CHAPTER 3

MATERIALS AND METHODS

3.1 Materials

3.1.1 Plant material

Senna alata leaves were collected from Songkhla Province, Thailand, in November 2006. The voucher specimen (specimen no. SKP 097.1 03 01 01) was deposited at the herbarium of the Faculty of Pharmaceutical Sciences, Prince of Songkla University, Thailand. The leaves were dried at 50 ºC for 24 h in a hot air oven and were reduced to coarse powder using a grinder. Dried plant powder was kept in well-closed container protected from light at 4 °C.

3.1.2 Chemicals and reagents

3.1.2.1 For extraction and purification

- Methanols, commercial grade (High Science distributor, Thailand)
- Ethyl acetate, commercial grade (High Science distributor, Thailand)
- Hydrochloric acid, AR grade (Lab scan Asia, Thailand)
- Acetic acid, glacial (Lab scan Asia, Thailand)
- Ferric chloride, AR grade (Riedel-de Haen, Germany)
- *n*-Hexane, commercial grade (High Science distributor, Thailand)
- Sodium hydroxide, AR grade (Lab scan Asia, Thailand)
- Silica gel 60, No. 9385 (Scharlau, Spain)
- Amberlite® IRA-67 (Sigma, U.S.A.)

3.1.2.2 For antifungal activity assay

- Sabouraud Dextrose Agar (Becton, Dickinson, France)
- Sodium chloride, AR grade (Lab scan Asia, Thailand)
- *di*-Methyl sulfoxide (DMSO) (Riedel-de Haen, Germany)
- Sodium chloride, AR grade (Lab scan Asia, Thailand)

3.1.2.3 For establishment of the chemical specification

- Methanol, AR grade (Lab scan Asia, Thailand)
- Methanol, HPLC grade (Lab scan Asia, Thailand)
- Ethyl acetate, AR grade (Lab scan Asia, Thailand)
- Ethanol, AR grade (Lab scan Asia, Thailand)
- Hexane, AR grade (Lab scan Asia, Thailand)
- Chloroform, AR grade (Lab scan Asia, Thailand)
- Acetic acid, glacial (Lab scan Asia, Thailand)
- *n*-Octanol (Riedel-de Haen, Germany)
- Eosin methylene blue agar (EMB) (Becton, Dickinson, France)
- Sabouraud Dextrose Agar (SDA) (Becton, Dickinson, France)
- Melted plate count agar (PCA) (Becton, Dickinson, France)
- Aloe-emodin 95%, HPLC grade (Sigma, Germany)
- Emodin 90%, HPLC grade (Sigma, Germany)
- Rhein 85%, HPLC grade (Fluka, France)
- Chrysophanol 98%, HPLC grade (Fluka, France)
- Anhydrous *di*-sodium hydrogen orthophosphate (Anala R®, England)
- Potassium *di*-hydrogen orthophosphate (M & B, England)
- *di*-Potassium hydrogen orthophosphate (M & B, England)

3.2 Instrumentations

Table 3-1 General instrumentations equipment

Instrument	Model	Company
UV-visible spectrophotometer	Genesis 5	Miltonroy, U.S.A.
Hot air oven	DIN 12880-KI	Memmert, Germany
HPLC	Agilent 1100 series	Palo Alto, U.S.A.
HPLC column	TSK-gel ODS-80Tm	Tosho Bioscience, Japan
pH meter	PHM 82	Radiometer, Denmark
Evaporator	N-1000	Tokyo Raikakikai Co., Japan
Reciprocator	RK 20-VS	Heto-Holten AIS, Denmark
Centrifugal vacuum evaporator	AS 260	Global Medical Instrumentation, Inc. , U.S.A.
Muffle furnace	85P	Barkmey Division, U.S.A.
Vortex	G-560E	Scientific industries, U.S.A.
Laminar air flow	HT-122	International scientific supply Co., Ltd. Bangkok
Hot plate	SLR	Schott Gerate, Germany
Sartorius Moisture Analyzer	MA 100	Scientific promotion Co.,Ltd, Germany

3.3 Methods

3.3.1 Optimization of solvent system for extraction

3.3.1.1 Determination of optimum hydrochloric acid concentration

S. alata leaf powder (20 mg) was extracted with methanol (20 ml) consisting of several concentration of hydrochloric acid (0, 3, 5, and 10 %v/v) under reflux condition for an hour. The extract was then filtered and concentrated under reduced pressure. The sample was adjusted to 5 ml with methanol and subjected to HPLC analysis. The experiments were in triplicate.

