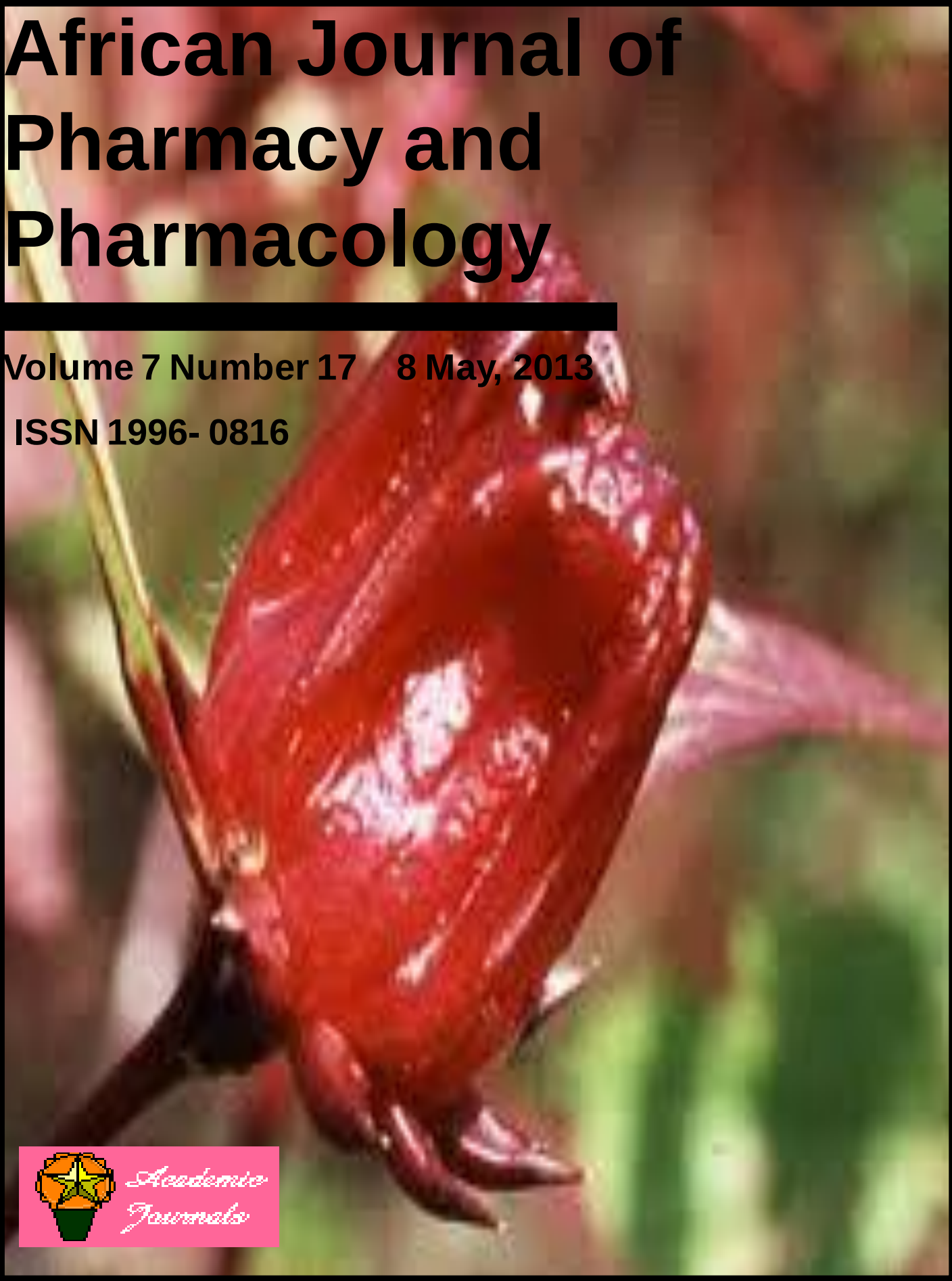
African Journal of Pharmacy and Pharmacology

Volume 7 Number 17 8 May, 2013

ISSN 1996- 0816



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Vol. 7(17), pp. 873-881, 8 May, 2013
DOI 10.5897/AJPP12.1329
ISSN 1996-0816 © 2013 Academic Journals
http://www.academicjournals.org/AJPP

African Journal of Pharmacy and Pharmacology

Review

Microsponges as promising vehicle for drug delivery and targeting: Preparation, characterization and applications

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Accepted 18 April, 2013

Development of novel drug delivery systems for optimization of drugs' efficacy and cost-effectiveness has become highly competitive and rapidly evolving area of interest. Controlling the delivery rate of drugs to a predetermined site has been one of the biggest challenges faced by formulators. Amongst the novel drug delivery systems that proved their efficacy in achieving controlled drug release are microsponges. They are used mostly for topical use and have recently been used for oral administration. Microsponges are sponge-like polymeric microspheres with a large porous surface that can entrap a wide variety of active ingredients. They can then be further incorporated into a formulated product such as a gel, cream, liquid or even tablets. Microsponges are designed to deliver a pharmaceutical active ingredient efficiently at the minimum dose and also to enhance stability and reduce drugs side effects. This review introduces the potential features of microsponges along with their advantages. Moreover, it highlights the methods of preparation and characterization of microsponges and also covers their topical and oral applications.

Key words: Microsponges, drug delivery, preparation, characterization, topical, oral.

INTRODUCTION

Conventional topical formulations are designed to work on the outer layers of the skin. When the active ingredients of these formulations are released upon application, a highly concentrated layer of active ingredient is produced that is rapidly absorbed. Thus, there is a genuine need for delivery systems to prolong the time that active ingredients can be retained on the surface of the skin or within the epidermis while decreasing its transdermal penetration. Moreover, as a result of the high concentration of active agents employed in the conventional topical dosage forms, several side effects are recorded in significant users such as irritation and allergic reactions (Pradhan, 2011).

Recently, there has been considerable interest in the development of novel microsponge based drug delivery systems to achieve targeted and sustained release of drugs (Kaity et al., 2010). Microsponges are polymeric delivery systems consisting of porous microspheres that are mostly used for extended topical administration of a variety of active ingredients such as emollients, fragrances, essential oils, sunscreens, and anti-infective, anti-fungal, and anti-inflammatory agents. Microsponges offer many advantages such as delivering the active ingredients at minimum dose, enhanced stability, reduced side effects, and the ability to modify drug release profiles (Nacht and Kantz, 1992). Just like a real sponge, each

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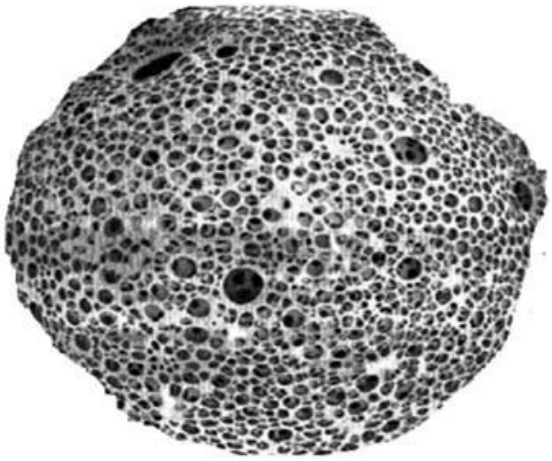

Figure 1. Highly porous nature of a microsponge. Source: Pradhan (2011).

microsphere consists of a myriad of interconnecting voids within a non-collapsible structure and a large porous surface. The resultant microsponge spheres are uniform, with a particle size range of 5 to 300 µg (Figure 1) (Pradhan, 2011).

Microsphere surrounded by the vehicle acts like microscopic sponges, storing the active ingredient until its release is triggered by skin application. Micropores within the spheres are employed for extensive drug retention. Microsponges consisting of non-collapsible structures with porous surface through active ingredients are released in a controlled manner. Release of drug into the skin is triggered by a variety of stimuli, including rubbing and higher skin temperature than ambient one. Their high degree of cross-linking results in particles that are insoluble, inert and of sufficiently strong strength to withstand the high shear commonly used in manufacturing of creams, lotions, and powders. Their characteristic feature is the capacity to load a high amount of active materials into the particle and on to its surface. Its large capacity for entrapment of drugs, up to three times its weight, differentiates microsponge products from other types of dermatological products. The active payload is protected in the formulation by the microsponge particle; it is delivered to skin via controlled diffusion. The sustained release of activities to skin over time is an effective tool to extend the efficacy and reduce the irritation commonly associated (Pradhan, 2011).

The microsponge technology was developed by Won in 1987, and the original patents were assigned to Advanced Polymer Systems, Inc. (Won, 1987). This company developed a large number of variations of the technique and applied it to the cosmetic as well as over the counter (OTC) and prescription pharmaceutical products (Pradhan, 2011). Nowadays, there are several Food and Drug Administration (FDA)-approved products such as Retin-A Micro® (0.1 or 0.04% tretinoin) and Carac (0.5% 5-flurouracil) that are used for acne treatment and actinic keratoses, respectively (Amrutiya et al., 2009).

Potential features of microsponge drug delivery systems

The potential features are as listed (Aritomi et al., 1996; Jain et al., 2011; Vyas and Khar, 2002):

1. Microsponges show acceptable stability over pH ranging from 1 to 11 and at high temperatures (up to 130°C).
2. Microsponges exhibit good compatibility with various vehicles and ingredients.
3. Microsponges have high entrapment efficiency up to 50 to 60%.
4. Microsponges are characterized by free flowing properties.
5. The average pore size of microsponges is small (0.25 µm) in a way to prevent the penetration of bacteria, thus they do not need sterilization or addition of preservatives.
6. Microsponges are non-allergenic, non-irritating, non-mutagenic and non-toxic.
7. Microsponges can absorb oil up to 6 times their weight without drying.

Advantages of microsponges over other technologies and delivery systems

The advantages include (Kaity et al., 2010; Pradhan, 2011):

1. Microsponges offer better control of drug release than microcapsules. Microcapsules cannot usually control the release rate of the active pharmaceutical ingredients (API). Once the wall is ruptured, the API contained within the microcapsules will be released.
2. Microsponges show better chemical stability, higher payload and easier formulation compared with liposomes.
3. In contrast to ointments, microsponges have the ability to absorb skin secretions, therefore, reducing greasiness and shine from the skin. Ointments are often aesthetically unappealing, greasy and sticky, resulting in lack of patient compliance.

Characters of drugs to be entrapped in the microsponges

There are certain requirements that should be fulfilled (or

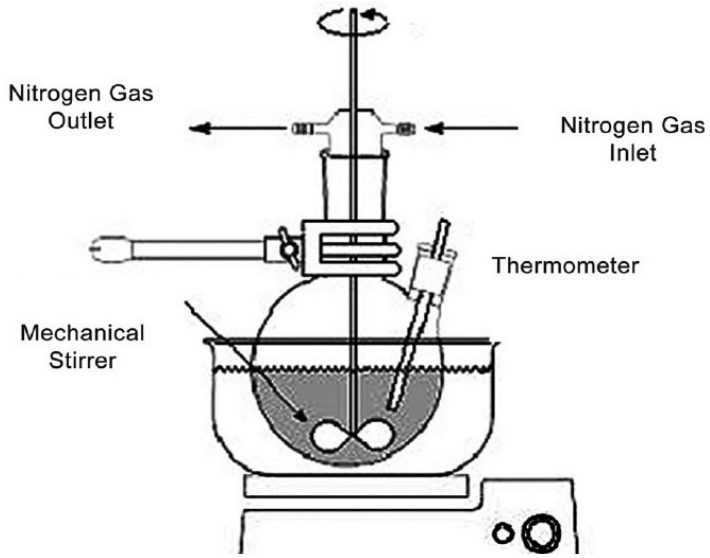

Figure 2. Reaction vessel for microsponge preparation by liquid-liquid suspension polymerization. Source: Kaity et al. (2010).

considered) when active ingredients are entrapped into microsponge (Jain et al., 2011; Pradhan, 2011):

1. It Should exhibit complete miscibility in monomer or have the ability to be miscible using the least amount of a water immiscible solvent.
2. Must be inert to monomers and do not increase the viscosity of the preparation during formulation.
3. It should be water immiscible or almost slightly soluble.
4. The solubility of active ingredients in the vehicle should be minimum; otherwise the microsponge will be diminished by the vehicle before application.
5. It should maintain (preserve) the spherical structure of microsponge.
6. It should be stable in polymerization conditions.
7. Only 10 to 12% w/w microsponge can be incorporated into the vehicle to eliminate cosmetic delinquent.
8. Payload and polymer design of the microsponges for the active must be adjusted to obtain the desired release rate of a given period of time.

