Available online at www.sciencedirect.com
SCIENCE DIRECT
European Journal of Pharmaceutics and Biopharmaceutics 60 (2005) 31–38
European Journal of Pharmaceutics and Biopharmaceutics
www.elsevier.com/locate/ejpb

Research paper

In vitro availability of kaempferol glycosides from cream formulations of methanolic extract of the leaves of *Melilotus elegans*

Tsige Gebre-Mariam[a,*], Kaleab Asres[b], Melkamu Getie[c], Abebe Endale[d], Reinhard Neubert[c], Peter C. Schmidt[d]

[a]*Department of Pharmaceutics, School of Pharmacy, Addis Ababa University, Addis Ababa, Ethiopia*
[b]*Department of Pharmacognosy, School of Pharmacy, Addis Ababa University, Addis Ababa, Ethiopia*
[c]*Institute of Pharmaceutical Technology and Biopharmaceutics, Martin-Luther University, Halle/Saale, Germany*
[d]*Institute of Pharmaceutical Technology, University of Tübingen, Tübingen, Germany*

Received 5 October 2004; accepted in revised form 10 January 2005
Available online 8 March 2005

Abstract

In Ethiopian traditional medicine, *Melilotus elegans* Salzm. ex Ser. (Leguminosae) is used for the treatment of haemorrhoids and lacerated wounds. In view of its wide spread use and proven anti-inflammatory activity, 80% methanolic extract of the leaves was formulated into creams. HPLC/UV and MS studies revealed the presence of flavonoids, of which kaempferol was the major aglycone. Quantitative estimation of kaempferol in the hydrolyzed extract as determined by HPLC/UV was found to be 16.3 ± 0.93 μg/mg ($n = 6$, range) of extract. The in vitro release profiles of kaempferol glycosides (quantified as kaempferol equivalent) from the cream formulations in a multilayer membrane system indicated that a lipophilic cream of the extract provides higher release of kaempferol glycosides than hydrophilic and amphiphilic ones. Over a study period of 4 h, the lipophilic cream released 66 ± 5.70% of kaempferol glycosides, while the hydrophilic and amphiphilic creams resulted in 55 ± 2.77 and 38 ± 2.30% release, respectively.

Keywords: *Melilotus elegans*; Flavonoids; Kaempferol glycosides; Kaempferol; Multilayer membrane; Creams; In vitro availability

1. Introduction

Melilotus elegans Salzm. ex Ser. (syn. *Melilotus abyssinica* Bak.) is one of the five species of the genus *Melilotus* (Leguminosae) that are known to occur in Ethiopia [1]. The mature plant of this erect annual herb is about 1 m in height. In Ethiopia, the plant is known by its Amharic vernacular names 'Egug' and 'Gugi' and it has a wide distribution, particularly in highlands where the altitude ranges between 1700 and 2800 m above sea level [2].

The polar and nonpolar extracts of the leaves of *M. elegans* have been tested for their anti-inflammatory activity on carrageenin-induced rat paw oedema. At a dose corresponding to 333.3 mg per kg body weight of dry plant material, the methanol extract has been found to display strong inhibition of oedema (40.4% inhibition, 4 h after carrageenin injection compared to the control group). This anti-inflammatory activity of the polar extracts has been found to be comparable to the inhibitory effect of 1 mg/kg of indomethacin in the same test system [3].

Furthermore, the bioactive aqueous extract was subjected to solid phase extraction on Isolute C-18 (EC) columns using hydroalcoholic solutions containing different proportions of MeOH and H_2O as eluent. Eluates obtained from water; 50 and 75% MeOH in water were shown to be active whilst the 25% MeOH in H_2O fraction was devoid of any significant anti-inflammatory action [3].

Flavonoids are ubiquitous secondary plant metabolites that are used as remedies for various ailments [4–11]. They are the most important phenolic compounds in medicinal plants, which consist mainly of flavones, flavonols and their glycosides. Their chemical structures consist of a basic skeleton of 2-phenylbenzopyrone, and the various

* Corresponding author. Department of Pharmaceutics, School of Pharmacy, Addis Ababa University, P.O. Box 1176 Addis Ababa, Ethiopia. Tel.: +251 55 85 65/66; fax: +251 1 55 85 66.
E-mail address: tsigegmw@phar.aau.edu.et (T. Gebre-Mariam).

0939-6411/$ - see front matter © 2005 Elsevier B.V. All rights reserved.
doi:10.1016/j.ejpb.2005.01.001

flavonoids differ in their degree of saturation, substituents, the number and position of hydroxyl- and methoxy groups as well as sugar residues [12–15].

Among the flavonoids, flavonols are one of the most important groups [16]. The isolation of two flavonol glycosides, kaempferol-3-O-(6″-α-L-rhamnosyl)-β-D-galactoside-7-*O*-α-L-rhamnoside (robinin) and kaempferol-3-*O*-β-D-galactoside-7-*O*-α-L-rhamnoside from the MeOH and aqueous extracts of the leaves of *M. elegans* has been reported elsewhere [3].

