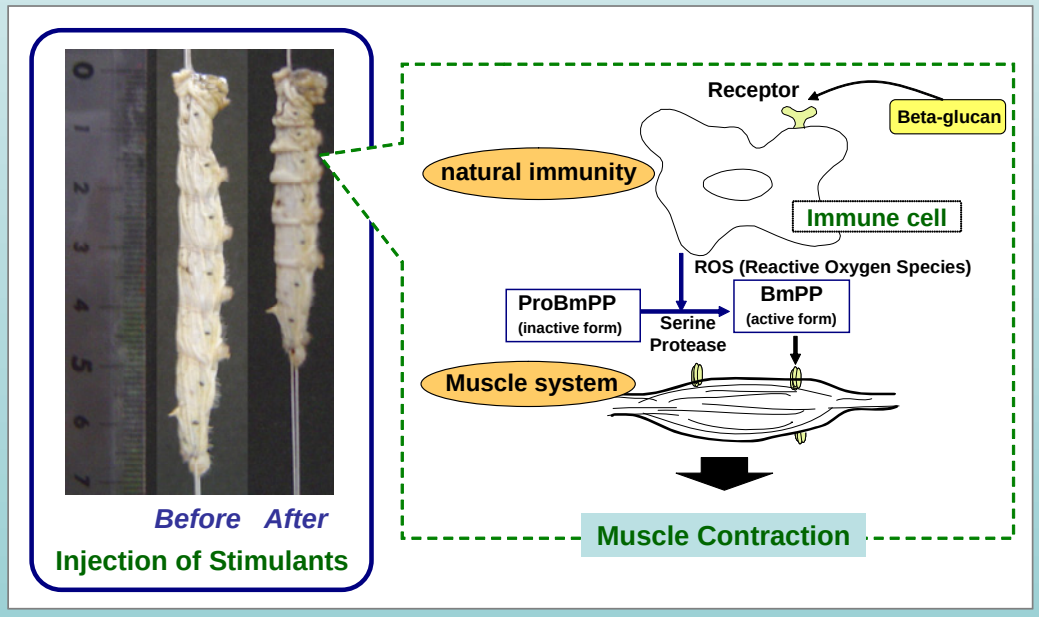
Drug Discoveries & Therapeutics

ISSN 1881-7831 Online ISSN 1881-784X
Volume 3, Number 5, October 2009
www.ddtjournal.com



Shandong University China-Japan Cooperation Center for Drug Discovery & Screen
International Advancement Center for Medicine & Health Research

Drug Discoveries & Therapeutics

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Subject Coverage: Basic and clinical pharmaceutical research including Pharmaceutical and therapeutical chemistry, Pharmacology, Pharmacy, Pharmacokinetics, Industrial pharmacy, Pharmaceutical manufacturing, Pharmaceutical technology, Drug delivery, Toxicology, and Traditional herb medicine.

Language: English
Issues/Year: 6
Published by: IACMHR and SDU-DDSC
ISSN: 1881-7831 (**Online ISSN** 1881-784X)
CODEN: DDTRBX

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Review

Intellectual property strategies for university spinoffs in the development of new drugs

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ABSTRACT: We will explain a new business model for university spinoffs involving the development of two types of products. The first are highly innovative, such as new drugs, while the second are typically less difficult to develop, such as functional foods. It is our belief that development of the second type of product can help solve the financial problems and stabilize management of Academic Start-ups. The key to development of several different types of products is accumulation of knowledge consisting not only of technical know-how, *e.g.* tips for use in injection, but also ideas obtained by researchers with the potential for future applications. Examination of the features of venture enterprises which have arisen from universities suggests that inventors, who are also professors, should participate in such start-ups.

Keywords: University spinoff, new drug, financial problem, second product

1. Introduction

We of Genome Pharmaceuticals Institute aim to develop new drugs and functional health foods using the silkworm as an experimental animal. We are a bio-venture company featuring cooperation between industry and academia that was established by Nobukazu Sekimizu, MBA, LLM, MOT and Dr. Kazuhisa Sekimizu, Professor of The University of Tokyo, in December 2000. The goal of our company is commercialization of the results of Professor Sekimizu's studies. Our unique feature is use of the silkworm as an experimental animal for the development of new drugs and health foods (*1-4*). We will explain the advantages of this technology.

There are two purposes of this paper. First, we will introduce advantages of the silkworm model for the study of bacterial infection and antibiotics. We will also show other results of our recent studies using silkworm models (*5*). Second, we will discuss an academic start-up model useful for overcoming funding problems and passing through the so-called "Valley of Death".

2. Assumption

Here, we explain the need for animal models in the development of new drugs, using as examples "Chemical A" and "Pathogenic bacteria B". Here, in the test tube (*in vitro*), "Chemical A" is effective against "Pathogenic bacteria B". However, if Chemical A is not stable in the animal body (*in vivo*), it will never exhibit therapeutic effects. A good drug should always be stable in the body. The stability of chemicals in the animal body is determined by 4 factors: Absorption, Distribution, Metabolism, and Excretion (ADME; Figure 1). Usually, medicines taken by mouth are absorbed through the intestine, enter the blood, and are distributed to the various organs of the body. They are mainly metabolized in the liver, and finally excreted. So, even when a chemical compound is for theoretical reasons considered likely to be effective, there are many cases in which it will be excreted without being absorbed or converted to new compounds called metabolites which do not exhibit medical effects. In

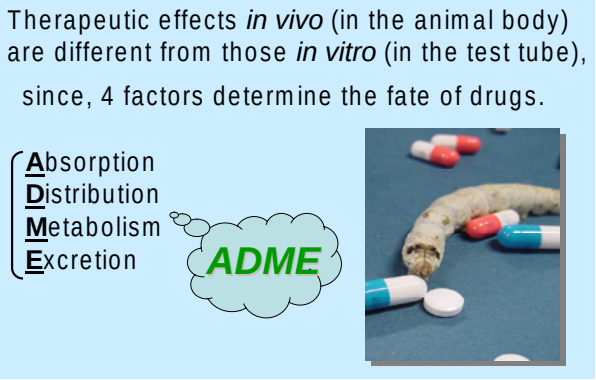

Figure 1. Need for animal models in development of new drugs.

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such cases, the compound will not be medically useful. It is therefore necessary to determine how medicines behave in the body in pharmacokinetic studies involving ADME.

Silkworms are, despite their appearance, biologically similar to humans in many respects, such as possessing analogous tissues or organs, having similar sensitivities to pathogens, and exhibiting comparable drug effects (*3*).

Here we show the advantages of the silkworm as an animal model (Figure 2). The first is cost which is much lower than with mice. The second is ethical problems. It is difficult to use mammals given the various issues related to animal protection. With silkworms, however, there are few ethical problems and no biohazards. In addition, it is easy to inject samples into their body fluids and gut accurately since they move little.

3. Technical aspects

3.1. *Silkworm models of infection for antibiotic screening*

We now consider technical aspects, as related to antibiotics (*1,2*). There are many candidate compounds for development as antibiotics. This makes searching for antibiotics among them extremely difficult. This challenging area is an ideal opportunity for establishing a company.

This study is based on a model of bacterial infection (*2*). As shown in Figure 3, Left is the control which

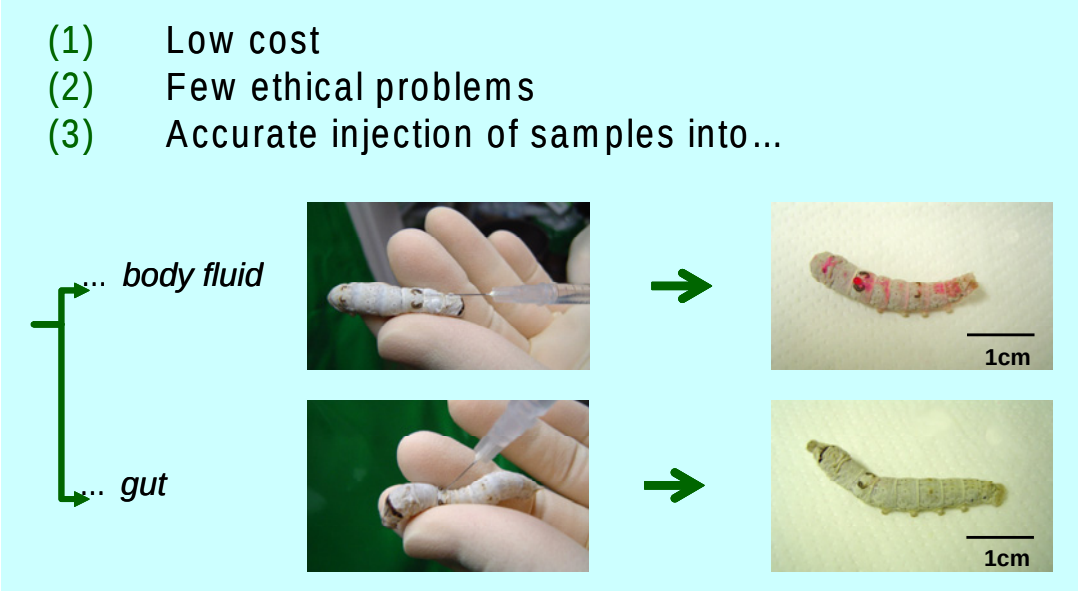
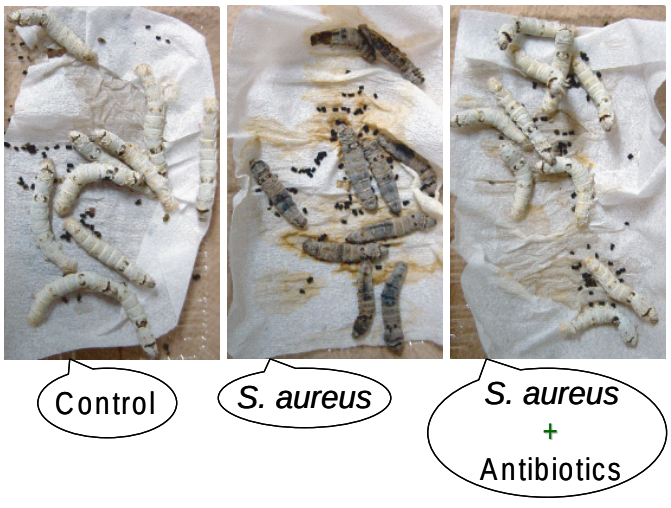


Figure 2. Advantages of the silkworm as an animal model.



Drugs	ED_{50}(μg/g · animal) [Strain · · · *S.aureus*]	
	silkworm	Mouse (reference)
teicoplanin	0.3	0.1
vancomycin	0.3	1
minocycline	3.9	1
flomoxef	0.2	0.3
linezolid	9	4

ED_{50}s of antibiotics

Figure 3. Therapeutic effects of antibiotics.

exhibits normal growth. The Center shows the condition in which bacteria (*Staphylococcus aureus*) were injected into silkworms which died. However, with treatment with appropriate antibiotics, they are still alive, at Right. Thus, although bacteria can kill silkworms, the infected silkworms can be successfully treated with antibiotics. Based on these findings, we are confident that this model will be very useful for searching for antibiotics, since the Effective Dose is the median dose that produces the desired pharmacological effect of a drug. In this experiment, the ED_{50} is the dosage in which half of the silkworms are cured and the others die.

Traditional methods of screening for antibiotics are shown in Figure 4. Normally, among many candidates, antibacterial activity is first examined in the test tube (*in vitro*); such tests may offer many discouraging attempts and finally yield candidate compounds. However, when compounds with antibacterial activity are injected into animals, in many cases there may be no therapeutic effects, depending on ADME, as already noted. Efficiency in screening candidates is thus of crucial importance to developing antibiotics. By comparison, the silkworm can be treated quickly and easily, at low cost, making it possible to experiment with a large number of candidates at once. This makes the silkworm an ideal tool for this type of study.

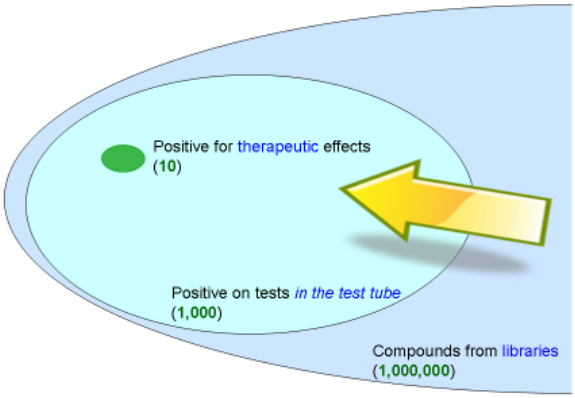


Figure 4. Traditional method for screening of antibiotics.

3.2. *Screening for stimulants of natural immunity*

We next consider screening for stimulants of innate immunity using the silkworm. There are two different systems of protection in mammals: acquired immunity, which depends on antibodies, and innate immunity, which is independent of them. Stimulation of innate immunity is useful for killing cancer cells, since this system is able to recognize them. Research can therefore be performed to determine the types of foods or agricultural products that can stimulate innate immunity.

For this purpose, we use silkworm muscle contraction as an index (*4*). If a sample stimulates innate immunity, then the silkworm muscle contracts.

This is a unique phenomenon, in which the muscle of the silkworm contracts and the silkworm's length gradually decreases (Figure 5). When stimulants of innate immunity activate immune cells, reactive oxygen species (ROS) are released. These act on serine proteases, which activate BmPP (Bombyx mori paralytic peptide), which in turn induces muscle

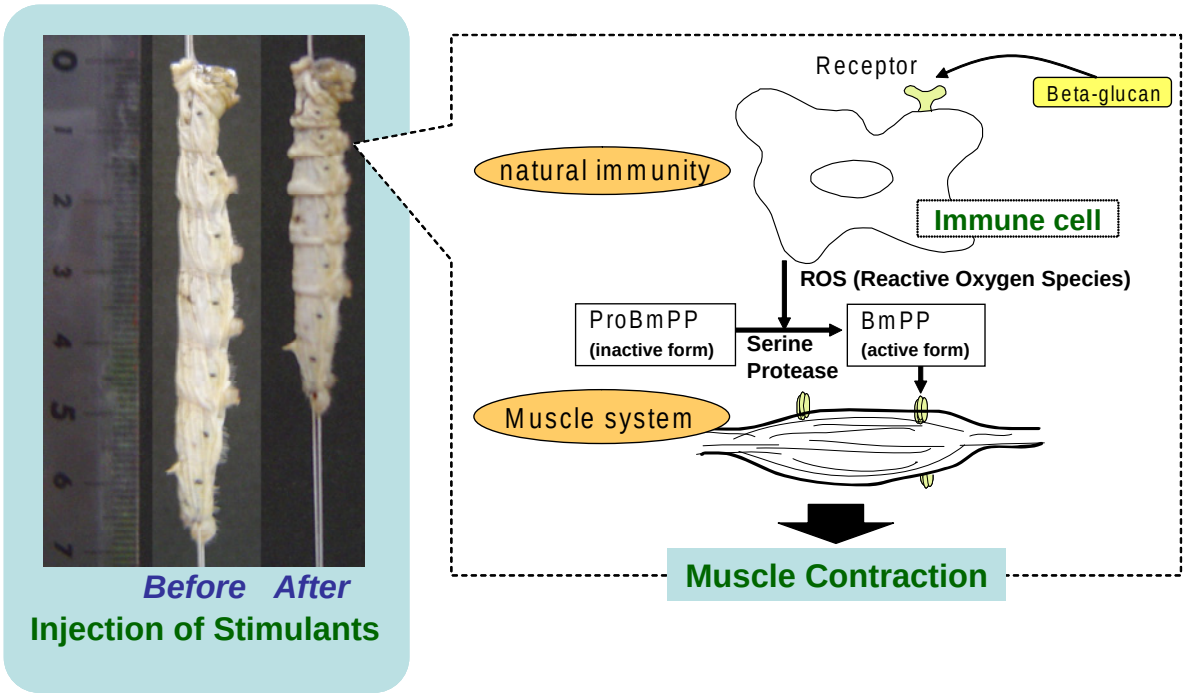


Figure 5. Muscle contraction of silkworms induced by stimulation of innate immunity.

contraction. Therefore, by observing the circumstances of muscle contraction valuable stimulants of innate immunity can be discovered.

Stimulation of innate immunity by some agricultural products and foods using this method is shown in Figure 6. In this way, we have developed effective food products, and have also developed a method for increasing specific activity.

3.3. *Other models and tests*

We have proposed safety tests for agricultural products, foods, and environmental agents using silkworms. Usually, in searching for poisonous materials in food, one chemical technique is required for each type of poison. However, testing for all substances that may be poisonous is not possible with this method. Silkworms, on the other hand, can function as a low-cost barometer for whether certain foods are dangerous for humans. The silkworm can thus function as a sort of "coal-mine canary" in such testing.

4. A proposed business model for academic start-ups

We have thus far considered some technical features of our work. We now consider a proposal for a new business model for academic start-ups. We will begin with aspects of academic start-ups in Japan. Then, based on our experience, we will propose a new model useful for overcoming problems with funding.

The numbers of new academic start-ups are shown in Figure 7. The horizontal axis shows years while the vertical axis shows the annual number of new academic start-ups. These data are based on the report "Plan for Improvement of Initial Conditions of Academic Start-Ups" (*6*).

Looking at changes over time, it can be seen that the number of Japanese start-ups has increased to a fair extent, and approached that of the U.S. However, it is still only half that of the U.S.

Cumulative numbers of Academic Start-Ups in Japan are shown in Figure 8 (*6*). The Japanese Government initiated the "1,000 Academic Start-Ups Plan" in 2001. The target was met in 2004 with several types of support from the Government. It should, however, be noted that the number of Start-Ups has recently been decreasing, due to decreases in government support and a decline in investment by venture capital.

We now consider some financial aspects (Figure 9). Funding is especially important for start-ups.

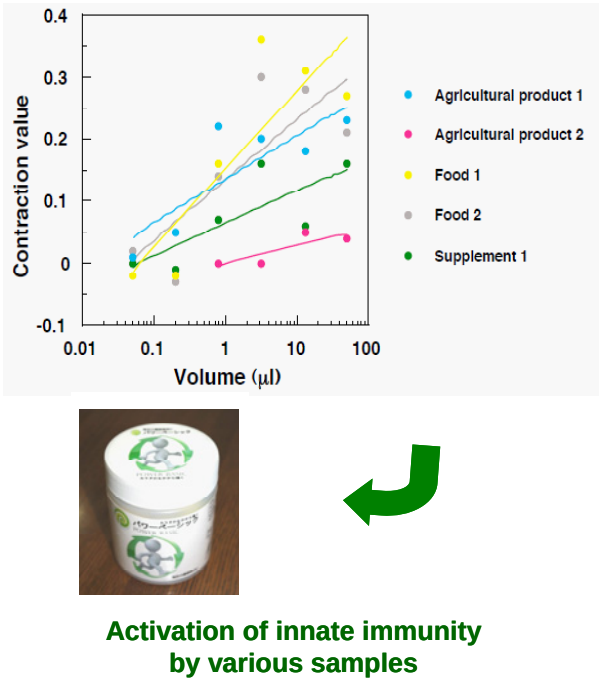


Figure 6. Induction of muscle contraction by activation of innate immunity.

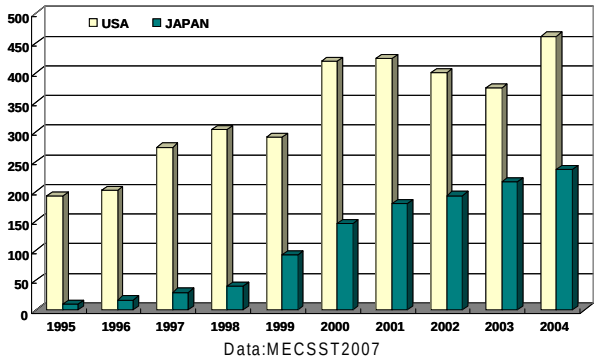


Figure 7. Number of new academic start-ups.

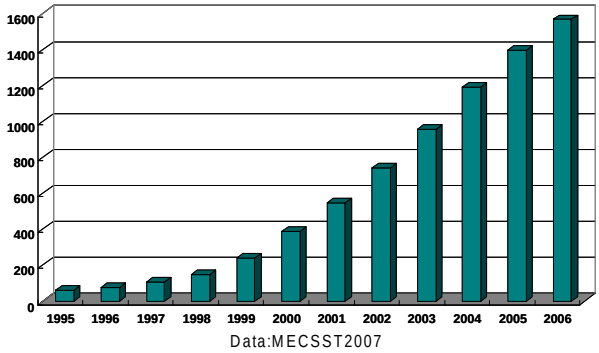


Figure 8. Cumulative numbers of academic start-ups in Japan.

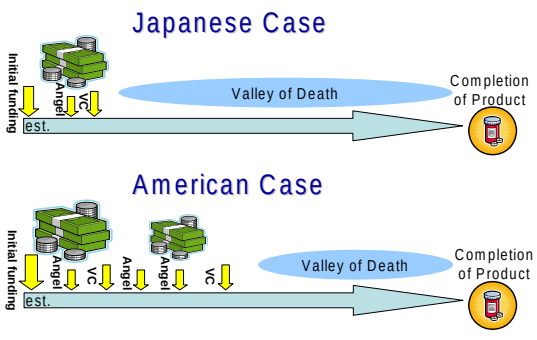


Figure 9. Model of funding for start-ups.

However, many venture enterprises have difficulty obtaining adequate research funds. They may eventually fall into the so-called "Valley of Death" and may go bankrupt. Here, the "Valley of Death" is the period until products are developed, after funding from venture capitalists, the "Angels". In Japan, since we do not have enough "Angels", or venture capitalists, the length of the "Valley of Death" is longer than in the U.S. (Figure 9).

We believe that the development of "second products" is one of the solutions to this problem. Quick development of such products can improve financial problems and stabilize management.

Next, we explain the key to the creation of second products. We believe that it involves the accumulation of knowledge consisting not only of technical know-how, *e.g.* tips for use in injection, but also ideas obtained by researchers with the potential for future applications. Since this type of knowledge is not usually able to receive patent protection, its real value is often unappreciated. We therefore simply call this type of knowledge "Know-How" here.

Examination of the features of venture enterprises which have arisen from universities suggests that inventors who are also professors have often participated in such start-ups (Figure 10). We also believe that this participation is of two types: cases in which the technology has been moved to a new enterprise by a TLO or something similar to that, and cases in which a professor continues to participate in a venture *as it is*.

In the former case, the model at Upper panel in Figure 10, since only the transfer of technology is involved, product development is limited to that closely related to the transferred technology. For example, during the development of health foods for humans, high-performance food for animals was also fortunately developed.

The latter case applies to Genome Pharmaceuticals Institute. In this case, the model at Lower panel in this figure, both "Know-How" and technology are involved in obtaining patentable products. It is thus possible to develop a wide variety of products using a Professor's new ideas along with "Know-How". This means that we can also develop second products unrelated to the technology used for first products.

Does any change occur with the existence of second products? We consider what happens. We can choose technologies which are easy to commercialize after taking marketing research into consideration, and sell the associated products. This will then provide funding for other products more difficult to develop.

In venture enterprises, especially those characterized by advanced technology, it is vital to company survival that funding continue until completion of the first main product (Figure 11). The development of second products should shorten the Valley of Death and make the venture enterprise viable.

We will now introduce our business model in greater detail (Figure 12). In the context of the model presented, in our company the first products are antibiotics and the second products are functional health foods. Although these products share use of the silkworm in their development, they are unrelated as regards to the technologies involved in their production. Notably, the second products are market-oriented and

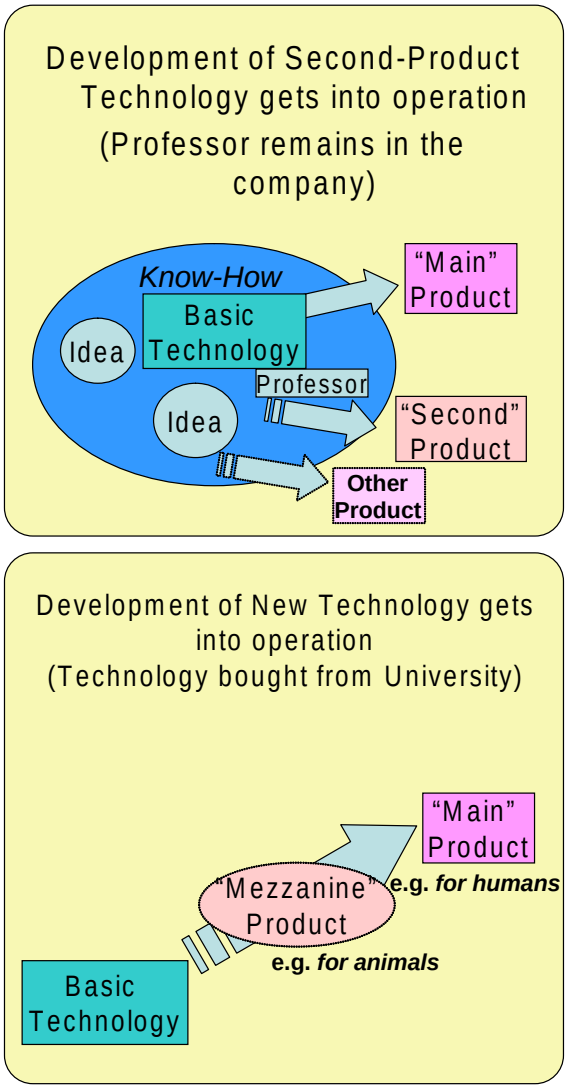


Figure 10. Intellectual property strategies for academic start-ups.

