

Antimicrobial Activity and Wound Healing Potential on Infected Rat of *Gracilaria Changii* Methanolic Extract

S. Sasidharan ^{a,b,*}, I. Darah ^a, and K. Jain^c

^aSchool of Biological Sciences, Universiti Sains Malaysia, Penang, Malaysia.

^bDepartment of Biotechnology, Faculty of Applied Sciences, Asian Institute of Medicine, Science and Technology, Kedah, Malaysia. E-mail: srisasidharan@yahoo.com

^cSchool of Chemical Sciences, Universiti Sains Malaysia, Penang, Malaysia.

Summary

The methanol extract of *Gracilaria changii* was analyzed for antiyeast activity against the *Candida albicans* and for infected wound healing activity. Formulated ointment was topically applied on the infected wound. The rate of wound healing was determined and histological analysis was performed in order to assess the healing pattern. The *G. changii* extract showed a significant antifungal activity against *Candida albicans* with an MIC of 1.56 mg/ml. *G. changii* extract treated rats showed, better wound closure, while increasing granulation tissue formation, increased collagenation, re-epithelialization and reconstitution of skin appendages, pertaining to wound healing. This investigation showed that the *G. changii* crude extract has a positive effect on the healing of infected skin wounds.

KEY WORDS: *Candida albicans*; *Gracilaria changii*; antifungal activity; wound healing activity

Introduction

Malaysia is endowed naturally with a very rich algae life. Among the algae with therapeutic properties in Malaysia, the *Gracillaria changii* of *Gracilariaceae* family found predominantly in mangrove areas of Malaysia. It grows abundantly in hot, humid equatorial countries such as Malaysia and Thailand. Although at one time it was considered to be a foodstuff because the algae are edible, the economic importance of the algae is now beginning to attract the attention of researchers. This is because the algae may have special properties including healing properties. The *Gracilaria sp* are widely used in the traditional medicine in Malaysia. Malay people administer the agar derived from *Gracilaria*, internally for coughs and in consumption (1). Beside that, *Gracilaria sp* boiled in vinegar used to treat swollen knees and unhealthy sores (1).

Wounds are physical injuries that result in an opening or breaking of the skin. Proper healing of wounds is essential for the restoration of disrupted anatomical continuity and disturbed functional status of the skin. Wound infections are most common in developing countries because of poor hygienic conditions. *Candida albicans*, *Staphylococcus aureus*, *Escherichia coli*, and *Pseudomonas aeruginosa* are some important organisms causing wound infection (2). Repair of injured tissues occurs as a sequence of events, which includes inflammation, proliferation and migration of different cell types (3). Infected wounds heal more slowly and have an increased incidence of scarring because of the toxic substance release by the pathogens (4)

A wide range of antibiotics are being used at present for treating wound infections, but they are now proved to have adverse effects in the human body, also these pathogens developed resistance to the antibiotics targeted against them (5). In view of this, so much recent attention has been paid to extracts of biologically active compounds isolated from plant species used in herbal medicine (6).

Topical applications of an extract with antimicrobial properties to the infected skin of the patient might significantly help in infected wound healing activity and protect the infected tissues from damage caused by the pathogens. In this context, antimicrobial potential of *G. changii* extract against dermatophyte *Candida albicans* was studied before the extract evaluate for wound healing potential.

Materials and Methods

Alga sample and extract preparation

Fresh *G. changii* sample was collected from Pantai Morib, Selangor Malaysia in January 2003. Approximately 100 g of dried algae sample was added to 200 ml of methanol and allowed to soak for 4 days. Removal of dried algae sample from extract done by filtration through cheesecloth, and the filtrate was further concentrated using a rotary evaporator. The dried extract were then re-dissolved in 10% DMSO (v/v) to yield the solution containing 100 mg of extract per ml solution for antimicrobial activity studies.

Microorganism

Candida albicans (B3648) was used as the test organism and was obtained from the laboratory stock culture. The yeast was cultured in Sabouraud Dextrose agar at 30°C for 24h. The stock culture was maintained on Sabouraud Dextrose agar slants at 4 °C.