3.3.1.2 Determination of optimum ferric chloride concentration

S. alata leaf powder (20 mg) was extracted with methanol (20 ml) consisting of 5 %v/v hydrochloric acid, which was suitable concentration (examined in section 3.3.1.1) and several concentrations of ferric chloride (0, 2.5, 5, and 10 %w/v) under reflux condition for an hour. The extracts were then filtered and concentrated under reduced pressure. The sample was adjusted to 10 ml with methanol and subjected to HPLC analysis. The experiments were in triplicate.

3.3.1.3 Determination of optimum water in extraction solvent

S. alata leaf powder (20 mg) was extracted with methanol (20 ml) consisting of 5 %v/v hydrochloric acid, 5 %w/v ferric chloride, which was suitable concentration (examined in section 3.3.1.2) and several concentrations of water (0, 5, 10, 15, and 20 %v/v) under reflux condition for an hour. The extracts were then filtered and concentrated under reduced pressure. The sample was adjusted to 10 ml with methanol and subjected to HPLC analysis. The experiments were in triplicate.

3.3.2 Increase of anthraquinone content by chromatographic techniques

S. alata leaf powder was successively extracted with methanol containing 5 %v/v hydrochloric acid, 5 %w/v ferric chloride, and 15 %v/v water under reflux for an hour (× 2). The filtrates were combined and concentrated *in vacuo*. The crude extract was partitioned between ethyl acetate and water. The ethyl acetate phase was concentrated and subsequently purified using silica gel vacuum column chromatographic technique compared with anion exchange chromatographic technique as the methods described below.

3.3.2.1 Silica gel vacuum column chromatography

3.3.2.1.1 Preparation of silica gel vacuum column

A sintered glass column (13 cm in diameter) was packed with silica gel, approximately 6-7 cm high. The column was connected with a suction flask, which connected to an aspirator (Figure 3-1)

Figure 3-1 Silica gel vacuum column chromatography

3.3.2.1.2 Sample loading and separation

The ethyl acetate fraction (25 g), which pre-adsorbed on silica gel, was loaded as thin layer on the surface of column. The column was eluted with a mixture of hexane and ethyl acetate (9:1 v/v) (500 ml) with the aid of vacuum pump. The pooled factions of anthraquinones were then dried *in vacuo*. The anthraquinone fraction (5 mg) was dissolved in methanol and adjusted to 10 ml, and then subjected to HPLC analysis. The experiments were in triplicate.

3.3.2.2 Anion exchange chromatography

3.3.2.2.1 Preparation of anion exchange

An adequate volume of methanol (500 ml) was added into 1000 g anion exchange resin (Amberlite® IRA-67) and gently stirred for a few minutes. After allowed to stand for 15 minutes, the methanol was decanted and the slurry was washed twice with distilled water (2 × 500 ml), and then allowed to stand in methanol for a future 5-10 min. The treated resin was slowly poured into a glass column (5 × 35 cm). The excess methanol was drained. A portion of methanol (500 ml) was then added to settle the resin. The column was then ready for sample loading.

3.3.2.2.2 Sample loading and separation

The ethyl acetate fraction of *S. alata* leaves (25 g) was dissolved in methanol (500 ml) and filtered. The filtrate was then loaded on to the Amberlite® IRA-67 column and allowed the solution passed through the column with a flow rate of 5 ml/min until finish. The column was then eluted with methanol until the green pigments were completely washed out. The anthraquinones were eluted from resin using 10% acetic acid in methanol. The eluents were combined and concentrated *in vacuo*. The residue (5 mg) was dissolved in methanol and adjusted to 10 ml and then subjected to the HPLC analysis. The experiments were in triplicate.