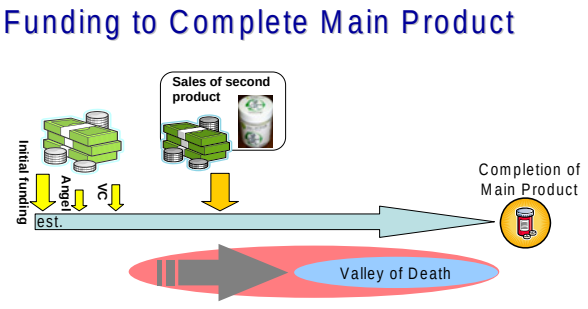


Figure 11. A proposed funding model.

tend to be closer to consumers.

This scheme is very attractive to our collaborating companies, and of course to us, since we usually need the cooperation of other pharmaceutical companies which have production lines enabling commercialization of products. It can be seen in this figure that this cooperation yields a variety of options.

We therefore believe that these various sources of financing assist cash flow in our company and enable continued development of antibiotics.

Data in Figure 13 is based on the "Basic report of academic start-ups" prepared by the Japanese Ministry of Economy, Trade, and Industry (*7*). According to this report, 13.8% of Academic Japanese start-ups develop second products. Of course, it might be thought that venture enterprises without sufficient funding would be unable to develop more than one product, and thus these data mean that few venture enterprises are able to develop second products. However, though they are few in number, they do exist.

Let us briefly consider the research situation in the United States. According to research by Scott Shane, development of general-purpose technology has good effects on cash-flow management. Such technology has many commercial applications and is close to consumers. Thus, this general-purpose technology and

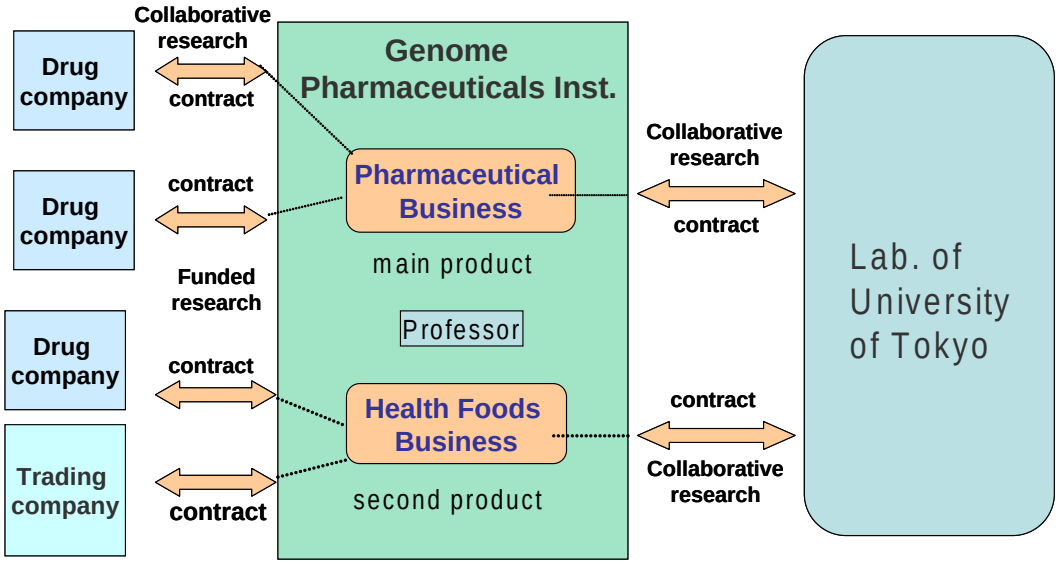


Figure 12. Our business model.

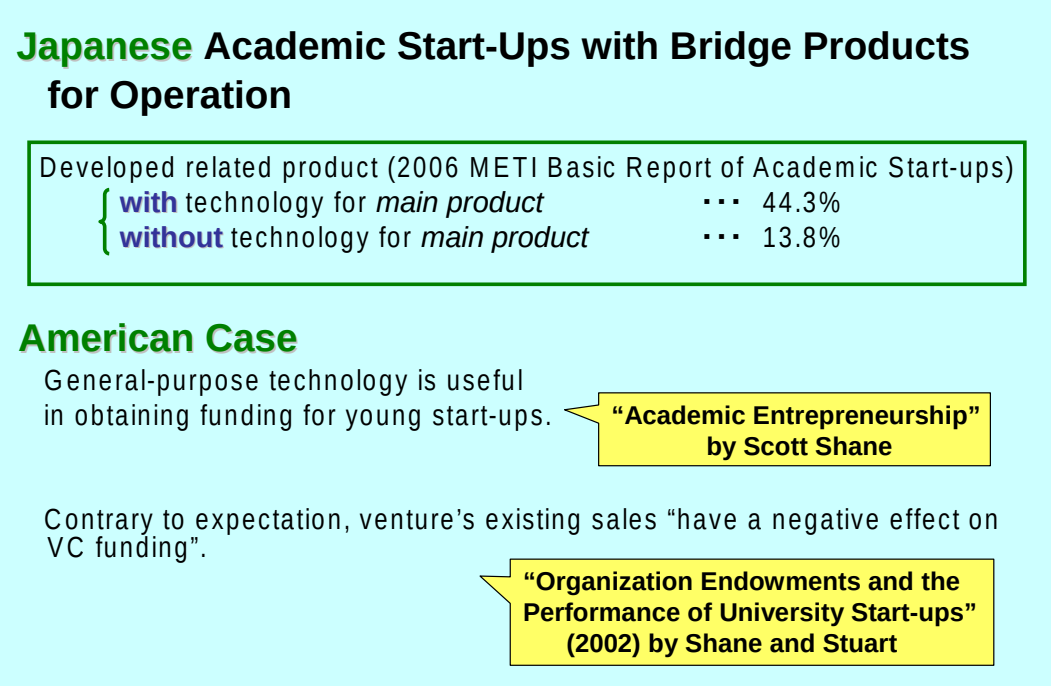


Figure 13. Obtaining sufficient funding for main products.

the second products we have explained are similar, and various sources of financing derived from product development are quite useful (*8*).

Shane also studied 134 ventures born at MIT, and concluded that, "Contrary to expectation, venture's existing sales have a negative effect on VC funding". He found the likely explanation for this result to be that "firms with substantial sales that have not yet received VC funding may not be actively looking for funding from venture capitalists because they are able to support their operations with internally generated cash flows" (*9*). We are confident that these findings support the importance of developing second products at an early stage of venture enterprises.

The overall conclusions of this paper are that, in the case of Academic Start-Ups: 1) professors *who invented* the original technology should, to the extent possible, continue to participate after the establishment of a start-up, 2) start-ups should obtain "Know-How" technology, and 3) start-ups should develop two or more products as a deliberate management strategy. This will enable passing through the Valley of Death and the development of very ambitious technology.

Acknowledgments

This study was supported in part by the Program for Promotion of Fundamental Studies in Health Sciences of the National Institute of Biomedical Innovation (NIBIO).

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(*Received July 7, 2009; Revised July 13, 2009; Accepted July 14, 2009*)

Review

Microarray analysis of gene expression in medicinal plant research

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ABSTRACT: Expression profiling analysis offers great opportunities for the identification of novel molecular targets, drug discovery, development, and validation. The beauty of microarray analysis of gene expression is that it can be used to screen the expression of tens of thousands of genes in parallel and to identify appropriate molecular targets for therapeutic intervention. Toward identifying novel therapeutic options, natural products, notably from medicinal plants used in traditional Chinese medicine (TCM), have been thoroughly investigated. Increased knowledge of the molecular mechanisms of TCM-derived drugs could be achieved through application of modern molecular technologies including transcript profiling. In the present review, we introduce a brief introduction to the field of microarray technology and disclose its role in target identification and validation. Moreover, we provide examples for applications regarding molecular target discovery in medicinal plants derived TCM. This could be an attractive strategy for the development of novel and improved therapeutics.

Keywords: Microarray, traditional Chinese medicine

1. Introduction

The 30,000-40,000 genes of the human genome project constitute the likely possible therapeutic targets for medicine (*1,2*). The continuous gain of information on the sequence of entire genomes has increasingly challenged researchers to identify the functions of these genes and their interaction pathways in health and disease (*3*). Ways to measure gene expression include: northern blotting, serial analysis of gene expression (SAGE) differential display, and dot-blot analysis. All previously mentioned techniques are inappropriate for the analysis of the expression of multiple genes at once. The emergence of new tools enables investigators to address previously intractable problems and to reveal novel potential targets for therapies. Nowadays, microarray technology can be used to test thousands of genes at the same time and to analyze the expression of those genes. Microscopic arrays of large sets of DNA sequences immobilized on solid substrates are becoming a standard technology applied in research laboratories all over the world. Since its first application (*4*), microarray technologies have been productively functional in almost each and every aspect of biomedical research (*5-9*). Arrays are ordered samples of DNA sequences with each sample representing a particular gene. These arrays can then be assayed for changes in gene expression of the representative genes after various treatments, various conditions or tissue origins, thus providing a functional aspect from sequence information in a given sample (*10*).

DNA microarrays are precious tools in the identification or quantification of many specific DNA sequences in complex nucleic acid samples (*11*) therefore, they have been used to identify cardinal aspects of growth and development, as well as to explore the underlying genetic causes of many human diseases (*5*). Microarray based studies have enormous potential in the exploration of disease processes such as cancer (*6*) and in drug design, response, and development (*7*). In addition, the technology is applied to a considerable extent to investigate several pathological conditions, such as inflammation (*12*), breast cancer (*8*), colon cancer (*13*), and pulmonary fibrosis (*14*). Microarrays, comprising thousands of genes at once, generate gene expression 'profiles'. Such profiles are comprehensive patterns that are characteristic of the responses of cells or tissues to drug treatment, to environmental changes, to differentiation into specialized tissues, or to dedifferentiation into tumor cells. Thus, microarrays document detailed responses of cells and tissues to both disease and the intended and unintended effects of drug treatments and hence facilitate medical research (*3,7,15*). Moreover, such trials expand the size of existing gene families, discover new patterns of coordinated gene expression across gene families, and disclose entirely new classes of genes.

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2. Microarray design

Most arrays used for gene expression profiling and molecular targets analysis in the biological sciences today can be divided into two groups: complementary DNA (cDNA) and oligonucleotide microarrays (*16*). This division refers to characteristics of the probes, the individual pieces of gene-specific DNA that are spotted on the array surface. cDNA probes are usually products of the polymerase chain reaction (PCR) generated from cDNA libraries or genomic DNA, and are typically more than 150 nucleotides in length. On the other hand, synthetic oligonucleotides have a maximum length of around 80 nucleotides, thus conferring greater specificity among members of gene families (*17,18*). Array fabrication involves either spotting of presynthesized probes using highly precise robotic spotters, or in situ synthesis on glass slides (*16*). High-density spotted cDNA microarrays can contain up to 40,000 probes on a conventional microscope slide. In contrast, oligonucleotide arrays, consisting of gene-specific oligonucleotides, are synthesized directly onto a solid surface by either photolithography or ink-jet technology (*19*). Probes can be designed that represent the most unique part of a given transcript, making the detection of closely related genes possible (*16*). A major advantage of oligonucleotide arrays over cDNA arrays is that they require no handling and tracking of cDNA resources (*19*). Furthermore, the use of synthetic reagents in the manufacturing of oligonucleotide arrays minimizes variation among arrays, thus ensuring a high degree of reproducibility between microarray experiments.

Sample preparation is similar for cDNA and oligonucleotide microarrays. In both cases, mRNA is extracted, purified, reverse transcribed to cDNA, labeled, and hybridized to probes on the surface of the array slide (*16*). Two fluorescent dyes allow cDNA from two treatment populations to be labeled with different colors (Figure 1). When mixed and hybridized to the same array, the differentially labeled cDNA results in competitive binding of the target to the probes on the array. After hybridization, the slide is imaged using a confocal laser scanner and fluorescence

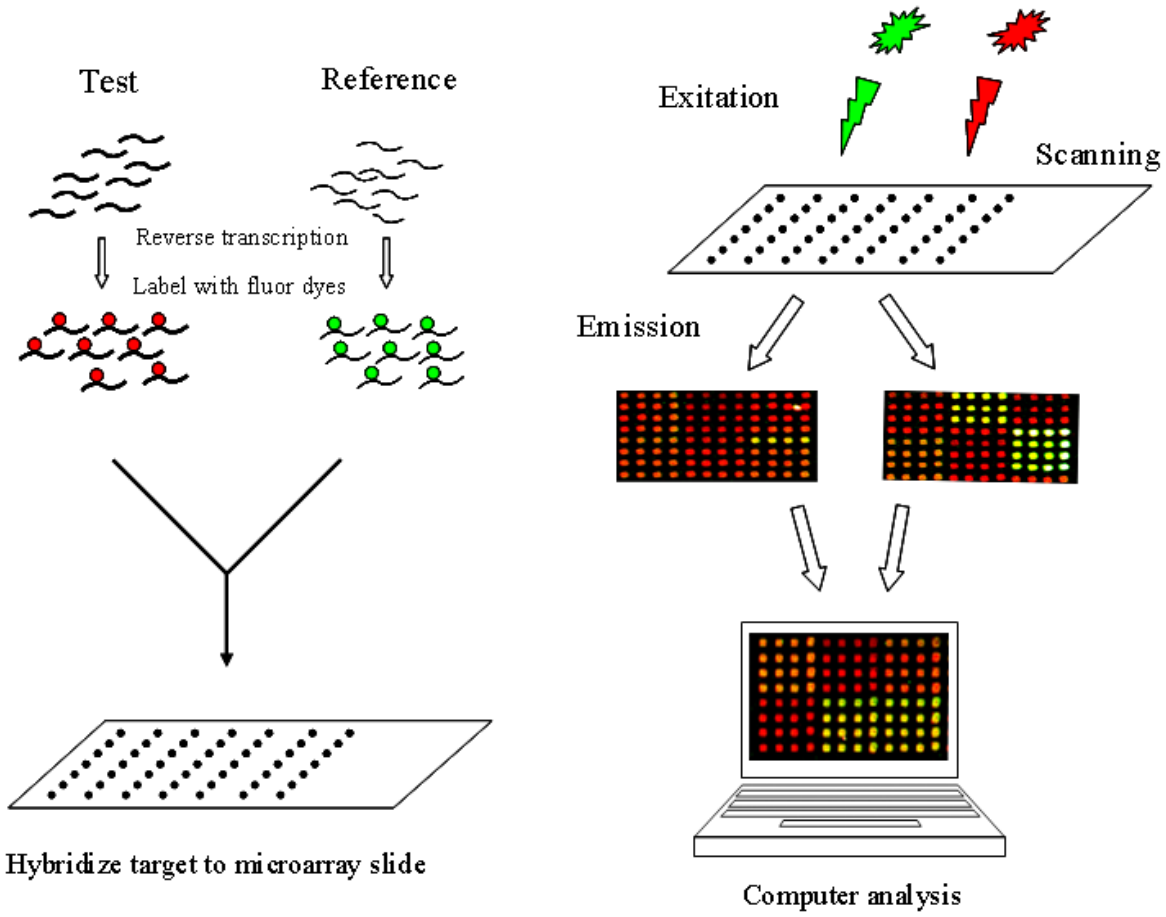

Figure 1. cDNA microarray procedure. Templates for genes of interest are amplified by PCR then printed on coated glass microscope slides. Total RNA from both the test and reference sample is fluorescently labeled with different fluor dyes. The fluorescent targets are pooled and allowed to hybridize to the clones on the array slide. Laser excitation of the incorporated targets yields an emission with a characteristic spectrum, which is measured using a scanning confocal laser microscope. Monochrome images are imported into software in which the images are merged. Data from a single hybridization experiment is viewed as a normalized ratio. In addition, data from multiple experiments can be examined using any number of data mining tools.

measurements are made separately for each dye at each spot on the array (Figure 1). This dual labeling enables the ratio of transcript levels for each gene on the array to be determined (*16,20*). Specialized software and data management tools are then used for data extraction, normalization, filtering, and analysis (*21*).

3. Identification of potential therapeutic targets

Recently, the search for disclosing therapeutic targets and discovering new therapeutic options has relied mainly on approaches based on large-scale genomics including the sequencing of expressed-sequence tags (ESTs), serial analysis of gene expression (SAGE) differential display, homology cloning, and related approaches (*22*). These EST sequencing efforts resulted in availability of databases containing information on the majority of human genes (*23*), most of which were of unknown therapeutic significance. Although these bioinformatics approaches, based on sequence homology and structural motifs, are conveniently and extraordinarily valuable, investigators still need an experimental approach to prioritize potential therapeutic targets. Microarray technology provides an excellent solution because it facilitates the identification of novel potential therapeutic targets from tens of thousands of genes in a single experiment. Using microarray technology to screen for differentially expressed genes is widely acknowledged as a valuable approach in the target discovery process (Table 1).

Forward pharmacology is an approach that can assist in identifying the mechanisms of action of poorly understood drugs or other bioactive compounds. It involves using microarrays to monitor mRNA changes induced by drugs or other bioactive compounds in order to deduce previously unknown actions of these compounds (*7*). For example, Hughes *et al.* (*24*) assessed the levels of more than 6,000 transcripts including 279 gene knockout strains under 300 experimental conditions. The global expression profiles of these diverse experimental conditions were then used for monitoring the effects of several drugs on yeast where dyclonine, a topical anesthetic of unknown action, was found to be implicated in perturbation of the pathway of ergosterol metabolism. This hypothesis was further confirmed by similar observations of the effects of haloperidol on the same pathway (*24*).

4. Microarray analysis after therapeutic target validation

Several approaches can be taken to validate and to prioritize candidate therapeutic targets once a shortlist has been identified. Among the most important approaches to validate and to prioritize candidate therapeutic targets are gene knockout and knock-in strategies in cells, model organisms and mice. In this context, microarrays can be used to assess the primary and secondary consequences of genetic manipulation.

5. Microarray in TCM

TCM has been used for thousands of years in China and is currently widely practiced in Chinese cancer centers. Nevertheless, it is a brand new area for formal scientific evaluation. TCM represents a holistic approach and lacks high-quality scientific evidence for its effectiveness. Therefore, TCM is frequently regarded with some skepticism by western academic medicine (*25*). Since DNA chip technologies has been introduced into medical sciences (*4,26*), many researchers have applied this technology to pharmacological analyses (*10,12*). Recent studies tried to cross the bridge between TCM and modern western medicine through applying modern technologies to identify molecular mechanisms and novel targets involved in TCM action on different

Table 1. Types of microarray and examples of current applications

Microarray Type	Sample	Objective	Applications
Expression profiling (cDNA, oligonucleotide arrays)	mRNA	To determine the changes in gene expression between different disease or different treatment conditions	Tumor classification Identification of prognostic and predictive markers Drug responce
SNP analysis	Genomic DNA	To detect mutations or polymorphisms in a gene sequence	Determination of genetic predisposition to a disease Monitoring of disease progression Drug development
Resequencing arrays	Genomic DNA	To sequence portions of the genome	Evaluation of germline mutations in individuals Identification of somatic mutations in cancer
Array CGH	Genomic DNA	To identify genetic amplifications, deletions or copy number changes	Tumor classification Risk assessment Development of prognostic and predictive markers
Protein microarrays	Antibody	To determine the changes in protein expression between different disease or different treatment conditions	Tumor classification Identification of prognostic and predictive markers Drug development

Abbreviations: CGH, comparative genomic hybridization; SNP, single nucleotide polymorphisms.

disorders. Some of these studies are presented here to pinpoint the significance of applying high throughput technologies in medicinal plant research.

6. Berberine and microarray

Berberine, a natural isoquinoline alkaloid, has been found in many clinically important plants including *Berberis aquifolium* (Oregon grape), *Berberis vulgaris* (barberry), *Coptis chinensi*s (goldenthread or Coptis), and *Coscinium fenestratum* (*27,28*). Berberine exhibits a wide range of biochemical and pharmacological activities (*29,30*). Berberine has been reported to be used as an anti-arrhythmia, anti-hypertension, anti-diarrhea, and anti-inflammatory agent (*31-33*). Additionally, the natural product was reported to possess an anti-tumor activity against different tumor types (*34-36*). To better understand the physiological actions and the molecular targets of berberine in pancreatic cancer, Iizuka *et al.* (*37*) used an oligonucleotide array which contained approximately 11,000 genes and undertook a gene expression profiling study to monitor the expression changes associated with sensitivities to berberine and *Coptidis rhizoma* in 8 human pancreatic cancer cell lines. From the oligonucleotide array data, 20 and 13 genes with strong correlations ($r^2 > 0.81$) to ID_{50} values for berberine and *C. rhizoma* were selected, respectively. Among these 33 genes, the levels of expression of 12 were correlated with the ID_{50} values of both berberine and *C. rhizoma.* They concluded that these genes are associated with the tumor-killing activity of berberine in *C. rhizoma* (*37*). Moreover, expression of the remaining 21 genes was correlated with the ID_{50} value of either purified berberine or *C. rhizoma*. Such an approach allowed common and distinct genes responsible for anti-proliferative activities of purified berberine and *C. rhizoma* to be identified (*37*).

Recently, DNA microarray chips were utilized to investigate the transcriptional changes of *Y. pestis*, a gram negative coccobacillar bacterium that causes plague, in response to berberine. The analysis was done after exposing *Y. pestis* to berberine. A total of 360 genes were differentially expressed; 333 genes were up-regulated, and 27 were down-regulated. The up-regulation of genes that encode proteins involved in metabolism was a remarkable change. Genes encoding cellular envelope and transport/binding functions represented the majority of the altered genes in addition to a number of genes of unknown encoding or unassigned functions. Furthermore, a number of genes related to iron uptake were also induced (*38*).

7. PC-SPES and microarray

PC-SPES is a preparation of eight Chinese herbs used in the treatment of prostate cancer and exhibits a promising antiproliferative and antitumor activity *in vivo* and *in vitro* in diverse cancer types (*39*). Applying DNA chip technology, Bonham *et al.* investigated the molecular effects of the herbal compound PC-SPES on prostate carcinoma cells. cDNA microarray analysis was utilized to identify expression profiling changes in LNCaP prostate carcinoma cells treated with PC-SPES and estrogenic agents including diethylstilbestrol. Interestingly, PC-SPES altered the expression of 156 genes following 24 h of exposure. Of particular interest, transcripts encoding cell cycle-regulatory proteins, alpha- and beta-tubulins, and the androgen receptor were significantly down-regulated. After comparing the gene expression profiles patterns resulting from these treatments, they concluded that the herbal preparation PC-SPES exhibits activities distinct from those of diethylstilbestrol and suggested that alterations in specific genes involved in modulating cell structure, cell cycle, and androgen response could be responsible for PC-SPES-mediated cytotoxicity (*40*).

8. Curcumin and microarray

Curcumin (diferuloylmethane), a major chemical component of turmeric (*Curcuma longa*), is used as a spice to give a specific flavor and yellow color to curry. It has also been used as a cosmetic and in some medical preparations (*41*). Curcumin has been shown to display anticarcinogenic properties in a mouse model system, as indicated by its ability to inhibit phorbol ester-induced skin tumors (*42*). In addition to its anticarcinogenic effect, curcumin exhibits antioxidant, antiangiogenic, antiproliferative, and anti-inflammatory properties (*43*). The previously mentioned effects of curcumin may be mediated by its inhibitory effects on a host of cell-signaling factors, including c-Myc, transcription factor AP-1, NF-κB, protein kinase C, Egr-1, epidermal growth factor receptor tyrosine kinase, c-Jun *N*-terminal kinase, protein serine/threonine kinases, protein tyrosine kinases, and IκB kinase (*42,44,45*).

A microarray analysis of gene expression profiles were used to characterize the anti-invasive mechanisms of curcumin in the highly invasive lung adenocarcinoma cells (CL1-5) (*46*). Using microarray chips containing 9,600 PCR-amplified cDNA fragments, 81 genes were down-regulated and 71 genes were up-regulated after curcumin treatment. Interestingly, below sublethal concentrations of curcumin (10 μM), several invasion-related genes were down-regulated, including: neuronal cell adhesion molecule (0.54-fold), matrix metalloproteinase 14 (MMP14; 0.65-fold), and integrins α6 (0.67-fold) and β4 (0.63-fold). In addition, several heat-shock proteins (Hsp) [Hsp27 (2.78-fold), Hsp70 (3.75-fold), and Hsp40-like protein (3.21-fold)] were up-regulated by curcumin treatment.