The study of flavonoids involves the separation of complex mixtures of compounds, often of very similar chemical structures. Chromatographic techniques such as paper chromatography (PC) and thin-layer chromatography (TLC) have played crucial roles in the analysis of such mixtures [17]. Whilst these techniques can be structurally informative when used in conjunction with a variety of detection agents, they are limited in their ability to provide ready and reliable quantification of components. High performance liquid chromatography (HPLC), in contrast, offers good separation of flavonoids through the optimisation of mobile and stationary phases, and is ideally suited to the quantification of the separated compounds [18–21].

Reports confirm that plants containing flavonoids in sufficiently large concentrations are effective in the topical treatment of skin or mucous membrane inflammation in folk medicine [22,23]. In Ethiopia, the powdered leaves of *M. elegans* are mixed with butter and applied topically against haemorrhoids, mouth inflammation and lacerated wounds. In view of its proven anti-inflammatory activities, the current work reports on the identification and estimation of kaempferol glycosides content in 80% methanol extract using MS and HPLC/UV techniques, respectively, as well as the release profiles of the glycosides from formulated extract of hydrophilic, amphiphilic and lipophilic creams.

2. Materials and methods

2.1. Collection of plant materials

The leaves of *M. elegans* were collected in October 2003 in and around the city of Addis Ababa, Ethiopia. Its identity was confirmed by the National Herbarium, Department of Biology, Addis Ababa University, Addis Ababa, Ethiopia. The plant material was air dried at room temperature, powdered using mortar and pestle and kept in airtight and umber containers for extraction.

2.2. Chemicals and solvents

Methanol and petroleum ether (40–50 °C) used for extraction, (Riedel-de Haën, Sigma-Aldrich Laborchemikalien, GmbH, Seelze, Germany); methanol HPLC grade (J.T. Baker, Deventer, The Netherlands); hydrochloric acid (Grüssing GmbH, Füsum, Germany); authentic samples of kaempferol, quercetin, isorhamnetin and rutin, as well as glycerol, emulsifying cetostearyl alcohol type A, glycerol monostreate 60 and polyoxyethylene glycerolmonostearate (Carl Roth GmbH & Co., Karlsruhe, Germany); ethanol and ether (Condea Chemie GmbH, Herne, Germany), propylene glycol (Woelm Pharma GmbH & Co., Eschwege, Germany); liquid paraffin, cetostearyl alcohols and wool alcohols (Beiersdorf AG, Hamburg, Germany); white soft paraffin (SYNOPHARM GmbH & Co. KG, Barsbüttel, Germany); 4% collodion solution and medium-chain triglycerides (Caesar & Loretz GmbH, Hilden, Germany) were used as received. Water used was deionized and double distilled. All other solvents used were of analytical grade unless specified.

2.3. Extraction

Powdered leaves (100 g) were placed in a glass percolator and successively defatted with 3×500 ml of petroleum ether (50–60 °C) over a period of 24 h. The petroleum ether extract was then percolated, filtered and then concentrated using rota vapour at 40 °C and 200 mbar. The marc was dried in a hood and extracted with 3×500 ml of 80% methanol by percolation over a period of 36 h. The percolates were collected, filtered and concentrated using rota vapour at 40 °C and 100 mbar. The aqueous concentrates were then deep-frozen at −20 °C overnight (14 h) in a cold room. The frozen mass was then transferred into a freeze drying equipment consisting of a Lyovac GT 2 freeze drier (Finn Aqua Santasalo-Sohlberg Co., Tuusula, Finland), a programmable thermostat FP 50-MH (Julabo Labortechnik GmbH, Seelbach, Germany) and a vacuum pump Trivac D8B (Leybold-Heraeus AG, Cologne, Germany). The mass was further frozen at −35 °C for a period of 4 h and subsequently subjected to lyophilization. The primary drying was carried out by a stepwise increase of the temperature to +5 °C over a period of 10 h. The secondary drying was carried out by gradually increasing the temperature to +20 °C over a period of 10 h. Immediately after lyophilization, the dried extract was homogenized using pestle and mortar in order to obtain a uniform dried extract and kept in an airtight container over a desiccator. The average yield obtained from three batches of extraction was 35.1±0.3 g.

2.4. Detection of flavonoids in the crude extract of M. elegans by MS

Since kaempferol glycosides were previously reported in the leaves of *M. elegans* [3], their presence in the 80% methanolic extract was confirmed by mass spectrometry. In this, an ion trap mass spectrometer Finnigan LCQ (ThermoFinnigan, San Jose, CA, USA) was used. Ionisation was carried out in the negative ion mode by applying an electrospray voltage of 4.5 kV and a capillary temperature of 220 °C.

Initially, solutions containing 1 μg of each of kaempferol, quercetin, isorhamnetin and rutin were prepared in 80% methanol and filtered (0.2 μm). The solution was injected with a syringe pump at a flow rate of 20 μl/min and ionisation was carried out (as described above) and mass spectra for the respective flavonoids were obtained, which were used as references.