Techniques of microsponges preparation

Preparation of microsponge can take place in a one-step or two-step process based on the physicochemical properties of drug to be loaded. If the drug is porogen, (that is an inert non-polar substance which will generate the porous structure), it will not deter the polymerization process or become activated by it and also is stable to free radicals. A porogen drug can be entrapped with one-step process (liquid-liquid suspension polymerization) (Pradhan, 2011). Microsponges are prepared by the following methods:

Liquid-liquid suspension polymerization

Suspension polymerization process in liquid-liquid systems is utilized for the preparation of microsponges in a one step process (Figure 2). At first, the monomers are dissolved with the active ingredients (non-polar drug) in a proper solvent. The prepared solution is then dispersed in the aqueous phase containing surfactants and dispersants to facilitate the formation of suspension. Once the suspension is formed with droplets of the required size, then polymerization is initiated by the addition of catalyst, increasing temperature, or irradiation. As the polymerization process continues, a spherical structure is produced containing thousands of micro-sponges bunched together. During the polymerization process, an inert water-immiscible liquid but completely miscible with monomer is used to form the pore network in some cases, which is then removed once the process is complete. The particles are then washed and processed until they are substantially ready for use (Kaity et al., 2010; Pradhan, 2011).

Quasi-emulsion solvent diffusion

Microsponges can be prepared by quasi-emulsion solvent diffusion method. In this method, an internal phase is used containing polymer such as eudragit RS

100 or ethyl cellulose dissolved in organic solvent. The drug is then dissolved into the polymer solution under ultrasonication. The inner phase is then poured into external phase containing polyvinyl alcohol and distilled water with continuous stirring for adequate period of time (Shah et al., 1989). Microsponges are then separated by filtration. Finally, the microsponges are washed and dried in an air heated oven at 40°C for 12 h (Comoglu et al., 2003).

Characterization of microsponges

Measurement of particle size

Various formulation and process variables can greatly affect the particle size of microsponge formulations. Measurement of particle size of loaded and unloaded microsponges can be performed using laser light diffractometry or any other suitable method. Results can be expressed in terms of mean size range. Cumulative (%) drug release from microsponges of different particle sizes should be plotted against time to study the effect of particle size on drug release.

Particles larger than 30 µm can impart grittiness and hence particles of sizes between 10 and 25 µm are preferred to be used in topical formulations (Chadawar and Shaji, 2007).

Morphology and Surface topography

The surface structure of microsponges can be examined using scanning electron microscopy (SEM) technique. The prepared microsponges are coated with gold-palladium under an argon atmosphere at room temperature, and then SEM images are recorded at the required magnification. SEM images may also be recorded for a fractured microsponge to study its ultra-structure (Emanuele and Dinarvard, 1995; Nokhodchi et al., 2007).

Production yield and entrapment efficiency

Percentage yield can be calculated using the equation (Kilicarslan and Baykara, 2003; Sensoy et al., 2009):
Percentage yield (PY) = (Final obtained mass of microsponges / initial mass of polymer and drug) × 100

The entrapment efficiency of the microsponges can be computed using the equation:

Entrapment Efficiency (EE%) = (Actual drug content / Theoretical drug content) × 100

Determination of true density

True density can be measured by an ultra-pycnometer using helium gas, and calculated as a mean of repeated determinations (Bertrand et al., 2007).

Pore structure

Porosity parameters of microsponges are essential in monitoring the intensity and the duration of active ingredient effect. Average pore diameters, shape and morphology of the pores can be determined by using mercury intrusion porosimetry technique. The effect of pore diameter and volume on the rate of drug release from microsponges can also be studied using the same technique (D'souza, 2008).

Viscoelastic properties

Microsponges with varying viscoelastic properties can be produced according to the needs of the final formulation. The degree of cross-linking affects the drug release from the prepared microsponges, where increased cross-linking tends to decrease the release rate. Hence, viscosity measurements should be done so that the viscoelastic properties of microsponges can be modified and adjusted to obtain the desired release properties (Jelvehgari et al., 2006; D'souza, 2008).

Physicochemical characterization

Thermoanalytical methods: Thermal analysis using differential scanning calorimetry (DSC) is carried out for the pure drug, polymer and the drug-polymer physical mixture to confirm compatibility. DSC is also performed for the microsponge formulations to ensure that the formulation process does not change the nature of the drug. Samples (approximately 2 mg) are placed in aluminum pans, sealed and operated at a heating rate of 20°C/min over a temperature range 40 to 430°C. The thermograms obtained by DSC for the physical mixtures, as well as microsponges, should be observed for broadening, shifting and appearance of new peaks or disappearance of certain peaks. The peak corresponding to the melting of the drug should be preserved in all thermograms (Ceschel et al., 2003; Mishra et al., 2011).

Fourier transform infrared spectroscopy (FTIR): Fourier transform infrared spectroscopy (FTIR) is carried out for the pure drug, polymer and the drug-polymer physical mixture and microsponge formulations.

The samples are incorporated in potassium bromide discs and evaluated using FTIR spectrometer. The peaks corresponding to the characteristics bands of the drug should be preserved in the spectra of the microsponges to indicate that no chemical interaction or changes took place during the preparation of the formulations (Jain and Singh, 2010a, 2011).

Powder X-ray diffraction (XRD): Powder X-ray diffraction (XRD) can be performed for both pure drug, polymer and microsponge formulation to investigate the effect of polymerization on crystallinity of the drug. The disappearance of the characteristic peaks of the drug in the formulation could indicate that the drug is dispersed at a molecular level in the polymer matrix (Bodmeier and Chen, 1989; Singla et al., 2001).

In vitro release studies, release kinetics and mechanism

In vitro release studies can be performed using United States Pharmacopeial (USP) dissolution apparatus equipped with a modified basket consisted of 5 µm stainless steel mesh at 37°C. The release medium is selected according to the type of formulation that is, topical or oral, while considering solubility of active ingredients to ensure sink conditions. Sample aliquots are withdrawn from the medium and analyzed by suitable analytical method at regular intervals of time. The drug release from topical preparations (for example, creams, lotions and emulgels) containing microsponges can be carried out using Franz diffusion cells. Dialysis membrane is fitted into place between the two chambers of the cell. A predetermined amount of formulation is mounted on the donor side of Franz cell. The receptor medium is continuously stirred at and thermostated with a circulating jacket. Samples are withdrawn at different time intervals and analyzed using suitable method of assay (Embil and Nacht, 1996; Jelvehgari et al., 2006). To determine the drug release kinetics and investigate its mechanism from microsponges, the release data are fitted to different kinetic models. The kinetic models used are; first order, zero order, Higuchi and Korsmeyer-Peppas models (Higuchi, 1963; Wagner, 1969; Korsmeyer et al., 1983; Peppas, 1985). The goodness of fit was evaluated using the determination coefficient (R^2) values.

Applications of microsponges

Topical drug delivery

Topical formulations aim to deliver drugs to the outer layers of the skin. Conventional topical formulations release their active ingredients upon application, producing a highly concentrated layer of active ingredient that is rapidly absorbed. However, microsponge systems are designed to deliver a pharmaceutical active ingredient efficiently at the minimum dose. They consist of non-collapsible structures with porous surface through which active ingredients are released in a controlled manner. Therefore, such systems can prevent excessive accumulation of active ingredients within the epidermis and the dermis, thus they can significantly reduce the irritation and side effects caused by drugs without reducing their efficacy. In addition to modification of drug release and reduction of side effects, microsponges are also capable of enhancing the stability of many drugs. The drug loaded porous microsponges can further be incorporated into creams, lotions or powders (Patel and Patel, 2006). Microsponges are applied for the topical delivery of several drugs and cosmetic agents as shown in Table 1 (Kaity et al., 2010).

Several studies have been performed for the development of microsponges loaded with topically applied drugs. A formulation of hydroquinone (HQ) 4%, with retinol 0.15%, entrapped in microsponge reservoirs, was developed by Grimes (2004) for the treatment of melasma and postinflammatory hyperpigmentation. The formulation was intended to release HQ gradually in order to prolong exposure to drug and to decrease skin irritation. The safety and efficacy of this product were evaluated in a 12-week, open-label study. In this open-label study, the microentrapped HQ 4% with retinol 0.15% was proved to be safe and effective. A microsponge system for retinoic acid was also developed and tested for drug release and anti-acne efficacy. The greater reductions in inflammatory and non-inflammatory lesions obtained with tretinoin entrapped in the microsponge was found to be statistically significant (James et al., 2005).

Topical application of benzoyl peroxide (BPO), a drug that is mainly used in the treatment of mild to moderate acne and athlete's foot, is commonly associated with skin irritation. It has been shown that controlled release of BPO from a delivery system to the skin could reduce irritation due to reduction of drug release rate from formulation (Wester et al., 1991; D'souza, 2001). Jelvehgari et al. (2006) developed a microspongic delivery system of BPO using an emulsion solvent diffusion technique, by adding an organic internal phase containing benzoyl peroxide, ethyl cellulose, and dichloromethane into a stirred aqueous phase containing polyvinyl alcohol. BPO microparticles were then incorporated into standard vehicles for release studies. It was found that the presence of emulsifier was essential for microsponge formation and that the drug to polymer ratio, stirring rate and volume of dispersed phase influen-

Table 1. Topical applications of microsponges.

Active agent	Application
Sunscreens	Long lasting product efficacy, with improved protection against sunburns and sun related injuries even at elevated concentration and with reduced irritancy and sensitization
Anti-acne for example, benzoyl peroxide	Maintained efficacy with decreased skin irritation and sensitization
Anti-inflammatory for example, hydrocortisone	Long lasting activity with reduction of skin allergic response and dermatoses
Antifungals	Sustained release of actives
Antidandruffs for example, zinc pyrithione	Reduced unpleasant odor with lowered irritation with extended safety and efficacy
Antipruritics	Extended and improved activity
Skin depigmenting agents for example, hydroquinone	Improved stabilization against oxidation with improved efficacy and aesthetic appeal
Rubefacients	Prolonged activity with reduced irritancy greasiness and odor

Source: Kaity et al. (2010).

ced the particle size and drug release behavior of the formed microsponges. Generally, an increase in the ratio drug to polymer resulted in a reduction in the release rate of BPO from microsponges which was attributed to a decreased internal porosity of the microsponges. Further studies showed that the morphology and particle size of BPO microsponges were also affected by drug to polymer ratio, stirring rate and the amount of emulsifier used (Nokhodchi et al., 2007).

Fluocinolone acetonide (FA) is a corticosteroid used in dermatological preparations to lessen skin inflammation and relieve itching, however, the percutaneous absorption increases risk related with systemic absorption of the drug from topically applied formulation. D'souza and Harinath (2008) developed topical anti-inflammatory gels of fluocinolone acetonide entrapped in eudragit based microsponge delivery system. The fluocinolone acetonide loaded microsponges were prepared using the quasi-emulsion solvent diffusion method aiming to control the release of drug to the skin which in turn reduces the drug percutaneous absorption and thus lessens its side effects. The prepared microsponges were evaluated for several parameters including particle size analysis, loading efficiency, production yield and surface morphology. Microsponges were then incorporated into carbopol 934, and comparative anti-inflammatory studies were performed with gels containing the free dug.

A microsponge based topical delivery system of mupirocin, a topical antibiotic used for skin infections, was developed by Amrutiya et al. (2009), aiming to achieve sustained drug release and enhanced deposition in the skin. Microsponges containing mupirocin were prepared by emulsion solvent diffusion method. A 3^2 factorial design was applied to examine and optimize the effect of formulation and process variables, namely; internal phase volume and stirring speed, on the physical characteristics of microsponges. The optimized microsponges were incorporated into an emulgel base. The mupirocin-loaded formulations were evaluated for *in vitro* drug release, *ex vivo* drug deposition, and *in vivo* antibacterial activity. Drug release studies showed diffusion-controlled release pattern, and drug deposition studies using abdominal rat skin demonstrated significant retention of the drug in skin from microsponge-based formulations. The optimized formulations were stable and nonirritant to skin according to Draize patch test.

In addition, microsponges-based emulgel formulations exhibited prolonged efficacy in mouse surgical wound model infected with *Staphylococcus aureus*. The enhanced retention of mupirocin in the skin from the microsponge based formulations suggests the formulation to be an efficient delivery system for treatment of primary and secondary skin infections as compared with marketed mupirocin ointment and conventional mupirocin emulgel.

Saboji et al. (2011) developed microsponges containing ketoconazole drug with six different proportions of Eudragit RS 100 as polymer using quasi-emulsion solvent diffusion method. The microsponge formulations were evaluated for particle size, loading efficiency and production yield. The microsponge formulations showing the best properties were then incorporated into 0.35% w/w carbopol gel. The ketoconazole microsponges incorporated into gel formulations showed acceptable physical parameters, appropriate drug release profile and marked *in vivo* antifungal activity on guinea pig skin.

Administration of hydroxyzine HCl, an antihistaminic drug used in oral formulations for the treatment of urticaria and atopic dermatitis, is usually associated with

dizziness, blurred vision, and anticholinergic responses. Therefore, Zaki et al. (2011) investigated the formulation of eudragit RS-100 microsponges of hydroxyzine HCl with the objective of producing an effective drug-loaded dosage form that is able to control the release of the drug into the skin.