Using whole-genome microarrays, Nones *et al.* (*47*) investigated the effects of dietary curcumin on

colonic inflammation and gene expression in multidrug resistant gene-deficient (*mdr1a*$^{-/-}$) mice, a model of inflammatory bowel diseases (*47*). Microarray and pathway analyses suggested that the effect of dietary curcumin on colon inflammation could be mediated *via* an induction of xenobiotic metabolism and suppression of pro-inflammatory pathways, probably mediated by pregnane X receptor (Pxr) and peroxisome proliferator-activated receptor α (Ppara) activation of retinoid X receptor (Rxr). These results indicated the potential role of global gene expression and pathway analyses to study and better understand the effect of foods in modulating colonic inflammation (*47*).

Yan C *et al.* undertook a transcriptional profiling study to identify novel targets of curcumin. A cDNA array comprised of 12,625 probes was used to compare total RNA extracted from curcumin-treated and untreated MDA-1986 cells for differential gene expression. 202 up-regulated mRNAs and 505 down-regulated transcripts were identified. The proapoptotic activating transcription factor 3 (ATF3) was significantly up-regulated > 4-fold. In addition, two negative regulators of growth control [antagonizer of myc transcriptional activity (Mad) and p27kip1] were induced 68- and 3-fold, respectively. Furthermore, two dual-activity phosphatases (CL 100 and MKP-5), which inactivate the c-jun-NH_2-kinases, showed augmented expression, matching the reduced expression of the upstream activators of c-jun-NH_2-kinase (MEKK and MKK4). Of the down-regulated genes, the expression of Frizzled-1 (Wnt receptor) was strongly suppressed (8-fold). Additionally, two genes implicated in growth control (*K-sam*, encoding the keratinocyte growth factor receptor, and *HER3*) as well as the E2F-5 transcription factor, which regulates genes controlling cell proliferation, also showed decreased expression. Moreover, they identified activating transcription factor 3 (AFT3) as a novel contributor to the proapoptotic effect of curcumin (*48*). Sentrix Human WG-6 BeadChips were used to perform a large-scale gene-expression profiling during curcumin-triggered apoptosis (8-36 h) in follicular lymphoma HF4.9 cells (*49*). The comprehensive transcriptional response included differential expression of genes encoding apoptotic signaling proteins, transcription and splicing factors, tumor and metastasis suppressors, proteins involved in regulation of cell adhesion, lymphoid development, migration (*e.g.*, CXCR4) or B-cell activation (*e.g.,* CD20), and others (*49*).

In another study, to extend the knowledge on pathways or molecular targets already reported to be affected by curcumin (cell cycle arrest, phase-II genes) and to explore potential new candidate genes and pathways that could play a role in colon cancer prevention, a gene expression analysis in response to curcumin treatment was investigated in two human colon cancer cell lines (HT29 and Caco-2). Using cDNA microarrays containing four thousand human genes, HT29 colon cancer cells were exposed to two different concentrations of curcumin and gene expression changes were monitored in time (3, 6, 12, 24, and 48 h). Changes in gene expression after short-term treatment (3 or 6 h) with curcumin were also investigated in a second cell type, Caco-2 cells. Gene expression changes (> 1.5-fold) were observed at all time points. HT29 cells were more sensitive to curcumin than Caco-2 cells. Early response genes were involved in signal transduction, cell cycle, gene transcription, DNA repair, xenobiotic metabolism, and cell adhesion. A number of cell cycle genes, among them several that have a role in transition through the G_2/M phase, were modulated in HT29 cells after curcumin treatment. Furthermore, the observed changes in G_2/M cell cycle arrest genes were confirmed by flow cytometry. Moreover, they showed that some cytochrome P450 genes were downregulated by curcumin in both cell lines. In addition, curcumin affected expression of metallothionein genes, p53, tubulin genes, and other genes involved in colon carcinogenesis (*50*).

Ramachandran *et al.* compared the expression profiles of apoptotic genes induced by curcumin in the MCF-7 human breast cancer cell line and the MCF-10A mammary epithelial cell line (*51*). Microarray hybridization of Clontech apoptotic arrays which consisted of 214 apoptosis-associated genes was performed. Of the 214 apoptosis-associated genes, the expression of 104 genes was significantly altered after curcumin exposure. They reported that gene expression was altered up to 14-fold in MCF-7 as compared to MCF-10A (up to 1.5-fold) and concluded that the effect of curcumin was higher in MCF-7 cells compared to the MCF-10A mammary epithelial cell line. In MCF-7 cells, curcumin up-regulated 22 genes and down-regulated 17 genes. The up-regulated genes included CRAF1, GADD45, TRAF6, HIAP1, CASP2, CASP1, CASP3, CASP4, and TRAP3. The down-regulated genes included TNFR, TRIAL, PKB, and TNFRSF5 (*51*).

At the microRNA level, array analysis showed that curcumin alters the expression profiles of microRNAs in human pancreatic cancer cells. An oligonucleotide microarray chip was used to profile microRNA (miRNA) expression in pancreatic cells treated with curcumin. Curcumin altered miRNA expression in human pancreatic cells, up-regulating miRNA-22, and down-regulating miRNA-199a. They suggested that the biological effects of curcumin may be mediated through modulation of miRNA expression (*52*).

9. Artesunate and microarray

Artesunate (ART) is a semisynthetic derivative of artemisinin, the active principle of *Artemisia annua* L. ART and other artemisinin derivatives are novel drugs

in the treatment of malaria (*53*). Large clinical studies with malaria patients showed that ART is well tolerated, with insignificant side effects (*54*). In addition to the well known antimalarial activity of ART, we have previously identified a profound cytotoxic action of ART against cancer cell lines of different tumor types (*55*).

Previously, we identified mRNA expression profiles associated with the response of tumor cells to ART, Arteether, and Artemether (artemisinin derivatives) (*56*) and performed correlation and hierarchical cluster analyses of the inhibition concentration 50% (IC_{50}) values and mRNA expression levels of 464 genes deposited in the database of the National Cancer Institute (*57*). The mRNA expression of 208 out of 464 genes (45%) correlated significantly with IC_{50} values of at least one artemisinin derivative. These genes belong to different biological classes (oncogenes, apoptosis-regulating genes, drug resistance genes, tumor suppressor genes, DNA damage and repair genes, proliferation-associated genes and cytokines). Hierarchical cluster analysis identified two different gene clusters. One cluster contained genes which correlated significantly to all artemisinin derivatives. The second cluster contained genes differentially associated with the response of artemisinin derivatives to cancer cells.

Additionally, in order to confirm that Genome-wide microarray analyses provide an attractive approach to identify genes involved in the response of cancer cells to natural products, Anfosso *et al.* (*58*) investigated artemisinin and six derivatives and found a significant correlation of the sensitivity of these compounds to genes regulating tumor angiogenesis. Indeed, among mechanisms governing effects of artemisinins towards tumors is the inhibition of angiogenesis as shown by us and others (*59,60*).

Recently, we undertook a gene expression profiling study to identify novel molecular targets modulating the effect of artesunate on MiaPaCa-2 and BxPC-3 pancreatic cancer cell lines. cDNA microarray chips containing some 7,000 genes representing apoptotic, angiogenic, growth factors, anti-apoptotic, and metastasis-associated genes were used. Results showed that artesunate mediated growth inhibitory effects and induced apoptosis in pancreatic cancer cells through modulation of multiple signaling pathways. Moreover, we discovered that artesunate is a novel topoisomerase IIα inhibitor. Several molecular targets involved in the intrinsic and extrinsic apoptotic pathways were affected after treatment with ART. Among those molecular targets are *APAF1*, *BAX*, *BAK*, *CASP 2*, *CASP 3*, *CASP 4*, *CASP 5*, *CASP 6*, *CASP 8*, *CASP 9*, and *CASP 10*. Moreover, we have shown that the cytotoxic effect of ART on pancreatic cancer cells could be mediated, in part, through up-regulation of *DDIT3*, and *NAG-1* genes and down-regulation of *PCNA* and *RRM2* genes (*21*).

10. Cantharidin and microarray

Cantharidin is a vesicant product of Chinese blister beetles and Spanish flies (*61*). All body fluids of blister beetles have cantharidin, and the dried bodies have been used as an anticancer agent in traditional Chinese medicine for a long time (*62*). It was found that Cantharidin induces apoptosis of human multiple myeloma cells *via* inhibition of the JAK/STAT pathway (*63*). Zhang *et al.* (*64*) used cDNA microarrays (12,800 chip; United Gene Holdings, Ltd., PRC) to identify gene expression changes in HL-60 promyeloid leukemia cells treated with cantharidin. Cantharidin-treated cells decreased expression of genes coding for proteins involved in DNA repair (*e.g.*, *FANCG*, *ERCC*), DNA replication (*e.g.*, *DNA polymerase delta*), energy metabolism (*e.g.*, *isocitrate dehydrogenase alpha*, *ADP/ATP translocase*). Moreover, Cantharidin also decreased expression of genes coding for proteins that have oncogenic activity (*e.g.*, *c-myc*, *GTPase*) or show tumor-specific expression (*e.g.*, *phosphatidylinositol 3-kinase*). In addition, they suggested that cantharidin could be used as an oncotherapy sensitizer after reporting that exposure of HL-60 cells to cantharidin resulted in the decreased expression of multidrug resistance-associated protein genes (*e.g.*, *ABCA3*, *MOAT-B*). The increased expression of genes involved in modulating cytokine production and inflammatory response (*e.g.*, *NFIL-3*, *N-formylpeptide receptor*), partly explained the stimulating effects on leukocytosis (*64*).

In an attempt to identify key molecular determinants that involve sensitivity or resistance of tumor cells to cantharidin, we analyzed the microarray database of the National Cancer Institute (USA) in 60 tumor cell lines (*65,66*). Out of 9,706 genes identified, 21 genes whose mRNA expression correlated with the highest correlation coefficients to inhibition concentration 50% (IC_{50}) values were selected by COMPARE analysis and false discovery rate calculation (*66*). These genes were subjected to hierarchical cluster analysis to reveal whether the expression profiles of these genes could be used to predict sensitivity or resistance of cell lines to cantharidin. While the specific functions of the proteins encoded by the 21 identified genes were diverse, nevertheless, it is intriguing that many of them are involved in DNA repair, DNA damage response, and/or apoptosis (*66*).

11. Conclusion

DNA microarrays are a powerful and easy-to-use genomic tool. The genomic-wide data provided after microarray applications can provide potential information that helps to find the causes of disease, the mechanism of drug action, and the discovery of gene products that are targets for therapy in various diseases. With the use of this approach, novel molecular

targets and new therapeutic options can be identified. In this paper we have reviewed recent experiments using microarray technology involving medicinal plants derived from TCM as examples. Furthermore we showed that expression analysis was effective in identifying novel pathways and molecular targets mediating their effects.

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(*Received June 22, 2009; Accepted July 25, 2009*)

Review

Structure analysis of short peptides by analytical ultracentrifugation: Review

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ABSTRACT: Short peptides are potential drug candidates for pharmaceutical and biotech industries. Short peptides are natural ligands for numerous G-protein coupled receptors (GPCR) and hence constitute a large number of drug candidates. Synthetic short peptides are also extensively developed as agonistic or antagonistic ligands that function in a similar manner to antibodies, soluble receptors and protein ligands. Characterization of the peptides in solution is often performed in the presence of organic solvents, which can presumably generate the structure bound to the target surface and also enhance the solubility of the peptides. Analytical ultracentrifugation (AUC) technique should provide information on the state of self-association of the peptide in solution. Its application for short peptides has been far less than the applications for proteins. We believe that AUC should be used to show the associated state of the peptides, as reviewed in this paper.

Keywords: Analytical ultracentrifugation, sedimentation, peptide, aggregation, disordered structure

1. Introduction

Requirement of high quality analysis of protein solution has lead to extensive applications of analytical ultracentrifuge (AUC) for pharmaceutical proteins (*1-5*). This technology measures the homogeneity of proteins in solution, in particular the amount of aggregated proteins and their sizes (*1-5*). It appears that the same scrutiny has not been extended to the low molecular weight peptides, with a few exceptions (*6-9*), while peptides constitute a major part of drug developments (*e.g.*, peptide mimetics developed by Affymax Inc., USA). Many G-protein coupled receptors (GPCR) use short peptides as a natural ligand (*10-12*). Synthetic short peptides are developed as an agonistic or antagonistic ligand that targets various cell surface receptors, protein ligands and cell surface markers in a similar manner that antibodies are used (*14,15*). At first glance, AUC may not be a versatile technology for short peptides, as they would not sediment due to their small mass as in proteins (*16*). We show here that AUC is informative for the analysis of short peptides in solution using a neuroprotective peptide, Humanin and ADNF9, as a model.

Humanin and ADNF9 are 24 and 9 amino acid peptides and protect neurons from various cell toxic stresses (*17-19*). The mechanism of their protective effects is not clear, in part due to lack of information on the conformational state in solution. We have used AUC to characterize the self-association of these peptides in different aqueous solutions and shown that their biological activities must be evaluated in the context of the solution structure (*8,9,20-22*). Here we review such data to demonstrate the value of AUC for peptide research and development.

2. Principle of AUC

Numerous publications are available for the operative principle of AUC (*1-3,23*). It uses centrifugal force to sediment (or float) the solutes whose density differs from the medium. Proteins and peptides generally have a higher density than aqueous medium and hence sediment toward the bottom of the cell (Figure 1). The sedimentation of the solutes (here peptides) is monitored by absorbance (or refractive index), while sedimenting, as a function of cell position and time (see middle row of Figure 1). The rate of the sedimentation of the solute is a function of the mass and shape. A typical sedimentation profile of Humanin (HN) peptide is shown in Figures 2A and B. Each curve

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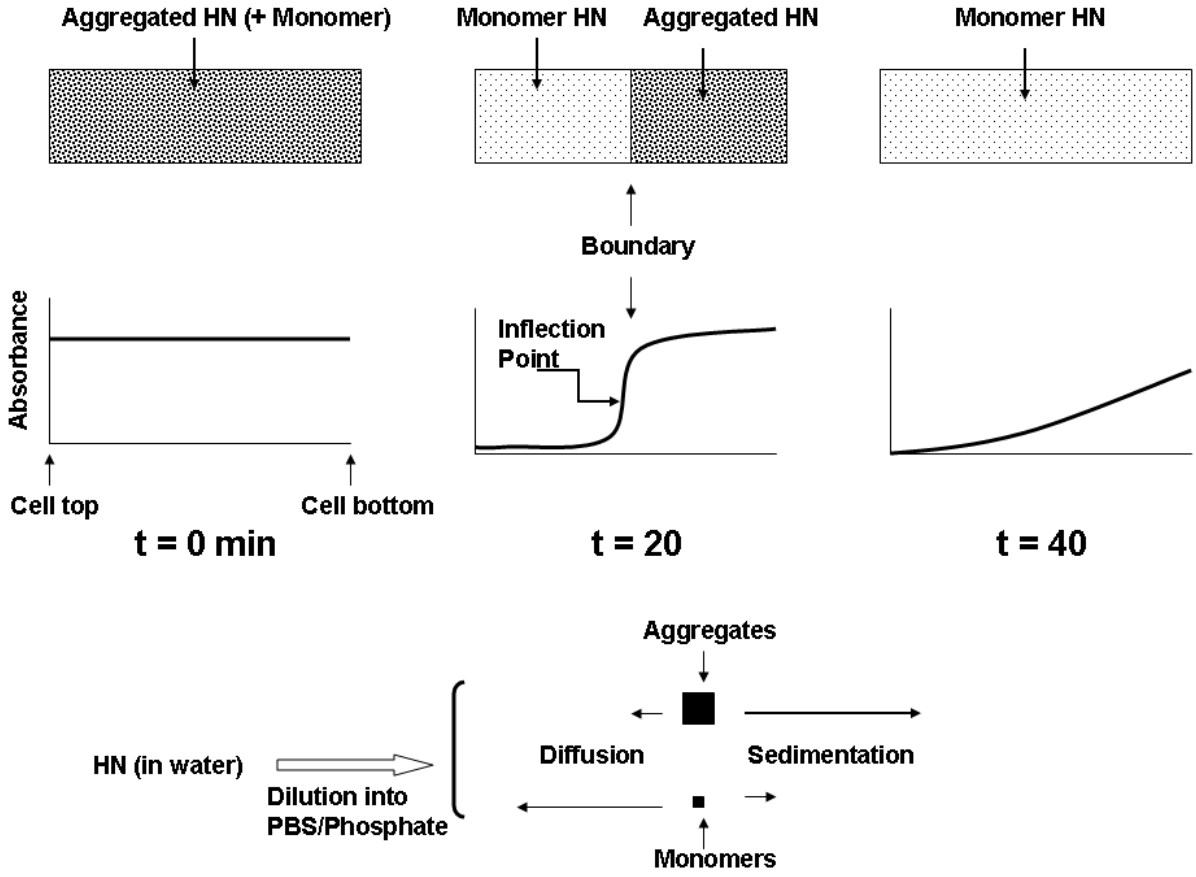

Figure 1. Schematic illustration of sedimentation profile. First row shows the distribution of monomeric and aggregated HN in the centrifuge cell as a function of sedimentation time. Second row shows the absorbance profile of the cell at different sedimentation time. Third row shows the results of dilution of HN dissolved in water into buffers.

corresponds to UV absorbance at different positions in the centrifuge cell. As the peptide sediments, the curve moves toward the bottom of the cell (from left to right), reflecting sedimentation of the peptide. The shape of the curve indicates the homogeneity (or heterogeneity) of the sample. If it contains one species (for example, all in monomer), then it should show an inflection point, as depicted in Figure 1 (see middle row). If the sample contains many different forms (*e.g.*, monomer, dimer, *etc.*), then the curve shows a broad boundary or multiple inflection points. Analysis of the curve gives the information about the distribution of various self-associated species of the peptide samples in solution.

3. Sedimentation analysis of example peptides

Preparation of peptide samples appears to contribute to the outcome of the self-association of HN peptides and possibly other peptides. As far as Humanin peptides are concerned, the standard procedure for their biological analysis has been to dissolve them first in water and then dilute into the assay media (*17*). In fact, wild type HN peptide is insoluble when directly dissolved in phosphate-buffered saline (PBS) (*20*). HN and HN derivatives are fully soluble, upon visual inspection, in water. Although it would be interesting to know the self-association of the HN peptides in water, hydrodynamic technologies, including AUC, cannot be applied to such system due to unscreened charges on the peptides: the addition of electrolytes screens the charges and makes the peptides behave normally in hydrodynamic measurements. While insoluble upon direct dissolution of the HN in PBS, the samples once dissolved in water lead to an apparently transparent solution when diluted into PBS or other aqueous buffers. It should be noted that apparent transparency does not mean that they are in functional structure or state in buffer solution. This appears to be the case for HN.

The functional structure of HN has been proposed to be a dimer, based on co-precipitation of two different forms of the peptide (*24,25*). As schematically shown in Figure 3, first, a HN peptide was conjugated to beads. When this bead was mixed with a presumably dimeric Humanin fused with immunological detection tag (HN-tag), the HN-tag was detected in the bead fraction, indicating that HN-tag was trapped by the bead through its binding to HN attached on the bead (Figure 3, line 1). When the above bead fraction containing both HN and HN-tag was incubated with excess HN, no HN-tag was observed in the bead fraction by immunostaining

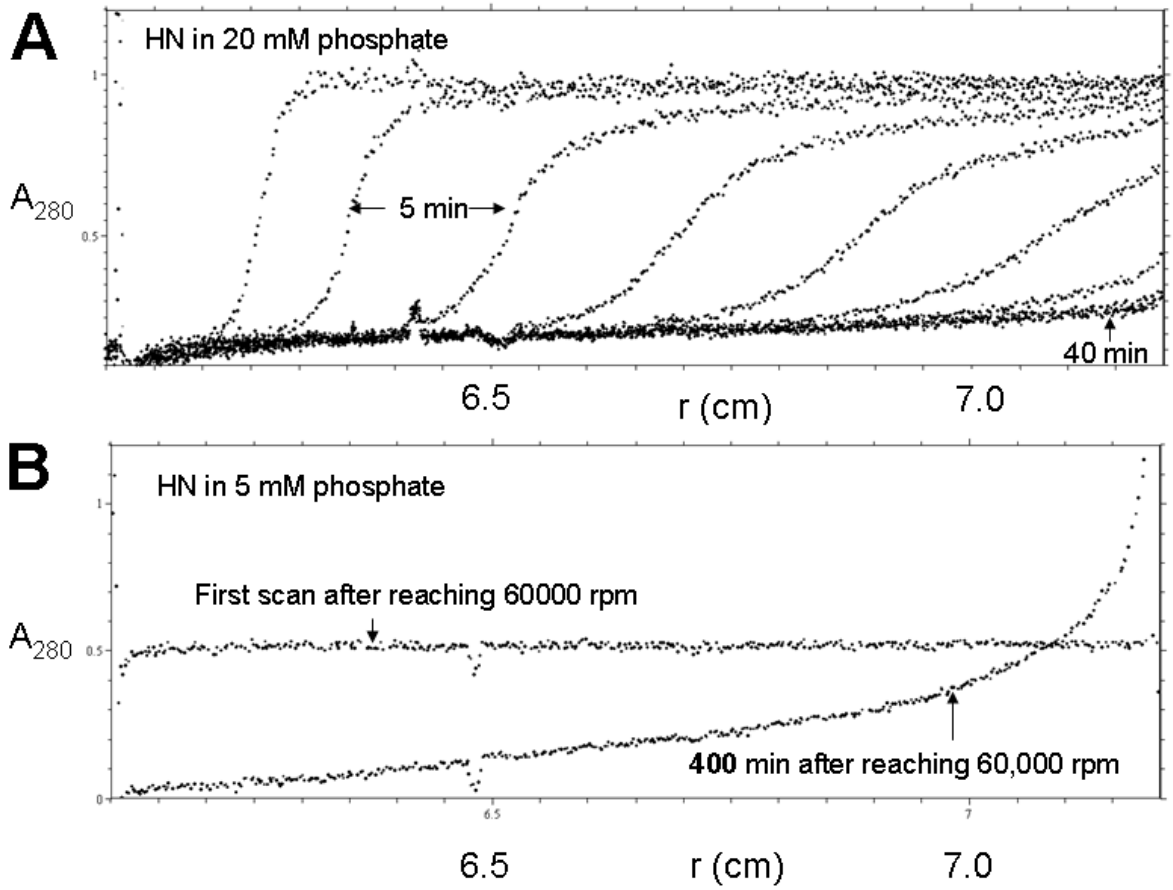

Figure 2. Sedimentation profile of HN in 20 (A) and 5 (B) mM phosphate, pH 6.0. Reformatted from ref. *22*.

(line 2). These observations were used to hypothesize that HN functions as a dimer. However, the same data can equally be explained from aggregated HN. HN extensively aggregates in PBS (*20*). Figure 3 (line 3) assumes that HN-tag also aggregates. Aggregated HN-tag may further self-associate with the HN on the bead, resulting in both HN and HN-tag aggregated on the bead. When this bead was mixed with excess HN aggregates (line 4), HN-tag aggregates are displaced with the HN aggregates, releasing HN-tag into solution. Based on this analysis, it appears that dimerization does not appear to be the only explanation for the observed co-precipitation of HN with HN-tag. If dimerization is not the mechanism of the HN function, then what is the structure of HN that exhibits neuroprotective activity.