Antimicrobial activity

The antimicrobial activity test was performed by disk diffusion method (7). Sterile blank discs (6-mm diameter) were impregnated with 100 mg of the extract per ml from the *G. changii*. Extract impregnated discs were placed in Muller-Hinton agar plates inoculated with the test organism and incubated at 37°C for 24 to 48 h. Discs with plain 10% DMSO were used as negative control and Miconazole Nitrate (30 µg/ml) served as positive controls. The minimal inhibitory concentration (MIC) of the extract was determined by the broth tube dilution method (7). Double dilution was made from higher dilution 100 mg/ml to lower dilution in a series of test tubes. Each tube was inoculated with 10⁵ CFU/ml microbial suspensions. The tubes were incubated at 37°C for overnight and the Minimum Inhibition Concentration value was determined as the lowest concentration of the crude extract in the broth medium that inhibited the visible growth of the test microorganism and also confirmed by plating. The sample was tested in triplicate.

Wound healing activity

Crude extract formulation

A 10% (w/w) crude extract was prepared by mixing the extract in yellow soft paraffin obtained from Pharmacy (8)

Animal

Rat weighing between 150 to 200 g from the Animal House of the University of Science Malaysia were used. The rats were placed in a room with controlled cycles of 12 h of light and

12 h of darkness; light went on at 7 am. Water and food were provided to animals ad libitum. Experiments were conducted in accordance with the internationally accepted principles for laboratory animal use and care (EEC Directive of 1986; 86/609/EEC).

In vivo wound healing activity

Rats were anaesthetised using 45 mg/kg of sodium thiopental given by the intraperitoneal route. Full thickness wound (1.5 × 1.5 cm) was made on a shaved dorsal area; the wounds were infected with the 0.1 ml *Candida albicans* (approximately 10⁹ CFU). After 24 h, the wounds were treated topically with 10% formulated crude extract, while the control rat were treated only with yellow soft paraffin containing same quantity of methanol without extract for 12 days. The decrease in wound diameters during the healing process was measured with an analytical pakimeter.

Microbiological examination of skin tissue

Superficial skin tissues were excised on days 4, 8 and 12. One mg of excised tissue was placed in 10 ml of sterile saline, vortex for few minutes and the total yeast cells count was analyzed by serial dilution method.

Histological analysis

Skin tissues were collected and transferred to 10% neutral buffered formalin (NBF) for 24 h at 4°C. The formalin fixed tissues were dehydrated through grades of alcohol and cleared in xylene and then embedded in paraffin wax (58–60° mp). The molds were labeled and stored until use. There were 5 to 7 µm sections deparaffinized and stained with hematoxylin following counterstained with eosine (9).

Statistical analysis

All results have been expressed as mean ± S.D. and the results were compared statistically by Student's independent *t*-test and Tukey-Kramer test using SPSS software (student version 7.01). The *P* value <0.05 was considered statistically significant.

Results

Antimicrobial activity

Anti yeast activity results is shown in Table 1. The extract was inhibitory against the yeast tested. The agar dilution method showed the Minimal Inhibitory Concentration values of 1.56 mg/ml.

Wound healing activity

The total yeast cells count from the skin tissue on different days of analysis is shown in Figure 1. Application of algae extract resulted in a diminishing level of total yeast cells count in the infected wound. There was major reduction from 10^9 CFU to 10^5 CFU in extract treated rat on day 4 when compared to yellow soft paraffin treated rat, which records 10^6 CFU.

Table 1. Antimicrobial Activity (Zone Of Inhibition And Mic^a) of Crude Methanolic Extract of the *Gracilaria Changii* Compared to Commercial Antibiotic Miconazole Nitrate.

Yeast	Methanol extract		
	Zone of Inhibition (mm) ^b		MIC ^c (mg/ml)
	Crude Extract	Miconazole Nitrate	
<i>Candida albicans</i> (B3648)	18.00	20.00	1.56

^a Agar dilution method, mean value $N = 3$.

^b The values (average of triplicate) are diameter of zone of inhibition at 100 mg/ml crude extract and 30 µg/ml Miconazole Nitrate.

^c MIC: Minimal Inhibitory Concentration of the *G. changii* extract.