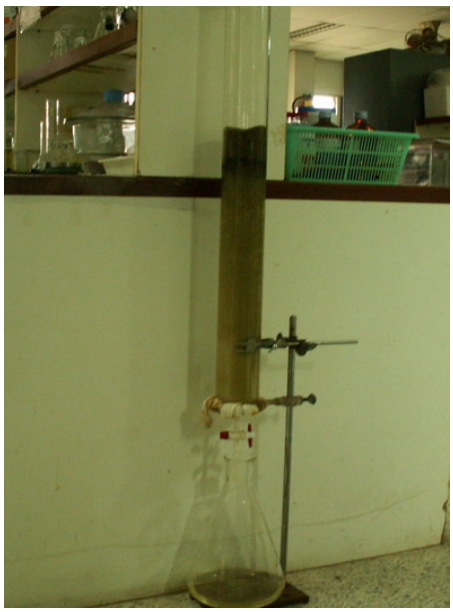

Figure 3-2 Anion exchange chromatography

3.3.3 Quantitative analysis of anthraquinones

Quantitative analysis of anthraquinone in *S. alata* leaf extract was achieved using the HPLC method that has been previously established and validated (Panichayupakaranant *et al.*, accepted).

3.3.3.1 HPLC Conditions

HPLC analysis was carried out using Agilent 1100 series equipped with a Agilent 1100 series Photodiode-array detector (PDA) and autosampler. Data analysis was performed using Agilent software (Agilent, U.S.A). Separation was achieved isocratically at 25 ºC on a 150 mm x 4.6 mm i.d. TSK-gel ODS-80Tm column. The mobile phase consisted of methanol-2% aqueous acetic acid (70:30, v/v) and was pump at a flow rate of 1 ml/min. The injection volume was 20 µl. The quantification wavelength was set at 254 nm.

3.3.3.2 Calibration curves

Separate stock solutions of the reference standards, rhein, aloe-emodin, emodin and chrysophanol were made in methanol. Working solution of the combined standards was subsequently prepared in methanol and diluted to provide series of the standards as shown in Table 3-2 for use in constructing calibration curves for each of the target analytes. Calibration curves were constructed on three consecutive days by analysis of a mixture containing each of the standard compounds at five concentrations and plotting peak area against the concentration of each reference standard (Figure 3-2). The linearity of the detector response for the standards was assessed by means of linear regression (Table 3-2).

Table 3-2 Linear ranges and correlation coefficients of the calibration curves

Anthraquinones	**Y = aX+b linear model**[a]	**r^2**	**Concentration (µg/ml)**
Rhein	Y = 60.603X - 5.6766	1.0000	2.2 - 34.8
Aloe-emodin	Y = 83.021X + 3.7335	1.0000	3.2 - 51.2
Emodin	Y = 58.187X - 1.2942	1.0000	2.7 - 43.6
Chrysophanol	Y = 85.871X + 0.1900	1.0000	3.3 - 53.0