Let us go back to sedimentation profile of HN in 20 mM phosphate, pH 6.0 (Figure 2A). The HN peptide sediments as large aggregates, as can be seen by a movement of sedimentation boundary in 5 min span (an indication of fast sedimentation). Careful examination of sedimentation pattern showed a fraction of the peptide remaining in the cell after 40 min spin at 60,000 rpm. Sedimentation profile at 40 min has a shallow slope ascending from the top to the bottom of the cell. This can be explained from the distribution of HN into aggregates and monomers (see Figure 1, bottom row). Here we assumed that the structure of HN in water (though unknown for self-association) converts to aggregates (shown a large black square) and monomers (small square) upon dilution into PBS or phosphate buffer, pH 6.0. At $t = 0$ (*i.e.*, before centrifugation), both monomers and aggregates are distributing in the cell as a homogeneous solution (see top row of Figure 1) and hence has a flat absorbance from the top to the bottom of the cell (middle row). After ~20 min centrifugation ($t = 20$) at 60,000 rpm, the aggregates sediment half-way to the bottom leaving the monomer-only phase behind. Boundary between the monomer-only phase and sedimenting aggregates is broader than depicted due to diffusion of aggregates (though slow). Monomer-only phase also should have a concentration gradient of monomers that also sediment, but at a much slow rate than aggregates (see bottom row, small arrow). At $t = 40$, all aggregates sediment to the bottom, leaving only the monomers in the cell. Slow sedimentation and fast diffusion of the monomer, as depicted in the bottom row (Figure 1), cause a shallow concentration gradient. Namely, the observed shallow slope at $t = 40$ in Figure 2A means that there is a fraction of HN that does not sediment fast enough to form a boundary. This was not observed in PBS: namely, no absorbance gradient, such as shown in Figure 1 (middle row, far right)

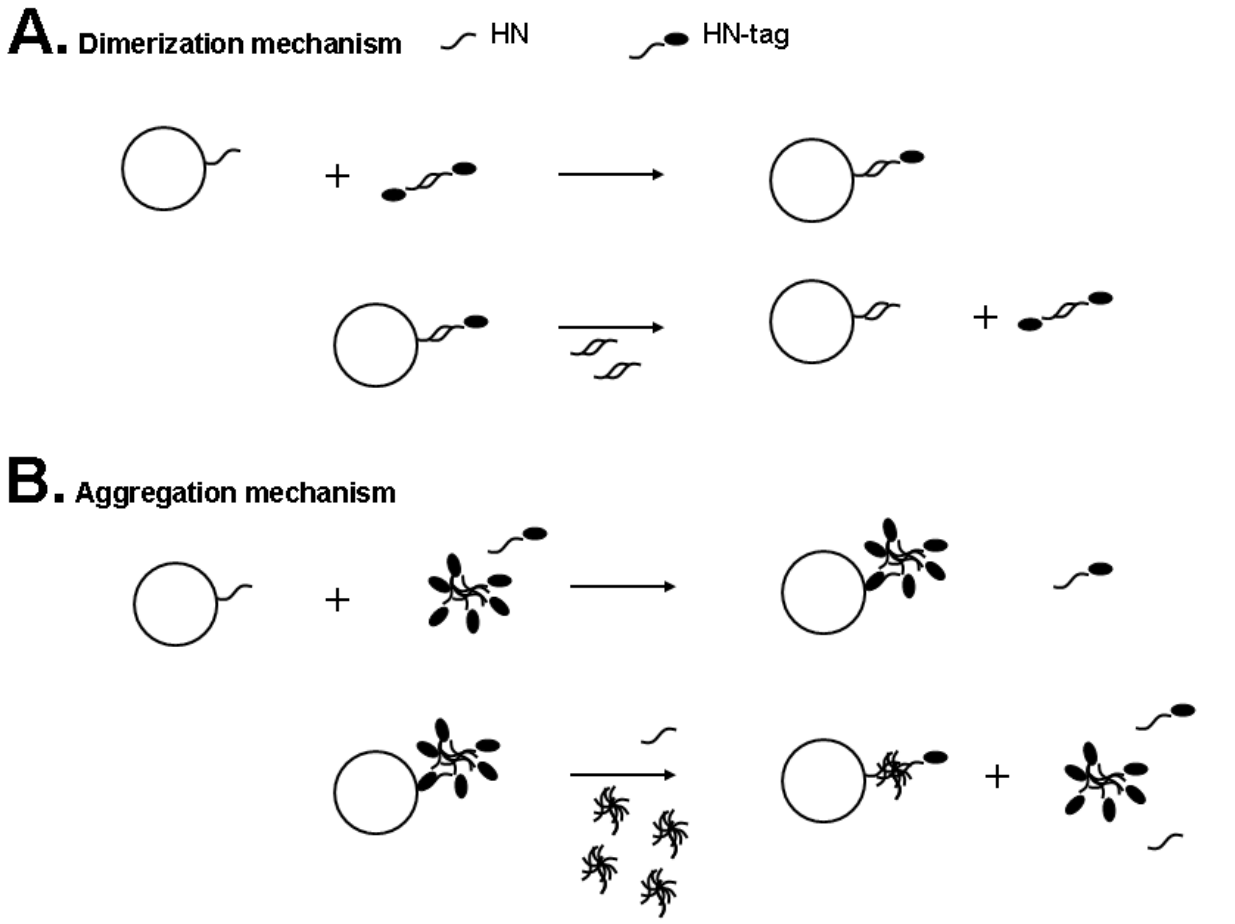

Figure 3. Schematic illustration of co-precipitation experiments. First line shows binding of dimeric HN-tag with HN on the bead. Second line shows dissociation of HN-tag in the presence of excess HN dimers. Third and fourth lines show the above binding and dissociation experiments with the aggregated HN-tag or HN.

or Figure 2A, was observed in PBS. This indicates that while a majority of the HN peptide aggregated in PBS, a fraction of HN had a smaller mass species in 20 mM phosphate, pH 6.0. Although farther away from physiological pH, the phosphate concentration was reduced to 5 mM, which increased the amount of the protein remaining in the cell after spin. Figure 2B shows the concentration gradient after 400 min spin at 60,000 rpm. Comparison with Figure 2A shows a steeper concentration gradient after 400 min spin, indicating that 5 mM phosphate clearly increased the amount of low mass species. The molecular weight of that fraction can be more clearly determined by sedimentation equilibrium.

Sedimentation equilibrium experiment is done at lower centrifugal speed and smaller cells. After reaching sedimentation equilibrium (*i.e.*, complete balance between centrifugal force and diffusion), the concentration gradient formed, such as shown in Figure 4A for HN, is used to determine the molecular weight. The observed concentration gradient in Figure 4 represents the peptide species after spinning down the aggregated species and that remain in the centrifuge cell. The molecular mass thus determined showed that the peptide in that remaining fraction is a monomer. Namely, the HN peptide either extensively aggregates or is monomeric in phosphate buffer at pH 6.0. Assuming that the aggregated species is not the functional form, the observed results suggest that the active structure is monomer.

4. HN analogs

It appears likely that the monomeric form of HN observed in 5 and 20 mM phosphate, pH 6.0, is the functional state or at least the precursor of the HN activity. We further addressed this possibility from the analysis of various HN analogs. Structure-function mutational analysis resulted in several analogs that are more active than the wild type HN. One of the analog that has a mutation of Ser14Gly had a 1,000-fold higher activity that the parent HN (*22*). One possibility may be that this analog is more soluble in PBS. Sedimentation analysis showed otherwise, as this analog also did aggregate extensively. More informative is another analog with multiple mutations that also had a similarly enhanced activity (*20*). The most significant observation for this analog is its molecular weight. Sedimentation

equilibrium analysis clearly demonstrated that this analog is a monomer in PBS and contains little aggregates. Thus, it is more likely that the monomeric state in PBS is at least the molecular species in PBS that leads to the biological function of HN, although a transition to other structures cannot be excluded under physiological conditions.

Structure-function analysis also created another analog with a single mutation of Ser7Ala, which is devoid of activity (*22*). This analog behaves similarly to HN in sedimentation analysis: *i.e.*, extensive aggregation in 20 mM phosphate, pH 6.0 and a large fraction remaining in the cell after 400 min spin at 60,000 rpm. What is remaining in the cell was determined to be a monomer similarly to HN and S14G analog: the sedimentation equilibrium data plotted in Figure 4B fit a single monomeric species. Thus, the lack of activity for this analog is not due to its aggregation state and must be due to the sequence difference.

5. Cicular dichroism

Circular dichroism (CD) is a commonly used spectroscopy for peptide samples. CD analysis showed that HN and HN analogs (both active S14G and inactive S7A analogs) are all disordered at low peptide concentrations. As shown in Figure 5, the CD spectra of the HN peptides below 0.05 mg/mL show no indication of α-helical or β-sheet structures and correspond to more or less disordered secondary structures. However, at higher peptide concentration (0.1 mg/mL), there appears to be a transition into a β-sheet structure. Such structure transition is enhanced at higher peptide concentration and must be due to extensively aggregation. Namely, it appears that the monomeric structure of HN is disordered and the aggregated structure is β-sheet.

6. Perspective

Sedimentation analysis is a standard technology for aggregation analysis of therapeutic proteins. Such aggregation analysis is essential for pharmaceutical proteins, as aggregated proteins may cause immunogenicity. A small amount of protein aggregates, *e.g.*, 1-5%, can generate antibodies against therapeutic proteins: numerous examples exist for such immunogenicity problems: *e.g.*, bone morphgenic proteins (*26*), monoclonal antibody (*27*), tumor necrosis factor antagonist (*28*) and growth hormone (*29*). Sedimentation analysis is used to quantitatively show

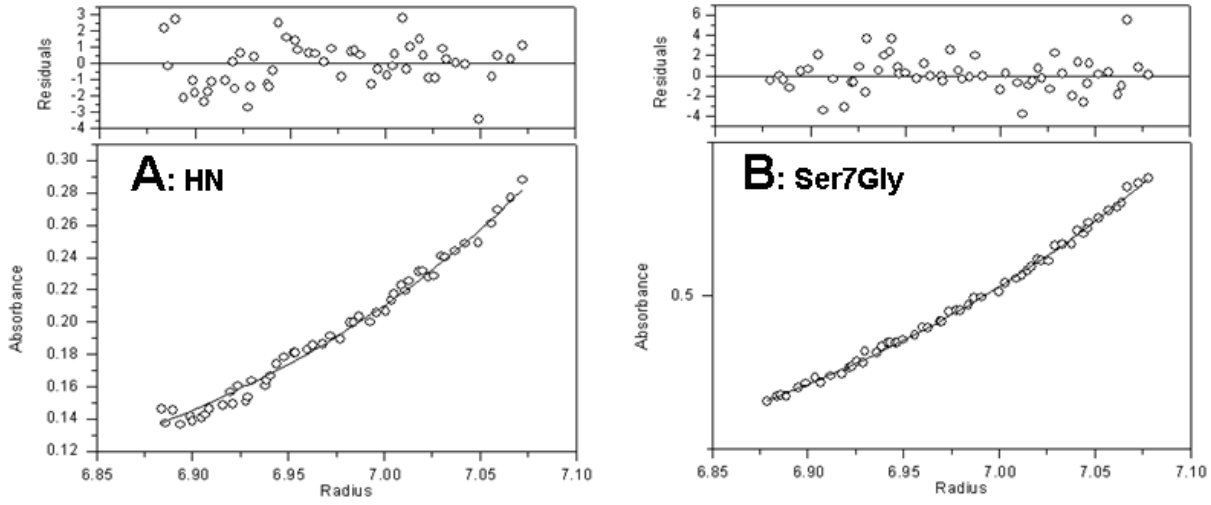

Figure 4. Sedimentation equilibrium of HN (A) and Ser7Ala analog (B) in 5 mM phosphate, pH 6.0. The data represent the peptide left in the cell after centrifugation. Reformatted from ref. *22*.

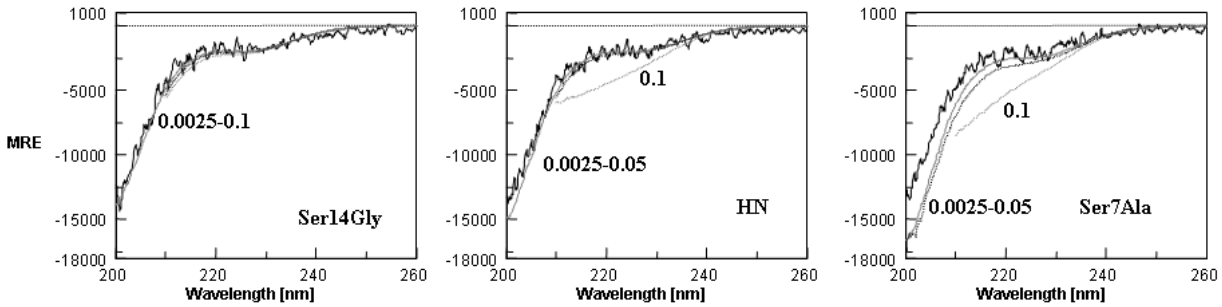

Figure 5. Circular dichroism spectra of HN, Ser14Gly analog and Ser7Ala analog in 5 mM phosphate, pH 6.0. Reformatted from ref. *22*.

the amount of aggregated species in the pharmaceutical proteins. Size exclusion chromatography is also used for this purpose with a caveat that SEC columns often non-specifically bind proteins, leading to incorrect estimate of aggregate content. AUC has no such problems. It does not appear, however, that AUC is also commonly used for therapeutic peptides. Due to small mass, it would be difficult to determine the aggregation if the peptides distribute into the monomers and small oligomers. However, certain less soluble peptides, such as HN, may be subjected to AUC analysis for structure-function analysis. In the case of HN, it was made clear that the original dimer hypothesis was unlikely and such interpretation occurred due to the tendency for HN to heavily self-associate in buffer solutions.

We have applied AUC for other short peptides, ADNF9 (*8*) and NAP (*9*). These are also 9 and 8 amino acid neuroprotective peptides. It was apparent that these are more soluble judged from the dissolution kinetics in PBS. AUC analysis demonstrated that these peptides are monomeric. CD analysis showed that their structures are disordered as in HN. They showed no indication of β-sheet structure at higher peptide concentration, consistent with no apparent aggregation even at increased concentration. Value of AUC for structure-function analysis was also demonstrated for the neuropeptide head activator containing 11 amino acids (*30*) and the neuropeptide Y containing 13 amino acids (*31*). Although earlier studies suggested a dimer for both the full length head activator peptide and the shorter fragment as a functional structure, AUC analysis revealed otherwise: while the full length was a monomer, the fragment extensively aggregated (*30*). The monomeric neuropeptide Y self-associated into oligomers in 40% trifluoroethanol, a helcal inducing organic solvent (*31*). In conclusion, AUC can be used for characterization of short peptides that should help understand the mechanism of their biological functions and avoid confusion about the solution structure responsible for the activities. With further clinical developments of peptides, AUC may also find greater applications for quality control of the therapeutic peptides.

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(*Received July 24, 2009; Accepted August 8, 2009*)

Original Article

Beneficial effects of a standardized *Hypericum perforatum* extract in rats with experimentally induced hyperglycemia

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ABSTRACT: The main aim of this study was to test the therapeutic potential of a standardized *Hypericum perforatum* extract in treating metabolic disturbances commonly associated with type-2 diabetes mellitus. Daily oral administration of the *Hypericum perforatum* extract (100, 200, and 300 mg/kg/day) for 14 consecutive days counteracted in a dose-dependent manner the alterations in blood glucose levels and lipid profile as well as liver glycogen content and body weight changes observed in a rat mode of nicotinamide-streptozotocin-induced diabetes. In general, effects of the highest dose of the extract in this model were quite similar, but not identical, to those of a 10 mg/kg/day dose of glibenclamide. The effects of single oral doses of the extract in a rat oral glucose tolerance test conducted in fasted animals were also analogous to those of an antidiabetic drug therapeutic use. These observations not only further expand the therapeutic potentials of Hypericum extracts but also indicate that stimulation of insulin release could be involved in their modes of actions. The importance of an extract with diverse, therapeutically interesting pharmacological properties is also briefly discussed.

Keywords: *Hypericum perforatum*, streptozotocin, nicotinamide, diabetes mellitus, NIDDM

1. Introduction

Different types of concoctions obtainable from the perennial herb *Hypericum perforatum* (also commonly called St. John's wort) have been known to have diverse medicinal uses for centuries (*1*). However, most reports dealing with the herb's clinical efficacy and bioactivities of its extracts over the past few decades have largely focused only on their antidepressant-like and other CNS-modulating effects (*2*,*3*). Information available on activity profiles of a hydroalcoholic extract of *Hypericum perforatum* (Hp) in animal models has revealed that such extracts could have other diverse therapeutic potentials and that hyperforin is likely to be a major bio-active constituent involved in their therapeutically interesting bio-activities (*4*). Although hyperforin is quantitatively the major secondary metabolite of the plant, several other therapeutically interesting bio-active phenolic components of the herb are also known. Since such components of the extract possess strong antioxidant properties (*5*-*7*), several reports based on this property of Hypericum extracts have also appeared in recent years. However, two facts that should be emphasized are that the bio-active constituents and activity profiles of diverse types of extracts vary considerably and that observations made with a specific type of extract may not be necessarily valid for other types as well.

Like in other parts of the globe, the diverse healing properties of *Hypericum perforatum* found on the Indian subcontinent have long been known (*8*,*9*). Unlike in the Western world, however, the herb was not traditionally considered to be a psychoactive plant in India. The very first report on antidepressant-like effects of an Indian Hypericum extract appeared only at the end of the past century (*10*). Although the extract tested in this study was prepared by a procedure analogous to those commonly used in the Western world to obtain hydroalcoholic Hypericum extracts, its analytically definable chemical constituents were unlike those widely in commercial use outside India. Unlike other Hypericum extracts, it was rich in xanthones (*11*,*12*). Like other extracts in commercial use, however, it also contained hyperforin and other bio-active phenolics and flavonoids.

In light of observations made with diverse types of Hypericum extracts, efforts are underway at the authors' laboratories to better pharmacologically and toxicologically characterize different types of Hypericum extracts and their known bio-active constituents. The ultimate goal of these efforts is to be able to design appropriate clinical studies for Hypericum extracts

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and/or their chemical constituents based on their broad preclinical activity profile. Therefore, earlier studies were conducted to define the activity profile of a standardized extract of *Hypericum perforatum* growing in India in a battery of animal models currently in wide use for drug discovery purposes or to define the activity profiles of known drugs (*13-19*).

The present paper describes the results of several experiments designed to test the effects of a Hypericum extract commonly in therapeutic use in the Western world (*i.e.,* standardized to containing at least 3% hyperforin and 0.3% hypericines) on metabolic disturbances often encountered in type-2 diabetic patients. In the first set of reported experiments, possible effects of repeated oral doses of the extract in the nicotinamide-streptozotocin rat model were tested. During the course of these experiments, a report (*20*) describing beneficial effects of hyperforin and Hypericum extract on insulin release came to the authors' attention. Since that study used *in vitro* models, testing whether the same would also hold true in intact animals was an interesting question. Consequently, the second set of reported experiments quantified the possible effect of single acute doses of the extract in a glucose tolerance test. Results of this experiment indicate that stimulation of insulin release could be involved in the observed effects of the extract in the type-2 diabetes model used.

2. Materials and Methods

2.1. *Animals*

Adult Charles-Foster rats (180 ± 10 g) were obtained from the Central Animal House of the Institute of Medical Sciences, Banaras Hindu University, Varanasi. The animals were housed in groups of six in polypropylene cages at an ambient temperature of 25 ± 1ºC and 45-55% relative humidity with a 12:12 h light/dark cycle. Except when otherwise mentioned, animals were always provided with commercial food pellets and water *ad libitum*. All animals were acclimatized to laboratory conditions for at least one week before their use in experiments, and each animal was subjected to experimental procedures only once. Principles of laboratory animal care (NIH publication number 85-23, revised 1985) guidelines were followed.

2.2. *Plant extract*

The tested hydro alcoholic extract of *Hypericum perforatum* (Hp) was obtained from Indian Herbs Research & Supply Co. Ltd., Saharanpur, UP, India. It was standardized (HPLC) to contain no less than 3.0% hyperforin and 0.3% hypericines. Thus, the tested extract can be considered to be a representative of Hypericum extracts commonly used in the Western world for therapeutic purposes as an antidepressant.

2.3. *Drug administration*

The extract was suspended in 0.3% carboxy methyl cellulose (CMC) and was orally administered through stainless steel oral gavage at three dose levels, *viz.* 100, 200, and 300 mg/kg of body weight. In hyperglycaemic rat experiments, daily single treatment continued for 14 consecutive days. Doses of the extract were chosen on the basis of earlier studies by this laboratory (*10,14,15*). Control rats received an equal volume of CMC, while glibenclamide (10 mg/kg body weight) was orally administered to another group of animals (positive control group). For an oral glucose tolerance test, the same doses of the extract as well as those of the standard drug were orally administered only once.

2.4. *Oral glucose tolerance test*

An oral glucose tolerance test was performed to evaluate the effects of the extract on peripheral glucose utilization in normal rats. Albino rats of either sex were divided into five groups (n = 6), fasted overnight, and orally administered 0.3% CMC, Hypericum extract (100, 200, and 300 mg/kg), or glibenclamide (10 mg/kg). Thirty minutes after the treatment, glucose (2 g/kg) was orally administered to all treatment groups. Blood glucose levels were quantified in blood samples collected by retro-orbital puncture prior to glucose administration as well as 30, 60, and 120 min thereafter.

2.5. *Induction of NIDDM*

A standardized procedure was used to induce hyperglycemia in rats (*21*). NIDDM was induced in animals fasted overnight by a single intraperitoneal injection of 65 mg/kg streptozotocin (Merck, Germany) 15 min after the *i.p.* administration of 120 mg/kg nicotinamide (SD fine Chem, India). Current pharmacology protocols (*22*) were followed for preparation and administration of a streptozotocin solution. Hyperglycemia was confirmed by blood glucose level determination conducted on the 3rd and 7th day after the streptozotocin injection. Rats with consistent hyperglycemia on the 7th day (fasting blood glucose levels > 250 mg/dL) were used to test the effects of the extract on type-2 diabetes.

Animals were divided into six groups of six rats each: Group I – Normal control rats treated with 0.3% CMC; Group II – Diabetic control rats treated with 0.3% CMC; Group III – Diabetic rats treated with Hp 100 mg/kg/day; Group IV – Diabetic rats treated with Hp 200 mg/kg/day; Group V – Diabetic rats treated with Hp 300 mg/kg/day; and Group VI – Diabetic rats treated with glibenclamide 10 mg/kg/day. Blood samples were collected by retro-orbital puncture and

the fasting blood glucose level was estimated on day 0 before the start of treatment, and on the 14th day of treatment, with a commercially available biochemical kit (Span Diagnostics Ltd., India). On the 14th day, plasma lipid profiles were also estimated using biochemical kits (Span Diagnostics Ltd., India). The liver glycogen content of rats was estimated using an anthrone reagent (*23*). Body weight changes of rats were periodically recorded throughout the experiment.

2.6. *Statistical analysis*

The mean ± standard error of the mean (SEM) was calculated for the values observed in each experimental group. Statistical analysis was performed by one-way analysis of variance (ANOVA) followed by Tukey-Kramer multiple comparison test. GraphPad InStat (version 3.06) software was used for statistical analysis.

3. Results

3.1. *Glucose tolerance test*

Results of this experiment are summarized in Figure 1. Mean blood glucose levels of all groups before glucose loading were not statistically different from one another. After oral glucose loading, however, blood glucose levels in the group treated with the extract and the group treated with glibenclamide were significantly lower than those of the control group. Such was the case at every time point after glucose loading. These results reveal that like a standard anti-diabetic drug used in this experiment, the extract probably facilitates glucose utilization by stimulating glucose induced insulin release. Since blood insulin levels were not measured, such intervention must be experimentally verified in later studies. In any case, effects of the extract were dose-dependent, and the extract's maximum effects were observed within the first hour after an oral glucose challenge. Observed effects of a 200 mg/kg dose of the extract were more pronounced than those of lower doses. However, there was no statistically significant difference between the effects of 200 and 300 mg/kg doses of the extract. The maximum ceiling dose of the extract in this test is apparently 200 mg/kg and the effect of higher doses of the extract is qualitatively and quantitatively comparable to that of 10 mg/kg glibenclamide.

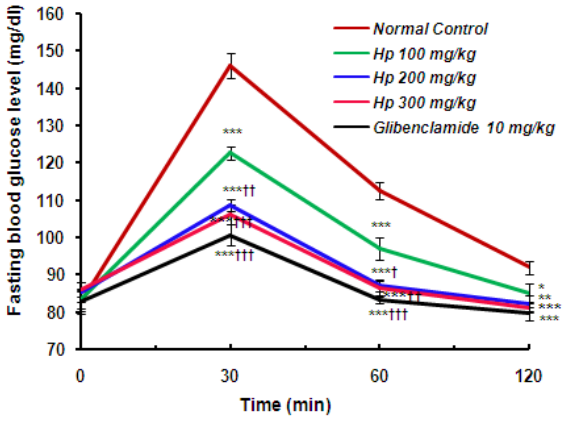

Figure 1. Effect of the Hp extract on an oral glucose tolerance test in normal rats. Values are mean ± SEM of 6 animals in each group. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ *vs.* normal control; † $p < 0.05$, †† $p < 0.01$, ††† $p < 0.001$ *vs.* Hp 100 mg/kg.

3.2. *Blood glucose levels in an NIDDM model*

Compared to the normal control group, fasting blood glucose levels on day 0 of all groups subjected to a nicotinamide-streptozotocin challenge were significantly and equally higher (see Figure 2). In the diabetic control group, blood glucose levels remained elevated until day 14 of the experiment. However, diabetic animals receiving oral administration of an Hp extract (100, 200, and 300 mg/kg/day) for 14 consecutive days reduced fasting blood glucose levels in a dose-dependent manner. On day 14, there was no statistically significant difference between the blood glucose levels of the diabetic group treated with 300 mg/kg/day extract and the normal control group, and such was also the case for the diabetic group treated with 10 mg/kg/day glibenclamide. Analogous to findings of the glucose tolerance test, no statistically significant difference between the effects of 200 and 300 mg/kg/day doses of Hp extract was observed.