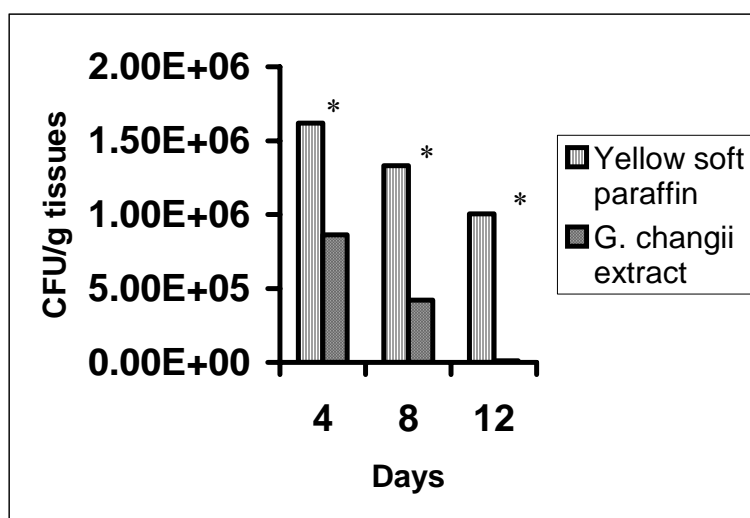


Figure 1. Yeast cells count present in the skin tissue of infected Rat ($n = 6$). *Significant difference at $P < 0.05$.

The CFU recoded for 12 days was 10^3 CFU in extract treated rat and 10^6 CFU in yellow soft paraffin treated rat. The cicatrizing activity of animals treated with 10% *G. changii* extract in the rat model is presented in Fig. 2. Animals treated with 10% *G. changii* extract resulted in a faster reduction of the wound diameter than the animals treated only with yellow soft paraffin. At day 12 of the experiment, the animals treated with 10% *G. changii* extract wounds were smaller than the animals treated only with yellow soft paraffin wounds ($P < 0.001$, Tukey–Kramer) (Figure 2).

Figure 3 to 5 shows the histology of control, infected and extract treated skin tissue of rat. Complete loss of superficial epithelium and inflammatory exudates were observed in the infected rat skin compare to the control rat (Figure 3B). Extract treated rat skins have shown marked epithelialization and new blood vessel formation. Furthermore, the number of hair follicle and sebaceous glands (SG) in the control rat were less than extract-treated rat in the healed area (Figure 3C). Histological studies of granulation tissue of the *G. changii* extract treated animals showed a significant increase in the collagen deposition with few macrophages (Figure 5) compared with the infected rat (Figure 4)

Discussion and Conclusion

One of the major factors affecting non-healing infected wounds is the presence and persistence of pathogen burden, which can interfere with the normal process of healing (10). Since there is a definite role of applications of a crude extract with antimicrobial properties in skin infected patient (10), the antimicrobial activity of the *G. changii* extract also studied. The results indicate that the crude extract possesses good antiomicrobial activity by inhibiting *Candida albicans* with the MIC value 1.56 mg/ml (Table I). This again validates the effective wound healing activity of *G. changii* extract. Thus the infected wound healing activity observed may be because of the good antimicrobial activity showed by the crude extract which protects the infected tissues from damage course by the pathogens.

Topical (applied directly to the skin) applications of drugs are effective in wound healing because of its availability at the infected wound site compared to systemic application (11, 12). The ability of microorganisms in the wound bed to create massive damage depends on the virulence capacity of the organism, the amount of inoculums present in the wound site along with the host immune response.