The oil in an oil emulsion solvent diffusion method was applied for the production of eudragit RS-100 microsponges of the drug using acetone as dispersing solvent and liquid paraffin as the continuous medium. Sucrose and pregelatinized starch were used as pore inducers to enhance the rate of drug release. The produced microsponges showed nearly 98% encapsulation efficiency and 60 to 70% porosity. The pharmacodynamic effect of the chosen preparation was tested on the shaved back of histamine-sensitized rabbits. Histopathological studies were also driven for the detection of the healing of inflamed tissues. The prepared systems proved their efficacy for relieving histamine-induced inflammation.

A xanthan gum-facilitated ethyl cellulose microsponges loaded with diclofenac were prepared by Maiti et al. (2011) using the double emulsification technique. The prepared microsponges were subsequently dispersed in a carbopol gel base for controlled delivery of the active to the skin. Scanning electron microscopy revealed the porous, spherical nature of the microsponges. Increasing the drug to polymer ratio positively influenced the production yield, drug entrapment efficiency and mean particle diameter. However, compared to the microsponges with high drug to polymer ratio, the permeation of entrapped drug through excised rat skin was reduced significantly for the microsponges prepared at low and intermediate drug to polymer ratios. In addition, the microsponges prepared at the lowest drug to polymer ratio exhibited a comparatively slower drug permeation and thus, were considered most suitable for controlled delivery of diclofenac sodium to the skin. The gel containing these optimized microsponges was comparable to that of a commercial gel formulation and did not show serious dermal reactions.

Deshmukh and Poddar (2012) have recently developed a glabridin microsponge-loaded gel for treating various hyperpigmentation disorders.

The microsponges were prepared using the emulsion solvent evaporation method and characterized for drug loading and morphology. SEM and porosity studies confirmed spherical and porous nature. *In vitro* diffusion studies of gel formulation depicted highest correlation with Higuchi treatment.

Animal studies carried out using brownish guinea pigs with ultra violet (UV)-induced pigmentation model supported the better depigmenting activity of the microsponges incorporated gels as compared to plain gel.

Oral drug delivery

A microsponge system offers several advantages for oral drug delivery, such as:

1. Preserve the active ingredients within a protected environment and offer oral controlled delivery to the lower part of the gastrointestinal tract (GIT).
2. Microsponge systems improve the solubility of poorly soluble drugs by entrapping these drugs in their porous structure.
3. As the porous structure of the microsponge is very small in size, the drugs entrapped will be reduced to microscopic particles with higher surface area, and consequently improved rate of solubilisation.
4. Maximize the amount of drugs to be absorbed, as the time it takes the microsponge system to pass through the intestine is considerably increased.

Several studies have been investigated for the development of microsponges loaded with topically applied drugs. Jain and Singh (2010b) prepared colon specific formulations by loading paracetamol in eudragit RS 100 based microsponges using quasi-emulsion solvent diffusion method. Compression coating of microsponges with pectin: hydroxypropyl methylcellulose (HPMC) mixture followed by tableting was used. The *in vitro* drug release studies were done on all the formulations and the results were evaluated kinetically and statistically. The study concluded that the release data followed Higuchi matrix but diffusion was the main mechanism of drug release from microsponges. *In vitro* studies showed that compression coated colon specific tablet formulations started the release of drug at the 6th h resultant to the arrival time to proximal colon.

In another study, Gonul et al. (2002) studied the effects of pressure and direct compression on tableting of microsponges using ketoprofen as a model drug. ketoprofen microsponges were prepared by two methods: quasi-emulsion solvent diffusion method with eudragit RS 100 and direct compression method. Different pressure values were investigated with the tablet powder mass to determine the optimum pressure value for the compression of the tablets. Results of the study indicated that microsponge compressibility was superior compared to the physical mixture of the drug and polymer. It was concluded that microsponges can produce mechanically strong tablets due to the plastic deformation of sponge like structure.

Jain and Singh (2010a) studied the potential of formulating dicyclomine loaded eudragit based microsponge by means of a quasi-emulsion solvent diffusion method for colonic delivery. The compatibility of the drug with various formulation components was studied. Surface morphology and shape of the microsponges were demonstrated using SEM.

The compatibility studies showed that there was no chemical interaction throughout the preparation of the formulations and that the drug remains stable in all the formulations. The release rate of the drug from the microsponges was decreased with increasing the drug: polymer ratio. Kinetic studies showed that the main mechanism of drug release followed Higuchi matrix controlled diffusion. An initial burst effect showed that the drug release was bi-phasic with 16 to 30% of the drug released in the 1st h. Cumulative release for the microsponges over 8 h ranged from 59 to 86 %. The authors concluded an approach for the alteration of microsponges of dicyclomine for prolonged drug release. The distinctive compressibility of microsponges can be applied to get efficient local action, as microsponges may be taken up by macrophages which are present in colon.

Colon specific drug delivery system containing flurbiprofen (FLB) microsponges was investigated by Orlu et al. (2006). The authors formulated microsponges containing FLB and eudragit RS 100 using quasi-emulsion solvent diffusion method. Also, FLB was loaded into a commercial Microsponge® 5640 system by means of entrapment method. Compression coating and pore plugging of microsponges with pectin: HPMC mixture followed by tableting was used to prepare colon specific formulations. The prepared microsponges were spherical in shape and found to be 30.7 to 94.5 µm in diameter and showed high porosity values (that is, 61 to 72%). The pore shapes of microsponges prepared by quasi-emulsion solvent diffusion method were found as spherical whereas by entrapment method it was found as cylindrical holes.

Due to the plastic deformation of sponge-like structure of microsponges, mechanically strong tablets were produced for colon specific drug delivery. *In vitro* studies revealed that colon specific tablet formulations prepared by compression coating started to release the drug at the 8th h resultant to the proximal colon arrival time due to the addition of enzyme which could follow a modified release pattern, whereas the drug release from the colon specific formulations prepared by pore plugging the microsponges showed an increase at the 8th h which was the time where the enzyme was added.

Conclusion

Microsponge has become a rapidly evolving technology that can be widely applied in the pharmaceutical field. Owing to their microporous structure and their ability to release their active ingredients in a controlled manner, microsponges can effectively target drugs to their desired sites of action. The most prominent advantages of this delivery system is the diminished side effects and improved stability. Thus, microsponge delivery systems are regarded as a promising vehicle for the controlled and targeted release of various topical and oral active agents. More recently, researches are focused on merging the attractive characteristics of microsponges with the revolutionized nanotechnology trend to enhance their performance.

REFERENCES

Amrutiya N, Bajaj A, Madan M (2009). Development of microsponges for topical delivery of mupirocin. AAPS Pharm. Sci. Tech. 10:402-409.

Aritomi H, Yamasaki Y, Yamada K, Honda H, Koshi M (1996). Development of sustained release formulation of chlorpheniramine maleate using powder coated microsponges prepared by dry impact blending method. J. Pharm. Sci. Technol. 56(1):49-56.

Bertrand N, Leclair G, Hildgen P (2007). Modeling drug release from bioerodible microspheres using a cellular automaton. Int. J. Pharm. 343: 196-207.

Bodmeier R, Chen, H. (1989). Preparation and characterization of microspheres containing the anti-inflammatory agents, indomethacin, ibuprofen, and ketoprofen. J. Control. Release. 10:167–75.

Ceschel GC, Badiello R, Ronchi C, Maffei P (2003). Degradation of components in drug formulations: a comparison between HPLC and DSC methods. J. Pharm. Biochem. Ana .32:1067-1072.

Chadawar V, Shaji J (2007). Microsponge delivery system. Curr. Drug, Del. 4: 23-129.

Çomoğlu T, Gönül N, Baykara T (2003). Preparation and in vitro evaluation of modified release ketoprofen microsponges. Il Farmaco, 58:101-106.

D' souza JI (2008). The Microsponge Drug Delivery System: For Delivering an Active Ingredient by Controlled Time Release. Pharma.Info.net 6(3):62.

D'souza JI (2001). In-vitro antibacterial and skin irritation studies of microsponges of benzoyl peroxide. Ind. Drugs. 38:361-362.

D'souza JI, Harinath NM (2008). Topical anti-inflammatory gels of fluocinolone acetonide entrapped in eudragit based microsponge delivery system. Res. J. Pharm. Tech. 1:502-506.

Deshmukh K, Poddar SS (2012). Tyrosinase inhibitor-loaded microsponge drug delivery system: new approach for hyperpigmentation disorders. J. Microencaps 29(6):559-68.

Emanuele AD, Dinarvand R (1995). Preparation, characterization and drug release from thermo responsive Microspheres. Int. J. Pharm. 118:237–242.

Embil K, Nacht S (1996). The microsponge drug delivery system (MDS): a topical delivery system with reduced irritancy incorporating multiple triggering mechanisms for the release of actives. J. Microencaps, 13:575-588.

Grimes PE (2004). A microsponge formulation of hydroquinone 4%and retinol 0.15% in the treatment of melasma and post-inflammatory hyperpigmentation. Cutis. 74:362-368.

Higuchi T (1963). Mechanism of sustained-action medication theoretical analysis of rate of release of solid drugs dispersed in solid matrices. J. Pharm. Sci. 52:1145–1149.

Jain V, Singh R (2010). Dicyclomine-loaded eudragit®-based microsponge with potential for colonic delivery: preparation and characterization. Trop. J. Pharm. Res 9: 67-72.

Jain N, Sharma PK, Banik K (2011). Recent advances on microsponge delivery system. Int. J. Pharm. Sci. Rev. Res. 8:13-23.

Jain V, Singh R (2010). Development and characterization of eudragit RS 100 loaded microsponges and its colonic delivery using natural polysaccharides. Acta Poloniae Pharmaceutica-Drug Res. 67:407-415.

Jain V, Singh R (2011). Design and characterization of colon-specific drug delivery system containing paracetamol microsponges. Arch. Pharm. Res. 34:733-740.

James J, Leyden J, Alan S, Diane T, Kenneth W, Guy W (2005). Topical Retinoids in Inflammatory Acne: A Retrospective, Investigator-Blinded, Vehicle-Controlled, Photographic Assessment. Clin. Therapeut. 27:216-224.

Jelvehgari M, Siahi-Sahbad MR, Azarmi A, Gary P, Nokhodchi A (2006). The microsponge delivery system of benzoyl peroxide: Preparation, characterization and release studies. Int. J. Pharm. 308:124-132.

Kaity S, Maiti S, Ghosh AK, Pal D, Ghosh BS (2010). Microsponges: A novel strategy for drug delivery system. J. Adv. Pharm. Technol. Res. 1(3):283-290.

Kilicarslan M, Baykara T (2003). The effect of the drug/polymer ratio on the properties of Verapamil HCl loaded microspheres. Int. J. Pharm. 252:99–109.

Korsmeyer RW, Gurny R, Doelker E, Buri P, Peppas NA (1983). Mechanisms of solute release from porous hydrophilic polymers. Int. J. Pharm. 15:25-35.

Maiti S, Kaity S, Ray S, Sa B (2011). Development and evaluation of xanthan gum-facilitated ethyl cellulose microsponges for controlled percutaneous delivery of diclofenac sodium. Acta Pharm .61:257-270.

Mishra MK, Shikhri M, Sharma R, Goojar MP (2011). Optimization, formulation development and characterization of Eudragit RS 100 loaded microsponges and subsequent colonic delivery. Int. J. Drug Disc. Herb Res 1:8-13.

Nacht S, Kantz M (1992). The microsponge: A novel topical programmable delivery system. Top. Drug Deliv. Syst. 42:299–325.

Nokhodchi A, Jelvehgari M, Siahi MR, Mozafari MR (2007). Factors affecting the morphology of benzoyl peroxide microsponges. Micron, 38:834-840.

Orlu M, Cevher E, Araman A (2006). Design and evaluation of colon specific drug delivery system containing flurbiprofen microsponges. Int. J. Pharm .318:103-117.

Patel G, Patel JK (2006). Use of a Microsponge in Drug Delivery Systems, Pharmaceutical Processing, 158. http://www.pharmpro.com/Archives/2006/02/Use-of-a-Microsponge-in-Drug-Delivery-Systems

Peppas NA (1985). Analysis of Fickian and non-Fickian drug release from polymers. Pharm. Acta Helv 60:110.

Pradhan SK (2011). Microsponges as the versatile tool for drug delivery system. Int. J. Res. Pharm. Chem. 1(2):243-258.

Saboji JK, Manvi FV, Gadad AP, Patel BD (2011). Formulation and evaluation of ketoconazole microsponge gel by quassi emulsion solvent diffusion. J. Cell. Tiss Cult. 11:2691-2696.

Sensoy D, Cevher E, Sarıcı A, Yılmaz M, Ozdamar A, Bergişadi N (2009). Bioadhesive sulfacetamide sodium microspheres: Evaluation of their effectiveness in the treatment of bacterial keratitis caused by Staphylococcus aureus and Pseudomonas aeruginosa in a rabbit model. Eur. J. Pharm. Biopharm. 72:487-495.

Shah VP, Elkins J, Lam S, Skelly JP (1989). Determination of in vitro drug release from hydrocortisone creams. Int. J. Pharm. 53:53-59.

Singla AK, Sharma ML, Dhawan S (2011). Nifedipine loaded chitosan microspheres: characterization of internal structure. Biotech. Histochem. 76:65-171.