3.3. *Lipid profile of diabetic rats*

On the 14th day of the experiment, rats subjected to a nicotinamide-streptozotocin challenge (diabetic control

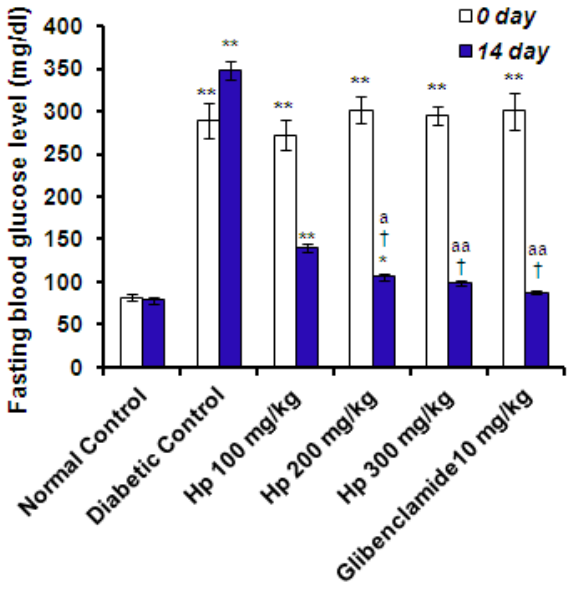

Figure 2. Effect of the Hp extract on the blood glucose level of rats with nicotinamide-streptozotocin-induced diabetes. Values are mean ± SEM for 6 animals in each group. * $p < 0.05$, ** $p < 0.001$ *vs.* normal control; † $p < 0.001$ *vs.* diabetic control; [a] $p < 0.01$, [aa] $p < 0.001$ *vs.* Hp 100 mg/kg.

Table 1. Effect of the Hp extract on lipid profile of rats with nicotinamide-streptozotocin-induced diabetes

Group (n = 6)	TC (mg/dL)	TG (mg/dL)	HDL-C (mg/dL)	LDL-C (mg/dL)
Normal control	79.53 ± 2.29	46.85 ± 1.47	38.67 ± 0.77	31.48 ± 2.59
Diabetic control	143.27 ± 6.68**	115.31 ± 6.58**	24.47 ± 1.43**	95.74 ± 7.1**
Hp 100 mg/kg	103.8 ± 1.78**†††	78.07 ± 3.24**†††	28.18 ± 0.40**†	60.00 ± 2.36**†††
Hp 200 mg/kg	85.67 ± 2.69$^{\dagger\dagger a}$	59.16 ± 1.7$^{\dagger\dagger aa}$	31.74 ± 0.3$^{**\dagger\dagger a}$	42.09 ± 3.2 $^{\dagger\dagger a}$
Hp 300 mg/kg	81.29 ± 3.26$^{\dagger\dagger aa}$	55.86 ± 3.22$^{\dagger\dagger aa}$	32.24 ± 0.76$^{**\dagger\dagger a}$	37.88 ± 3.94$^{\dagger\dagger aa}$
Glibenclamide 10 mg/kg	73.1 ± 2.55$^{\dagger\dagger aaa}$	69.37 ± 2.4*††	33.94 ± 0.77$^{\dagger\dagger aaa}$	25.28 ± 2.71$^{\dagger\dagger aaa}$

Values are mean ± SEM, n = number of animals in each group. $^{*}p < 0.01$, $^{**}p < 0.001$ *vs.* normal control, $^{\dagger}p < 0.05$, $^{\dagger\dagger}p < 0.001$ *vs.* diabetic control, $^{a}p < 0.05$, $^{aa}p < 0.01$, $^{aaa}p < 0.001$ *vs.* Hp 100 mg/kg.

group) showed a significant elevation in plasma total cholesterol (TC), triglycerides (TG), and low-density lipoprotein-cholesterol (LDL-C) levels while their plasma high-density lipoprotein-cholesterol (HDL-C) levels decreased significantly. Administration of an Hp extract or glibenclamide effectively restored these parameters to the physiological values observed in the normal control group (see Table 1). Here again, the effects of Hp 200 and 300 mg/kg/day doses of extract were comparable to those of a dose of glibenclamide (10 mg/kg/day).

3.4. *Liver glycogen of diabetic rats*

In comparison to the normal control group, a significant reduction in the liver glycogen content of the control diabetic group was observed. The results summarized in Figure 3 demonstrate that like glibenclamide, the extract reversed this effect of the nicotinamide-streptozotocin challenge and that its observed effect was dose-dependent. Quantitatively, the effect of Hp 300 mg/kg/day was again similar to that of 10 mg/kg/day glibenclamide.

3.5. *Body weight*

The body weight changes in control, diabetic control, and diabetic rats treated with the extract or with glibenclamide are summarized in Table 2. Animals in the normal control group gained about 4 g body weight over 14 days, whereas those in the diabetic control group lost about 24 g during this period. The mean loss in body weights of diabetic groups treated with Hp 100 and Hp 200 mg/kg/day were about 14 and 11 g, respectively, which was significantly less than that observed in the diabetic control group. In contrast, the diabetic group treated with Hp 300 mg/kg/day gained about 3 g body weight during the treatment period. The body weight loss of the diabetic group treated with glibenclamide (10 mg/kg/day) was significantly less than that of diabetic control group. Glibenclamide's effect on this parameter was comparable to that of Hp 200 mg/kg/day. Thus, a treatment regimen of Hp 300 mg/kg/day compensated for almost all of the quantified metabolic effects of a nicotinamide-streptozotocin challenge and also facilitated a return in body weight to normal levels.

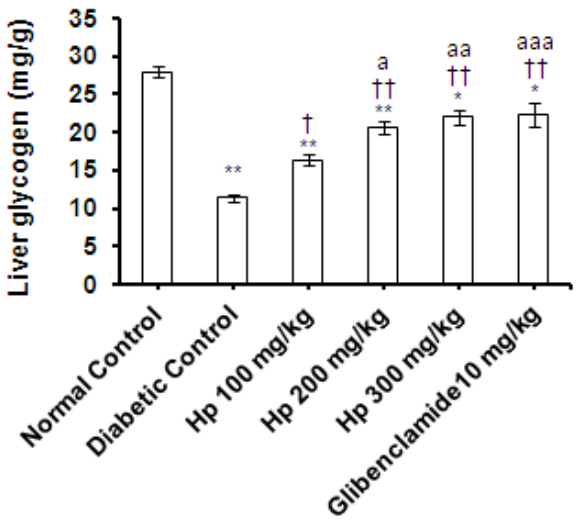

Figure 3. Effect of the Hp extract on liver glycogen content of rats with nicotinamide-streptozotocin-induced diabetes. Values are mean ± SEM for 6 animals in each group. $^{*}p < 0.01$, $^{**}p < 0.001$ *vs.* normal control; $^{\dagger}p < 0.01$, $^{\dagger\dagger}p < 0.001$ *vs.* diabetic control; $^{a}p < 0.05$, $^{aa}p < 0.01$, $^{aaa}p < 0.001$ *vs.* Hp 100 mg/kg.

Table 2. Effect of the Hp extract on body weight in rats with nicotinamide-streptozotocin-induced diabetes

Group (n = 6)	Body weight (g)	
	Initial (0 day)	Final (14th day)
Normal control	186 ± 2.19	190.67 ± 2.06
Diabetic control	183 ± 2.37	159 ± 3.52$^{*\$\$}$
Hp 100 mg/kg	184 ± 3.57	170.67 ± 3.5$^{*\dagger\$}$
Hp 200 mg/kg	188.17 ± 3.54	177 ± 4.09*†
Hp 300 mg/kg	182.54 ± 4.27	185.83 ± 2.69$^{\dagger ab}$
Glibenclamide 10 mg/kg	185.16 ± 3.8	176.5 ± 3.08*†

Values are mean ± SEM, n = number of animals in each group, $^{*}p < 0.001$ *vs.* normal control, $^{\dagger}p < 0.001$ *vs.* diabetic control, $^{a}p < 0.001$ *vs.* Hp 100 mg/kg, $^{b}p < 0.01$ *vs.* Hp 200 mg/kg, $^{\$\$}p < 0.001$, $^{\$}p < 0.05$ *vs.* day 0 for the same group.

4. Discussion

Taken together with the known pharmacological activity profile of Hypericum extracts in animal models, the observations of this study provide further experimental

evidence that the therapeutic potential of the herb is broader than that commonly expected based on existing knowledge of the medicinal uses of this plant. Indeed, findings revealed that the treatment regimen generally used in animal models to observe antidepressant-like and other CNS-modulating effects of Hypericum extracts is also effective in counteracting several metabolic disturbances encountered in type-2 diabetic animals. The effects of the extract on blood glucose levels and other metabolic parameters used in this study were dose-dependent. In general, the observed effects of a 300 mg/kg/day dose were almost identical to those of 10 mg/kg/day glibenclamide. However, the beneficial action of this dose of extract on the body weights of diabetic animals was more pronounced than that of a recognized anti-diabetic agent with potent ability to stimulate insulin release. Thus, the extract could have beneficial effects independent of its possible stimulation of insulin release.

In an oral glucose tolerance test conducted in normal rats after single oral doses of the Hp extract (100, 200, and 300 mg/kg) and glibenclamide, the effects of the extract were comparable to those of glibenclamide. These observations strongly suggest that like many therapeutically used anti-diabetic drugs (*24*), stimulation of insulin release could be involved in the effects of the extract observed in the type-2 diabetes model. A recent finding that hyperforin modulates insulin secretion under *in vitro* conditions (*20*) agrees with this assumption. However, the question whether only hyperforin is involved in the spectrum of effects observed with the Hp extract in a diabetes model or in an oral glucose tolerance test cannot be answered with certainty.

In line with the strategy being developed based on other medicinal plant-based drug discovery projects (*25*), previous studies have revealed diverse activity involving CNS modulation (*10,15-17*) by Hypericum extracts as well as their potential anti-inflammatory (*14*) and glibenclamide-like antidiabetic action (*18*). Initially, experiments examining the effect of a Hypericum extract in diabetes models were planned to test the possibility that their phenolic components with antioxidant and/or free radical scavenging properties could have beneficial effects on oxidative damage in the pancreas and other peripheral tissues. Taken together, the results presented in this and an earlier report (*18*) strongly suggest that such mechanisms may not be necessarily the only ones involved in its observed effects on metabolic disorders.

Research has established that hyperforin, one of the known bio-active components of the Hp extract used in this study, is a potent stimulator of diverse types of neurotransmitters under *in vitro* conditions (*26*). The effects of this extract component are not due to its direct interactions with specific neurotransmitter transporters and are probably due to its effects on cellular ionic homeostatic mechanisms (*27*). Since such mechanisms are also known to be involved in the control of glucose metabolism and insulin release (*28,29*), hyperforin may, by virtue of its modulation of cellular ionic concentration, also be involved in the observed effects of Hp extracts in diabetic animals.

Recent observations of the authors' laboratories (*30*) have revealed that, in a rat model of streptozotocin-induced diabetes, the effects of pure hyperforin (10 mg/kg/day; *i.p.*) are somewhat analogous to those of the Hypericum extract used in this study. Hyperforin has been reported to release acetylcholine in the rat brain (*31,32*). Acetylcholine may subsequently stimulate muscarinic M3 receptors in pancreatic cells (*33,34*) and augment insulin release, resulting in anti-hyperglycemic activity. Hyperforin has also been reported to activate cation channels of the transient receptor potential (TRP) family (*35*). Interestingly, the TRP channels of pancreatic β cells are also activated during glucose-stimulated insulin secretion (*29*). Thus, TRP channels could be a possible target of hyperforin in stimulating insulin secretion.

In conclusion, standardized extracts of Hp have a beneficial effect in a rat model of NIDDM and preliminary observations indicate that hyperforin may be at least partially responsible for the observed activity of Hp extracts though additional evidence will be provided by future studies.

Acknowledgements

The authors wish to thank Indian Herbs Research & Supply Co. Ltd., Saharanpur, India, for providing standardized extracts of Indian *Hypericum perforatum*. G M Husain wishes to thank the University Grants Commission, New Delhi, for its financial assistance.

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(*Received July 24, 2009; Revised August 17, 2009; Accepted August 19, 2009*)

Original Article

Sedative and anxiolytic effects of different fractions of the *Commelina benghalensis* Linn

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ABSTRACT: The present study was designed to investigate sedative and anxiolytic properties of the four different fractions (chloroform, pet ether, *n*-butanol and hydromethanol soluble fractions, coded as CFCB, PECB, NBCB and HMCB, respectively) of the aerial parts of *Commelina benghalensis* using rodent behavioral models, such as hole cross, open field and thiopental sodium induced sleeping time tests for sedative property and elevated plus-maze (EPM) test for anxiolytic potential, respectively. All fractions, at the doses of 200 mg/kg, *p.o.* and 400 mg/kg, *p.o.*, displayed dose dependent suppression of motor activity, exploratory behavior (in hole cross and open field tests) and prolongation of thiopental induced sleeping time in mice; maximum effect was shown by chloroform (CFCB) and pet ether (PECB) fractions. In EPM test, chloroform (CFCB) and pet ether (PECB) fractions with similar doses significantly ($p < 0.05$) increased exploration to and time spent by the treated mice in EPM open arms in a way similar to that of diazepam while the effect of NBCB and HMCB fractions on entry to and time spent in open arms was not found to be statistically significant. These findings provide *in vivo* evidence that aerial parts of *C. benghalensis* in general, and chloroform (CFCB) and pet ether (PECB) soluble fraction has significant sedative and anxiolytic effects. Furthermore, these results may justify the scientific basis for the use of this plant in traditional medicine as a modality for anxiety and related disorders.

Keywords: Medicinal plant, *Commelina benghalensis*, sedatives, anxiolytics, elevated plus-maze

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1. Introduction

Anxiety and depressive disorders are the most frequent psychiatric conditions encountered today. Modern life stress, associated trials and tribulations are suspected to be responsible for the upsurge of such psychiatric derangements. It is reported that more than 20% of the adult population suffer from these conditions at some stage during their life (*1*,*2*). Since long the benzodiazepines remain to be the most frequently prescribed synthetic drugs of choice for acute anxiety and other allied disorders including depression, epilepsy and insomnia. But chronic use of these drugs have very serious side effects ranging from respiratory, digestive and immune system dysfunctions to deterioration of cognitive function, physical dependence and tolerance (*3*). In this context, there has been a resurgence of interest in medicine from natural sources (mainly from plant kingdom) with the hope that drugs of plant origin will have significantly lesser side effects than that observed with synthetic drugs while having comparable efficacy. Thus the search for novel pharmacotherapy from medicinal plants for psychiatric illnesses has advanced significantly in the past decade (*4*). This is reflected in the large number of herbal medicines whose psychotherapeutic potential has been assessed in a variety of animal models. These studies have provided useful information for the development of new pharmacotherapies from medicinal plants for successful use in modern clinical psychiatry.

Commelina benghalensis (family Commelinaceae) is a perennial herb native to tropical Asia and Africa, used in the Indian subcontinent as a folk medicine for the treatment of leprosy, headache, fever, constipation, jaundice and snake bite (*5*-*7*). The plant is also used for mouth thrush (*8*), inflammation of the conjunctiva, psychosis (*9*), epilepsy, nose blockage in children (*10*), insanity (*11*) and exophthalmia. *C. benghalensis* is used medicinally as a diuretic, febrifuge and anti-inflammatory (*12*-*15*). It is used as an animal fodder,

eaten by humans as a vegetable in Pakistan, also used there medicinally, but with different purported effects, including as a laxative and to cure inflammations of the skin as well as leprosy (*16*). The plant is also reported to have antitumor, anticancer and antioxidant activity (*5*,*17*,*18*). Previous phytochemical investigations of the Commelina genus were reported on *C. undulata* R.Br., *C. benghalensis* L. and *C. communis* L. from which several types of compounds such as alkaloids, steroids, terpenoids, iridoids, flavonoids, lignans, aliphatic alcohols, polyols, and phenolic acids were obtained (*19-27*). Moreover, the whole plant of *C. benghalensis* was reported to contain alkaloid, volatile oil, wax (*28*), vitamin-C and higher levels of both lutein and β-carotene (*29*,*30*).

However, only a few biological works of medicinal interest have so far been carried out on this plant to substantiate the traditional claims. Thus, we have evaluated the various fractions aerial parts of *C. benghalensis* for sedative and anxiolytic activities in rodent behavioral models. Additionally, we have determined the pharmacological basis for the use of the plant in traditional medicine for the treatment of major neuropsychiatric disorder involving anxiety.

2. Materials and Methods

2.1. *Drugs and chemicals*

The following drugs and chemical were used in this study: Diazepam (Square Pharmaceutical Ltd., Bangladesh), thiopental sodium, quercetin, *etc*. (Sigma Chemicals Co., USA).

2.2. *Plant material*

The plant was collected from Old Elephant Road, Eskaton Garden, Dhaka in April 2008 when weed beds were in their maximum densities. The whole plant with leaves, stems and roots was collected and identified by experts in Bangladesh National Herbarium, Mirpur, Dhaka, where a voucher specimen (Accession No.32784) has been deposited for future reference. The plant was thoroughly washed with water; roots were discarded and the aerial parts were dried in hot air oven at 50°C for 3 days and at 40°C for the next 4 days.

2.3. *Extraction and solvent-solvent partitioning*

The dried aerial parts were coarsely powdered from which 500 g was extracted with a mixture of methanol: water (7:3, v/v) in a Soxhlet apparatus. The solvent was completely removed and obtained 18 g (yield 3.6%) dried crude extract. Solvent-solvent partitioning was done using the protocol as described by Rahman *et al*. (*18*). The crude extract was dissolved in 10% aqueous methanol to make the mother solution which was partitioned off successively by three solvents namely pet ether (3 × 100 mL), chloroform (3 × 100 mL), *n*-butanol (3 × 100 mL). All the three fractions and the residual hydromethanol fraction were subjected to dryness under reduced pressure. The dried extracts thus obtained were used for investigation (*18*).

2.4. *Animal*

For the experiment male Swiss albino mice, 3-4 weeks of age, weighing between 20-25 g, were collected from the Animal Research Branch of the International Center for Diarrheal Disease and Research, Bangladesh (ICDDR, B). Animals were maintained under standard environmental conditions (temperature: (24.0 ± 1.0°C), relative humidity: 55-65% and 12 h light/12 h dark cycle) and had free access to feed and water *ad libitum*. The animals were acclimatized to laboratory condition for one week prior to experimentation.

2.5. *Phytochemical screening*

The freshly prepared crude extract was qualitatively tested for the presence of chemical constituents. These were identified by characteristic color changes using standard procedures (*31*).

2.6. *Total flavonoid content determination*

Total flavonoid contents of the fractions were estimated using a method previously described by Kumaran and Karunakaran (*32*) using quercetin as the reference. Each 1 mL of the extracts in methanol (250 µg/mL) was mixed with 1 mL aluminium trichloride in ethanol (20 mg/mL and a drop of acetic acid, and then diluted with ethanol to 25 mL. The absorbance at 415 nm was read after 40 min. Blank samples were prepared from 1 mL of plant extract and a drop of acetic acid, and then diluted to 25 mL with ethanol. These data were used to estimate the flavonoid contents using a standard curve obtained from various concentration of quercetin.

2.7. *Thiopental sodium induced sleeping time test*

The animals were randomly divided into ten groups consisting of five mice each. The test groups received different fractions of the aerial parts of *C. benghalensis* at the doses of 200 mg/kg and 400 mg/kg body weight (b.w.) while positive control was treated with diazepam (1 mg/kg) and control with vehicle (1% Tween 80 in water). Thirty minutes later, thiopental sodium (40 mg/kg) was administered to each mouse to induce sleep. The animals were observed for the latent period (time between thiopental administration to loss of righting reflex) and duration of sleep *i.e.* time between the loss and recovery of righting reflex (*33*).

Table 1. Result of chemical group test of the crude aqueous-methanolic extract of the aerial parts of *C. benghalensis*

Extract	Steroid	Alkaloid	Reducing sugar	Tannin	Gum	Flavonoid	Saponin
C. benghalensis	–	++	–	–	–	+++	–

(+): Present, (–): Absent.

2.8. *Hole cross test*

The method was carried out as described by Takagi *et al*. (*34*). A steel partition was fixed in the middle of a cage having a size of 30 × 20 × 14 cm. A hole of 3 cm diameter was made at a height of 7.5 cm in the center of the cage. The animals were divided into control, positive control, and test groups containing five mice each. The test groups received four different fractions of the aerial parts of *C. benghalensis* at the doses of 200 mg/kg and 400 mg/kg b.w. orally whereas the control group received vehicle (1% Tween 80 in water). The number of passage of a mouse through the hole from one chamber to other was counted for a period of 3 min at 0, 30, 60, 90, and 120 min after oral administration of the test drugs (*34*).

2.9. *Open field test*

In open field test, the animals were divided into control, positive control, and test groups containing five mice each. The test groups received different fractions of the aerial parts of *C. benghalensis* at the doses of 200 mg/kg and 400 mg/kg b.w. orally whereas the control group received vehicle (1% Tween 80 in water). The floor of an open field of half square meter was divided into a series of squares each alternatively colored black and white. The apparatus had a wall of 40 cm height. The number of squares visited by the animals was counted for 3 min at 0, 30, 60, 90, and 120 min after oral administration of the test drugs (*35*).

2.10. *Elevated plus-maze (EPM) test*

The apparatus consist of two open arms (5 × 10 cm) and two closed arms (5 × 10 × l5 cm) radiating from a platform (5 × 5 cm) to form a plus-sign figure. The apparatus was situated 40 cm above the floor. The open arms edges were 0.5 cm in height to keep the mice from falling and the closed-arms edges were 15 cm in height. Sixty minutes after administration of the test drugs, each animal was placed at the center of the maze facing one of the enclosed arms. During the 5-min test period, the number of open and enclosed arms entries, plus the time spent in open and enclosed arms, was recorded (*36*). Entry into an arm was defined as the point when the animal places all four paws onto the arm. The procedure was conducted in a sound attenuated room; observations made from an adjacent corner.

Table 2. Total flavonoid contents of the different fractions of the aerial parts of *C. benghalensis*

Sample	Total flavonoid (in mg/g, quercetin equivalents)
CFCB	838.72 ± 3.25
PECB	105.82 ± 4.33
NBCB	305.31 ± 2.16
HMCB	51.99 ± 16.23

Values are the mean of duplicate experiments and represented as mean ± SD. CFCB = Chloroform fraction, PECB = Pet ether fraction, NBCB = *n*-butanol fraction and HMCB = Hydromethanol fraction of the aerial parts of *C. benghalensis*.

2.11. *Statistical analysis*

Statistical analysis for animal experiment was carried out using one-way ANOVA followed by Dunnet's multiple comparisons. The results obtained were compared with the vehicle control group. p values < 0.05, 0.001 were considered to be statistically significant.

3. Results

3.1. *Phytochemical screening*

Phytochemical analyses of the crude extract revealed the presence of alkaloid and flavonoid (Table 1).

3.2. *Total flavonoid content*

The fractions were found to contain high amounts flavonoids (expressed as quercetin equivalents) as measured by total flavonoid content determination assay (Table 2). Flavonoid contents were of the following order: CFCB > NBCB > PECB > HMCB.

3.3. *Thiopental sodium induced sleeping time test*

In thiopental induced hypnosis test, CFCB and PECB fractions of the aerial parts of *C. benghalensis*, at the doses of 200 and 400 mg/kg, induced the sleep at an earlier stage while NBCB and HMCB fractions were found to have little effect on the onset of thiopental induced sleep. But all fractions dose dependently prolonged the duration of sleeping time in test animals compared to control (Table 3).

3.4. *Hole cross test*

All fractions, at 200 mg/kg and 400 mg/kg b.w. doses,

produced significant ($p < 0.05$, $p < 0.001$) decrease of locomotion from its initial value during the period of experiment (Figure 1). Maximum suppression of locomotor activity was displayed by chloroform fraction (CFCB), which was comparable to the reference drug diazepam.

3.5. *Open field test*

The number of squares traveled by the mice was suppressed significantly from the second observation period at both dose levels (200 mg/kg and 400 mg/kg b.w.) of the four fractions of the aerial parts of *C. benghalensis*. The results were dose dependent and statistically significant (Figure 2). The locomotor activity decreased in the following order: CFCB > PECB > NBCB > HMCB.