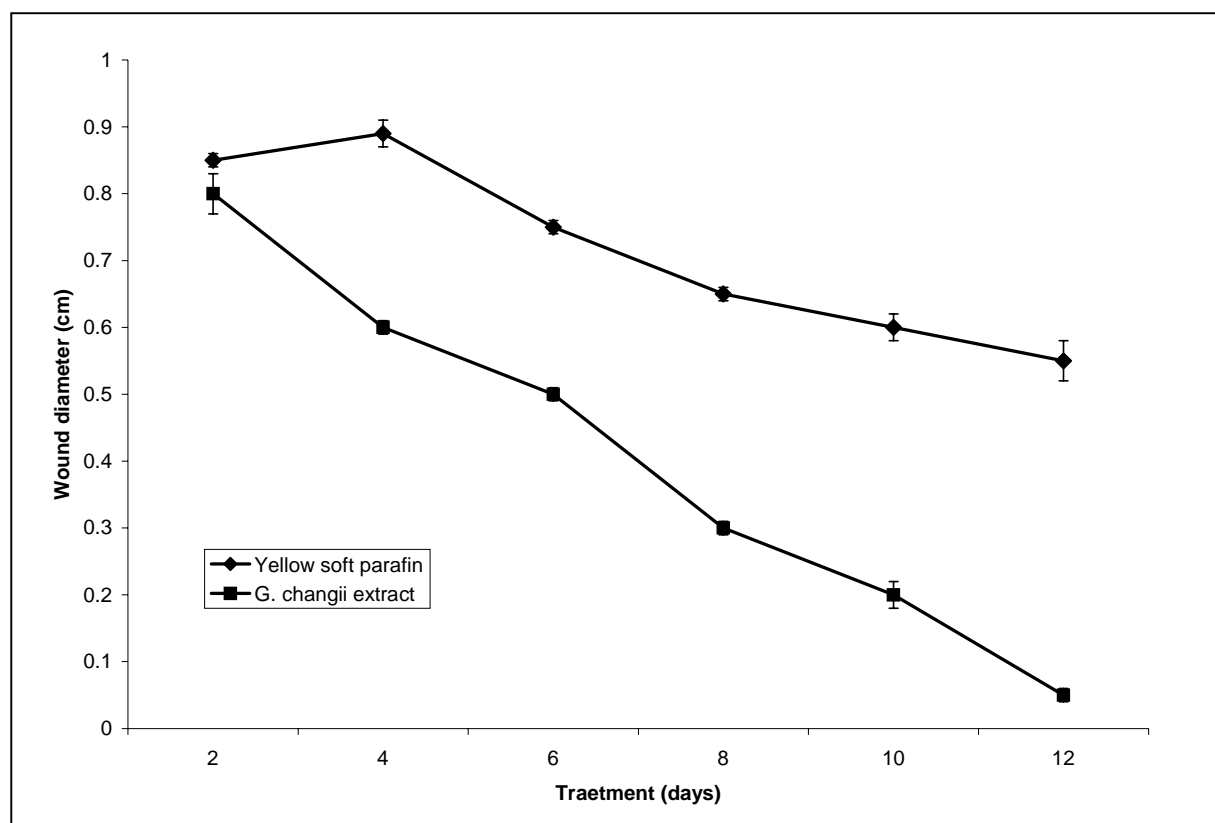


Figure 2. Cicatrizing activity of *G. changii* in skin lesions of animals inoculated with 1×10^9 CFU/mL of *C. albicans*. Data represent control animals (♦) and animals treated with 10% *G. changii* extract (■).

In this study, significant wound closure was observed in *G. changii* treated rat. Significant reductions of yeast count in the treated rat were observed on day 12 from 10^9 CFU to 10^3 CFU/g tissues, further confirms the effectiveness of *G. changii* treatment. Well-correlated microbiological examinations of skin tissue studies were observed with histological examinations. A close examination of skin tissue sections revealed that the tissue regeneration occurred in the treated group compared to control wounds. Epidermal regeneration in treated rat also confirmed that the extract had a positive effect toward epithelialization. The treatment also helps the regeneration of skin appendages such as sebaceous gland and hair follicles. In conclusion, the crude extract of *G. changii* exhibited significant pro-healing activity in the infected wound when topically applied on rat.