For test solutions, 10 mg of crude extract of *M. elegans* were dissolved in 80% methanol (vortexed for about a minute followed by sonication for about 30 min). The solution was filtered (0.2 μm) and injected at a flow rate of 20 μl/min and ionisation was carried out in the negative ion mode under identical conditions described above for the standard solutions. Mass spectra corresponding to various components of the extract including flavonoids were obtained. Using an ion trap analyser, MS^n mass spectroscopic studies were also carried out which resulted in the spectrum of kaempferol. Following hydrolysis, the amount of kaempferol in the crude extract was estimated by HPLC.

2.5. Quantitative estimation of kaempferol glycosides in the crude extract of M. elegans by HPLC

The amount of kaempferol glycosides present in the extract was estimated using HPLC/UV (HP 1100 HPLC System, Agilent, Waldbronn, Germany) equipped with a binary gradient pump, UV diode array detector, autosampler and solvent degasser. Initially, standard solutions of kaempferol (0.5–50 μg/ml) were prepared and filtered (0.2 μm). Twenty micrilitres of the standard kaempferol solution were injected into a stationary phase comprising of Nucleosil® RP 18 column, 125×2 (Macherey-Nagel, Düren, Germany). The mobile phase consisted of Solvent A (HPLC grade water and 0.1% formic acid) and Solvent B (HPLC grade methanol and 0.1% formic acid) which was run under gradient conditions following the schedule: 10, 70, 70 and 10% of Solvent B at 0, 10, 20 and 22 min, respectively. The flow rate was 0.3 ml/min. The run time was 30 min. Each of the solutions was analyzed at the detection wavelength of 275 nm three times from separately prepared fresh solutions.

Solutions of 10 mg/ml of *M. elegans* extract in 80% methanol were prepared in an ultrasonic bath at 30 °C for 30 min, cooled and filtered (0.2 μm). In order to facilitate hydrolysis of the kaempferol glycoside, to each 2 ml of the solutions, 0.5 ml of 5.5% hydrochloric acid was added and heated over a boiling water bath for 1 h. The solutions were then allowed to cool and analyzed by HPLC/UV under conditions described above. The results are the mean average of at lease six determinations.

2.6. Formulations of the extract into creams

Creams of the dried extract of *M. elegans* were prepared using hydrophilic, amphiphilic and lipophilic cream bases. Hydrophilic Cream Base containing emulsifying cetostearyl alcohol: liquid paraffin: white soft paraffin (30:35:35) (German Pharmacopoeia, DAB 1999) and Lipophilic Cream Base composed of cetostearyl alcohol: wool alcohols: white soft paraffin (0.5:6:93.5) (German Pharmacopoeia, DAB 1999) were prepared as outlined in the respective monographs. Amphiphilic Cream Base (German Pharmaceutical Codex, DAC 2003) was obtained from SYNOPHARM GmbH & Co. KG (Barsbüttel, Germany) and it consisted of Glycerol monostearate 60, Cetyl alcohol, Medium-chain triglycerides, White soft paraffin, Macrogol 20 glycerol monostearate, Propylene glycol and Purified water in the ratio of 4:6:7.5:25.5:7:10:40, respectively.

Each of the cream formulations contained 10% w/w dried extract of *M. elegans*. This was achieved by levigating the dry extract (20 g) with the cream base (180 g) in a mortar and pestle until a smooth cream was obtained. The creams were then filled into 15 g aluminum collapsible tubes (Wepa GmBH, Höhr-Grenzhausen, Germany) using laboratory-made cream-filler.

2.7. Preparation of membranes

Model membranes, described elsewhere [24,25] were prepared as follows. One hundred grams of 4% collodion solution, accurately weighed, were added to 100 g of 4% w/w glycerol solution in ether/ethanol (85:15, w/w ratio, respectively). The mixture was placed on a membrane preparation apparatus [25] that provides membrane sheets of uniform thickness and glycerol content. The membrane sheets were then cut into discs of uniform diameter (4 cm) by a hollow sharp-edge stainless steel cylinder designed for this purpose.

2.8. In vitro release studies of kaempferol glycosides from creams containing extract

The release of kaempferol glycosides from creams of *M. elegans* extract was evaluated using a multi-layer membrane system described in the literature [25–28]. The apparatus consisted of 5 polyacrylate (Piacryl®, Piesteritz, Germany) cells each containing four membranes, fitted together vertically. In this study, four cells each containing three membranes were prepared and used for each experiment. To an exposed membrane area (4 cm^2) of each cell, about 100 mg (accurately weighed) of cream were applied. The four cells were fitted together vertically and placed in a thermostatic chamber maintained at 32 ± 0.2 °C [26]. The apparatus was then removed from the chamber at selected time intervals (0.5, 1, 2, 3 and 4 h). The cells were separated and the creams remained on the surface of the first membranes of the three cells were removed completely. The membranes were then extracted with 5 ml of 80% methanol for 30 min in ultrasonic bath at room temperature. The creams that remained on the membranes of the fourth cells were extracted together with the membranes and used as controls. The solutions obtained from extraction of

the membranes were filtered, hydrolyzed as described above, cooled and filtered. The kaempferol content was determined by HPLC/UV (see above) and the release profiles determined. The results are the mean average of at least three determinations. Solutions of the membrane and the bases alone were also analyzed separately with HPLC/UV under the same condition.