Vyas SP, Khar RK (2002). Targeted and controlled drug delivery: novel carrier systems. CBS Publications, 1st ed., New Delhi, 453.

Wagner JG (1969). Interpretation of percent dissolved-time plots derived from in vitro testing of conventional tablets and capsules. J. Pharm. Sci. 58:1253–1257.

Wester RC, Patel R, Nacht S, Leyden J, Melenders J, Maibach H (1991). Controlled release of benzoyl peroxide from a porous microsphere polymeric system can reduce topical irritancy. J. Am. Acad. Dermatol .24:720–726.

Won R (1987). Method for delivering an active ingredient by controlled time release utilizing a novel delivery vehicle which can be prepared by a process utilizing the active ingredient as a porogen. Patent No 4690825 US.

Zaki Rizkalla CM, Latif Aziz R, Soliman II (2011). In vitro and in vivo evaluation of hydroxyzine hydrochloride microsponges for topical delivery. AAPS Pharm. Sci. Tech. 12:989-1001.

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Vol. 7(17), pp. 882-885, 8 May, 2013
DOI 10.5897/AJPP09.176
ISSN 1996-0816 © 2013 Academic Journals
http://www.academicjournals.org/AJPP

African Journal of Pharmacy and Pharmacology

Full Length Research Paper

The effects of aqueous extracts of *Hibiscus sabdariffa* Linn. calyces of *var. ruber and var. intermedius* on intestinal transit in rats

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Accepted 4 April, 2013

The effects of aqueous extracts of *Hibiscus sabdariffa l.* calyces of *var. ruber* and *var. intermedius* on intestinal transit were determined in experimental rats. The dried calyces of *H. sabdariffa l.* were pulverized and 10% extracts of both *var. ruber* and *var. intermedius* were made and administered orally at varying doses. Test rats were given the 10% extracts of both the species of *H. sabdariffa* at 0.5 ml/100 g,1 ml/100 g and 2 ml/100 g body weight, while control rats received normal saline. After 30 min, each animal was then given 1.5 ml of the dye solution orally. About 1 h after administering the dye, each rat was sacrificed and the intestine carefully dissected out. The length of the intestine and the transit point of the orally administered dye were then measured. The transit point was calculated as a percentage of the total length of the intestine. The extracts of both *var. ruber* and *var. intermedius* of *H. sabdariffa l.* caused a reduction in transit points of the dye. The extract of *var. intermedius* was more effective than *var. ruber*. The reduction in transit point and the increase in transit time by both extracts indicates that the plant possess astringent effect and could be used at appropriate dose as constipating agent or for stabilizing diarrhea. The acute toxicity study of *H. sabdariffa l.* in rats was found to be above 5,000 mg kg^{-1}.

Key words: Astringent, aqueous extract, *Hibiscus sabdariffa*, intestinal transit, *var. ruber, var. intermedius.*

INTRODUCTION

Hibiscus sabdariffa has been reported to be antiseptic, aphrodisiac, astringent, cholagogue, demulcent, digestive, diuretic, emollient, purgative, refrigerant, sedative, stomachic and tonic (Olaleye, 2007). *H. sabdariffa Linn* is a herb belonging to the malvaceae family, growing about 0.5 to 3.0 m high, with a strong tap-root. It is cultivated for leaf, fleshy calyx, seed or fibre according to the respective properties of the two major varieties *var. ruber* (red) and *var. intermedius* (green) (Dalziel 1973). *H. sabdariffa l.* is grown in Central and West Africa, South East Asia, and elsewhere in parts of West Indies, Jamaica and Central America. It is commonly known as roselle or red sorrel (English), karkade (Arabic), yakuwa (Hausa), amukan (Yoruba) and okworo ozo (Ibo). The thick, red and fleshy, cup-shaped calyces of the flower are consumed worldwide as a cold beverage and as a

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hot drink (sour tea). These extracts are also used in folk medicine against many complaints that include high blood pressure, liver diseases and fever (Dalziel, 1973; Wang et al., 2000; Ross, 2003; Bako et al., 2009). The red anthocyanin pigments in the calyces are used as food colouring agents (Esselen and Sammy, 1975). In light of this, the study is designed to evaluate and compare the astringent effect of *H. sabdariffa l.* species extracts of *var. ruber* and *var. intermedius.*

MATERIALS AND METHODS

Animals

Thirty five albino rats of both sexes weighing between 130 to 150 g were used. These rats were obtained from the Department of Human physiology, Ahmadu Bello University, Zaria. They were randomly divided into seven groups of five rats per group (n = 5). Each group was kept in separate standard cage with 12 h light/dark cycle condition in the animal room of the Department of Human Physiology, Ahmadu Bello University Zaria. They were fed on commercial feeds with tap water *ad libitum*. The cages were cleaned every day, with food and water changed daily. The animals were starved 24 h before the experiment but water was allowed.

Plants

The two *H. sabdariffa l*. species (*var. ruber and var. intermedius*) were bought in Samaru-Zaria market Kaduna state, Nigeria. The plants was identified in the Department of Biological Sciences, Ahmadu Bello University Zaria, authenticated with a voucher number 1056 and deposited in the Herbarium section. Extraction was conducted using maceration method in the Department of Pharmacognosy and drug development, Faculty of Pharmaceutical Sciences, Ahmadu Bello University, Zaria. The respective calyces of both plants were sun dried separately, pulverized and then sieved. Twenty five grams (25 g) of both the powdered calyces were weighed out and dissolved in 250 ml of distilled water to make 10% extract. The resultant extracts were filtered and kept in refrigerator before use.

Dye

The dye was prepared by a modified method of Uwagboe and Orilikwe (1995). 95 ml of 10% aqueous suspension of charcoal (BDH, England) was mixed with 5 ml of Giemsa stain (BDH, England).

Intestinal transit study

The thirty five albino rats were grouped into control (Group I), *var. ruber*-treated (Group II to IV) and *var. intermedius*-treated (Group V to VII) as shown in Table 1. About 24 h before the experiment, food was withdrawn from the animals but water was allowed. During the experiment, each group received the appropriate dose of the respective extracts using oral cannula. Control group (normal saline), Group II to IV (*var. ruber*-extract) and Group V to VII (*var. intermedius*-extract) received the extracts, respectively.

Thirty minutes after administration of the extract, 1.5 ml of the dye was administered orally to each rats using oral cannula. The rats were then kept without food and water for one hour before the determination of transit point of the dye. The rats were sacrificed by overdose of chloroform at the end of the time. The peritoneum of the rats was opened and the entire length of the small intestines were carefully stretched and cut open. The lengths of the intestines from the pyloric junction as well as the distance transversed by the dye from the pyloric junction were measured.

Statistical analysis

The data collected are expressed as mean ± standard error of mean (SEM). The data obtained were analyzed using one way analysis of variance (ANOVA) and Turkey-Kramer *post hoc* test for multiple comparisons. The results were considered statistically significant if the p values were 0.05 or less (Betty and Jonathan, 2003).

RESULTS

The distance transversed by the dye from the pyloric junction was calculated as percentages of the entire length of the intestines. This was regarded as the percentages of the transit point of the dye at each dose of the extract. The mean and standard error of the mean (mean ± SEM) of these percentages were calculated for each group. Data from of the control group were regarded as zero administration of the extract. The level of significance between the transits point of each dose were determined using Turkey-Kramer post hoc test.

DISCUSSION

The results of the present study showed that both extracts of *var. ruber and var. intermedius* produced a significant reduction ($P < 0.01$) of the percentage transit point when compared with that of the control as shown in Table 2 and Figure 2. *Var. intermedius* seems to be more effective because it has activity at the lowest dose ($P < 0.05$) but the response is not dose-dependent. *Var. ruber* responded in a dose-dependent manner but the response was observed at the second dose as shown in Table 2 and Figure 1. The extracts of *H. sabdariffa* exhibited a relaxing activity by reducing the transit point. Reduction in the percentage transit point indicates a reduction in intestinal motility and an increase transit time (Oluwade et al., 2004).

H. sabdariffa is reported to inhibit the tone of various isolated muscle preparations that included rabbit aortic strip (Obiefuna et al., 1994) and rat ileal strip (Salah et al., 2002). The extract also rhythmically contracted rat uterus, guinea-pig tracheal chain and rat diaphragm. However, as the extracts contain organic acids and minerals, the effect of the extract on different smooth muscle preparations would be expected to be variable. The mechanism of action of *H. sabdariffa* aqueous extract on smooth muscles cannot be ascertained from this study. Although studies carried out by coworkers suggested that the overall effect is a direct relaxation of the smooth muscles, which is blocked by atropine through

Table 1. Animal groupings and treatments.

Extracts-treatment group	Animal groups	n	Dose of 0% aqueous extract of *var. ruber* (ml/100 g)	Dose of 10% aqueous extract of *var. intermedius* (ml/100 g)	Dose of 0.9% Nacl (ml/100 g)
Control group	I	5	-	-	1.0
Var. ruber-treated groups	II	5	0.5	-	-
	III	5	1.0	-	-
	IV	5	2.0	-	-
Var. intermedius-treated groups	V	5	-	0.5	-
	VI	5	-	1.0	-
	VII	5	-	2.0	-

Table 2. Mean percentage transit points of dye in the intestines of rats administered extracts.

Parameter / Group	Control normal saline 1.0 ml (5)	*Hibiscus sabdariffa l.*					
		Dose of 10% aqueous extract of *var. ruber* (ml/100 g)			Dose of 10% aqueous extract of *var. intermedius* (ml/100 g)		
		0.5 ml (5)	1.0 ml (5)	2.0 ml (5)	0.5 ml (5)	1.0 ml (5)	2.0 ml (5)
Mean (%) transit point	73.0±4.1	67.0±1.3	55.4±7.6	52.6±6.0	61.2±1.5	53.2±4.7	55.8±4.2
Level of significance		NS	S**	S**	S*	S**	S**

NS = Not significant; S* = significant (P < 0.05); S**=significant (P < 0.01).

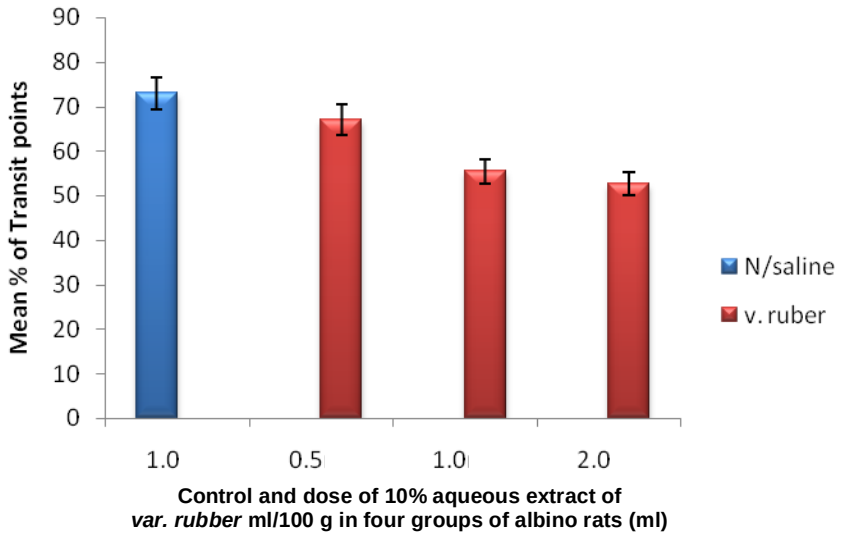

Figure 1. Mean percentage transit points of dye in the intestines of rats administered with *v. ruber*.

cholinoceptors as cholinergic antagonist. These responses may explain antibacterial activity by Olaleye (2007), the hypotensive action of the extract (Ali et al., 1991; Adegunloye et al., 1996) and the astringent effect of gastrointestinal smooth muscles.

Pectin, one of the phytochemical constituents (Odebiyi and Sofowora, 1978) of *H. sabdariffa* used as anti-diarrhea (Swinyard, 1975), could be responsible for the astringent effect. The acute toxicity study of *H. sabdariffa* in rats was found to be above 5,000 mg kg^{-1} according to Lorke's method (1983) which characterizes it to be safe for consumption. The astringent effect of the plant may

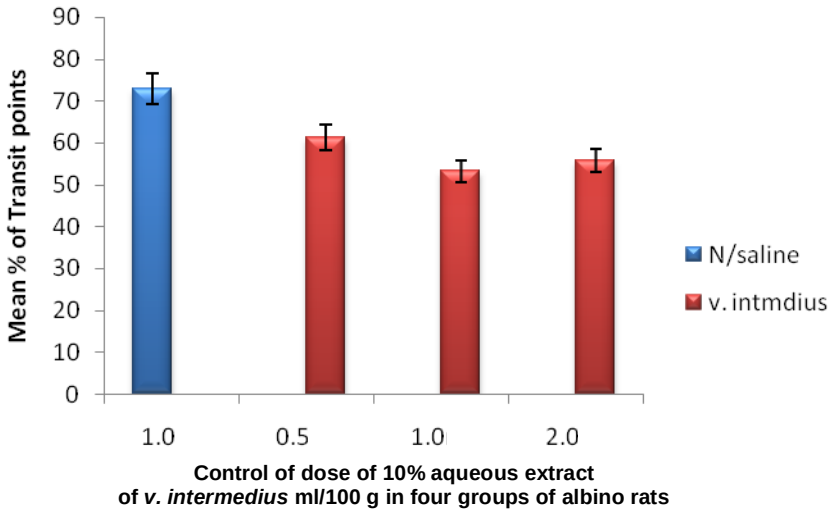

Figure 2. Mean percentage transit points of dye in the intestines of rats administered with *v. intermedius.*

be mediated through cholinoceptors as cholinergic antagonist, because atropine blocked the effect of the extract. *H. sabdariffa l.* species (*var. ruber* and *var. intermedius*) reduces the intestinal motility, though *var. intermedius* seems to be more effective because it responded at the lowest dose.