3.6. *Elevated plus-maze test*

CFCB and PECB treatment, at 400 mg/kg b.w., significantly increased the percentage of entries (Figure 3A) of mice into the open arms, and the percentage of time spent (Figure 3B) in the open arms of the elevated plus-maze. However, the effects of treatment of mice with NBCB and HMCB fractions on open arm entries and time spent in open arms were not statistically significant.

Table 3. Effects of the different fractions of the aerial parts of *C. benghalensis* on thiopental induced sleeping time in mice

Treatment	Dose (mg/kg)	Onset of sleep (min)	Duration of sleep (min)
Control	Vehicle	15.4 ± 4.207	47.2 ± 6.485
Diazepam	1	6.8 ± 2.584	98.6 ± 7.059**
CFCB1	200	9 ± 2.716	84.8 ± 9.588*
CFCB2	400	7.2 ± 2.608	102.4 ± 13.53**
PECB1	200	9.4 ± 2.971	79.6 ± 7.735*
PECB2	400	7.4 ± 3.328	94.4 ± 10.545**
NBCB1	200	15.8 ± 2.608	66.2 ± 7.684
NBCB2	400	12.6 ± 3.328	73.6 ± 7.497*
HMCB1	200	14.6 ± 3.114	63.8 ± 12.295
HMCB2	400	13.6 ± 3.978	69.4 ± 4.102

Values are mean ± SEM, (n = 5); * $p < 0.05$, ** $p < 0.001$, Dunnet test as compared to control [Vehicle = 0.4 mL/mouse, *p.o.*; CFCB = Chloroform fraction, PECB = Pet ether fraction, NBCB = *n*-butanol fraction and HMCB = Hydromethanol fraction of *C. benghalensis*; 1 = 200 mg/kg b.w., 2 = 400 mg/kg b.w.]

4. Discussion

It was observed from the present study that CFCB and PECB fractions of the aerial parts of *C. benglalensis* (200 mg/kg and 400 mg/kg) show strong sedative and antianxiety properties. Both CFCB and PECB fractions dose dependently potentiated the sleep induced by thiopental suggesting that the aerial parts of the plant possesses sleep inducing property. Thiopental, a hypnotic agent, when given at appropriate dose, induces sedation or hypnosis by potentiating GABA mediated postsynaptic inhibition through allosteric modification of GABA receptors. Substances which possess CNS depressant activity either decrease the time for onset of sleep or prolong the duration of sleep or both (*37*). Moreover, the study on locomotor activity, as measured by hole cross and open field tests, showed that all

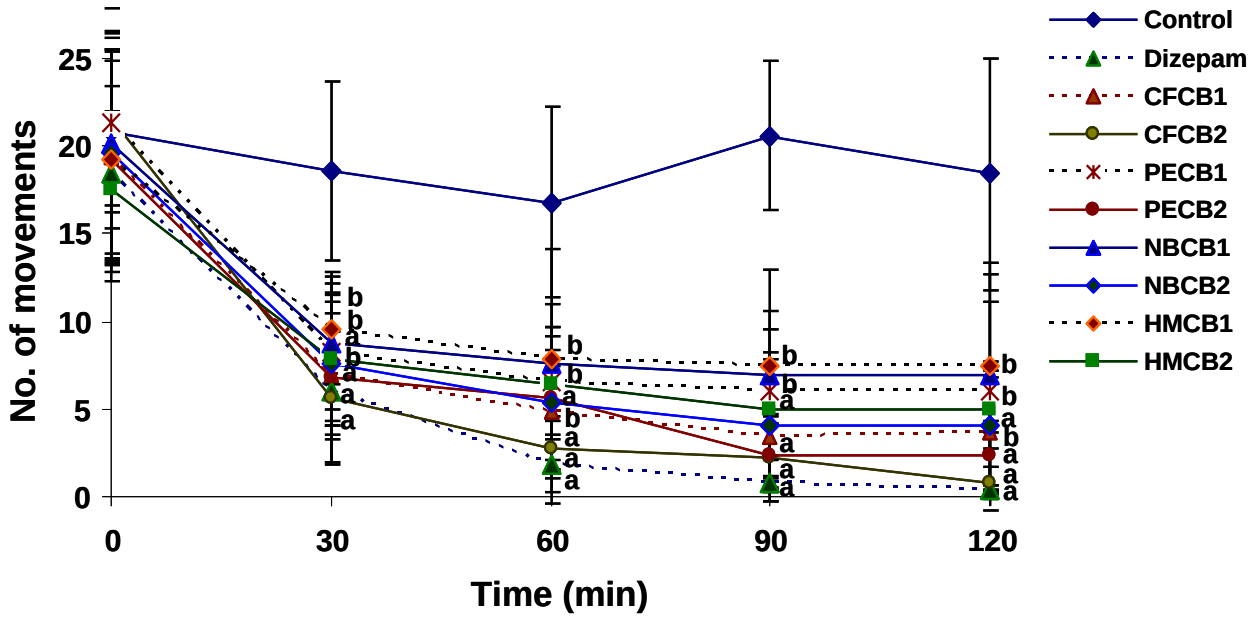

Figure 1. Effects of the different fractions of the aerial parts of *C. benghalensis* on Hole cross test in mice. Values are mean ± SEM, (*n* = 5); **b**, $p < 0.05$; **a**, $p < 0.001$, Dunnet test as compared to control [Vehicle = 0.4 mL/mouse, *p.o.*; CFCB = Chloroform fraction, PECB= Pet ether fraction, NBCB = *n*-butanol fraction and HMCB = Hydromethanol fraction of *C. benghalensis*; 1 = 200 mg/kg b.w., 2 = 400 mg/kg b.w.].

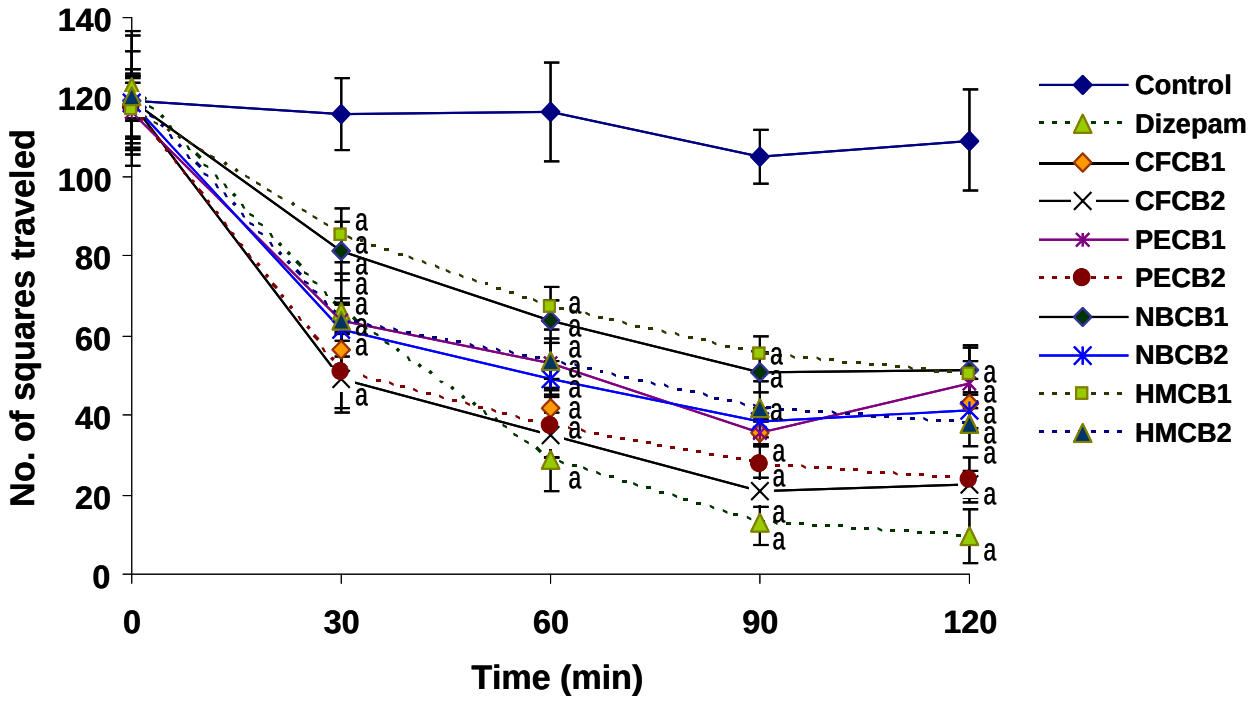

Figure 2. Effects of the different fractions of the aerial parts of *C. benghalensis* on Open field test in mice. Values are mean ± SEM, (n = 5); **a**, $p < 0.001$, Dunnet test as compared to control [Vehicle = 0.4 mL/mouse, *p.o.*; CFCB = Chloroform fraction, PECB = Pet ether fraction, NBCB = *n*-butanol fraction and HMCB = Hydromethanol fraction of *C. benghalensis*; 1 = 200 mg/kg b.w., 2 = 400 mg/kg b.w.].

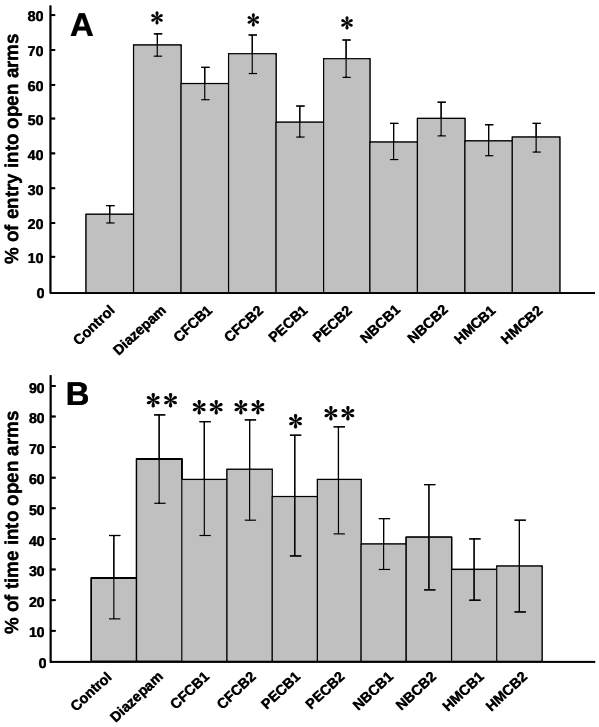

Figure 3. Effects of the different fractions of the aerial parts of *C. benghalensis* on the percentage of entries (A) and the time spent (B) in open arms of the elevated plus-maze during the 5-min test session. Values are mean ± SEM, (n = 5); * $p < 0.05$, ** $p < 0.001$, Dunnet test as compared to control [Vehicle = 0.4 mL/mouse, *p.o.*; CFCB = Chloroform fraction, PECB = Pet ether fraction, NBCB = *n*-butanol fraction and HMCB = Hydromethanol fraction of *C. benghalensis*; 1 = 200 mg/kg b.w., 2 = 400 mg/kg b.w.].

fractions of the aerial parts of *C. benghalensis* (at both dose levels) decreased the frequency and the amplitude of movements. Since locomotor activity is a measure of the level of excitability of the CNS (*38*), this decrease in spontaneous motor activity could be attributed to the sedative effect of the plant extracts (*39*,*40*). All fractions significantly decreased the locomotion in mice. The locomotor activity lowering effect was evident at the 2nd observation (30 min) and continued up to 5th observation period (120 min). Maximum depression of locomotor activity was observed from 3rd (60 min) to 5th (120 min) observation period. The results were also dose dependent and statistically significant (Figures 1 and 2).

However, the evaluation of the putative anxiolytic activity of the four different fractions of the aerial parts of *C. benghalensis* was performed using elevated plus-maze (EPM). The primary measures in the EPM are the percentage of entries into the open arms and of the time spent on the open arms. An anxiolytic effect is suggested when the test drug increases open arms entries without altering the total number of arm entries (*41*). Although CFCB and PECB treatment, at 200 mg/kg b.w., in mice did not display significant increase in the percentage of entries into open arms, both fractions at 400 mg/kg b.w. showed a significant increase in the percentage of time spent in the open arms of the maze, similar to the effects observed following treatment with the reference anxiolytic drug diazepam, in a dose

dependent manner. These results could indicate an anxiolytic-like activity of the chloroform and pet-ether soluble fractions (CFCB and PECB respectively) of the aerial parts of *C. benghalensis*.

Phytochemical analyses of the crude aqueous methanolic extract of the aerial parts of *C. benghalensis* revealed the presence of alkaloid and flavonoid. Again, total flavonoid content determination assay indicates high amounts of flavonoids in the fractions. There are also reports on the presence of anthocyanins (a kind of flavonoids) in this plant (*20*,*21*,*31*). So the observed bioactivities may be attributed to flavonoid compounds. However, many flavonoids were found to be ligands for the gamma aminobutyric acid type A ($GABA_A$) receptors in the central nervous system (CNS); which led to the hypothesis that they act as benzodiazepine-like molecules. Thus the sedative and anxiolytic effects observed might be due to the interaction of flavonoids with the GABA/benzodiazepine receptor complex in brain (*42*). This is supported by their behavioral effects in animal models of anxiety, sedation and convulsion (*43*,*44*). Electrophysiological experiments with flavone and flavanone derivatives have shown that some of them can modulate GABA-generated chloride currents, either positively or negatively. Due to the increased knowledge of the diversity of $GABA_A$ receptor subtypes, the number of studies with cloned receptors of defined subunit composition has recently risen, and experiments with some natural and synthetic flavones and flavonones have shown that they can modulate gamma aminobutyric acid (GABA)-generated chloride currents, either positively or negatively (*45*-*48*). Thus decreased spontaneous motor activity could be attributed to the CNS depressant activity of the aerial parts of *C. benghalensis*.

5. Conclusions

Using behavioral pharmacology models, we have demonstrated that the aerial parts of *C. benghalensis*, in particular chloroform (CFCB) and pet ether (PECB) soluble fractions, possesses strong sedative and anxiolytic potential. Therefore, these fractions could have significant therapeutic utility for the treatment of anxiety and related neuropsychiatric disorders. Furthermore, evidence obtained from the present study may justify the use of this plant in traditional medicine for the treatment of excited mental disorders such as psychosis, insanity, epilepsy, *etc*.. However, further studies are warranted to understand the underlying mechanism of sedative and anxiolytic activities and to isolate the active phytochemical ingredient(s) responsible for the observed bioactivities in animal models.

Acknowledgements

This research was supported in part by the Ministry of Science, Information and Communication Technology, Bangladesh. Authors are grateful to Professor Dr. Abdul Ghani, Chairman, Department of Pharmacy, Stamford University Bangladesh.

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(*Received August 12, 2009; Revised September 10, 2009; Accepted September 11, 2009*)

Original Article

Vaginal delivery of protein drugs in rats by gene-transformed *Lactococcus lactis*

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ABSTRACT: A probiotic bacterium, *Lactococcus lactis* subsp. *lactis* (*L. lactis*) transformed with plasmid ss80, which made it capable of synthesizing and secreting β-lactamase, a 29 kDa protein, was used to deliver β-lactamase *via* vaginal route. The vaginal absorption of β-lactamase in rats was studied when delivered by this *L. lactis* system and compared to the β-lactamase solution with or without the untransformed *L. lactis*. The vaginal administration of 1.2×10^7, 3×10^7, and 8×10^7 colony forming units (cfu) of *L. lactis* resulted in the amount absorbed of 77, 194, and 216 mU, with the respective doses. C_{max}, mean retention time and mean absorption time of β-lactamase were also increased with the increase in the cfu of *L. lactis* administered. These results have demonstrated that *L. lactis* can significantly increase ($p < 0.01$) the β-lactamase vaginal absorption as compared to the β-lactamase solution, which is probably due to the adhesion of *L. lactis* to and continuous synthesis and delivery of β-lactamase directly to the vaginal mucosa. In conclusion, transformed normal flora may be an efficient method to deliver protein drugs through the vaginal route.

Keywords: *Lactococcus lactis*, β-lactamase, normal flora, protein delivery, vaginal, pharmacokinetics

1. Introduction

Protein drugs are generally administered by parenteral route because of their low bioavailability through the other routes. There is a great need for an alternate non-invasive means for the delivery of the protein drugs. The non-invasive delivery routes that have been explored are oral, nasal, buccal, rectal and vaginal routes. The vagina has been focused as a favorable alternative site for the systemic delivery of protein drugs because of the relatively high permeability of the vaginal epithelium, by passage of the hepatic first-pass metabolism, large surface area and rich blood supply. In addition, a prolonged contact of a delivery system with the vaginal mucosa may be achieved more easily than at other absorption sites like rectum or intestinal mucosa. In a study where absorption of a potent luteinizing hormone-releasing hormone analog (leuprolide) from various routes of administration was compared, vaginal route showed the greatest potency as compared to the other non-parenteral routes (*1*). In post menopausal women, the reduced epithelial thickness may further increase the absorption (*2*).

We have proposed that normal flora may be used as a delivery system for the vaginal protein delivery. Normal flora consists of the non-pathogenic bacteria that exist in the open tracts of the human body such as intestine, nostril, and vagina. By recombinant DNA technology, the normal flora can be genetically engineered to synthesize and secrete the protein drugs. Their natural tendency to adhere tightly to the epithelial cell surface (*3*) of the channels where they normally reside will result in delivering sufficient amount of protein at the site of absorption and will also minimize the enzymatic and bacterial degradation of the protein drugs. This will result in the concentrate of protein drugs on the absorption surface to improve the bioavailability.

Lactobacillus is the most prevalent organism in the vaginal environment together with many other facultative and obligate aerobes and anaerobes. The acidic pH of 4-5 of healthy women of reproductive age is maintained by the lactobacilli (*4*). When the vaginal pH becomes alkaline it leads to various kinds of infections as the protective barrier provided by acidic layer becomes less effective.

L. lactis, one of the safest strains in the LAB (Lactic acid bacteria) family, is used in the present study. This strain has been transformed with plasmid ss80 (*5*).

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Therefore it can synthesize and secrete β-lactamase, a 29 kDa protein, which is the non-therapeutic model protein used in our study. β-Lactamases, produced by some of the gram-positive as well as gram-negative bacterium, are the enzymes that catalyze the hydrolysis of β-lactam ring and are responsible for the bacterial resistance to penicillin, cephalosporin and many other antibiotics. Previously, we have reported that the oral delivery of β-lactamase by this *L. lactis* could significantly increase the β-lactamase oral bioavailability by 2~3 folds ($p < 0.01$), and the mean transit time (MTT) by 3~4 times ($p < 0.01$), as compared to the solution form with/without the untransformed *L. lactis* (*6*). In our another study (*7*) we have found that *L. lactis* could significantly increase the transportation of β-lactamase through C-33A monolayer (human cervical cell monolayer) and almost double the transportation rate as compared to the solution form. The present study was carried out to examine the feasibility of this *L. lactis* to secrete and deliver β-lactamase *in vivo* and its bioavailability through vaginal route in rats. In addition, the effect of different doses of *L. lactis* on the plasma profile of β-lactamase was also investigated.

2. Materials and Methods

2.1. *Materials*

Lactococcus lactis subsp. *lactis*, transformed with plasmid ss80 (thereafter referred as *L. lactis*) encoding for β-lactamase and its secretion signal was generously provided by Dr. Soile Tynkleynen (Valio Ltd. Helsinki, Finland). M17 broth and agar were purchased from Becton Dickinson (Sparks, MD, USA). Ampicillin, β-lactamase (from Bacillus cereus EC 3.5.2.6), ascorbic acid, ethylenediamine tetraacetic acid (EDTA), trichloroacetic acid (TCA) and all other chemicals were purchased from Sigma Chemical Co. (St. Louis, MO, USA). All the chemicals were of analytical grade.

Female Sprague-Dawley rats weighing 300 ± 25 g, purchased from Taconic Farms, Germantown, NY, USA, were used in these experiments. The rats were acclimated to their surrounding for at least one week before the experiment. The micro-isolator cages were used with two animals in each cage. They were housed on a 12-h light/dark cycle and a relative humidity of 40 to 60%. All the experiments were performed under a vertical Laminar Flow Hood.

2.2. *Vaginal delivery of β-lactamase in rats*

A total of 36 female Sprague-Dawley rats were evenly randomized into six groups. The rats were restrained from food and water for 12 h prior to the dosing. The rats were given 0.2 mL of β-lactamase free solution or *L. lactis* in log-growing phase according to the schedule in Table 1. For the intravaginal administration, the solution was deposited deep into the vagina, using a 1 mL disposable syringe attached to a mouse gavaging needle. The blood samples (about 0.4 mL of each) were collected in an Eppendorf tube containing 25 mg of EDTA at the predetermined time points. The research protocol was approved by Institutional Animal Care and Use Committee (IACUC) at the St. John's University.

2.3. *β-lactamase HPLC assay*

β-Lactamase concentration in the plasma was assayed by an HPLC method reported previously (*8*). To a 0.2 mL of the plasma sample, 0.4 mL of 6.25 mM ampicilllin (substrate) was added. The reaction mixture was incubated at 37°C for 30 min and then 0.1 mL of 60% TCA at 4°C was immediately added to cease the reaction. The solution was centrifuged at 9,000 × g for 5 min and 0.5 mL of the supernatant was added to 2 mL

Table 1. Experimental design and pharmacokinetic parameters of β-lactamase in rats after vaginal administration (mean ± S.D., *n* = 6)

Group	Dose and route	$AUC_{0-\infty}$ mU•h•mL^{-1}	C_{max} mU	T_{max} h	MRT h	MAT h	AB[1] mU
I	1,008 mU of β-lactamase, vaginal	24.8 (8.9)	1.08 (0.09)	4-8	14.1 (2.2)	9.0 (2.2)	78.7 (28.2)
II	1,008 mU of β-lactamase and 3×10^7 cfu of the untran-*L. lactis,* vaginal	25.5 (4.0)	1.32 (0.2)	2-8	14.8 (1.1)	9.7 (1.1)	81.0 (12.6)
III	1.2×10^7 cfu of *L. lactis,* vaginal	24.4 (5.8)	1.3 (0.2)	4-8	14.7 (3.0)	9.6 (3.0)	77.4 (18.6)
IV	3×10^7 cfu of *L. lactis*, vaginal	61.0* (17.0)	2.8* (0.7)	4-8	20.0* (4.4)	14.9 (4.4)	193.6* (53.9)
V	8×10^7 cfu of *L. lactis*, vaginal	68.1* (18.5)	4.1* (0.6)	6-8	22.5* (4.1)	17.4* (4.1)	216.1* (58.7)
VI	252 mU of β-lactamase, *i.v.*	79.4 (12.4)	-	-	5.1 (0.9)	-	100

[1]: absolute bioavailability; *: significant difference from Group II and III ($p < 0.05$).

solution of 0.5 M acetate buffer at pH of 5 containing ascorbic acid (0.5 mg/mL) and EDTA (50 mM). The resulting solution was heated at 100°C for exactly 30 min. The processed samples were then analysed by an HPLC method after cooling them to the room temperature.

The HPLC system consisted of a Waters 600E system controller, a Waters 717 Autosampler, and a Waters 470 Scanning fluorescence detector. The separation was done on a μ Bondapak C18 cartridge column (300 × 3.9 mm I.D.). The injection volume was 10 μL. The mobile phase was 80% of 0.1 M phosphate buffer (pH = 5.0) and 20% of acetonitrile with a flow rate of 1.5 mL/min. The column effluents were monitored at excitation and emission wavelengths of 410 nm and 475 nm, respectively, for a run time of 11 min, and the peak of interest was seen at the retention time of 9.2 min. Two standard curves were constructed by analysis of the peak area against the concentration of the β-lactamase in 2 different concentration ranges of 0.252-1.26 mU/mL and 1.26-12.6 mU/mL, which were prepared by spiking the blank plasma with β-lactamase standard solution. The concentration of β-lactamase in the rat plasma samples was determined by the standard curve method.

2.4. *Pharmacokinetic (PK) parameters and statistics*

The PK parameters were calculated by the noncompartmental analysis (*9*). The area under the plasma concentration versus time curve from beginning to the last measurable concentration time point, $AUC_{0\text{-last}}$, was determined by the linear trapezoidal method, AUC from the last measurable concentration time point to infinite, $AUC_{last\text{-}\infty}$, was calculated as C_{last}/k, where C_{last} is the last measurable concentration and k the elimination rate constant. The area under the first moment curve (AUMC) was computed from time zero to infinity. MRT (Mean Residence Time) and MAT (Mean Absorption Time) were also calculated. The amount absorbed (AB) vaginally were determined as:

$$AB = \frac{AUC_{(vaginal)}}{AUC_{(i.v.)}} \times Dose_{(i.v.)}$$

The maximum plasma concentration reached (C_{max}) and the time at which it was reached (T_{max}) were observed from the β-lactamase plasma concentration-time profile. Statistical analysis (ANOVA) was performed with $\alpha = 0.05$ as the minimal level of significance.