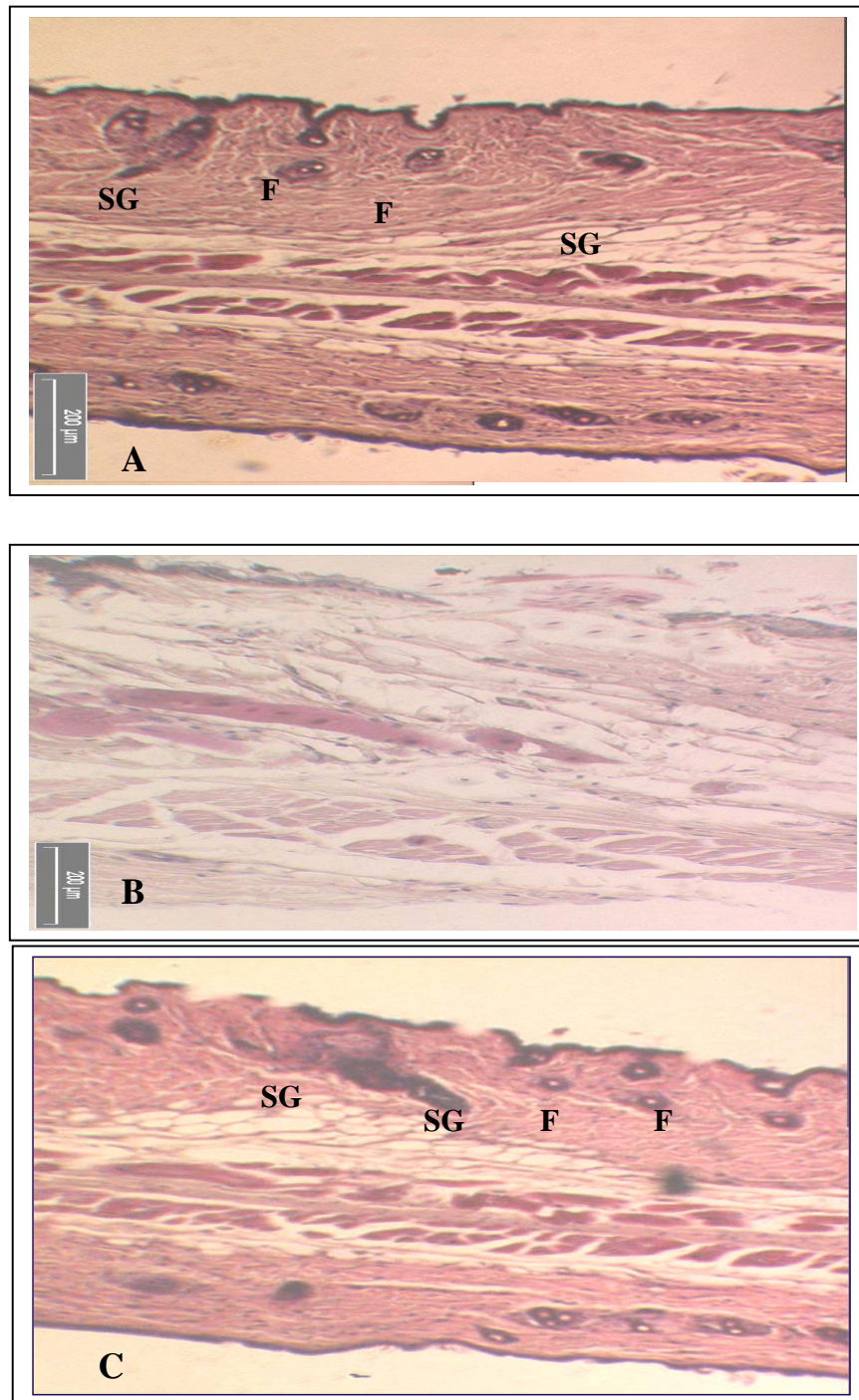


Figure 3. Hematoxylin and eosine stained skin tissue section of the control rat (A), infected rat (B) and *G. changii* extract-treated rat is observed on day 12 (C). The sebaceous gland (SG), coarse hair follicle (C) and bundle of collagen fibers were reconstituted in the healed area. The number of hair follicle (F) and sebaceous glands (SG) in the control rat were less than extract-treated rat in the healed area.

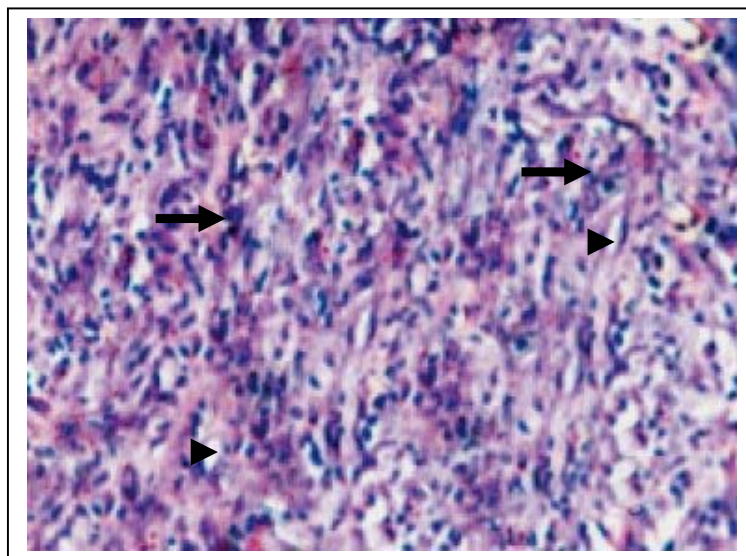


Figure 4. Histological section of granuloma tissue of infected rat showing decreased collagen deposition (Arrows head) and aggregation of macrophages (Arrow) (H & E, 150 \times).