ACKNOWLEDGEMENTS

The authors are grateful to Mohammad Nura of the Department of Human Physiology, Ahmadu Bello University, Zaria for the role he played in the laboratory during this research. We also acknowledge McArthur Foundation, Ahmadu Bello University, Zaria and University Board of Research of Ahmadu Bello University, Zaria for supporting this research work.

REFERENCES

Adegunloye BJ, Omoniyi JO, Ajabonna OP (1996). Mechanisms of the blood pressure lowering effects of the calyx extract of *Hibiscus sabdariffa* in rats. Afr. J. Med. Sci. 25:235–238.

Ali MS, Salih WM, Mohamed AH, Homeida AM (1991). Investigation on the antispasmodic potential of *Hibiscus sabdariffa* calyces. J. Ethnopharmacol. 31:249–257.

Bako IG, Mabrouk MA, Mohammad IM, Buraimoh AA, Abubakar MS (2009). Hypotensive effects of aqueous seed extract of *Hibiscus sabdariffa linn (Malvaceae)* on normotensive cat. Int. J Anim. Vet. Sci. 2:23-29

Betty RK, Jonathan AC (2003). Essential medical statistics. Second edition. Blackwell science, USA. pp. 15-409.

Dalziel JM (1973). The Useful Plants of West Tropical Africa. Crown Agent for Overseas Government and Administrations, London. pp.129–130.

Esselen WB, Sammy GM (1975). Roselle: a natural red colorant for foods. Food Prod. Dev. 7:80–82.

Lorke D (1983). A new approach to practical acute toxicity testing. Arch. Toxicol. 54:275-287.

Obiefuna PCM, Owolabi OA, Adegunloye BJ (1994). The petal extract of *Hibiscus sabdariffa* produces relaxation of the isolated rat aorta. Int. J. Pharmacog. 32:69–74.

Odebiyi OO, Sofowora EA (1978). Phytochemical screening of Nigeria medicinal plants. Lioydia 41:234-235.

Olaleye MT (2007). Cytotoxicity and antibacterial activity of methanolic extract of *Hibiscus sabdariffa*. J. Med. Plants Res. 1(1):009-013.

Oluwade MO, Eghianruwa KI, Daramola FO (2004). Effects of aqueous extract of *Hibiscus sabdariffa* calyces and *Ocimum gratissimum* leaves on intestinal transit in rats. Afr. J. Biomed. Res. 7:31-33.

Ross IA (2003). *Hibiscus sabdariffa*. In Medicinal Plants of the World, Vol 1, 2nd edn. New Jersey, Humana Press. pp. 267–275.

Salah AM, Gathumbi J, Vierling W (2002). Inhibition of intestinal motility by methanolic extracts of *Hibiscus sabdariffa L.* (Malvaceae) in rats. Phytother. Res. 16:283–285.

Swinyard EA (1975). Surfacing-acting drugs. In: Goodman AG, Gilman A. The pharmacological basis of therapeutics, 8th edition. Pergamon Press. pp.200-939.

Uwagboe PE, Orimilikwe SO (1995). Effect of histamine H_2 receptor blocker on gastrointestinal transit in conscious albino rats. Niger. J. Physiol. Sci. 11:56-59.

Wang CJ, Wang JM, Lin WL, Chu CY, Chou FP, Tseng TH (2000) Protective effect of *Hibiscus anthocyanins* against tert-butyl hydroperoxide-induced hepatic toxicity in rats. Food Chem. Toxicol. 38(5):411-416.

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Vol. 7(17), pp. 886-892, 8 May, 2013
DOI 10.5897/AJPP10.234
ISSN 1996-0816 © 2013 Academic Journals
http://www.academicjournals.org/AJPP

African Journal of Pharmacy and Pharmacology

Full Length Research Paper

Assessment of the knowledge, attitude and awareness of residents of Jos, Plateau State, Nigeria, towards worm infestation and de-worming

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Accepted 6 June, 2012

Due to the poor socioeconomic conditions and severe lack of good hygienic living conditions in developing countries of the world, worm infestation has become widely prevalent and is a major public health problem. The purpose of this study was to assess the knowledge, attitude and practice of residents of Jos towards worm infestations and de-worming. This involved a cross-sectional study in urban slum of Jos North L.G.A of Plateau State, North Central Nigeria. The Yamane method was used to randomly select 399 residents of Jos, which included adults from age 15 to 60 years of both sexes. The results obtained were analyzed using Chi-square test and descriptive statistics. Most respondents (about 50.0%) had moderate level of knowledge about worm infestation, which was only associated with educational level (*p*-value = 0.034) and age group (*p*-value = 0.0021). Attitude towards worm infestation was good, while that towards de-worming was very poor. However, since attitude towards de-worming was poor, only knowledge provision is inadequate, hence attitudinal change must be imbibed to reduce risk and incidence of worm infestation.

Key words: Assessment, worm infestation, de-worming, knowledge, attitudes.

INTRODUCTION

Worm infestations are very common in developing countries of which Nigeria is one, causing much diseases in both humans and domestic animals. A parasite is any living organism that lives on a host without benefiting the host in return and in most cases, causes damage to the host in the process (Centre for Disease Control, 2000). Parasites occur in two distinct forms: single-celled protozoa and multicellular metazoan also called helminths or worms. Metazoa are further subdivided into two phyla: the platyhelminthes (flatworms) and the nemathelminthes (roundworms and nematodes). The phylum platyhelminthes contains two medically important classes; cestoda (tapeworms) and trematoda (flukes) (Warren, 2002). Helminths are the largest intestinal human parasites and are usually long-lived, increasing the chance of re-infection and chronic disease course. For example, in the epidemiology of schistosomiasis, over 200 million people are said to be affected in the tropics and subtropics, mostly concentrated in the sub-Saharan region of Africa (Kumar and Clark, 2005).

A person or object from which the infectious agent passes to a host is known as the source of the infection and this source of infection may or may not be a portion of the reservoir. For example, humans are the reservoir of shigella infection; a cook who is a carrier may infect food that is served at a party; that item of food, rather than the reservoir is the source of infection in that particular outbreak (Adetokumbo et al., 2003). The infectious agent may be transmitted from one person to another or from the reservoir to a new host through different mechanism

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such as: Penetration of skin, Ingestion, and Contact. Many research results have shown relationship between certain dietary intake and worm infestations (Taweesak, 2002).

De-worming (sometimes known as worming or drenching) is the giving of an anthelmintic drug (a wormer, de-wormer, or drench) to an animal or human being to rid it of intestinal parasites such as roundworm and tapeworm (Wikipedia, 2010). De-worming, being a preventive and curative form of therapy for helminthiasis has been extensively carried out in various public health campaigns. However, there still exists the need for better and effective implementation of community directed treatment in areas endemic for worm infestation. It is in this respect that it was documented that community-directed treatment with ivermectin (CDTi) was very successful among 60 million Africans in rural African communities. The CDTi experience prompted the board of the African Programme for Onchocerciasis Control (APOC) to commission a study, to examine an expanded strategy of community directed interventions. In 2005, the 3-year multi-country study was launched, examining to what extent the CDI process could be used for the integrated delivery of other health interventions with vary degrees of complexity, alongside ivermectin (Community Directed Interventions, 2008).

In a related study in Kwanji district, Kebbi State, North-Eastern Nigeria, infection rate was comparatively lower than in some other endemic villages in Nigeria. This was attributed to a programme of community directed mass treatment of onchocerciasis with Mectizan which had been underway in the area (Ameh et al., 2008). In low and middle-income countries where de-worming policies have been adopted, it has generally proven to be a highly effective and economically efficient public health intervention. Due to its proven effectiveness and the relatively low cost of intervention, de-worming has attracted the attention of public health officials, developmental experts, and others concerned with global health. Chemotherapy, therefore, provides the single most efficient practical and inexpensive strategy to control helminthic infections (Hardman et al., 2001).

It can be seen from the above references that intestinal helminthic infestations among others, is one of the commonest cause of chronic infection in humans in the developing countries. The impure drinking water, low socio-economic state, poor sanitation coupled with low literacy levels are some of the major causes. The socio-economic peculiarity of Jos North as an urban slum and its positioning in the tropics makes it vital to this study. Jos as a town is known for the production of a variety of arable vegetable crops which are potent sources for worm infestations, considering the fact that most farmers use very contaminated water from rivers as source of irrigation. The poor hygiene and inadequacies associated with solid waste disposal (including faecal matter) is also an implicating factor for high chances of worm infestation. Over the past four (4) years, water supply in many parts of Jos-Bukuru metropolis had been in a deplorable state, leaving people with the only option of buying water from tanks that feed from dams, which have been linked to schistosomiasis (Kumar and Clark, 2005), while those in inner rural areas even drink from direct ground water.

The objective of this work was to access the current level of awareness, knowledge and attitude regarding worm infestations and to evaluate how effective residents of Jos carry out de-worming programme on themselves.

METHODOLOGY

This cross sectional study was conducted to assess the knowledge, attitude and practice towards worm infestation and de-worming using quantitative survey interview forms (questionnaires) which were pre-tested before the commencement of survey.

Population and sample group

The cosmopolitan population of Jos was the target population; people of both sexes within the age range of 15 to 60 years were used. Estimated population of Jos in year 2010 from 1996 census result was calculated according to the National Population Commission guideline of 1996 at an average growth rate of 2.8%, using the following formula:

$$\text{Population (2010)} = \text{Population (1996)} \left[1 + \frac{R}{100}\right]^{T}$$

Where R is the average population growth rate and T is the time interval from 1996 to 2010, which is 13 years. Using the population of 1996 (526,676 people), the estimated population could be calculated as:

$$\text{Population (2010)} = 526{,}767\left(1 + \frac{2.8}{100}\right)^{13}$$

Hence, the population for 2010 is equal to 754,200 people.

Sample size and selection

Sample size was calculated according to Yamane, with an alpha error of 0.05 and a precision of 5% thus:

$$N = \frac{n}{1 + ne^2}$$

Where N is the sample size, n is the population and e is the alpha error (0.05).

$$\therefore N = \frac{754{,}200}{1 + [754{,}200 \times (0.05)^2]}$$

Thus, N = 399 people. With this sample size, Jos was randomly zoned into four zones: Zone A (Terminus/Farin Gada), Zone B(Angwan Rukuba), Zone C (Rayfield/Hwolshe) and Zone D (Miango/Bukuru).

Procedure of the study

First, we co-ordinated with concerned sectors, e.g. National Population Commission, state coordinating office which provided population data for Jos. Next, questionnaires where pre-tested among five (5) respondents. These questionnaires were then dispatched within the zones for a period of 7 weeks running. Finally, the filled questionnaires were then retrieved and coded for analysis.

Data analysis

After a concise rechecking procedure, questionnaires were then encoded and analysed on Microsoft Excel, 2003. Comparison between variables was done using Chi-square test. A *p*-value of less than 0.05 was considered statistically significant. Descriptive statistics (percentages) were used to describe the frequency.

RESULTS

Three hundred and ninety-nine (399) residents of Jos were assessed during the study, out of which 56% (n = 226) were males and 43.6% (n = 173) were females. Minimum age was 15 years, while maximum age was 60 years. Age group between 15 – 35 years were the highest contacted, 85.20% (n = 340). Age between 36 - 50 years constituted 13.30% (n = 53), while 51 - 60 years group were the least. Overall, 94.70% of the respondents were Christians, while 5.30% were Muslims. About 66.2% (n = 264) of the respondents were ethnic groups from the North Central zone of the country, while the core North and South East were in the proportion of 3.5 and 15.0%, respectively. The South-South ethnic groups made up 5.8%, while those from the South-West were 9.5%. Moreover, 77.70% (n = 310) were single, while 20.3% (n = 81) were married. Only 2% were either widows/widowers.

In addition, the educational status showed 2.68% to have had only primary school education, while 28.32% had secondary school education as their highest qualification. Also, 69.0% of the population comprises those that had passed through tertiary institution. Furthermore, 5.8% were unemployed, 54.4% were students, especially of higher institution, 19% were civil servants, 10% were into businesses and 16.6% were into other occupations; 73.43% reside in Zone A (Terminus/Farin Gada), 19.80% reside in Zone B (Angwan Rukuba), Zone C (Rayfield/Hwolshe) had 3% while Zone D (Miango/Bukuru) comprised 3.77%.