2.9. Data analysis

The Higuchi model was used to determine the drug release profiles from the hydrophilic, amphiphilic and lipophilic cream bases. The results of drug release after 4 h of the study period were analysed using the Student *t*-test and ANOVA. A difference in the release results with $P<0.05$ was considered significant. Data are presented as mean average of at least three determinations.

3. Results and discussion

Defatting of the leaves of *M. elegans* with petroleum ether yielded a dried mass of 4.5 ± 0.1 g ($n=3$). Extraction of three batches of 100 g each of the leaves with 80% methanol resulted in 35.1 (±0.3) g of dark-green crude lyophilized extract. A study reported elsewhere [3] showed that the hydroalcoholic extract of *M. elegans* possessed anti-inflammatory activities. Therefore, in this study 80% methanol was employed to prepare the crude extract.

3.1. Detection of flavonoids in the crude extract by MS

Fig. 1a shows the negative ion mass spectrum obtained from 80% methanolic solution of the standard solutions of the flavonoids. Ion peaks are observed at (m/z) = 285.7, 301.4, 315.3 and 609.2 for kaempferol, quercetin, isorhamnetin and rutin, respectively.

Using an ion trap analyser MS^n, mass spectroscopic studies of the crude extract of *M. elegans* revealed the presence of a mass (m/z) = 739.5 (see Fig. 1b), which is most probably the quasi-molecular ion peak of the flavonol glycoside, kaempferol-3-*O*-(6″-α-L-rhamnosyl)-β-D-galactoside-7-*O*-α-L-rhamnoside (robinin), isolated from the plant [3]. Moreover, the MS^2 spectrum showed a fragment ion peak at m/z 593.2 $[(M-H)-146]^-$, that resulted from the loss of a terminal rhamnose unit (Fig. 1c). A subsequent fragmentation (MS^3) revealed an ion peak at m/z 285.1 $[(M-H)-146-146-162]^-$ corresponding to kaempferol after the loss of the second rhamnose and galactose units (Fig. 1d).

Fig. 1e shows the negative ion mass spectrum obtained from hydrolysed 80% methanolic solution of *M. elegans* extract, where an ion peak at $m/z=285.6$ is seen. Thus, the fragmentation in the spectroscopic studies and the hydrolysis of the extract provided the same ion peak ($m/z=285.6$), confirming the presence of kaempferol. Once the presence of kaempferol was verified, its amount in the crude extract was estimated by HPLC (see Section 2 above).

3.2. Quantitative analysis of kaempferol by HPLC

Plant extracts contain mixtures of numerous compounds and it is difficult to develop specific HPLC conditions for each of these substances. Therefore, the identification of these compounds requires isolation and purification of substances and to have a combination of different spectral data and physical constants of these substances. In this study, the presence of kaempferol glycosides in the extract obtained from the mass spectra was also confirmed by HPLC.

Under the chromatographic conditions described above, kaempferol was detected at 275 and 315 nm at a retention time of 13.73 min. The HPLC/UV chromatogram of a standard kaempferol solution is shown in Fig. 2a. The calibration curve for kaempferol standard solutions (0.5, 2, 5, 10, 20 and 50 μg/ml) was determined by HPLC/UV as described above. Linear Regression of the calibration curve of kaempferol standard solutions at 275 nm provided a linear-fit: Concentration of kaempferol = peak area − 37.3/153 with a correlation coefficient (R^2) of 0.9999. This linear fit was employed to quantify the amount of kaempferol in the hydrolyzed extract and the amount of kaempferol glycoside released (estimated as equivalent kaempferol) from the creams with time.

Flavonoids in plants may be present as free flavonol aglycones or as glycosides (usually at C-3 or C-7) or both. Rutin (quercetin-3-rutinoside), isoquercetin (quercetin-3-glucoside), quercimeritrin (quercetin-7-glucoside) and robinin (kaempferol-3-robinoside-7-rhamnoside) are examples of such glycosides [29]. In this study, the presence of flavonol aglycones was evaluated after hydrolysis of the extract solutions with 5% HCl (to liberate flavonol aglycones).

