harborne. Dried plant material (60 g) from *H. helix* was mechanically ground and weighed in a brown glass bottle and added to 900 mL of 30% ethanol water and stored for seven days at room temperature. The bottle was shaken after 24 hours occasionally and then filtered. The filtrate was put in the rotary evaporator to reduce the pressure until a thick residue formed. This residue was washed in the separating funnels with ethyl ether to remove the fatty materials and chlorophyll. This procedure was continued with ethyl ether until no colored matter was present. A thick viscous residue obtained was re-dissolved in 200-mL methanol and ethyl ether was added. A yellowish-white saponin extract formed. Addition of ethyl ether was continued until no more saponin formed. The saponin was obtained by decantation and air-dried at room temperature, yielding the extract (7.0 g).

**Isolation and Identification by Thin-Layer Chromatography (TLC)**
To identify the necessary component of *H. helix* extract, three different developing solvents were used as the mobile phase using a prepared glass and aluminum plate of silica gel (20 x 20 cm, GF254) as the coating substance.

- Methanol: chloroform: water (50:40:10)
- Methanol: glacial acetic acid: chloroform: water (30:10:50:10)
- N-butanol: water: glacial acetic acid (940:50:10)

The best separation of the active marker of hederacoside C in the chromatogram was fully identified at 254 and 365 nm. Each zone was scrapped, isolated, and dissolved in ether, which gave the desired compounds after filtration and removal of the solvent. The identification was also done by comparing Rf values for the standard and plant extract under the same conditions.

**Quantification of Hederacoside C from *H. helix* Extract by HPLC**
The high-performance liquid chromatography (HPLC) method was performed using an Agilent 1100 HPLC system (Agilent Technologies, Santa Clara, CA, USA) equipped with manual injector system, Shimadzu (Kyoto, Japan) LC-10ATvp pump, DGU-14A degasser, and SPD-10A VP UV–VIS detector, and Hypersil ODS C8 (150 x 4.6 mm) column (Thermo Scientific, Waltham, MA, USA). Agilent ChemStation software was used for data collection and processing. The chromatographic conditions used for analysis were as follows. The mobile phase consisted of acetonitrile, water, and orthophosphoric acid (150:850:2). The flow rate was 1.0 mL/min, injected volume was 20 µL, and samples were detected by an ultraviolet-visible detector at a wavelength of 210 nm. The retention time of hederacoside C was 9.6 and 10.0 min.

**Preparation of Microemulsion**
Preparation of microemulsion was done according to Chen et al. (12). A polysorbate 80 mixture of a surfactant and ethanol (co-surfactant) in a 2:1 ratio was mixed vigorously. A 4.6-g sample of the surfactant mixture was added to the oil (oleic acid, 0.5 g) and mixed together along with plant extract (0.5 g) using a magnetic stirrer. Distilled water (4.4 g) was slowly added under continuous stirring (1200 rpm) at room temperature (13, 14).

**Preparation of Gel**
The gel was prepared according to Proniuk et al. (15). Carbopol 934p (1 g) was weighed and dissolved slowly in distilled water while stirring continuously (1200 rpm) at ambient temperature to form a 1% solution. Triethanol amine was added slowly drop-wise to the prepared gel to increase the pH to 5.7. Then, we added plant extract (5 g) and measured the characterization (13, 14).

**Preparation of Ointment**
Formulation of the simple ointment B.P. was done according to Marriot et al by first melting the hard paraffin (4.75 g) at 60 °C then adding wool fat (4.75 g) followed by cetostearyl alcohol (4.75 g) (16). The mixture was stirred and cooled at ambient temperature. Soft white paraffin (80.75 g) and plant extract (5.0 g) was added and the characterization was measured (13, 14).

**Analysis of Drug Content**
Drug content of all formulations was analyzed. A 10-mg sample was weighed and added to 100 mL of hydroalcoholic solvent in a conical flask and stirred with a
magnetic stirrer for 1 hour until dissolved completely. The
dissolved solution was filtered through a membranous
filter (0.2 µm) and assessed by a validated HPLC method,
and the drug concentration was calculated.

**Preparation of Rabbit Skin for Franz Diffusion Cells**

Albino rabbit skin was used for ex vivo permeation studies
of the plant extract. The rabbit was anesthetized with
chloroform and hair was carefully shaved from the dorsal
region with an electric razor. The skin was washed with a
cotton swab. We waited 24 hours so that the skin would
become normal. The rabbits were scarified, and the skin
was excised carefully with the help of sharp blade. The
epidermis was removed by dipping it in hot water of
almost 600 °C. The dermis was teased from the other two
layers and the layers were separated. The epidermis layer
was cleaned with distilled water, covered with aluminum
foil, and stored at –500 °C until further use (14, 17).