DISCUSSION

From Table 1, most respondents like and consume vegetables often (62.3%), which are often bought from the market of which most of the farm are irrigated with contaminated water placing the consumers at risk of worm infestation if not properly prepared before consumption. The result also showed that most respondents only treat their water sometimes (n = 216, at 54.10%), while 21.80% (n = 87) do not treat water at all, leaving only 24.10% who do it always. Of these individuals, most of them drink tap water from dams, 43.4% (n = 173), which have been linked to spread of schistosomiasis (Kumar and Clark, 2005). It was also seen that 49.6% (n = 198) of the respondents consume beef, fish 49.1% (n = 196) and snail (45) which have been linked to taeniasis and even schistosomiasis, respectively. Moreover, about 1.80% (n = 7), consume pork which has been linked with trichinosis. Though a majority claimed they prepare their meat well cooked (98.50%), barbecue and grills sellers by the road side only prepare beef to undercooked level, increasing risk for taeniasis because they are highly patronised.

Table 2 showed that about 87.5% (n = 293) believed they could be infected by worms which was quite high. However, 62.70% (n = 210) of the people did not go for periodic medical check-up to know their worm status, while 28.90% (n = 97) normally did it sometimes. A few proportion, 12.50% (n = 92) did not think they could be infected by worms because of religious belief and their self acclaimed sense of good hygienic practice.

As regards knowledge, most respondents have heard about worm infestation, and knew that they were the largest intestinal human parasites. However, 47.80% (n = 160) provided correct response on occupational or lifestyle risk for worm infestation; 43.30% (n = 145) did not know, while 8.9% (n = 36) provided a wrong response like eating overripe fruit and drinking alcohol. Similar study have been done on knowledge, attitude and practice regarding liver fluke (Taweesak, 2002), which showed that 63% provided correct response on foods containing liver fluke and 37% with incorrect responses. Food sources for worm infestation in this study had 65.10% correct response, 12.2% incorrect response and 22.7% for those that do not know such foods. In the same study, 79% provided correct response about severe symptoms of liver fluke infection, while only 21% gave incorrect response. In this survey in Jos, 78.8% provided correct response on manifestations of worm infestation. Wrong response came up to 11.0%, while 10.2% did not know of any such manifestations. Table 3 shows that the respondents had good knowledge about de-worming, but attitude was generally poor. About 70% knew about de-worming, providing correct idea to the tone of 81.60%. Concerning attitude however, 75.3% respondents had carried out de-worming before but 55.0% (n = 117) could not remember the last time they did it.

From Table 4, it can be observed that more females were at risk for worm infestation than males. The proportion of males at low risk was higher (48.70%) than those at low risk for females (45.67%) at *p*-value = 0.025. This was contrary to study on knowledge, attitude and practice by Taweesak (2002) who found that more males were at risk due to high rate of consumption of half-cooked or raw fish than with females. However, the results agree with a study reported by Taweesak (2002)

Table 1. Food selection habit and general hygiene.

Variable	N	Percentage (%)
How often do you eat vegetables?		
(a) Very often	149	37.70
(b) Often	246	62.30
(c) Not at all	0	0.00
Preparation of vegetables before eating		
(a) Raw and wash	106	26.84
(b) Raw, wash and cook	229	58.00
(c) Raw or cook	60	15.16
Source of drinkable water		
(a) River	2	0.50
(b) Dam	4	1.00
(c) Well	60	15.00
(d) Borehole	105	26.30
(e) Tap	173	43.40
(f) Others	55	13.80
How often do you treat your water?		
(a) Always	96	24.10
(b) Sometimes	216	54.10
(c) Not at all	87	21.80
How do you treat your water?		
(a) Chemical	121	38.80
(b) Chemical and Boiling	56	18.00
(c) Boiling	135	43.20
What kind of meat do you eat?		
(a) Beef	198	49.60
(b) Goat	159	40.00
(c) Fish	196	49.10
(d) Chicken	156	39.10
(e) Snail	45	11.30
(f) Bush meat	12	3.00
(g) Pork	7	1.80
(h) Others	5	1.30
How do you prepare your meat?		
(a) Well cooked	393	98.50
(b) Undercooked	6	6.00
(c) Raw	0	0.00

that women were at more risk because they consumed rawer or half-cooked fish than males. On the other hand, Table 5 shows that knowledge about worm infestation does not mean low risk, because those at higher risk had better knowledge than those at low risk at *p*-value = 0.0184, which was highly significant. Those at low risk only had lower knowledge level because they felt they were not involved with foods and habits that would make them prone to worm infestation. This finding agrees with epidemiological survey on risk factors of liver fluke carried out by Kunjana which revealed that the higher the knowledge, the poorer the attitude due to high risk

Table 2. Information about worm infestation.

Variable	N	Percentage (%)
Have you ever heard about worm infestation?		
(a) Yes	335	84.00
(b) No	64	16.00
Mention three (3) human worms you know		
(a) Correct response	271	78.80
(b) Wrong response	97	11.00
(c) Do not know	31	10.20
Mention three manifestations of worm infection		
(a) Correct response	264	78.80
(b) Wrong response	37	11.00
(c) Do not know	34	10.20
Do you think you can be infected by worms?		
(a) Yes	293	87.50
(b) No	42	12.50
Do you go for periodic medical check-up to know your worm status?		
(a) Yes	28	8.40
(b) No	210	62.70
(c) Sometimes	97	28.90
(i) Have you ever been diagnosed of a worm infection?		
(a) Yes	113	33.70
(b) No	222	66.30
ii) If yes in (i), which worm was it?		
(a) Knows worm(s)	43	38.10
(b) Do not know worm(s)	70	61.90

Table 3. Knowledge on the concept of de-worming.

Variable	N	Percentage (%)
Do you know about the concept of de-worming?		
(a) Yes	283	70.90
(b) No	116	29.10
If 'yes' in 1, state a sentence about what you know?		
(a) Correct idea	231	81.60
(b) Wrong idea	52	18.40
Have you ever carried out de-worming?		
(a) Yes	213	75.30
(b) No	70	24.70
If yes in 3, when was the last time		
(a) 3 months ago and above	58	27.20
(a) 2 months ago and above	38	17.80
(b) Can not remember	117	55.00

Table 3. Contd.

How would you classify de-worming		
(a) Preventive	83	29.30
(b) Curative	30	13.80
(c) Preventive/curative	161	56.90
How often do you de-worm?		
(a) Daily	0	0.00
(b) Weekly	3	1.40
(c) Monthly	30	14.10
(d) Bi-monthly	24	11.30
(e) Quarterly	156	73.20
Who educated you on de-worming?		
(a) Self education	45	16.00
(b) Heal professional	162	57.20
(c) Non-health professional	76	26.80
What type of anthelmintic have you been taking?		
(a) Correct drug	150	70.40
(b) Can not remember	43	20.20
(c) Wrong drug	5	20.30
(d) Do not know	15	7.10
In what form does the anthelmintic exist?		
(a) Correct dosage form	205	96.20
(b) Wrong dosage form	2	0.94
(c) Do not know	6	2.86
How much of medicine do you take per dose and for how long?		
(a) Correct use	160	75.12
(b) Wrong use	53	75.12

Table 4. Association between gender and risk of worm infestation.

Characteristic		Risk level						Total	X^2	df	p-value
		Low		Moderate		High					
		n	%	n	%	n	%				
Gender	Male	110	48.70	116	51.30	0	0	226	0.118	2	0.025
	Female	79	45.67	94	54.33	0	0	173			

Table 5. Association between knowledge about worm infestation and risk for worm infestation.

Characteristic		Level of knowledge						Total	X^2	df	p-value
		Low		Moderate		High					
		n	%	n	%	n	%				
Risk level	Low	48	25.40	93	49.20	48	25.40	189	3.442	4	0.0184
	Moderate	38	18.10	118	56.20	54	25.70	210			
	High	0	0	0	0	0	0	0			

chances (Taweesak, 2002; Awasthi et al., 2008).

Conclusion

This study showed that the residence of Jos had good knowledge of worm infestation with females at higher risk of infection than males, although the females had a better knowledge and good attitude about de-worming than the males.

ACKNOWLEDGEMENT

The authors wish to appreciate all who participated in this study by responding to the questions outlined in the questionnaires which made this work a reality.

REFERENCES

Adetokumbo O, Lucas, Herbert MG (2003). Short Textbook of Public Health Medicine for the Tropics. Chavon Tec Ltd, Chennai, India. pp. 1-2, 27, 34, 36-39, 49-51, 77, 84, 86-89, 92, 137-150, 227-231.

Ameh IG, Ukatu VE, Adamu T, Muazu S (2008). Skin Microfilariae and Clinical Manifestations of Onchocerciasis in some villages at Kwanji District of Kebbi state, Nigeria. Jos J. Med. (3):36.

Awasthi S, Verma T, Kotecha V, Venkatesh V, Joshi V, Roy S (2008). Prevalence and Risk Factors Associated with Worm Infestatiion in Pre-school children (6-23months) in selected blocks of Uttar Pradesh and Jharkhand, Ind. J. Med. Sci. 12(62): 484-490.

Center for Disease Control (2000). Parasite Removal in Humans. http://www.ehow.com/how-dves_5233229http://www.ehow.com/how-dves_5233229.

Community Directed Interventions Study Group (2008). Community-directed interventions for priority health problems in Africa; results of a multicountry study. http://www.ncbi.nlm.nih.gov/pmc. Posted 1st December, 2009; Accessed; 15th July, 2010.

Hardman JG, Limbird LE, Gilman AG (2001). Goodman and Gilman's Pharmacological Basis of Therapeutics. McGraw Hill, Medical Publishing Division Inc. United States of America. p 1136.

Kumar P, Clark ML (2005). Kumar and Clark Clinical Medicine. Elsevier Saunders, London, pp. 106-116.

Taweesak F (2002). Knowledge, Attitude and Practice Regarding Liver Fluke Infestation in Hill Tribe Community: A study at Ban Tonpeung, Tumbol Romyen, Chiang Kham District, Phayao Province, Thailand. Thesis for Major Health Systems Development Programme, Thailand, pp. 28-31, 42, 50-57, 59-60.

Warren L (2002). Medical Microbiology and Immunology, Examination and Board Review. Lange medical books/McGraw-Hill, Medical Publishing Division. United States of America. pp. 342-380.

Wikipedia, the free encyclopaedia (2010). Deworming. http://www.en.wikipedia.org/wiki posted 1st December, 2009; assessed 15th July, 2010.

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Vol. 7(17), pp. 893-897, 8 May, 2013
DOI 10.5897/AJPP11.756
ISSN 1996-0816 © 2013 Academic Journals
http://www.academicjournals.org/AJPP

African Journal of Pharmacy and Pharmacology

Full Length Research Paper

Effect of seawater immersion on the NF-κB, IκB and TLR4 expression in small intestinal tissues in rats with abdominal open injury

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Accepted 16 April, 2012

The aim of this study was to observe the impact of seawater immersion on the dynamic expressions of NF-κB and IκB mRNA in small intestinal tissues in rats with abdominal open injury. Wistar rats were randomly divided into three groups: the control group (n = 7), the abdominal open injury (AOI) group and abdominal open injury plus seawater immersion (AOI+SI) group. The dynamic expressions of NF-κB and IκB mRNA in small intestinal tissues were detected in each group/subgroup by real-time PCR method. After 72 h, NF-κB mRNA level of AOI+SI group was increased obviously compared to that of AOI group (P<0.01). The IκB mRNA expression of both AOI+SI and AOI groups were increased obviously after 48 h compared to that of the control, but the difference between AOI+SI group and AOI group showed that the increase of AOI+SI group was more significant (P<0.01). In the long time of seawater immersion (>72 h), the NF-κB mRNA expression was significantly up-regulated, which promoted the synthesis of NF-κB protein and further magnified NF-κB signal pathways to lead to the development of continued inflammatory course ultimately; meanwhile, the obviously up-regulated IκB-α mRNA level increased the expression of IκB-α protein to down-regulate the activity of NF-κb as much as possible.

Key words: Abdominal open injury, seawater immersion, real-time PCR, NF-κB, IκB.

INTRODUCTION

Abdominal trauma is one of the most common injuries in modern sea battles with an incidence rate of 3 to 4%. Seawater immersion after abdominal trauma can lead to severe disorders in metabolism and hemodynamic, it can activate and cause the release of inflammatory cytokines in a large quantity to induce inflammatory reactions. And during the process, the barrier function of intestines is destroyed and a large amount of bacteria and endotoxin (ETX) invade into organic circulation systems to induce more serious immune imbalance and induce the initiation and development of MODS (multiple organ dysfunction syndrome), which might also serve as an important reason for the aggravation of injury (Sen and Baltimore, 1986; Ghosh et al., 1998).