[a] Y = peak area, X = concentration

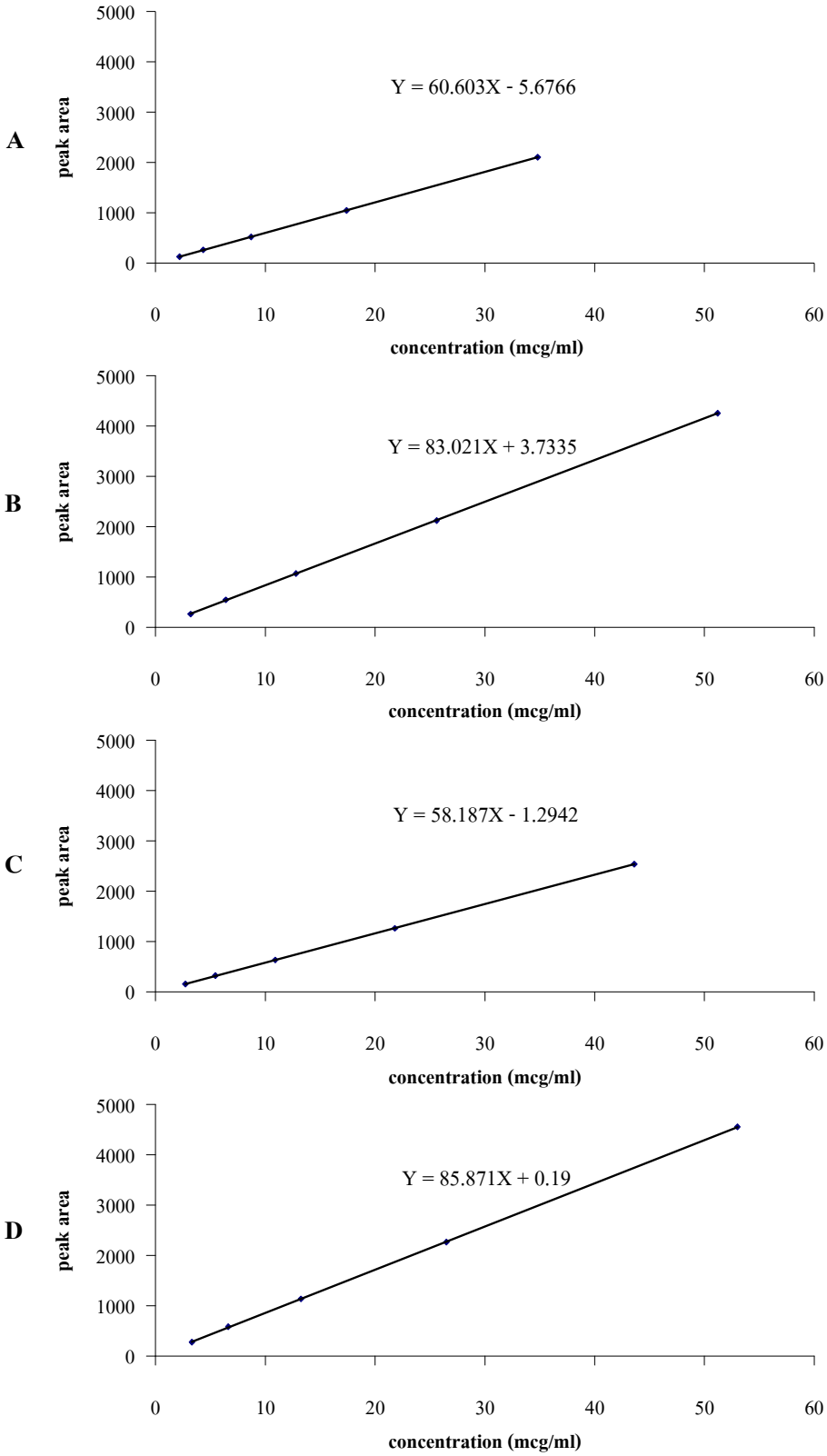

Figure 3-3 Calibration curves of standards; rhein (A), aloe-emodin (B), emodin (C), and chrysophanol (D)

3.3.4 Evaluation of antifungal activity against dermatophytes (Alcala *et al*., 1998)

3.3.4.1 Preparation of samples

The stock solution of the anthraquinone high yielding *S. alata* leaf extract was prepared at the concentration of 100 mg/ml in DMSO. The sample solutions were prepared in the concentration between 0.049 and 100 mg/ml by serial two-fold dilution. The samples were sterilized by filtration through a 0.45 µm membrane filter before testing. Clotrimazole and DMSO (1%) were used as positive and negative controls, respectively.

3.3.4.2 Preparation of test dermatophytes

Trichophyton mentagrophytes, *Trichophyton rubrum* and *Microsporum gypseum* were grown in Sabouraud dextrose agar slant. The selected colonies were mixed with sterile physiological saline and the turbidity was adjusted by adding sterile physiological saline until obtained a McFarland turbidity standard of 0.5 (10^6 colony forming units per ml).

3.3.4.3 Determination of minimum inhibition concentration

Minimum inhibition concentration (MIC) was determined using agar dilution method (Lorian, 1996). The sample solutions were diluted with Saburaud dextrose agar (1:100) to obtain the final concentrations between 0.49 and 1,000 µg/ml, and transferred into a 96-wells plate (150 µl/well). The tested dermatophytes were also diluted with Saburaud dextrose broth (1:100) to achieve 10^4 cells/ml, and transferred into the 96-wells plate (2 µl/well). The plate was incubated at 30 ºC for 7 day. The lowest concentration that did not show any growth of dermatophytes was taken as the MIC.