Various solutions (0.25–10 mg/ml) of the 80% methanolic extract of *M. elegans* were hydrolyzed and analyzed by HPLC/UV as described above. A typical chromatogram obtained for the hydrolyzed extract is displayed in Fig. 2b, indicating the presence of the aglycone, kaempferol. The relationship between concentration of the extract and peak area of the chromatograms was also rectilinear, peak area = $2550X+384.7$, where X is the concentration of extract, with (R^2) = 0.999. Using the linear-fit of the authentic standard solutions of kaempferol, the content of kaempferol was found to be 16.3 ± 0.93 μg/mg ($n=6$) of the extract.

Similarly, solutions obtained from the extraction of the membranes were hydrolyzed (see above) and analyzed by HPLC/UV. The amount of kaempferol glycosides released from the creams at a given time was quantified (as equivalent kaempferol) using the linear-fit equation of the calibration curve. The HPLC/UV analysis of solutions of the membrane and the three cream bases were carried out separately. Comparison of chromatograms of the bases

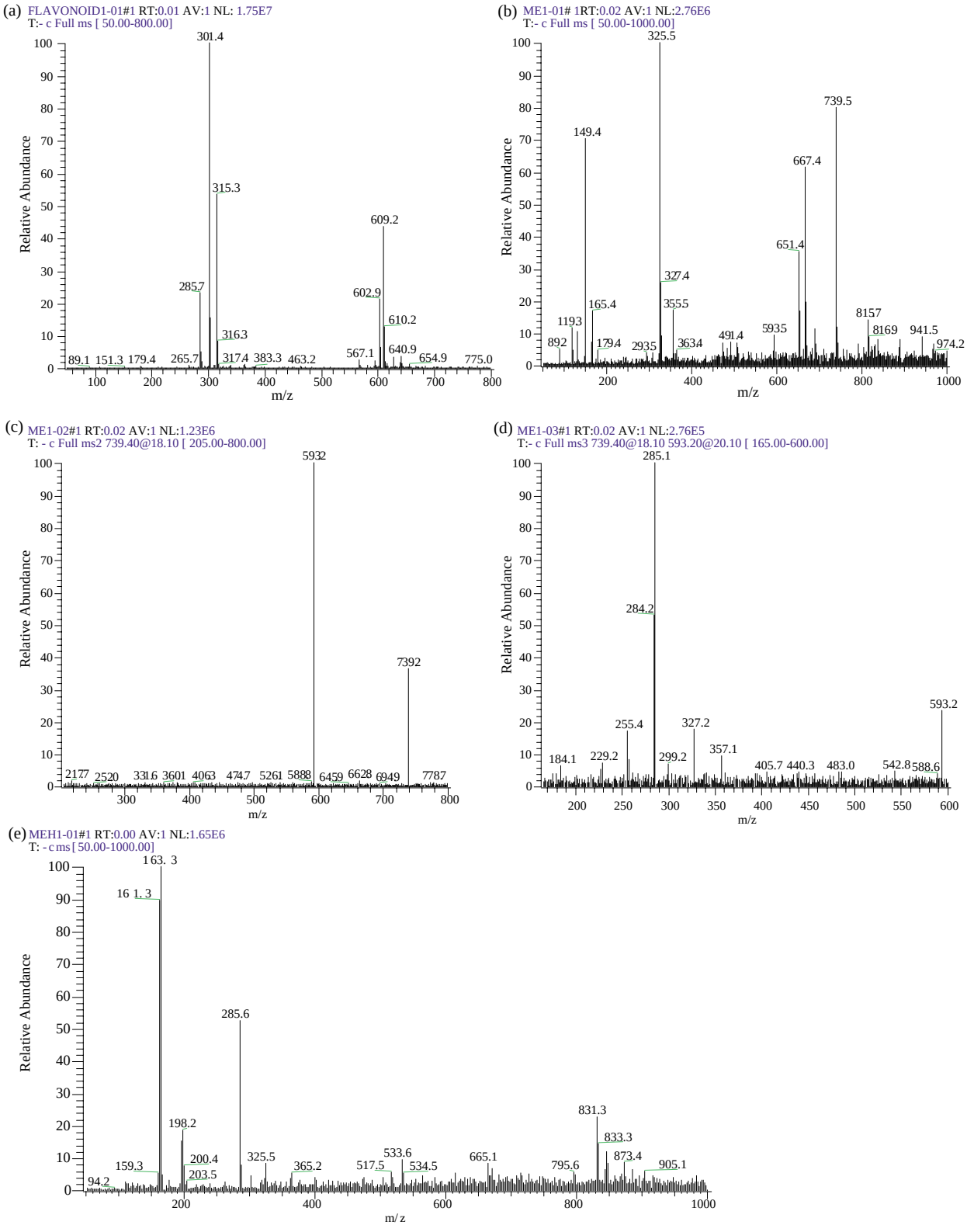

Fig. 1. The negative ion mass spectra of (a) standard solutions of kaempferol, quercetin, isorhamnetin and rutin, (b) the crude extract of *M. elegans* showing a glycoside flavonoid at (m/z) = 739.5, and (c) MS^2-fragmentation (593.2); (d) ion mass spectrum of kaempferol (m/z = 285.1) obtained upon fragmentation of *M. Elegans*, and (e) ion mass spectrum of kaempferol (m/z = 285.1) obtained upon hydrolysis of 80% methanolic solution of *M. elegans* extract.