3. Results and Discussion

3.1. *Vaginal absorption of β-lactamase in rats*

In the present experiment the feasibility of *L. lactis* to secrete β-lactamase in the cervico-vaginal ecosystem and investigate the absorption efficiency of β-lactamase into the systemic circulation by the delivery of *L. lactis* to the rat's vagina was studied. Figures 1 and 2 show the plasma concentration profile of β-lactamase after the vaginal administration. The pharmacokinetic parameters are listed in the Table 1.

For this study the free solution of β-lactamase was used as control. The vaginal administration of 1,008 mU of β-lactamase in free solution resulted in the mean C_{max} of 1.98 mU/mL, T_{max} between 4-8 h, MRT of 14 h, MAT of 9 h and no β-lactamase was detected at 48 h (Figure 1). The co-administration of the untransformed *L. lactis* with β-lactamase free solution showed similar ($p > 0.05$) β-lactamase plasma concentration profile as the free solution alone, which indicates that the untransformed *L. lactis* did not affect the absorption of β-lactamase *via* vaginal route.

In case of *L. lactis*, three doses were used to study not only the delivery efficiency but also the dose-absorption relationship. As shown in the Figure 2, the administration of 1.2×10^7, 3×10^7, and 8×10^7 cfu, resulted in C_{max} of 1.28 mU/mL, 2.79 mU/mL, and 4.07 mU/mL, respectively; T_{max} in the range of 4-8 h; MRT of 14.7 h, 20.0 h, and 22.5 h, respectively; MAT of 9.6 h, 14.9 h, and 17.4 h, respectively; and there was still a quantifiable amount of β-lactamase in the plasma at 72 h after dosing. The T_{max} for all the three doses was between 4-8 h. After the oral administration of 1.2×10^7 *L. lactis* cfu, β-lactamase was still detectable in plasma at 24 h, while 48 h after the 3×10^7 and 8×10^7 *L. lactis* cfu administration. MRT of the β-lactamase when delivered by the three doses of *L. lactis* cfu was 14.7, 20.0, and 22.5 h, respectively; and the MAT 9.6, 14.9, and 17.4 h, respectively. The amount of β-lactamase absorbed when delivered vaginally by 1.2×10^7, 3×10^7, and 8×10^7 of *L. lactis* was 77.4, 193.6, and 216.1 mU of *i.v.* dose, respectively.

There was a 40% increase in the MRT and MAT when 3×10^7 *L. lactis* cfu were administered ($p < 0.05$) as compared to the free solution. This increase was most probably due to the ability of *L. lactis* to adhere to the vaginal mucosa, and continuously multiply and secrete β-lactamase right onto the absorption epithelium. Our previous *in vitro* study (*7*) has also demonstrated that there was a 50% increase in the β-lactamase transport across the C-33A monolayer when delivered by the *L. lactis* as compared to the free solution.

The concentration of β-lactamase on the absorption surface is the major factor for absorption enhancement. As the transformed *L. lactis* adhered to the epithelial layer, it secreted β-lactamase directly onto the absorption surface, resulting in a locally high concentration. Over the time, this localized β-lactamase would diffuse through these membranes. The transformed *L. lactis* is thus able to significantly enhance the β-lactamase absorption *in vivo*. First,

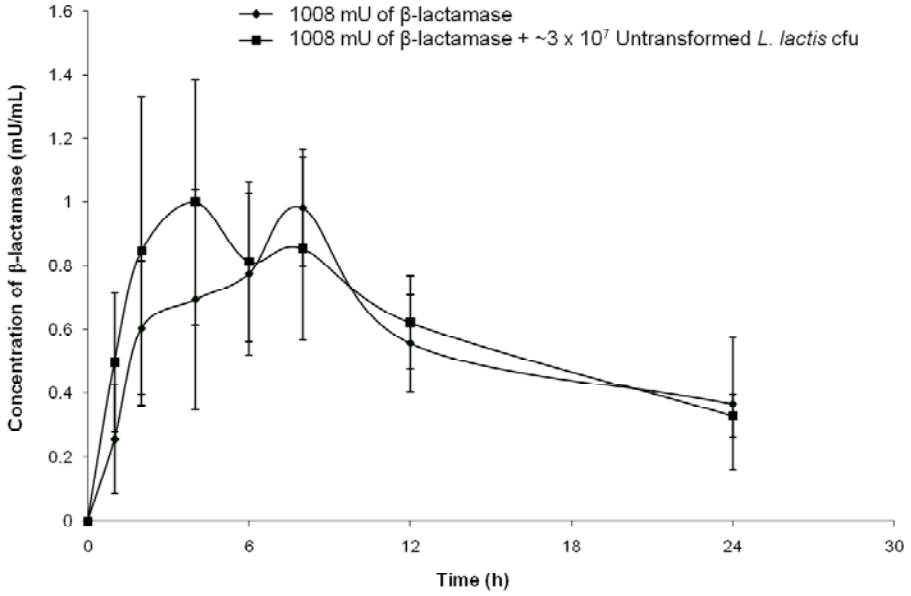

Figure 1. β-Lactamase plasma concentration after the vaginal administration of 200 µL of β-lactamase solution with or without untransformed *L. lactis* to the rats (*n* = 6).

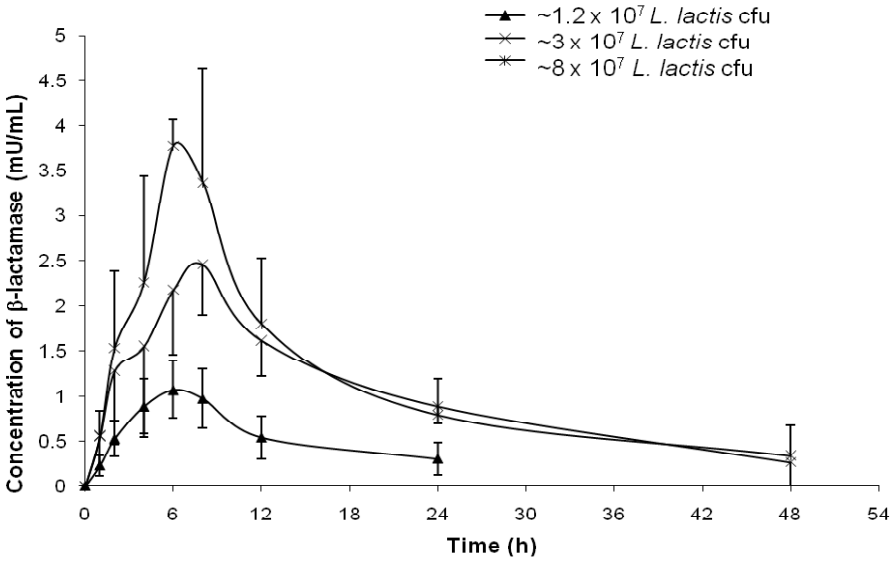

Figure 2. β-Lactamase plasma concentration after the vaginal administration of 200 µL of different doses of *L. lactis* to the rats (*n* = 6).

through the adherence to the vaginal epithelium, the transformed *L. lactis* will secrete β-lactamase onto the vaginal epithelium to concentrate the protein drug on the absorption surface and reduce the exposure of the protein drug to a hostile environment. Second, the transformed *L. lactis* will continuously produce and secrete β-lactamase, and due to its adhesive property it usually can stay in the vagina for a certain period before being eliminated, so that the transformed *L. lactis* can provide a prolonged delivery mechanism.

The amount of protein that can be delivered through this delivery system can be controlled by controlling the number of bacteria that is being delivered. Thus it would be of significant interest to compare the absorption profiles by the three different doses of the *L. lactis* (Figure 3). When the dose was increased from 1.2×10^7 cfu to 3×10^7 cfu (a 1.5-fold increase), the AUC and C_{max} were increased by 1.5 and 1.2 folds, respectively, showing a direct dose-absorption relationship. However, the further increase of the dose to 8×10^7 cfu (a 5.7-fold increase), did not resulted a proportional increase in absorption, although there was a 1.8-fold increase in AUC and a 2.2-fold increase in C_{max}. These results can be explained by the limited nutrients and space *in vivo*. In overall, the results demonstrate the relationship between the dose *L. lactis* and the protein drug entering into the systemic circulation, providing some guidance for dosing

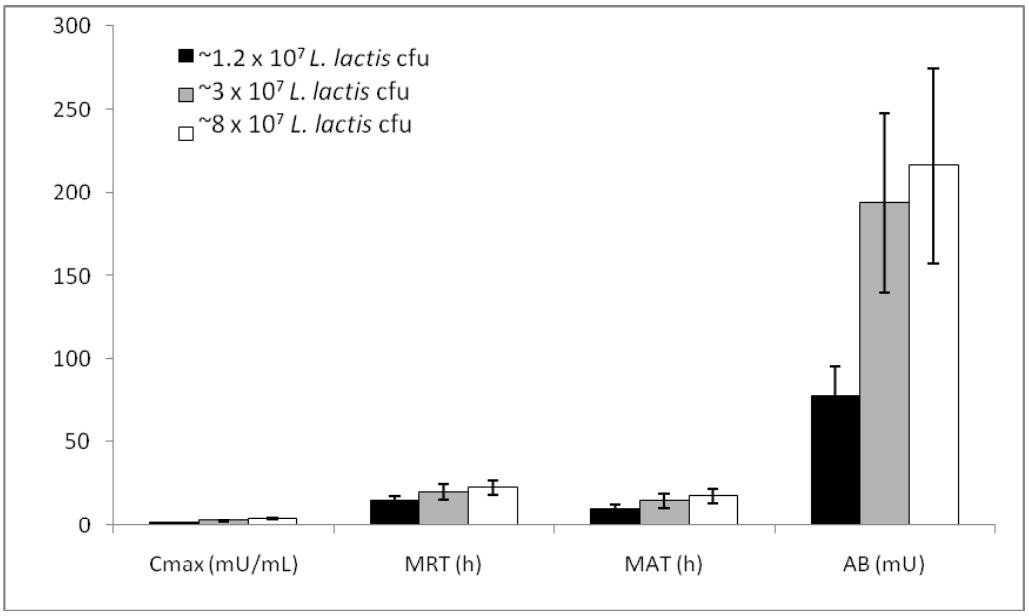

Figure 3. Relationship between *L. lactis* dose and some of the absorption PK parameters.

consideration in the future.

Since no β-lactamase was detected at 72 h after the administration of *L. lactis*, it can be assumed that most of *L. lactis* were either dead or eliminated out of the body by that time. This kind of phenomenon is actually desired in terms of drug delivery. Normal flora delivery system can provide a prolonged delivery mechanism, but to a certain degree, so that uncontrolled and undesired long-term actions can be avoided. Based on our previous study (*10*), we have observed that *L. lactis* are eliminated out of the body after oral administration. This phenomenon is supported by another report, which showed that *L. lactis* was a non-colonizing and was transient bacteria in the body (*11*). Thus this would terminate the drug delivery and also the possible risks of super bug development in the body after its administration.

The vagina extends from the vestibule to the uterus, and is situated behind the bladder and in front of the rectum; it is directed upward and backward, its axis forming with that of the uterus an angle of over 90°, opening forward. Its walls are ordinarily in contact, and the usual shape of its lower part on transverse section is that of an H, the transverse limb being slightly curved forward or backward, while the lateral limbs are somewhat convex toward the median line; its middle part has the appearance of a transverse slit (*12*). Drugs are transported across the vaginal membrane by the transcellular route, intracellular route or vesicular and receptor-mediated transport mechanisms (*13*). Its unique features in terms of secretion pH and microflora, and must be considered during the development and evaluation of vaginal delivery systems.

The vaginal route has been explored previously by many scientists for the delivery of various therapeutically active proteins such as insulin (*13*), calcitonin (*4*), and sex hormones (*4*). A very limited success has been achieved in the development of cervico-vaginal region as a potential systemic delivery site of these macromolecules. A safe and viable formulation is required to achieve a breakthrough in the field of this underutilized delivery route. One of the major concerns about vaginal delivery is the disturbance of the vaginal environment. For example, the depletion of vaginal lactobacilli can result in serious consequences which may lead to infection, thus maintenance of a normal microflora and the vaginal pH is important (*14*). In the complex vaginal ecosystem, lactobacilli are the most predominant bacterial species in healthy women. Delivery of the strains from lactobacilli family may be a choice from the safety view point. The adherence of normal flora to the mucosa provides a great advantage for the recombinant bacteria to deliver the protein drugs, since the bacteria will directly deliver the protein drugs onto the epithelial cell surface where the absorption takes place. The direct delivery of the protein drugs onto the epithelial surface will concentrate the protein drugs on the absorption surface to improve their absorption, and also minimize the exposure of the protein drugs to the degradation factors in the environment to reduce the pre-absorption degradation which usually is significant by other delivery methods.

The present study has further verified that the probiotics such as *L. lactis* when transformed by a special plasmid can be a living source for the protein drugs through vaginal route. This kind of delivery system provides a sustained delivery mechanism by which delivery period can be extended. It may be used for the delivery of suitable proteins which are capable of functioning locally or systemically.

3.2. *Conclusion*

There was an increase in the numerical value of the PK parameters, such as C_{max}, MAT, MRT, and AUC with the increase of dose of *L. lactis*.

Probiotics such as *L. lactis* when transformed by special plasmids can be a living source for efficient and sustained vaginal delivery of protein drugs. The amount delivered and the delivery period can be regulated by the number of the probiotics to be administered.

Acknowledgements

We would like to thanks Dr. Soile Tynkleynen, Valio Ltd., R&D (Helsinki, Finland) for generously providing the strain *Lactococcus lactis* subsp. *lactis* (ss80). The financial support from St. John's University is also acknowledged.

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(*Received September 2, 2009; Revised September 26, 2009; Accepted October 3, 2009*)

Original Article

Comparative development and evaluation of topical gel and cream formulations of psoralen

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ABSTRACT: The aim of the present investigation is to develop topical gel and cream formulations of psoralen for enhancing its transport through the skin, with the goal to shorten the delay between drug application and UVA irradiation. In our first studies, oil-in-water (O/W) creams of psoralen (0.05% concentration) were prepared using Apifil (PEG-8 Beeswax) and Plurol Stearique WL 1009 as emulsifying agents and aqueous cream (British Pharmaceutical Codex) as the cream base material. In our second studies, hydroalcoholic transparent gel formulations of this drug in a 0.05% concentration were prepared using hydroxypropylcellulose (HPC) as the gelling agent. The physicochemical compatibility between psoralen and formulation excipients used in the cream and gel formulations was confirmed by using differential scanning calorimetry and Fourier transform infrared spectroscopy. All prepared cream and gel formulations were evaluated for drug content uniformity, viscosity, pH, stability, and limpidity. The release of psoralen from all formulations *via* dialysis through a cellulose membrane into phosphate buffer pH 6.8 at 37°C was studied. The penetration enhancing effect of menthol (0-12.5%, w/w) on the percutaneous flux of psoralen through excised rat epidermis from gel and cream formulations was also investigated. The release profile of psoralen from gel formulations was higher than that from cream formulations. The percutaneous flux and enhancement ratio of psoralen across rat epidermis was significantly enhanced by the addition of menthol in both gel and cream formulations as compared to gel and cream formulations prepared without menthol ($p < 0.05$).

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Keywords: Psoralen, hydroalcoholic gel, cream formulation, *in vitro* skin permiation, vaginal, cosolvents

1. Introduction

Psoralen (*7H*-furo[3,2-g][1]benzopyran-7-one; PSO) is the parent compound of a family of naturally occurring furocoumarins used by plants as phytoalexins to combat attacks from fungi and insects (*1*).

PSO is increasingly used in dermatology for the photochemotherapy of diseases such as vitiligo, psoriasis, mycosis fungoides, atopic eczema and alopecia areata among others. The psoralens are currently employed in dematology (orally or topically), associated with ultraviolet A (UVA) irradiation. The combination of these previous compounds with UVA irradiation is known as PUVA therapy (psoralens plus UVA irradiation) (*2-7*).

According to the British Photodermatology Group, PSO is typically administered per os (1.2 mg/kg), 3 h before UVA irradiation. Topical application of psoralen can be advisable when the number and extension of affected areas are limited because it reduces the systemic side effects (*8*).

Topical and transdermal products are important classes of drug delivery systems, and their use in therapy is becoming more widespread. To be effective, topical dosage forms should conveniently deliver therapeutically useful drug concentrations at target sites (*i.e.*, basal layer of epidermis). Topical cream and gel formulations offer better patient compliance and hence are more acceptable to patients (*9*).

Hydroxypropylcellulose (HPC) is widely used in the pharmaceutical and cosmetic industries to give viscous or gel formulations. HPC possesses several desirable attributes as gelling agent including: high viscosity at low concentrations, stability to heat with negligible batch-to-batch variability, increased stability of formulations and has a pleasant texture. It is unaffected by aging, does not support bacterial or fungal growth, and is nonirritating (*10-12*).

Apifil® (PEG-8 Beeswax) and *Plurol*® *Stearique WL 1009* (polyglyceryl-6-distearate) were selected as O/W emulsifiers. Both of these emulsifiers can be used to formulate creams with varying concentration of oily phase without phase inversion. At higher concentrations, 5% to 15%, they form stable creams with a firm texture and a smooth, glossy appearance. They are particularly well-suited for the emulsification of vegetable oils (up to 15%) or short chain/medium polarity fatty acid esters. They perform well with other fatty acid esters, silicone oils, mineral oils and their substitutes.

The purposes of the present study are (*a*) to formulate an oil-in-water (O/W) cream and hydroalcoholic gel formulations containing psoralen, (*b*) to determine the effect of various additives on *in vitro* release of the drug from these formulations, and (*c*) to investigate the influence of permeation enhancers on drug permeation through rat skin.

2. Materials and Methods

2.1. *Materials*

PSO powder (purity: 90%) was procured from Yucca Laboratories, Bombay, India and used in the study without further purification. Two emulsifiers were chosen for cream PSO formulation: *Apifil*® (PEG-8 Beeswax) and *Plurol*® *Stearique WL 1009* (polyglyceryl-6-distearate), both were obtained as generous gift samples from GATTEFOSSE, France. Aqueous cream (British Pharmaceutical Codex) was used as the cream base material. HPC was procured from Colorcon Asia Pvt Ltd. (Mumbai, India). Other materials used in the study (2-propanol, propylene glycol, methanol, white soft paraffin, liquid paraffin, phenoxyethanol, potassium dihydrogen phosphate, *etc.*) were of analytical grade and procured from SD Fine Chemicals, India. Double-distilled water was used throughout the study.

2.2. *Investigation of physicochemical compatibility of drug and polymer*

The physicochemical compatibility between PSO and formulation excipients used in the cream and gel formulations was studied by using differential scanning calorimetry (DSC-Shimadzu 60 with TDA trend line software, Shimadzu Co., Kyoto, Japan) and Fourier transform infrared spectroscopy (FTIR-8300, Shimadzu Co., Kyoto, Japan).

In DSC analysis, the samples were hermetically sealed in flat-bottom aluminum pans, and heated over a temperature range of 35-300°C at a constant increasing rate of 10°C/min in an nitrogen atmosphere (50 mL/min). The thermograms obtained for PSO and physical mixtures of PSO with formulation excipients were compared.

Infrared (IR) spectra were recorded using an FTIR in the wavelength region between 4000 and 400 cm^{-1}. The spectra obtained for PSO and physical mixtures of PSO with formulation excipients were compared.

2.3. *Preparation of formulations*

The composition of different cream and gel formulations with and without permeation enhancer is shown in Tables 1 and 2.

2.3.1. *Oil-in-water cream samples*

All the aqueous phase material and the oil phase ingredients were placed in separate stainless steel containers and heated above 70°C. The water phase then was added to the oil phase with continuous agitation. The semisolid emulsions (O/W) were then cooled to approximately 40°C, and the PSO, previously dissolved in methanol, was incorporated. Other additives included in the formulation were also added at this stage. The batch was mixed to reach ambient temperature. The samples were then kept in airtight aluminum tubes.

2.3.2. *HPC gels*

The HPC powder was added to the required quantity of hot distilled water while being stirred, and the solution was allowed to cool. The methanolic solution of PSO (0.05%, w/v) was dissolved in 2-propanol and propylene glycol and mixed with hydrated HPC with continuous stirring at 37°C until the gel was formed (2 h).

2.4. *Analytical method*

All samples were analyzed for PSO content spectrophotometrically at a wavelength of 246 nm.

2.5. *Physicochemical properties*

2.5.1. *Drug content uniformity*

All samples were analyzed for PSO content prior to diffusion studies. Only samples with PSO contents within 100 ± 10% were used for diffusion studies. Drug content of the cream and gel formulations (1 g) was determined by dissolving an accurately weighed quantity of formulation in about 50 mL of pH 6.8 phosphate buffer containing 20% (v/v) ethanol. These solutions were quantitatively transferred to volumetric flasks and appropriate dilutions were made with the same buffer solution. The resulting solutions were then filtered through 0.45 µm membrane filters before subjecting the solutions to spectrophotometric analysis for PSO at λmax of 246 nm. Drug content was calculated from the linear regression equation obtained

Table 1. Composition of gel and cream formulations

Composition of gel formulations (%, w/w)							
Materials	Code						
	G	G1	G2	G3	G4	G5	G6
PSO	0.05	0.05	0.05	0.05	0.05	0.05	0.05
HPC	1.5	1.5	1.5	1.5	1.5	1.5	1.5
2-Propanol	–	10	15	20	–	–	–
Propylene glycol	–	–	–	–	10	15	20
Methanol	30	20	15	10	20	15	10
Distilled water q.s. to make	100	100	100	100	100	100	100
Composition of cream formulations (%, w/w)							
Materials	Code						
	C	C1	C2	C3	C4	C5	C6
PSO	0.05	0.05	0.05	0.05	0.05	0.05	0.05
Apifil®	–	3	6	9	–	–	–
Plurol®	–	–	–	–	3	6	9
Emulsifying wax	9	6	3	–	6	3	–
White soft paraffin	15	15	15	15	15	15	15
Liquid paraffin	6	6	6	6	6	6	6
Phenoxyethanol	1	1	1	1	1	1	1
Methanol	10	10	10	10	10	10	10
Distilled water q.s. to make	100	100	100	100	100	100	100

Table 2. Composition of gel and cream formulations with permeation enhancer

Composition of gel formulations with permeation enhancer (%, w/w)						
Materials	Code					
	G3	G3M1	G3M2	G3M3	G3M4	G3M5
PSO	0.05	0.05	0.05	0.05	0.05	0.05
HPC	1.5	1.5	1.5	1.5	1.5	1.5
2-Propanol	20	20	20	20	20	20
Propylene glycol	–	–	–	–	–	–
Methanol	10	10	10	10	10	10
Menthol	–	2.5	5.0	7.5	10	12.5
Distilled water q.s. to make	100	100	100	100	100	100
Composition of cream formulations with permeation enhancer (%, w/w)						
Materials	Code					
	C3	C3M1	C3M2	C3M3	C3M4	C3M5
PSO	0.05	0.05	0.05	0.05	0.05	0.05
Apifil®	9	9	9	9	9	9
White soft paraffin	15	15	15	15	15	15
Liquid paraffin	6	6	6	6	6	6
Phenoxyethanol	1	1	1	1	1	1
Methanol	10	10	10	10	10	10
Menthol	–	2.5	5.0	7.5	10	12.5
Distilled water q.s. to make	100	100	100	100	100	100

from the calibration data (*13-15*).

2.5.2. *Viscosity measurements*

A Brookfield Rotational Digital Viscometer DV II RVTDV-II was used to measure the viscosity (in cps) of the cream and gel formulations. The spindle was rotated at 10 rpm. Samples of the gels were allowed to settle over 30 min at the assay temperature (25 ± 1°C) before the measurements were taken.

2.5.3. *pH measurement*

The pH of all samples was measured using a pH meter (361, Systronics, India).

2.5.4. *Stability studies*

All cream and gel samples were stored at 4°C for 48 h and at 40°C for 48 h, respectively, and the physical aspects and homogeneity of the samples were investigated.

2.5.5. *Limpidity studies*

The limpidity of all gel samples was measured spectrophotometrically (transmittance) at 610 nm.