**Franz Diffusion Cell Setup and Receptor Media Prepa-
ratin**

A Franz diffusion cell apparatus (PermeGear, Hellertown,
PA, USA) was used for in vitro and ex vivo studies of *H.
helix* extract. The solution used in the receptor is called
receptor medium. It is selected based on its physical
and chemical compatibility with the artificial and natural
membrane, drug, and formulations. Because semi-solid
dosage forms from plant extracts were used in this
study, the receptor media was composed of ethyl alcohol
and phosphate buffer (25:75). The medium was filtered
through a 0.2-µm membrane, and the temperature of the medium was maintained at 32 °C in a circulating
temperature-controlled bath set to 32.5 °C. Franz
diffusion cells should be free from air bubbles. The system
was allowed to settle for 30 minutes before use (18–21).

**Sampling**

From each Franz diffusion cell, 1-mL samples were drawn
using a 1-mL syringe through the sampling point. At the
time of sampling, stirring was stopped and the sample
was taken and stirring continued afterwards with fresh medium. Throughout the sampling process, we ensured
that there were no bubbles in the receptor medium or
in the syringe because trapped air decreases the volume
and area available for permeation. If bubbles appear in
receptors compartments, it can be detached by gently
tilting the Franz cells. The sampling intervals were at 0.5,
1, 2, 3, 4, 5, 6, 12, and 24 hours, and recovered samples
were analyzed by HPLC (22–24). To accommodate
slight variations in the six Franz cells, experiments were
performed in triplicates.

**Pharmacokinetic Studies of hederacoside C across the
Rabbit Skin**

The quantity of drug in the receptor medium was
analyzed (0–24 h) by HPLC, and the released amount of
drug was identified and computed. Linear regression
analyses and parameters of drug permeation for each
formula were analyzed. The correlation coefficient ($R^2$)
was calculated for each formula by each kinetic equation
to assess whether the permeation of the drug through
the membrane or natural skin follows a zero order, first
order, Higuchi, Korsmeyer-Peppas, or Hixon-Crowell
diffusion release model. All calculations were carried
out according to the following kinetics equations using
DDSolver (a Microsoft Excel 2007 add-in program) (25). All
calculations were performed according to the following
Equations 1–5.

**Zero-order model:**

$$A = K_0 t + A_0$$  
Eq. (1)

where $A$ is the amount of drug released at time $t$ and $K_0$ is
the zero-order release rate constant.

**First-order model:**

$$\log A = Kt/2.3 + \log A_0$$  
Eq. (2)

where $A_0$ is the amount of drug release at time $t$ and $K$ is
the first-order rate constant.

**Higuchi-diffusion model**

$$Q = K_H t^{1/2}$$  
Eq. (3)

where $Q$ is the amount of drug released to the membrane
(in mg) at time ($t$) in minutes. $K_H$ is the Higuchi square root
of time release constant.
Korsmeyer-Peppas model

\[ \frac{C_t}{C_\infty} = K t^n \]  
\text{Eq. (4)}

where \( \frac{C_t}{C_\infty} \) is a fraction of drug released at time \( t \), \( K \) is the release constant, and \( n \) is the release exponent.

Hixson-Crowell diffusion release model

\[ \frac{Q_t}{Q} = K t^n \]  
\text{Eq. (5)}

where \( \frac{Q_t}{Q} \) is the amount of released drug at time \( t \), \( K \) is the constant comprising the structural and geometric characteristics of the formulations, and \( n \) is the release exponent.

Statistical Analysis

Two-way multivariate analysis (ANOVA) was used to evaluate the effect of dialysis cellulose membrane and natural rabbit skin using SPSS (version 18.0, IBM, Armonk, NY, USA) (26). For comparison of the formulations, the similarity factor (\( f_2 \)) analysis was used (27). The data from all formulations were fit to the Korsmeyer-Peppas kinetic release model and compared. Flux (\( J \)) was calculated as \( \mu g/h/cm^2 \) (28).

RESULTS AND DISCUSSION

Identification of hederacoside C by TLC

The identification of the compound was done by three different types of solvents (mobile phase) to confirm presence of the marker, hederacoside C. The Rf values of standard hederacoside C and the plant extract were observed in equal distance, as shown in Figure 1.

Quantification of hederacoside C by HPLC

The HPLC technique was used for the quantitative and qualitative analysis of hederacoside C from the plant extract and the formulations. The compound was confirmed with a retention time of 10.06 min for the standard and 9.66 min for the sample extract (Fig. 2). The hederacoside C content in the plant extract was 14.00%, and the drug content in the microemulsion, gel, and ointment formulation was 99.10%, 98.23%, and 97.45%, respectively (Table 1).