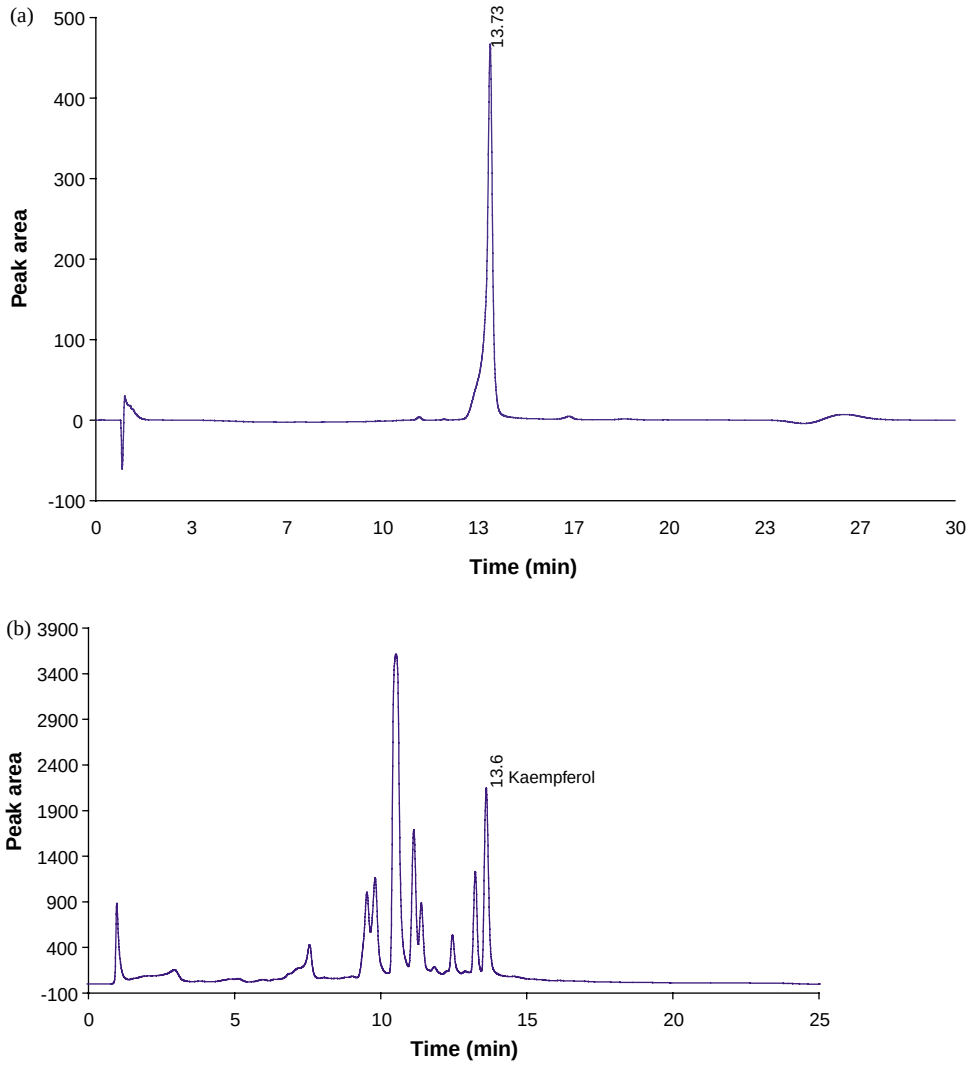

Fig. 2. Chromatograms of standard kaempferol solution (a) and the hydrolyzed solution of the crude extract of *M. Elegans* (b). Chromatographic conditions: stationary phase: Nucleosil® RP 18 column, 125×2; mobile phase: solvent A (water and 0.1% formic acid) and solvent B (methanol and 0.1% formic acid); flow rate: 0.3 ml/min; injection volume: 20 μl; run time: 30 min, detection wavelength: 275 nm.

and chromatogram of kaempferol indicated that there was no interference of the bases with kaempferol. Assuming homogeneous preparation, the amount of kaempferol equivalent applied on each cell was calculated from the known amount of cream applied and the control cells.