2.5.6. *In vitro skin permeation studies*

The animals used for the preparation of epidermis were male albino rats (150-200 g). All the experiments involving animals were conducted in accordance with institutional guidelines and were approved prior by the Institutional Ethics Committee. The institutional Ethics Committee approved the method of euthanasia. The animals had free access to food and water until used for the study. The care of the rats was in accordance with institutional guidelines. *In vitro* skin permeation studies were performed using a Franz diffusion cell with a receptor compartment capacity of 21 mL and an effective diffusion area of 2.54 cm^2. Dorsal hair was removed with a clipper and the full thickness of skin was surgically removed from each rat. Epidermis was prepared by a heat separation technique (*16*). The entire abdominal skin was soaked in water at 60°C for 60 sec, followed by careful removal of the epidermis. The epidermis was washed with water and used in the *in vitro* permeability studies. The excised rat epidermis was mounted between the donor and receptor compartment of the diffusion cell. One gram of each cream or gel formulations was placed over the skin and covered with paraffin film. The receptor compartment of the diffusion cell was filled with phosphate buffer (pH 6.8) containing 20% (v/v) ethanol (*17*). The whole assembly was fixed on a magnetic stirrer, and the solution in the receptor compartment was constantly and continuously stirred using magnetic beads at 50 rpm. Temperature was maintained at 37 ± 0.5°C. Samples were withdrawn at different time intervals and analyzed for drug content spectrophotometrically at a wavelength of 246 nm. The concentration of PSO in each sample was determined from a previously calculated standard curve. The receptor phase was replenished with an equal volume of phosphate buffer at each sample withdrawal. The cumulative percent amounts of drug permeated per square centimeter of skin were plotted against time.

2.6. *Data analysis and statistics*

The permeation parameters such as flux, permeability coefficient, and enhancement ratio were calculated for prepared cream and gel formulations.

The flux (μg/cm^2·h) of PSO was calculated from the slope of the plot of the cumulative amount of drug permeated per cm^2 of rat epidermal membrane at steady state against time using linear regression analysis (*18,19*).

The steady state permeability coefficient (K_P) of drug crossing rat epidermal membrane was calculated using the following equation (*20*):

$$K_P = \frac{J}{C} \quad (1)$$

where J is the flux and C is the concentration of PSO in donor compartment.

The penetration enhancing effect of menthol was calculated in terms of enhancement ratio (*ER*), and was calculated using the following equation (*21*):

$$ER = \frac{K_P \text{ with penetration enhancer}}{K_P \text{ without penetration enhancer}} \quad (2)$$

The data obtained in this study was subjected to statistical analysis using GraphPad-Prism 3.0 software, for one-way analysis of variance (ANOVA) following Student-Newman-Keuls multiple comparisons test. A P value of less than 0.05 was considered as evidence of a significant difference.

3. Results and Discussion

3.1. *Physicochemical compatibility of drug and polymer*

DSC and FTIR spectra of PSO and its physical mixture with cream and gel formulation polymers are shown in Figures 1 and 2.

The DSC analysis of pure PSO showed a sharp endothermal peak at 168.64°C, corresponding to its melting point (Figure 1). The DSC analysis of the

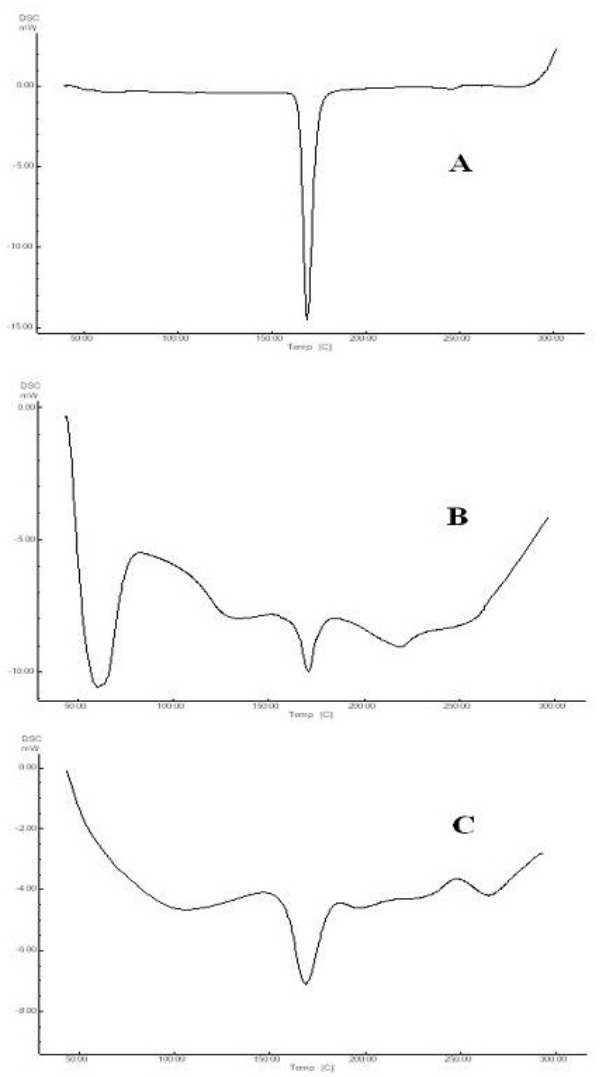

Figure 1. DSC thermograms of PSO (A), and its physical mixture with cream (B) and gel (C) formulation polymers.

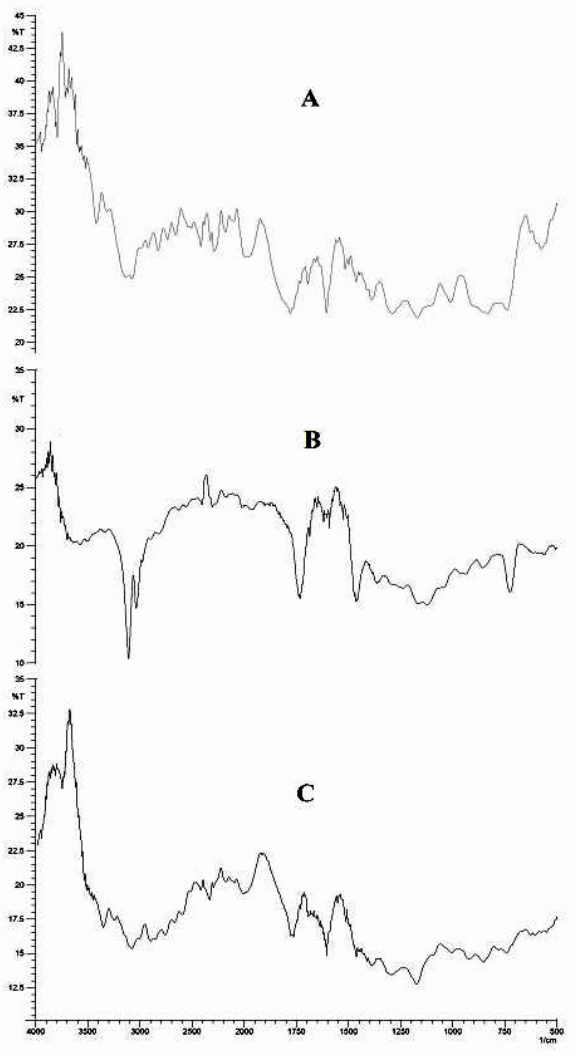

Figure 2. FTIR spectra of PSO (A), and its physical mixture with cream (B) and gel (C) formulation polymers.

physical mixture of PSO and the polymers used in cream and gel formulations revealed melting points of PSO at 167.72°C and 166.34°C, respectively. The negligible change in the melting point of PSO indicated compatibility of PSO with formulation polymers of cream and gel.

The IR spectral analysis of PSO alone showed principal peaks at wavenumbers 3057 (aromatic C-H stretch), 2402, 2284, 2002 cm^{-1} (overtone band), 1787 cm^{-1} (C=O stretching vibration of unsaturated lactones), 1609 cm^{-1} (C=C of ring), 1310 cm^{-1} (C-C(=O)-C stretching vibration), 1189 cm^{-1} (O-C-C band), 1052 cm^{-1} (symmetric C-O-C stretch) and 753 cm^{-1} (out of plane C-H bend), confirming the purity of the drug (Figure 2). In the IR spectra of the physical mixture of PSO with cream and gel formulation excipient the major peaks of PSO were observed at wavenumbers 3077, 2292, 1767, 1596, 1169, and 729 cm^{-1}; and 3054, 2296, 2010, 1776, 1603, 1320, and 752 cm^{-1}, respectively. However, some additional peaks were observed in the physical mixture, possibly because of the presence of polymers. The DSC and IR results suggest that the drug and polymers are compatible. Wade and Weller reported that HPMC, EC, PVP, and other common polymers are popular in controlled- and sustained release formulations because of their compatibility with several drugs (*22*).

3.2. *Physicochemical properties*

3.2.1. *Drug content and viscosity*

All prepared cream formulations of PSO were found to contain 98.7-101.3% of PSO. In gel formulations, increased concentrations of 2-propanol lead to decreased viscosity whereas increased concentrations of propylene glycol lead to increased viscosity of the formulations. In cream formulations, increased concentrations of emulsifying agent lead to increased viscosity of the formulation (Table 3).

Table 3. Drug content, viscosity, limpidity, amount of drug permeated in 3 h (Q_3), and % PSO released for different cream and gel formulations

Formulation code	Drug content[a]	pH	Viscosity η (cps × 10^3)[a]	Limpidity (T%)	Q_3[a] (μg cm^{-2})	% PSO released[a]
C	99.9 ± 3.7	6.8	108 ± 4.66	–	32.20 ± 2.6	16.36 ± 0.7
C1	101.1 ± 3.1	6.7	104 ± 4.87	–	51.10 ± 2.9	25.96 ± 1.2
C2	100.6 ± 3.7	6.8	111 ± 4.72	–	71.67 ± 3.4	36.41 ± 1.4
C3	98.1 ± 3.8	6.7	119 ± 4.65	–	101.82 ± 4.9	51.72 ± 2.4
C4	98.6 ± 3.5	6.7	107 ± 4.31	–	43.25 ± 2.1	21.97 ± 1.2
C5	100.6 ± 3.9	6.9	116 ± 5.06	–	63.57 ± 3.0	32.29 ± 2.8
C6	101.4 ± 4.2	6.9	125 ± 6.87	–	92.97 ± 4.5	47.23 ± 2.0
G	100.2 ± 4.5	6.0	92 ± 3.31	89 ± 3.4	78.50 ± 2.65	39.88 ± 1.5
G1	98.4 ± 4.4	6.1	89 ± 3.34	86 ± 3.1	94.70 ± 2.95	48.11 ± 2.1
G2	99.3 ± 2.1	6.1	79 ± 3.27	83 ±3.2	105.51 ± 4.49	53.60 ± 1.9
G3	101.4 ± 3.9	6.0	71 ± 3.29	81 ± 2.9	115.21 ± 4.94	58.53 ± 2.2
G4	98.3 ± 4.1	5.9	104 ± 3.67	88 ± 3.4	54.70 ± 2.12	27.79 ± 1.1
G5	101.1 ± 3.3	6.0	117 ± 4.52	93 ± 4.2	49.60 ± 2.10	25.20 ± 1.2
G6	99.5 ± 4.3	5.9	129 ± 5.64	96 ± 4.0	41.40 ± 4.51	21.03 ± 0.9

[a] Mean ± SD; n = 3.

3.2.2. *pH measurement*

All gel formulations showed a pH range of 5.9-6.1; however, the O/W creams exhibited a higher pH range, between 6.7 and 6.9.

3.2.3. *Stability studies*

During the study no change in physical aspects and homogeneity was observed in all cream and gel formulations. Moreover, all the formulations also showed no alteration in pH during this study.

3.2.4. *Limpidity studies*

Gels were classified in four categories, from limpid (T% > 80%) to very opaque (T% < 20%). All gel samples prepared were limpid as the T% values were more than 80% for all formulations. Gel formulations containing propylene glycol were more limpid than formulations containing 2-propanol (Table 3).

3.3. *In vitro drug permeation study*

3.3.1. *Cream and gel formulations without permeation enhancer*

3.3.1.1. *Oil-in-water cream samples*

The cumulative amount of PSO released from 0.05% creams were determined and plotted in Figure 3. Each data point represents the mean of 6 determinations. The amount of drug was constant in all different cream formulations.

Substitution of emulsifying wax with increasing concentration of *Apifil*® or *Plurol*® over the range of 3%, 6%, and 9%, exhibited a linear increase in PSO release

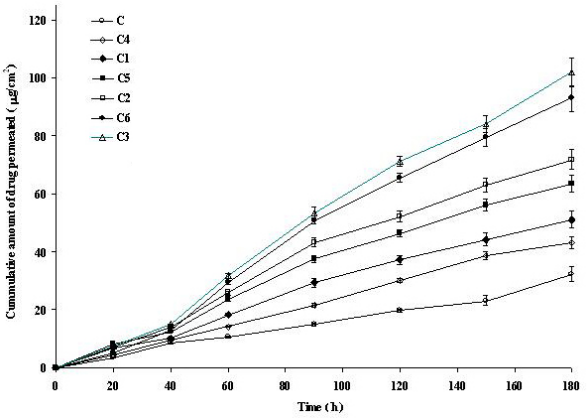

Figure 3. Cumulative amount of PSO permeated from cream formulations containing different concentrations of *Apifil*® or *Plurol*® as emulsifying agent therough rat epidermis (n = 3).

compared to formulations containing only emulsifying wax (9%). Complete substitution of emulsifying wax with *Apifil*® or *Plurol*® showed better release of PSO as compared to partial or no substitution of emulsifying wax with *Apifil*® or *Plurol*®. Among all cream formulations, formulations containing 9% *Apifil*® showed the highest release (101.82 ± 4.9 μg/cm^2) of PSO in 3 h. Hence, this concentration was selected for preparing cream formulations to study the effect of menthol as permeation enhancer.

3.3.1.2. *Gel samples*

Cosolvents were used in various topical formulations to aid the solubilization of the active substances in the vehicle. In this study, 2-propanol and propylene glycol were added as cosolvents to increase the solubility of PSO in the aqueous phase selectively rather than in the micellar portion of the gel. The effect of 2-propanol

on the release of PSO was studied using the gel with 0.05% PSO in 1.5% HPC and varying the 2-propanol concentration (10%, 15% or 20%) (Figure 4). In all of these formulations, 2-propanol concentration (10%, 15% or 20%) was substituted with methanol (20%, 15% or 10%), respectively.

Over the range of 2-propanol concentrations used, the cumulative amount of PSO increased linearly from 78.50 ± 2.65 μg/cm^2 for gels prepared without 2-propanol to 115.21 ± 4.94 μg/cm^2 for gels containing 20% of 2-propanol at 3 h. The enhanced drug release in the presence of 2-propanol could be due to the decreased viscosity of PSO gel. These results are in agreement with a previous investigation performed by Chi and Jun, 1991 (*23*), who demonstrated that ethanol increased release of ketoprofen from gel formulations due to a decrease in viscosity. Moreover, the relative increase in the release was probably due to the fact that, at these concentrations, 2-propanol augmented the solubility of the drug in the gel, causing an increase in the thermodynamic activity and enhanced permeation.

The effect of various concentrations of propylene glycol on the release of PSO from a gel formulation was also investigated. The concentration of HPC (1.5%) and the drug (0.05%) remained constant; however, the amount of methanol in the vehicle was substituted with an equal volume of propylene glycol (10%, 15%, and 20%) (Figure 4). Over the range of propylene glycol concentrations used, the diffusion coefficient of PSO decreased linearly from 78.50 ± 2.65 μg/cm^2 for the gel without propylene glycol to 41.40 ± 4.51 μg/cm^2 for the gel containing 20% propylene glycol (Table 3). The decrease in drug release from the gel containing propylene glycol might be due to the increase of viscosity of PSO gel. However, the addition of propylene glycol in gel formulations caused improvement of the limpidity of the gels when measured spectrophotometrically at 610 nm. The gel formulation containing 2-propanol (20%) was selected to study the effect of menthol as permeation enhancer.

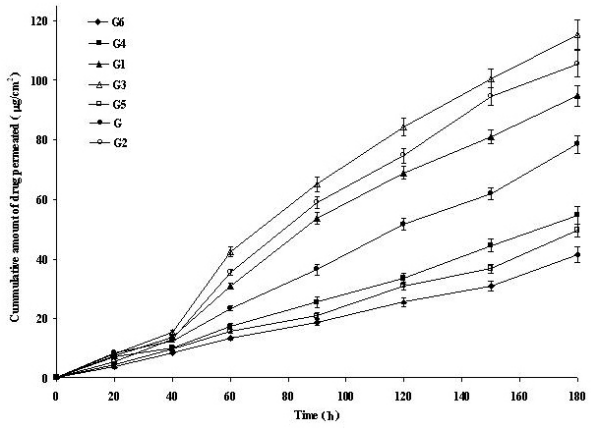

Figure 4. Cumulative amount of PSO permeated from gel formulations containing different concentrations of 2-propanol and propylene glycol as cosolvents therough rat epidermis (*n* = 3).

3.3.2. *Cream and gel formulations with permeation enhancer*

The penetration enhancing effect of menthol on the permeability of PSO across the excised rat epidermis from cream and gel formulations was investigated. Permeation parameters for PSO from the cream and gel formulations are shown in Table 4. The cumulative amount of drug crossing rat epidermis from both cream and gel formulations containing various amounts of menthol is shown in Figures 5 and 6.

The maximum amount of PSO that permeated during the 3 h ($Q3$) of the study was 101.82 ± 4.89 μg·cm^{-2} and 115.21 ± 4.94 μg·cm^{-2} from cream and gel formulations prepared without menthol, respectively (Table 4). The flux was obtained by dividing the

Table 4. Drug content, flux (*J*), permeability coefficient (*K*p), enhancement ratio (*ER*), amount of drug permeated in 3 h (Q_3), % PSO released, and Higuchi R^2 values for the *in vitro* permeation study across rat epidermal membrane from cream and gel formulations of PSO containing selected concentrations of menthol at the end of 3 h

Formulation code	Drug content[a]	*J* (μg·cm$^{-2\text{-}1}$·h^{-1})[a]	*K*p (cm·h^{-1} × 10^3)[a]	*ER*[a]	Q_3[a] (μg·cm^{-2})	% PSO released[a]	R^2 Higuchi
C3	99.9 ± 4.2	35.37 ± 1.4	72.18 ± 3.2	1.00 ± 0.048	101.82 ± 4.9	51.72 ± 2.4	0.9942
C3M1	101.1 ± 4.5	37.49 ± 1.6	76.51 ± 3.7	1.06 ± 0.040	110.40 ± 5.3	56.08 ± 2.9	0.9918
C3M2	98.8 ± 4.0	40.68 ± 1.9	79.76 ± 3.8	1.11 ± 0.042	123.20 ± 6.1	62.58 ± 2.7	0.9947
C3M3	101.5 ± 4.8	43.14 ± 1.8	86.28 ± 4.3	1.20 ± 0.052	134.14 ± 6.4	68.14 ± 3.2	0.9927
C3M4	100.2 ± 4.4	44.93 ± 1.9	89.86 ± 4.4	1.24 ± 0.54	139.60 ± 7.1	70.92 ± 3.4	0.9935
C3M5	99.4 ± 4.3	48.82 ± 2.2	95.72 ± 4.7	1.33 ± 0.048	158.57 ± 6.8*	80.55 ± 3.7	0.9902
G3	100.2 ± 4.1	41.10 ± 1.2	82.2 ± 3.2	1.14 ± 0.034	115.21 ± 4.9	58.53 ± 2.2	0.9872
G3M1	101.7 ± 3.7	45.51 ± 1.7	89.2 ± 3.6	1.24 ± 0.039	137.65 ± 5.4	69.92 ± 3.2	0.9915
G3M2	98.9 ± 3.9	46.65 ± 1.5	95.2 ± 4.2	1.32 ± 0.044	149.21 ± 5.9	75.78 ± 3.9	0.9901
G3M3	100.2 ± 4.5	49.39 ± 2.1	100.7 ± 4.7	1.40 ± 0.051	162.08 ± 6.3	82.33 ± 3.8	0.9945
G3M4	99.6 ± 3.3	51.48 ± 2.4	102.9 ± 4.9	1.43 ± 0.052	173.23 ± 6.8	88.00 ± 4.2	0.9936
G3M5	101.1 ± 3.8	52.59 ± 2.1	107.3 ± 5.1	1.49 ± 0.049	187.69 ± 7.5*	95.30 ± 4.2	0.9957

[a] Mean ± SD; $n = 3$; *Significant at $p < 0.05$ when compared with formulation C and G

cumulative amount of drug permeated per cm^2 of the skin with time. Thus, the corresponding flux of PSO was 48.82 ± 2.2 $\mu g \cdot cm^{-2} \cdot h^{-1}$ and 52.59 ± 2.1 $\mu g \cdot cm^{-2} \cdot h^{-1}$ for the cream and gel formulations without menthol, respectively.

A marked effect of menthol on PSO permeation was observed when it was incorporated in both types of cream and gel formulations in varying quantities. The cumulative amounts of PSO that permeated over 3 h (*Q*3) were found to have increased ranging from 110.40 ± 5.3 to 158.57 ± 6.8 $\mu g \cdot cm^{-2}$ for PSO cream and 137.65 ± 5.4 to 187.69 ± 7.5 $\mu g \cdot cm^{-2}$ for PSO gel formulations containing 2.5-12.5 % (w/w) of menthol. The corresponding flux values ranged from 37.49 ± 1.6 to 48.82 ± 2.2 $\mu g \cdot cm^{-2} \cdot h^{-1}$ and 45.51 ±1.7 to 52.59 ± 2.1 $\mu g \cdot cm^{-2} \cdot h^{-1}$ for PSO cream and gel formulations, respectively. However, a lag period of 40 min was observed in both cream and gel formulations in the permeation of the drug through the stratum corneum (Figures 4 and 5). It may be observed from the results that the flux of drug increased with an increase in concentration of menthol in cream and gel formulations (Table 4).

The increased permeability of PSO with increasing concentrations of menthol in both formulations from 0 to 12.5% (w/w) (Figures 5 and 6) indicated an increase in both the permeability coefficient and enhancement ratio (Table 4). There was a 1.33- and 1.49-fold increase in the permeability of the drug observed from the cream and gel containing 12.5% (w/w) of menthol, respectively. Both the permeability coefficient and enhancement ratio of PSO were increased linearly with all menthol concentrations in both cream and gel formulations (Table 4). When the data were analyzed, the amount of drug permeated fit Higuchi ($r^2 > 0.99$) from 20 min to 3 h with a lag period of about 40 min for both formulation types (Table 4).

The topical cream and gel formulations developed with menthol as permeation enhancer showed good transport of PSO as compared to formulations developed without menthol. Gel formulations of PSO showed good release and transport of PSO as compared to cream formulations. The novel topical gel formulation developed in this study can be used in PUVA therapy to achieve an adequate drug level at the target site at the time of UVA radiation.

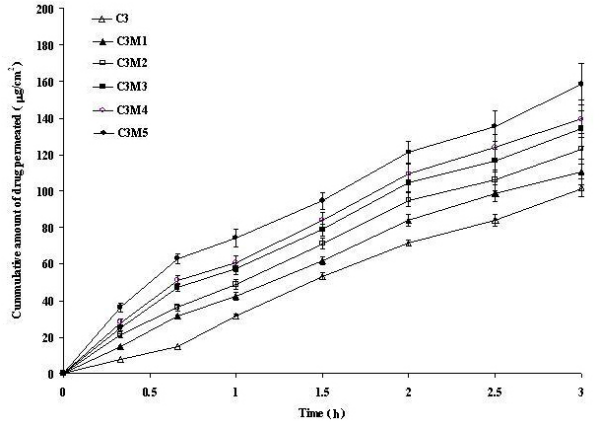

Figure 5. Effect of menthol concentration on permeation of PSO from cream formulations therough rat epidermis (*n* = 3).

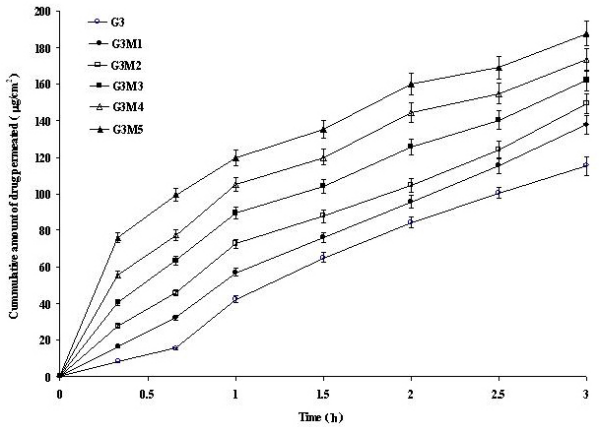

Figure 6. Effect of menthol concentration on permeation of PSO from gel formulations therough rat epidermis (*n* = 3).

4. Conclusions

Topical cream and gel formulations of PSO developed in this study have great potential as an effective and safe way to apply PSO to enhance its transport through skin, with the goal to shorten the delay between drug application and UVA irradiation. The *in vitro* permeation study crossing rat epidermal membranes showed that menthol enhanced the transdermal permeation of PSO from cream and gel drug reservoir systems. Gel formulations showed better permeation of PSO as compared to cream formulations. The topical gel formulations of PSO developed in this study have great utility and are a viable option for effective and controlled management of vitiligo and psoriasis. Further experiments will be conducted in other animal models and based on the results trials may be performed on humans.

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(*Received September 2, 2009; Revised September 22, 2009; Accepted October 3, 2009*)

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