Inflammatory reactions constitute an essential part in a secondary injury, and also, they are the major cause for the progressive aggravation of injury or even MODS after trauma. During the process, inflammatory mediators and cytokines play important roles. Among different transcription factors regulating inflammatory genes, nuclear factor kappa B (NF-κB) occupies the most dominant position, which is also the necessary cytokine for transcriptional activation of many genes regulating inflammatory reactions in cells.

Meanwhile, Toll-like receptor 4 (TLR), as the upstream receptor of NF-κB, could activate NF-κB-centered signal transduction pathways, induce inflammatory reactions and promote the activation of antigen presenting cells

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during the inflammatory reaction process (Abbasi et al., 2010; Ghosh et al., 1998; Shishodia et al., 2003). However, the detail research of these inflammatory factors in seawater-immersed abdominal injury has not found. In this study, the expressions of NF-κB, IκB (inhibitor protein of nuclear factor κB) and TLR4 in small intestinal tissues in rats with seawater-immersed abdominal injury were detected by SYBR Green real-time PCR technique, and the role of NF-κB-centered inflammatory systems in seawater-immersed abdominal injury was explored.

MATERIALS AND METHODS

Animal grouping

A total of 63 male Wistar rats were randomly divided into three groups. The control group: rats were normally fed only for index observations (n = 7); abdominal open (AOI) group: 28 rats underwent abdominal open injury for model building, and they were subdivided according to different detection time points (12, 24, 48 and 72 h subgroups, n = 7); and abdominal open plus seawater immersion (AOI+SI) group: 28 rats underwent seawater immersion after abdominal open injury, and they were subdivided (12, 24, 48 and 72 h subgroups, n = 7).

Animal model

In order to wipe out the influences of biorhythm on experimental results, all experiments were started at 8 a.m. After being fed at experimental animal centre for about one-week, rats were experimented. And before model building, they were fasted without food for 24 h and without water for 1h.

AOI group: rats were anesthetized with 3% pentobarbital sodium, and then fixed with supine position on a self-made plate; to create a surgical abdominal open injury, a midline lower abdominal incision of 3 cm was performed by eye scissors, and a self-made iron mesh was used to prop open the incision and then fixed in case of evisceration; and rats were erectly exposed to 22℃ for 1 h.

AOI+SI group: animal models with abdominal open injury were established following the same procedures as those in AOI model building; and then, rats were immersed in seawater at 22℃ for 1 h, and the seawater surface was even with the xyphoid. Intestinal tissues at the distance of 15 cm upward away from ileocecal junction were harvested for sample detections after rats were killed.

Primers and cDNA synthesis

Primer sequences were designed by Primer premier 5 software as follows: actin: 5'-CCC ATC TAT GAG GGT TAC GC-3' (upstream) and 5'-TTT AAT GTC ACG CAC GAT TTC-3' (downstream), and the amplified fragment was 150 bp; TLR: 5'-TGC TCA GAC ATG GCA GTT TC-3' (upstream) and 5'-TCA AGG CTT TTC CAT CCA AC-3' (downstream) , and the amplified fragment was 206 bp; NF-κB: 5'-AAC ACT GCC GAG CTC AAG AT-3' (upstream) and 5'-CAT CGG CTT GAG AAA AGG AG-3' (downstream) , and the amplified fragment was 163bp; and IκB: 5'-CCT CAC CCT TCC CCA ATA AT-3' (upstream) and 5'-GTG TGA ATG GTG CCT GTG AC-3' (downstream) with an amplified fragment of 199 bp.

RNA extraction was carried out according to the instructions of RNA extraction kit (Tiangen Biotech Co., Ltd, China) and the first strand of cDNA was synthesized also according to the instructions of kit (Promega M170A, USA).

Real-time PCR

The real-time PCR reaction system (50 μl) for NF-κB and IκB-α contained 25 μl 2×SYBR mixture (4 mM Mg^{2+}), PCR primers(10μM) each 1μl, 0.3μl Taq enzyme and 2 μl cDNA. The amplification conditions: pre-denaturation of 95℃ for 2 min followed by 45 cycles of 95℃ 20 s, 58℃ 25 s and 72℃ 30 s, and one cycle of 65℃ 10 s, 95℃ and 20℃ (0.5℃/s).

ΔCT = CT value in each group/subgroup-actin CT value in the same group. And $2^{-\Delta CT}$ was used for comparisons of copy numbers of TLR_4 mRNA by RT PCR among different groups/subgroups.

Western blot analysis

Total protein samples from intestinal tissues were extracted and determined by BCA method. The proteins were separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and the protein bands were then transferred to nitrocellulose membranes. Membranes were blocked in 5% skim milk for 2 h and incubated with a primary antibody in a 1:1000 dilution rabbit anti-human VEGF antibody overnight at 4℃. After TBST washing 3 times (10m for each), the membranes were incubated with 1:2500 dilution peroxidase-conjugated goat anti-rabbit IgG for 1 h at room temperature. After TBST washing 3 times (10 min for each), the membranes were stained by ECL and then exposed by autoradiography. The optical density of the film was scanned using a gel-scanner, and β-actin was used as the internal reference. The 10 times ratio of the integral optical density was calculated to represent the relative amount. The experiment was repeated three times, and the average was calculated.

Statistical analysis

Data were presented as $\dot{x} \pm s$ and analyzed by SPSS 16.0 statistical software. Repeated measure ANOVA was carried out for PCR $2^{-\Delta CT}$ value, and SNK-q method was used for pairwise comparison when a difference emerged. One-factor analysis of variance was used for differences among groups/subgroups, and SNK-q for pairwise comparisons between groups/subgroups. $P<0.05$ was considered statistically significant.

RESULTS

Amplification and melting curves of TLR, NF-κB and IκB

Amplification curves of TLR, NF-κB and IκB displayed that there were linear relationship between fluorescence intensities and initial copy numbers, and melting curves displayed that single peaks appeared, indicating RT-PCR products were at high purity and the reactions in our study were specific.

Changes of TLR, NF-κB and IκB mRNA expression

The expression of NF-κB mRNA in AOI+SI group was significantly increased at 72 h compared to that in the control or AOI group at the same time point ($P<0.01$); though the expressions of IκB-$_\alpha$ mRNA in both AOI and AOI+SI groups were obviously increased compared to

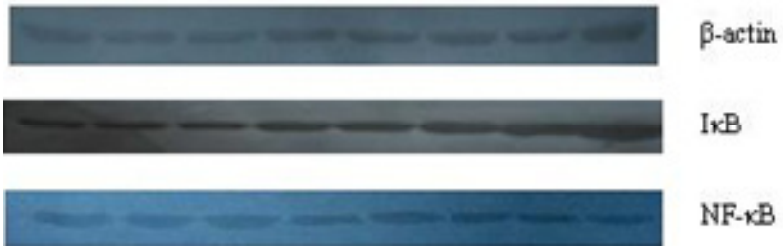

Figure 1. Electrophoresis results of the expressions of IκB and NF-κB in intestinal mucous membrane tissues 3 h later after injury. Lane 1-4: S+W group; Lane 5-7: W group; Lane 8: C group.

Figure 2. Electrophoresis results of the expressions of IκB and NF-κB in intestinal mucous membrane tissues 24 h later after injury. Lane 1 to 4: S+W group; Lane 5 to 7: W group; Lane 8: C group.

Figure 3. Electrophoresis results of the expressions of IκB and NF-κB in intestinal mucous membrane tissues 72 h later after injury. Lane S+W group; Lane 5 to 7: W group; Lane 8: C group.

that in the control at 48 h ($P<0.01$), the expression of IκB-$_{\alpha}$ mRNA in AOI+SI group was more significantly increased at 72 h compared to that in AOI group ($P<0.01$). And the expression of TLR_4 mRNA in AOI+SI group was also significantly increased compared to that in AOI or the blank group at 72 h ($P<0.01$).

Changes of NF-κB and IκB protein expression

Western blotting results showed that the expression of IκB in AOI+SI group was decreased at 3 h compared to that in the blank or AOI group, and such decrease was continued up to 72 h ($P<0.05$) while the difference of the expressions of IκB between AOI and the blank groups had no statistical significance though the expression in AOI group was a little lower than that in the blank ($P>0.05$); in addition, the expressions of NF-κB in different groups told totally different stories in contrast with those of IκB (Figures 1 to 3).

DISCUSSION

Sea battle injury belongs to a type of acute injury, which is far more complicated than injury in a matched field battle (Ghosh et al., 1998). Seawater immersion after abdominal trauma could lead to severe disorders in metabolism and hemodynamics (Shishodia et al., 2003). During this process, due to the devastating damage to the barrier function of intestines, a large amount of bacteria and endotoxin invade into organic circulations to

activate and induce the release of a large quantity of inflammatory cytokines, which in turn, causes inflammatory reactions. And in the process of inflammatory reactions, inflammatory mediator NF-κB promptly participated and played a central and unremitting role, which induced the increase of inflammatory cytokines such as TNF-α, blood vessel endothelial adhesion factors etc, leading to the aggravation of inflammatory reactions and even worse tissue injury (Schmitz et al., 2001; Ramakrishnan et al., 2004; Shenkar et al., 1996). But the mechanism underlying excessive inflammatory reactions and activation of NF-kB synthesis caused by seawater immersion is not known.

In our study, the expressions of TLR_4 mRNA, NF-κB mRNA and IκB-α mRNA in small intestinal tissues of rats immersed in seawater after abdominal injury were successfully detected by SYBR Green real-time PCR (Ethridge et al., 2002; Yan et al., 2006; Kuk et al., 2001). And our results showed that in AOI+SI group, the expression of TLR_4 mRNA was increased at 48 h and significantly increased at 72 h; the expression of NF-κB mRNA was significantly increased at 72 h; and the expression of IκB-α mRNA was more significantly increased compared to that in AOI group, though both groups exhibited significant increase in this respect compared to the blank.

TLR_4 (Toll-like receptror-4) is widely expressed in all cells, especially in monocytes. TLR_4 plays an important role in lipopolysaccharide (LPS) signal transduction pathways, which is the main component of bacteria in the natural immune system, like gram-negative bacteria. LPS, released by gram-negative bacteria, integrated with LPS binding proteins (LBP) in blood flow to form complexes, and these complexes interacted with mCD14s (LBP receptor) on the surfaces of monocytes and macrophagocytes to activate TLR_4 signal transduction pathways, which in turn, activated the increase of NF-κB activity and regulated related genes, leading to a series of pathological changes (Chen et al., 2008; Clemens, 2000; Medzhitov, 2001; Lin et al., 2004; Genovese et al., 2008).

Our study demonstrated that seawater immersion after injury, led to a damage of intestinal barrier function and translocation of bacteria and endotoxin through multiple pathways, causing bacterial translocation (BT) and gut original endotoxaemia. And due to this, LPS was released and the released LPS further activated TLR_4 signal transduction pathways (Rota et al., 2002; Bini et al., 2008; Wheeler and Bernard, 1999).

TLR_4 transducted signals and further activated a series of downstream responses via TIR domains: the structural domain of TIR interacted with that of MyD88 carboxyl terminus to activate MyD88, and through the interaction with the death domain (DD) of carboxyl terminus in serine/threonine protein kinase 4 ($IRAK_4$), the activated MyD88 recruited $IRAK_4$ into TLR signaling complex to cause the autophosphorylation of IRAK; the activated IRAK interacted with tumor necrosis factor receptor associated factor-6 (TRAF6), and the activated TRAF6 integrated with TGF-βassociated kinase (TAK1) and TAK binding protein (TAB) to form complex and to activate NF-κB induced kinase (NIK); the activated NIK further phosphorylate IκB multi-enzyme complex, and the activated IκB multi-enzyme complex exerted its effect on the inhibitor of NF-κB to phosphorylate two serine sites of IκB and lead to the degradation of IκB, by which transcription factor NF-κB was set free from IκB/ NF-κB complex. It immigrated into the nucleus and consequently, the immigrant led to the transcription of NF-κB mRNA, and induces the gradual increase of NF-κB synthesis, causing systemic inflammatory reactions, organ functional defects and multiple organ dysfunction syndromes (MODS) via the release of inflammatory factors (West et al., 2006; Abraham, 1999).

NF-κB is a eukaryotic nuclear transcription factor. After immigrating into the nucleus, it exerts its function of being a transcription factor by priming genetic transcription. Our results also indicated that the lasting existence of severe injury caused by seawater immersion after abdominal injury plus positive feedback stimulating factors synthetized and released by the promotion of NF-κB (such as inflammatory mediators TNF-α, IL-2β, etc.) lead to the increase of the expression of NF-κB mRNA, which further promotes the increase of NF-κB protein synthesis and magnify NF-κB-centered inflammatory signal transduction pathways. Meanwhile, 48 h later after seawater immersion, the intracellular negative feedback regulatory channel of NF-κB inflammatory signal transduction pathway had been initiated, and the manifest increase of $I\kappa B\text{-}_\alpha$ mRNA promoted the increase of $I\kappa B\text{-}_\alpha$ protein to make the dynamic balance of NF-κB between the nucleus and the cytoplasm tilt towards the side of cytoplasm, and in so doing, to down-regulate the activity of NF-κB in the nucleus thereby, terminating the transcription and generation of inflammatory mediators (Pegu et al., 2008; Hotchkiss et al., 2003).