When creams are applied topically to the skin, the active agent must be released from its carrier (vehicle) before it contacts the epidermal surface and be available for penetration into the stratum corneum and lower layers of the skin. Therefore, hydrophilic (Hydrophilic Cream Base, DAB 1999), amphiphilic (Amphiphilic Cream, DAC 2003) and lipophilic (Lipophilic Cream, DAB 1999) formulations of the extract of *M. elegans* were prepared and the release profiles of kaempferol glycosides from these formulations were studied. The percentage of kaempferol glycosides (expressed as kaempferol equivalent and determined relative to the mean of the amount of kaempferol from controls), released against the square root of time is shown in Fig. 3. As shown in the figure, the drug release from the various cream bases followed the Higuchi release kinetics with correlation coefficents $(r) \geq 0.9955$. Within 4 h of the study period, the lipophilic cream of the extract exhibited higher release of kaempferol glycosides (66%) as compared to the hydrophilic (55%) and amphiphilic (38%) creams. The release values are significantly different at $P < 0.05$ on the basis of an ANOVA test followed by a Newman–Keuls test when compared between each group. These differences could be explained on the basis of the properties of robinin, one of the main kaempferol glycosides of *M. elegans*, as follows: Robinin exists in two polymorphs, α- and β-robinin. α-Robinin is soluble in hot water, while β-robinin dissolves in hot ethanol of strength ≥95% [30]. The octanol/water partition coefficient of robinin was calculated to be 17.3 [31]. Therefore, robinin is considered as a more or less hydrophilic compound. The faster and higher release from the lipophilic cream is expected as the glycosides, due

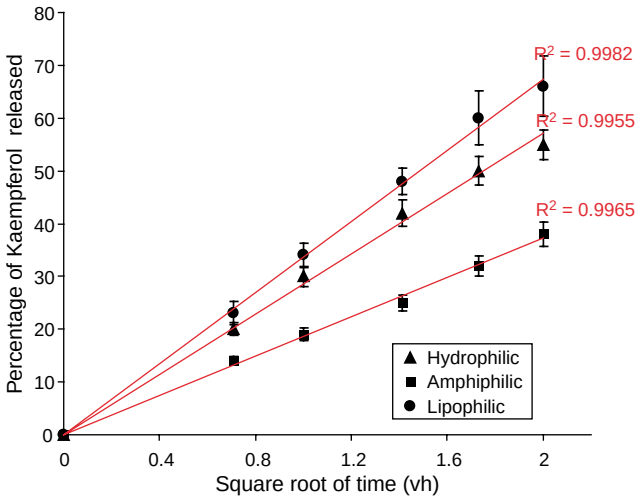

Fig. 3. The percentage release of kaempferol glycosides (expressed as kaempferol equivalent) from creams of the crude extract of *M. elegans* against the square root of time (the bars show the SD of the mean).

to their polarity have less affinity to the cream base. The hydrophilic cream is still a water free cream base containing the lipophilic components (liquid and white soft paraffin) and the hydrophilic emulsifier complex of cetosteatyl alcohol and sodium cetostearyl sulfate. Hence, the release from the hydrophilic cream base is reduced compared to the lipophilic cream. In the amphiphilic cream containing 40% of water, partition of robinin between the lipophilic and hydrophilic phase of the cream is possible, which leads to a further delay of the release. The more hydrophilic robinin is attracted by the aqueous phase of the system and thereby the partition of the total amount of robinin between the phases of the cream on one hand and the membrane on the other is changed.

4. Conclusion

Kaempferol glycosides are present in significant quantities (16.3 μg of per mg of 80% methanolic extract, as kaempferol equivalent) in the leaves of *M. elegans*. As the flavonoids of *M. elegans* were shown to have anti-inflammatory activities, the traditional use of the leaves of *M. elegans* for the treatment of skin diseases is justified. Lipophilic cream of *M. elegans* extract resulted in a higher release of kaempferol glycosides than hydrophilic and amphiphilic creams. Judicious choice of the cream base may be required to control the release of the flavonoid from creams.

Acknowledgements

The financial support of AvH to TGM and the technical assistance of Mrs M. Woigk are greatly acknowledged.