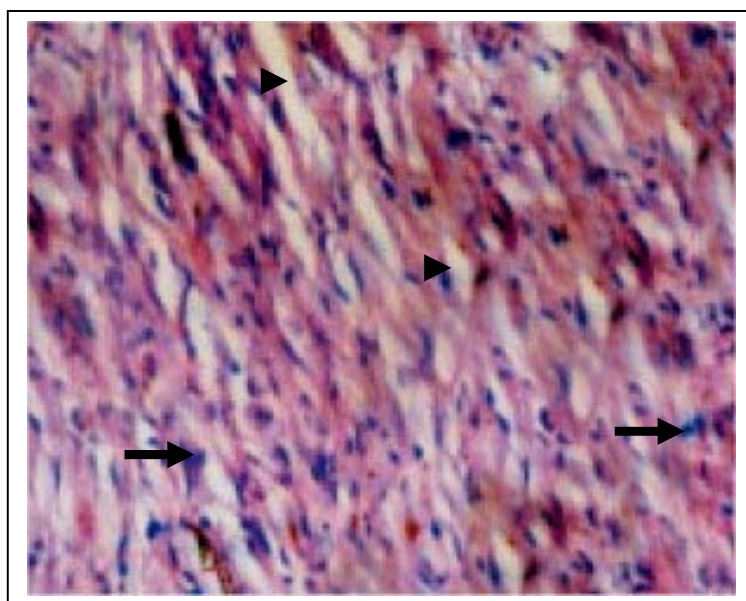


Figure 5. Histological section of granuloma tissue of the animal treated with methanolic extract of *G. changii* showing increased collagen deposition (Arrows head) and less macrophages (Arrow) (H & E, 150 \times).

The results presented in this report are a preliminary communication. Further studies using the extract of *G. changii* are indicated to identify optimal treatment routes, dosages, and which constituent(s) may be conferring its wound-healing potential on this natural product. That there is potential to heal wounds in an animal model is exciting, and future work should address what it offers wound healing in both veterinary and clinical practice.

References

1. Burkill IH. A Dictionary of the Economic Products of the Malay Peninsula. Government of Malaysia and Singapore by the Ministry of Agriculture and cooperatives, Kuala Lumpur. 1935; pp. 1127.
2. Kumar MS, Sripriya R, Raghavan HV, Sehgal PK. Wound Healing Potential of *Cassia fistula* on Infected Albino Rat Model. *J. Surg. Res.* 2006; 131: 283-289.
3. Sidhu GS, Mani H, Gaddipati JP, et al. Curcumin enhances wound healing in streptozotocin induced diabetic rats and genetically diabetic rat. *Wound Repair Regen.* 1999; 7: 362–374.
4. Robson MC. Wound infection. A failure of wound healing caused by an imbalance of bacteria. *Surg. Clin. North Am.* 1997; 77: 637- 650.
5. Ghaffari A, Miller CC, McMullin B, Ghahary A. Potential application of gaseous nitric oxide as a topical antimicrobial agent. *Nitric oxide* 2006; 14: 39-44.
6. Essawi T, Srouf M. Screening of some Palestinian medicinal plants for antibacterial activity. *J. Ethnopharmacol.* 2000; 70: 343-349.
7. NCCLS. Performance standards for anti-microbial susceptibility testing, eleventh informational supplement, Document M100-S11, NCCLS, Wayne (USA). 2001.
8. Carter SL. Cooper and Gunn's Dispensing for pharmaceutical students. CBS Publisher and Distributors, Delhi, 1987; pp.199–200.
9. Mcmanus JGA, Mowry RW. Staining methods: Histological and histochemical. Harper and Row, New York. 1984.
10. Stewart PS, Costerton JW. Antibiotic resistance of bacteria in biofilms. *Lancet* 2001; 358: 135–138.
11. Poste G. Liposomes targeting in vivo: Problems and opportunities. *Biology Cell* 1983; 47: 19–39.
12. Grislain L, Couvreur P, Lenaerts V, Roland M, Deprez-Decampeneere A, Speiser P. Pharmacokinetics and distribution of a biodegradable drug-carrier. *Int. J. Pharmacogn.* 1983; 15: 335–345.