Comparison of \( H. \) helix Formulations

A 1-g sample of herbal topical formulations containing hederacoside C were analyzed via dialysis cellulose membrane and natural rabbit skin, as shown in Figure

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Table 1. Drug Content in Three Formulations Containing Hedera helix L. Extract

<table>
<thead>
<tr>
<th>Formulations</th>
<th>Drug Content</th>
</tr>
</thead>
<tbody>
<tr>
<td>Microemulsion</td>
<td>99.10%</td>
</tr>
<tr>
<td>Gel</td>
<td>98.23%</td>
</tr>
<tr>
<td>Ointment</td>
<td>97.45%</td>
</tr>
</tbody>
</table>

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Figure 1. (A) Thin-layer chromatography (TLC) of hederacoside C. (real image); (B) TLC of hederacoside C at 256 nm; (C) TLC of hederacoside C at 365 nm.

Figure 2. Chromatogram of (A) hederacoside C (control retention time: 10.06 min) and (B) isolated hederacoside C (retention time: 9.66 min).
3A and B, respectively. The microemulsion and gel formulations showed visible release from the cellulose membrane and rabbit skin, and both formulations had similar release and permeability patterns at 6 h ($f_2 = 68$ and $80$, respectively) and up to 24 h ($f_2 = 60$ and $60$, respectively), as indicated in Table 2. The ointment formulation did not have a similar release and permeability pattern as the dialysis membrane and rabbit skin. The $f_2$ value for the ointment was less than 50 when compared to the microemulsion and gel formulations. These data indicate that the membrane nature had a considerable impact on the release and permeability of the formulations.

The amount of drug released from the different formulations (microemulsion, gel, and ointment) using the dialysis cellulose membrane and natural rabbit skin are listed in Table 3 and 4, respectively. The amount of hederacoside C released from different formulations using the cellulose membrane and rabbit skin can be arranged according in descending order as microemulsion > gel > ointment.

The similarity between both in vitro and ex vivo results illustrates the value of administrating hederacoside C as a topical dosage form, as shown in Figure 2A and B. The release profiles for the microemulsion and gel were clear within 24 h (Tables 3 and 4). Although drug release from the microemulsion formulation through the cellulose membrane and rabbit skin was 70.239% and 66.0571% after 24 h, respectively, the large amount of hederacoside C released may be due to the presence of enhancers (i.e., polysorbate 80 and ethanol). The large release from the gel formulation may be due to olive oil and swell-up in the first hour, and its first burst may increase the penetration of drug and then drop. Drug release from the gel through the cellulose membrane and rabbit skin was 63.9157% and 59.5%, respectively. The amount of drug released from the ointment through the cellulose membrane and rabbit skin was 40.9514% and 37.64%, respectively. The slow release of the drug from the ointment may be due to affinity of the drug to the base and its viscosity. Furthermore, slow release from the ointment may be due to the absence of the oily phase in this formulation. The drug release through the cellulose membrane and rabbit skin follows the Korsmeyer-Peppas model. Table 5 shows the flux ($J$) values for all dosage forms. The use of ethanol solvent as a receptor medium for the semisolid dosage form increases the rate of drug release between the donor and receptor compartments (23, 24). The penetration of hederacoside C through the cellulose membrane was more than the rabbit skin, which could be explained by the above-mentioned reason or the rabbit skin used in this study was thicker or had smaller pore size than the cellulose membrane, which could not allow more drugs to

<table>
<thead>
<tr>
<th>Formulations</th>
<th>Dialysis Cellulose Membrane</th>
<th>Natural Rabbit Skin</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0–6 h</td>
<td>0–24 h</td>
</tr>
<tr>
<td>Microemulsion vs. Gel</td>
<td>68</td>
<td>60</td>
</tr>
<tr>
<td>Microemulsion vs. Ointment</td>
<td>36</td>
<td>27</td>
</tr>
<tr>
<td>Gel vs. Ointment</td>
<td>41</td>
<td>32</td>
</tr>
</tbody>
</table>

Figure 3. Comparison of mean drug release profiles of hederacoside C from three formulations containing *Hedera helix* L. extract using (A) dialysis cellulose membrane and (B) natural rabbit skin.
Table 3. Hederacoside C Release from Three Formulations Using Dialysis Cellulose Membrane

<table>
<thead>
<tr>
<th>Amount of Drug Released after 24 h (%)</th>
<th>Base</th>
<th>Amount of Drug Release at Each Time Point (mg/1.5 cm²)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>0.5 h</td>
</tr>
<tr>
<td>70.239</td>
<td>Microemulsion</td>
<td>0.7320</td>
</tr>
<tr>
<td>63.9157</td>
<td>Gel</td>
<td>0.7575</td>
</tr>
<tr>
<td>40.9514</td>
<td>Ointment</td>
<td>5.5276</td>
</tr>
</tbody>
</table>