3.3.5 Determination of the moisture content (Subcommittee on the Establishment of the Thai Herbal Pharmacopoeia, 1998)

Moisture content of the *S. alata* leaf extract was performed using loss on drying method. The extract was accurately weighed to 0.5 g in the pan of the Sartorius Moisture Analyzer and dried at 105 ºC for 4-8 minutes until the weight was constant. The percentage loss on drying of the test sample was automatically recorded. The analyses were in triplicate.

3.3.6 Determination of the total ash content (Subcommittee on the Establishment of the Thai Herbal Pharmacopoeia, 1998)

The extract was accurately weighed to 0.5 g and placed in a tarred crucible, which was previously ignited, cooled and weighed. The sample was incinerated by gradually increasing the temperature not exceeding 450 ºC in muffle furnace until free from carbon, then cooled and weighed. The percentage of the total ash was calculated with reference to the weight of the dry powdered drug. The analyses were in triplicate.

3.3.7 Determination of the microbial contamination (British Pharmacopoeia commission, 2001)

3.3.7.1 Sample preparation

The extract was accurately weighed to 1 g and dissolved in 0.1% (v/v) peptone water 9 ml, then adjusted to 10 ml with 0.1% (v/v) peptone water. The stock solution was diluted with 0.1% (v/v) peptone water to provide five concentrations ranging from 10^{-1}-10^{-5} g/ml.

3.3.7.2 Determination of aerobic bacteria contamination

Determination of aerobic bacteria contamination was performed by pour plate method. The sample solution (1 ml) as well as melted plate count agar (15 ml to 20 ml) were

added into Petri dishes (9 cm in diameter), and mixed until homogenously. At least two Petri dishes were prepared for each level of dilution. The plates were incubated at 37 ºC for 3 days. The plates corresponding to one dilution and showing the highest number of colonies less than 300 were selected. The number of colony-forming units per gram was calculated.

3.3.7.3 Determination of *Escherichia coli* contamination

Determination of *E. coli* contamination was performed by pour plate method. The sample solution (1 ml) as well as eosin methylene blue agar (15 ml to 20 ml) were added into Petri dishes (9 cm in diameter), and mixed until homogenously. At least two Petri dishes were prepared for each level of dilution. The plates were incubated at 37 ºC for 1 day. Growth of red, non- mucoid colonies of gram-negative rods indicates the possible presence of *E. coli*. The plates corresponding to one dilution and showing the highest number of colonies less than 300 were selected. The number of colony-forming units per gram was calculated.

3.3.7.4 Determination of fungi contamination

Determination of fungi contamination was performed by pour plate method. The sample solution (1 ml) as well as Sabouraud Dextrose Agar (15 ml to 20 ml) were added into Petri dishes (9 cm in diameter), and mixed until homogenously. At least two Petri dishes were prepared for each level of dilution. The plates were incubated at 28 ºC for 5 days. The plates corresponding to one dilution and showing the highest number of colonies less than 300 were selected. The number of colony-forming units per gram was calculated.

3.3.8 Determination of solubility (British Pharmacopoeia commission, 2001)

The extract was accurately weighed to 10 mg and place in a vessel of at least 100 ml capacity. The vessel was placed in a constant temperature device, maintained at a temperature of 25 ± 0.2 ºC. Various solvents (water, DMSO, ethanol, methanol, ethyl acetate, chloroform, and hexane) were examined by adding of the strength prescribed in the monograph by increments of

10 µl, shaking frequently and vigorously for 10 minutes. Record the volume of solvent added when a clear solution was obtained. If the solution was becomes cloudy or undissolved. The sample was continuously added until 10 ml. After addition of 10 ml of solvents, the sample or parts of it remained undissolved, the experiment had to be repeated in a 100 ml volumetric flask. At lower solubilities the time required to dissolve a substance can be considerably longer, at least 24 hours should be allowed (British Pharmacopoeia commission, 2001).

Descriptive term of solubility and approximate volume of solvents required to completely dissolve a solute (in milliliters per gram of solute) are drawn as follow (Table 3-3).

Table 3-3 Solubility criteria of the extract in various solvents

Solubility term	**Volume of solvent required to dissolve 1 g of solute (ml)**
Very soluble	less than 1
Freely soluble	from 1 to 10
Soluble	from 10 to 30
Sparingly soluble	from 30 to 100
Slightly soluble	from 100 to 1,000
Very slightly soluble	from 1,000 to 10,000
Practically insoluble	more than 10,000

The term partly soluble is used to describe a mixture of which only some of the components dissolve.