References

[1] M. Thulin, Leguminosae of Ethiopia, Aio Print, Odense, Copenhagen, 1983. pp. 204–205.
[2] M. Thulin, The leguminosae, in: I. Heidberg, S. Edwards (Eds.), Flora of Ethiopia, Addis Ababa and Asmara, Ethiopia; Uppsala, Sweden, vol. 3 (1989), pp. 245–246.
[3] K. Asres, U. Eder, F. Bucar, Studies on the antiinflammatory activity of extracts and compounds from the leaves of *Melilotus elegans*, Ethiop. Pharm. J. 18 (2000) 15–24.
[4] S. Christie, A.F. Walker, G.T. Lewith, Flavonoids—a new direction for the treatment of fluid retention, Phytother. Res. 15 (2001) 467–475.
[5] K.P. Wilhelm, S. Biel, C.P. Siegers, Role of flavonoids in controlling the phototoxicity of *Hypericum perforatum* extracts, Phytomedicine 8 (2001) 306–309.
[6] P. Knekt, R. Jarvinen, R. Seppanen, M. Hellovaara, L. Teppo, E. Pukkala, A. Aromaa, Dietary flavonoids and the risk of lung cancer and other malignant neoplasms, Am. J. Epidemiol. 146 (1997) 223–230.
[7] J.O. Kuti, H. Konuru, Antioxidant capacity and phenolic content in leaf extracts of tree spinach (Cnidoscolus spp.), J. Agric. Food. Chem. 52 (2004) 117–121.
[8] G. Lombardi-Boccia, M. Lucarini, S. Lanzi, A. Aguzzi, M. Cappelloni, Nutrients and antioxidant molecules in yellow plums (*Prunus domestica* L.) from conventional and organic productions: a comparative study, J. Agric. Food Chem. 52 (2004) 90–94.
[9] H. Negishi, J.W. Xu, K. Ikeda, M. Njelekela, Y. Nara, Y. Yamori, Black and green tea polyphenols attenuate blood pressure increases in stroke-prone spontaneously hypertensive rats, J. Nutr. 134 (2004) 38–42.
[10] M. Gabor, Anti-inflammatory and anti-allergic properties of flavonoids, Prog. Clin. Biol. Res. 213 (1986) 471–480.
[11] U. Seitz, P.C. Oefner, S. Nathakarnkitkool, M. Popp, G.K. Bonn, Capillary electrophoretic analysis of flavonoids, Electrophoresis 1992; 35–38.
[12] K.E. Heim, A.R. Tagliaferro, D.J. Bobilya, Flavonoid antioxidants: chemistry, metabolism and structure–activity relationships, J. Nutr. Biochem. 13 (2002) 572–584.
[13] S.A. Aherne, N.M. O'Brien, Dietary flavonols: chemistry, food content, and metabolism, Nutrition 18 (2002) 75–81.
[14] N.C. Cook, S. Samman, Flavonoids: chemistry, metabolism, cardioprotective effects, and dietary source, Nutr. Biochem. 7 (1996) 66–76.
[15] H.M. Merken, G.R. Beecher, Measurement of food flavonoids by high-performance liquid chromatography, J. Agric. Food Chem. 48 (2000) 575–585.
[16] P.C.H. Hollman, E.J.M. Feskens, M.B. Katan, Tea flavonols in cardiovascular disease and cancer epidemiology, Proc. Soc. Exp. Biol. Med. 220 (1999) 198–202.
[17] K.R. Markham, Techniques of Flavonoid Identification, Academic Press, London, 1982.
[18] V.K. Vande Casteele, H. Geiger, C.F. Van Sumere, Separation of flavonoids by reverse-phase high performance liquid chromatography, J. Chromatogr. 240 (1982) 81–94.
[19] I. Erlund, G. Alfthan, H. Siren, K. Ariniemi, A. Aro, Validated method for the quantitation of quercetin from human plasma using high-performance liquid chromatography with electrochemical detection, J. Chromatogr. B Biomed. Sci. Appl. 727 (1999) 179–189.
[20] A. Kiehne, U.H. Engelhardt, Thermospray-LC-MS analysis of various groups of polyphenols in tea, Z. Lebensm-Unters, Forsch. 202 (1996) 48–54.
[21] P.L. Mauri, L. Iemoli, C. Gardana, P. Riso, P. Simonetti, M. Porrini, P.G. Pietta, Liquid chromatography/electrospray ionization mass

spectrometric characterization of flavonol glycosides in tomato extracts and human plasma, Rapid. Commun. Mass Spectrom. 13 (1999) 924–931.
[22] H.P. Hoermann, H.C. Korting, Evidence for the efficacy and safety of topical herbal drugs in dermatology, I: antiinflammatory agents, Phytomedicine 1 (1994) 161–171.
[23] R. Della-Loggia, Lokale antiphlogistische Wirkung der Kamillen-flavone, Dtsch Apoth Ztg. 125 (1985) 9–11.
[24] R. Neubert, C. Bendas, W. Wohlrab, B. Gienau, W. Fürst, A multilayer membrane system for modelling drug penetration into skin, Int. J. Pharm. 75 (1991) 89–94.
[25] S. Huth, R. Neubert, L. Boltze, A. Büge, Experimental determination and mathematical modelling of propylene glycol transport from semisolid vehicles, Chem. Pharm. Bull. 44 (1996) 1263–1266.
[26] R. Neubert, W. Wohlrab, C. Bendas, Modelling of drug penetration into human skin using a multilayer membrane system, Skin Pharmacol. 8 (1995) 119–129.
[27] M.T. Knorst, R. Neubert, W. Wohlrab, Release of urea from semisolid formulations using a multilayer membrane system, Drug Dev. Ind. Pharm. 23 (1997) 259.
[28] A. Schendzielorz, B.D. Hanh, R. Neubert, S. Wartewig, Penetration studies of clotrimazole from semisolid formulation using step-scan FT-IR photoacoustic spectroscopy, Pharm. Res. 16 (1999) 42–45.
[29] S. Budavari (Ed.), The Merck Index, 12 ed, Merck & Co., Inc., Whitehouse Station, NJ, 1996, pp. 1381–1428.
[30] C.E. Sando, The plant coloring matter, Robinin. J. Biol. Chem. 94 (1932) 675–680.
[31] CAS Registry Number 301-19-9 through SciFinder.