$R^2$ (coefficient of correlation)

<table>
<thead>
<tr>
<th>Formulations</th>
<th>Zero Order</th>
<th>First Order</th>
<th>Higuchi</th>
<th>Hixon-Crowell</th>
<th>Korsmeyer-Peppas</th>
<th>Best-Fitting Model</th>
</tr>
</thead>
<tbody>
<tr>
<td>Microemulsion</td>
<td>0.8197</td>
<td>0.9636</td>
<td>0.9493</td>
<td>0.9644</td>
<td>0.9599</td>
<td>Korsmeyer-Peppas</td>
</tr>
<tr>
<td>Gel</td>
<td>0.8207</td>
<td>0.9466</td>
<td>0.9496</td>
<td>0.9651</td>
<td>0.8502</td>
<td>Korsmeyer-Peppas</td>
</tr>
<tr>
<td>Ointment</td>
<td>0.8131</td>
<td>0.8693</td>
<td>0.9512</td>
<td>0.9789</td>
<td>0.8502</td>
<td>Korsmeyer-Peppas</td>
</tr>
</tbody>
</table>

Table 4. Hederacoside C Release from Three Formulations Using Natural Rabbit Skin

<table>
<thead>
<tr>
<th>Amount of Drug Released after 24 h (%)</th>
<th>Base</th>
<th>Amount of Drug Release at Each Time Point (mg/1.5 cm²)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>0.5 h</td>
</tr>
<tr>
<td>66.0571</td>
<td>Microemulsion</td>
<td>0.4833</td>
</tr>
<tr>
<td>59.5</td>
<td>Gel</td>
<td>0.5342</td>
</tr>
<tr>
<td>37.64</td>
<td>Ointment</td>
<td>0.4193</td>
</tr>
</tbody>
</table>

$R^2$ (coefficient of correlation)

<table>
<thead>
<tr>
<th>Formulations</th>
<th>Zero Order</th>
<th>First Order</th>
<th>Higuchi</th>
<th>Hixon-Crowell</th>
<th>Korsmeyer-Peppas</th>
<th>Best-Fitting Model</th>
</tr>
</thead>
<tbody>
<tr>
<td>Microemulsion</td>
<td>0.8924</td>
<td>0.9743</td>
<td>0.9809</td>
<td>0.9828</td>
<td>0.9581</td>
<td>Korsmeyer-Peppas</td>
</tr>
<tr>
<td>Gel</td>
<td>0.8595</td>
<td>0.9442</td>
<td>0.9661</td>
<td>0.9727</td>
<td>0.9212</td>
<td>Korsmeyer-Peppas</td>
</tr>
<tr>
<td>Ointment</td>
<td>0.8460</td>
<td>0.8927</td>
<td>0.9643</td>
<td>0.9767</td>
<td>0.8771</td>
<td>Korsmeyer-Peppas</td>
</tr>
</tbody>
</table>

Table 5. Flux Values of Three Formulations Containing Hedera helix L. Extract

<table>
<thead>
<tr>
<th>Formulations</th>
<th>Dialysis Cellulose Membrane (µg/cm²/h)</th>
<th>Natural Rabbit Skin (µg/cm²/h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Microemulsion</td>
<td>7.10</td>
<td>6.10</td>
</tr>
<tr>
<td>Gel</td>
<td>5.02</td>
<td>4.12</td>
</tr>
<tr>
<td>Ointment</td>
<td>3.80</td>
<td>2.40</td>
</tr>
</tbody>
</table>
penetrate. Additional studies are required to determine if the experimental effects were only the result of pore size, enhancer, or if the behavior of the membrane material also contributed to the inequitable power.

CONCLUSION
The current study demonstrated that dialysis cellulose membrane, natural rabbit skin, and Franz diffusion cells can be used to assess release of hederacoside C from microemulsion, gel, and ointment formulations containing H. helix extract. The in vitro and ex vivo release experiments showed similar release rates. The ointment showed a slower rate of drug release through dialysis cellulose membrane and natural rabbit skin. Statistical analysis proved that each parameter was significant with little variation. These formulations could be used for further studies, and in vitro and ex vivo diffusion cell experiments can be utilized to develop improved formulations of herbal medicines.

ACKNOWLEDGEMENTS
The authors would like to thank the Government of Balochistan Department of Health (for providing study leave), and Dr. Mahmood Ahmad; Dean and Faculty of Pharmacy at The Islamia University of Bahawalpur for support to complete this research work.

CONFLICT OF INTEREST
The authors disclosed no conflicts of interest related to this study.

REFERENCES