3.3.9 Partition coefficient (Organization for Economic Cooperation and Development, 1995)

3.3.9.1 Pre-saturation of the solvents

Before a partition coefficient was determined, the phases of the solvent system were mutually saturated by shaking at the temperature of the experiment. It was practical to shake two large stock bottles of either *n*-octanol or water with a sufficient quantity of the other solvent for 24 hours on a mechanical shaker and then let them stand long enough to allow the phases to separate and to achieve a saturation state.

3.3.9.2 Test substance

The stock solution of the anthraquinone high-yielding *S. alata* leaf extract was prepared in *n*-octanol that pre-saturated with water to produce three concentrations of the extract. The concentration of this stock solution should be precisely determined before it is employed in the determination of the partition coefficient. This solution should be stored under conditions which ensure its stability. Triplicate test vessels containing the required, accurately measured amounts of the two solvents together with the necessary quantity of the stock solution should be prepared for each of the test conditions. The *n*-octanol phases should be measured by volume. The test vessels were placed in a reciprocator and rotated quickly through 360° about its transverse axis so that any trapped air rises through the two phases. Experience has shown that 50 such rotations are usually sufficient for the establishment of the partition equilibrium. To be certain, 100 rotations in five minutes are recommended. For the determination of the partition coefficient, it is necessary to determine the concentrations of the anthraquinones in both phases. An aliquot of each of the two phases was taken and analyzed by HPLC. The total anthraquinone content in both phases should be calculated and compared with the anthraquinone content originally introduced by this equation.

$$P_{ow} = \frac{C_{n-octanol}}{C_{water}}$$

P_{ow} = Partition coefficient

$C_{n\text{-octanol}}$ = Concentration of anthraquinones in *n*-octanol phase (µg/ml)

C_{water} = Concentration of anthraquinones in water phase (µg/ml)

3.3.10 Stability tests

3.3.10.1 Effect of light on stability of the extract

The anthraquinone high-yielding *S. alata* leaf extracts were weighed to 100 mg and kept in well-closed containers. The extracts were then stored at room temperature (30 ± 2 ºC) and under exposed to light (36-watt fluorescent lamp and 40 cm distance from the containers). An aliquot of each sample was taken at 0, 1, 2, 3, 4, 6, 8, 12, and 17 weeks and subjected to quantitative analysis of the anthraquinones using HPLC. The experiments were in triplicate.

3.3.10.2 Effect of temperature on stability of the extract

The anthraquinone high-yielding *S. alata* leaf extracts were weighed to 100 mg and kept in well-closed containers, protected from light. The extracts were then stored at 4 ± 2 ºC and room temperature (30 ± 2 ºC). Prepare for each temperature at three replicates. An aliquot of each sample was taken at 0, 1, 2, 3, 4, 6, 8, 12, and 17 weeks and subjected to quantitative analysis of the anthraquinones using HPLC. The experiments were in triplicate.

3.3.10.3 Effect of accelerated condition for stability of the extract

The anthraquinone high-yielding *S. alata* leaf extracts were weighed to 100 mg and kept in well-closed containers, protected from light. The extracts were then stored in a stability chamber at 45 ºC, 75% humidity for 4 month. An aliquot of each sample was taken at 0,

1, 2, 3, 4, 6, 8, 12, and 17 weeks and subjected to quantitative analysis of the anthraquinones using HPLC. The experiments were in triplicate.

3.3.10.4 Effect of pH on stability of the extract

The anthraquinone high-yielding *S. alata* leaf extracts were accurately weighed to 100 mg and dissolved in phosphate buffer solution pH 5.5, 7.0, and 8.0. The sample solutions were kept in well-closed containers, protected from light and stored at room temperature (30 ± 2 ºC) for 4 month. An aliquot of each sample was taken at 0, 1, 2, 3, 4, 6, 8, 12, and 17 weeks and subjected to quantitative analysis of the anthraquinones using HPLC. The experiments were in triplicate.

3.3.11 Statistic

Values are expressed as mean ± S.D. Data were analyzed by student *t*-test. The level of statistical significance was taken at $P < 0.05$.