In acute injuries, NF-κB participates in the activation of macrophages and leucocytes, and manipulates the genetic expressions of many pro-inflammatory factors. Thus, the loss of manipulation will cause the magnification of inflammatory reactions and even tissue injuries. From the perspective of molecular biology, the causes for the activation of NF-κB in abdominal injury with seawater immersion also include the translocation of microbial populations, apart from factors such as trauma, stress, etc. With seawater immersion, after LPS activates TLR signal transduction pathways, TLR may participate in the process of the activation of NF-κB inflammatory reactions, which may also promptly and everlastingly participate in the process of subsequent inflammatory reactions after abdominal injury with seawater immersion. The activation of NF-κB in injury with seawater immersion was more prompt and everlasting, and the excessive expression of NF-κB in injury with seawater immersion

could lead to the excessive release of cytokines such as TNFα, etc, leading to an water fall effect (Suk et al., 2001), which may further lead to systemic inflammatory reactions, organ functional defects and multiple organ dysfunction syndrome (MODS) (Hoffmann et al., 1999; Stone, 1994).

In this study, the expressions of NF-κB and IκB in intestinal mucous membrane tissues were detected by western blotting. Our results showed that the expression of NF-κB was increased and that of IκB was decreased at 3 h in AOI+SI group, and such effects lasted until 72 h while the expressions of NF-κB and IκB in AOI group didn't display the same changes, indicating NF-κB promptly and lastingly participates in the process of subsequent inflammatory reactions in abdominal injury with seawater immersion. The continuous down-regulation of IκB indicated that the mechanism of NF-κB negative feedback was not built for a rather long period, and the severity of injury with seawater immersion was more serious than that of injury alone and factors causing injury continuously existed, during which NF-κB might play a leading role (Akira and Hemmi, 2003).

In the abdominal seawater-immersion injury process, inflammatory reactions constitute an important component of a secondary injury, which are also the major cause for the aggravation of injury and MODS. During the whole process, inflammatory mediators and inflammatory cytokines play important roles in the pathological reactions after injury. The severity of inflammatory reaction is correlated with the expressions of genes coding inflammatory mediators, and the expressions of these genes are regulated by transcription factors. Studies have shown that seawater immersion after injury can activate inflammatory cells and release a variety of cells as well as humoral factors, leading to an excessive stress and inflammatory reactions. And our results indicate that during the above-mentioned process, NF-κB instantly and lastingly takes part, whose mechanism might be associated with its participation in the activation of macrophages and leucocytes and its control on the genetic expressions of pro-inflammatory factors, which may ultimately cause the magnification of inflammatory reactions or even tissue injuries.

REFERENCES

Abbasi FM, Ahmad H, Perveen F, Inamullah M, Brar DS (2010). Assesment of genomic relationship between *Oryza sativa* and *Oryza australiensis*. Afr. J. Biotechnol. 9(12):1312-1316.

Abraham E (1999). Why immunomodulatory therapies have not worked in sepsis. Intensive Care Med. 25:556-566.

Akira S, Hemmi H (2003). Recognition of pathogen-associated molecular patterns by TLR family. Immunol. Lett. 85:85-95.

Bini R, Olivero G, Trombetta A, Castagna E, Cotogni P (2008). Effects of dimethyl sulfoxide,pyrrolidine dithiocarbamate,and methylprednisolone on nuclear factor-kappaB and heat shock protein 70 in a rat model of hemorrhagic shock. Trauma 64:1048-1054.

Chen G, Shi J, Qi M, Yin H, Hang C (2008). Glutamine decreases intestinal nuclear factor kappaB activity and pro-inflammatory cytokine expression after traumatic brain injury in rats. Inflamm. Res. 57:57-64.

Clemens JA (2000).Cerebral ischemia:gene activation, neuronal injury, and the protective role of antioxidants. Free Radic. Biol. Med. 28:1526-1531.

Ethridge RT, Hashimoto K,Chung DH, Ehlers RA, Rajaraman S, Evers BM (2002).Selective inhibition of NF-κB attenuates the severity of cerulean-induced acute pancreatitis. J. Am. Coll. Surg. 195:495-505.

Genovese T, Esposito E, Mazzon E, Muià C, Paola RD, Meli R (2008). Evidence for the role of mitogen-activated protein kinase signaling pathways in the developments of sinal cord injury. Pharmacol. Exp. Ther. 325:100-114.

Ghosh S, May MJ, Kopp EB (1998). NF-kappa B and rel proteins: Evalutionarily conserved mediators of immune responses. Annu. Rev. Immunol. 16:225-260.

Hoffmann JA, Kafatos FC, Janeway CA, Ezekowitz RA (1999). Phylogenetic perspectives in innate immunity. Science 284:1313-1318.

Hotchkiss RS, Karl IE (2003). The pathophysiology and treatment of sepsis. N. Engl. J. Med. 48:138-150.

Suk K, Kim SY, Kim H (2001). Regulation of IL-18 production by IFNγ and PGE2 in mouse microglial cells: involvement of NF-kB pathway in the regulatory processes. Immunol. Lett. 77:79-85.

Lin L, Spoor MS, Gerth AJ, Brody SL, Peng SL (2004). Modulation of Th1 activation and inflammation by the NF- kappaB repressor Foxj 1. Science 303:1017-1020.

Medzhitov R (2001). Toll-like receptors and innate immunity. Nat. Immunol. 1:135-145.

Pegu A, Qin S, Fallert BA, Nisato RE, Pepper MS, Reinhart TA (2008). Human lymphatic endothelial cells express multiple functional TLRs1. J. Immunol. 180:3399-3405.

Ramakrishnan P, Wang W, Wallach D (2004). Receptor specific signaling for both the altemative and the canonical NF-kappaB activation pathways by NF-kappaB induced kinase. Immunology 21:477-489.

Rota C, Bergamini S, Daneri F, Tomasi A, Virgili F, Iannone A (2002). N-Acetylcysteine negatively moulates nitric oxide production in endotoxin-treated rats through inhibition of NF-kappaB activation.Antioxid. Redox. Signal 4:221-226.

Schmitz Ml, Bacher S, Kracht M (2001). I-κB independent control of NF-κB activity by modulatory phosphorylations.Trends Biochem. Sci. 26:186-190.

Sen R, Baltimore D (1986). Inducibility of kappa immunoglobulin enhancer binding protein NF-κB by a posttranslational mechanism. Cell 47:921-928.

Shenkar R, Schwartz MD, Terada LS, Repine JE, McCord J, Abraham E (1996). Hemorrhage activates NF-kappaB in murine lung mononuclear cells *in vivo*. Am J. Physiol. 270:L729-L735.

Shishodia S, Majumdar S, Banerjee S, Aggarwal BB (2003). Ursolic acid inhibits nuclear factorκB activation induced by carcinogenic agents through suppression of IκB alpha kinase and p65 phosphorylation: correlation with down 2 regulation of cyclooxygenase, matrix metalloproteinase 9, and cyclinD1. Cancer Res. 63:4375-4383.

Stone R (1994). Search for sepsis drugs goes on despite past failures. Science 264:365-367.

West AP, Koblansky AA, Hosh S (2006). Recognition and signaling by Toll-like receptors. Ann. Rev. Cell Dev. Biol. 22:409-437.

Wheeler AP. Bernard GR (1999). Treating patients with severe sepsis. N. Engl. J. Med. 340:207-214.

Yan Yh, Jing XL, Chen YM, Chen Y (2006). Role and significance of nuclear factor-κB activity in systemic inflammatory response syndrome. Chin. J. Postgrad. Med. 29:23-25.

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Vol. 7(17), pp. 898-1008, 8 May, 2013
DOI 10.5897/AJPP11.789
ISSN 1996-0816 © 2013 Academic Journals
http://www.academicjournals.org/AJPP

African Journal of Pharmacy and Pharmacology

Full Length Research Paper

Towards a new economic model on biopharmaceutical companies

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Accepted 30 December, 2011

Intangible assets are one of the most important issues in biopharmaceutical companies today, due to the idiosyncrasies of this business. In such an innovative sector, companies have been investing heavily in research, development and innovation and the book value often does not accurately reflect the real value of the companies. It was necessary, therefore, a new accounting model that meets the needs of this sector. This work main contribution is the design of an intellectual capital report, particularly suited to the biopharmaceutical companies, a model that has been called 3C 2P 2R. The two main conclusions of the study have to do with the growing importance of intangibles as a new way to create value and new way of organizing economic activities in biopharmaceutical companies, and the need for new forms and quality of information provided, since no proper assessment of this asset has high costs. The proposal made here may become a fundamental tool to improve the valuation ofbiopharmaceutical companies.

Key words: Drug industry, pharmaceutical economics, economic value, economic models, healthcare market.

INTRODUCTION

The birth of the biopharmaceutical industry comes along with the discovery of recombinant DNA and monoclonal antibody technologies, 40 years ago (Walsh, 2007). Biopharmaceuticals are complex macromolecules created recombining DNA, using cell fusion or genetic manipulation. One of the main differences with traditional pharmaceutical industry is that biopharmaceuticals are usually administered by subcutaneous, intravenous or intramuscular injection, instead of orally.

From the appearance of human insulin, the first drug produced via genetic engineering; in 1982, more than 100 biopharmaceuticals have been marketed (Roche, 2006). Sales have grown enormously and the sector has little to do with that of the 1970s. During these years, the world has seen the discovery of the first recombinant vaccine (against hepatitis B) in 1986, the first therapeutic monoclonal antibody (against kidney transplant rejection), also in 1986, and the first and only oligonucleotide in 1998 (against cytomegalovirus retinitis in AIDS patients), among others (Alexander et al., 2011; Rydzewsky, 2008). Nowadays, cost of medicines is growing constantly as new medicines are marketed (Kulkarni et al., 2010).

One of the most important issues for the industry today is to reach a fair valuation of the intangible assets. In such an innovative sector, in which companies invest heavily in research, development and innovation, the book value often does not reflect the real value. Since the main purpose of accounting should be to help in making efficient and more successful decisions, this objective can be hindered in the case of technology-based innovating companies (EIBTs), as the accounting information provided by traditional financial statements does not constitute itself a useful tool to achieve that goal.

Only a few companies in the world produce

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biopharmaceuticals. This makes it impossible to assess industry performance using traditional measures, such as output, market share, export growth or productivity gains. In this situation, the use of measures, such as R&D and revenue gets more importance for these companies.

It seems clear that the main drivers of value creation in these companies are intangible (Holland, 2004). They are not directly observable, so their identification and measurement, which are crucial, is really difficult. Although, these assets may generate competitive advantages, there is little information about them. The result of all this is that in technology-based companies (EIBTs) in general and in biopharmaceutical companies in particular, financial statements do not reflect properly the financial situation. As a result, "*the informative capacity of financial statements on the current and future financial situation of companies is clearly decreasing*" (Cañibano et al., 1999). Both the European Union and the primary regulators of corporate accounting, the International Accounting Standards Board (IASB) and Financial Accounting Standards Board (FASB), have been active in recent years in projects regarding the disclosure on intangibles. In this sense, the European Union issued the "Guidelines for the Management and Dissemination of Information on Intangibles" (Project published by the Airtel-Vodafone Foundation in 2002).

The empirical studies conducted in the past two decades have revealed the progressive deterioration of the relationship between the market price of a company and other financial variables, such as earnings, book value or cash flows. One consequence is that the role of accounting information in investment decisions is declining. This concern about the usefulness of the accounting model has led various United State agencies like the Securities and Exchange Commission (SEC) and FASB to set up working groups to identify the inadequacy of the financial statements, suggesting ways to improve them. It is necessary, therefore, for a new model that meets the needs of companies operating in sectors like high technology, life sciences or the internet. They are committed to innovation, a process that generally can be divided into three stages, discovery/learning, implementation and marketing. The traditional accounting models do not provide relevant information about these processes of innovation, which are crucial for the survival and success of these companies (Gutiérrez de Mesa, 2004).

The objective of this work is to design a new model for biopharmaceutical companies, which improves the valuation possibilities for this industry. The aim of the model includes:

1. To increase financial support for the industry
2. To strengthen long term investment in biopharmaceuticals
3. To encourage forecasting initiatives in companies in the sector
4. To optimize financial situation of the firms
5. To reduce risks and enhance the viability of the industry.

METHODOLOGY

In order to develop the model, the first step will be to discuss how the R&D process works in biopharmaceutical companies. This will help to create the base from which the second step will be started. In a second phase, a deep analysis of the currently existing models of accounting information in the biotech sector will be done. This will include a review of major national and international contributions in terms of intellectual capital models in the sector. Once it has been done, we will make our own model from the most useful parts of those analyzed, creating the biopharmaceutical companies model.

RESULTS

The R+D+I process in biopharmaceutical industry

After analyzing all the material and sources available, it was found that the R+D+I process, in the case of biopharmaceutical companies, was substantially different to innovation processes in other areas. The role of innovation activities is to reach the economic realization of a process or product (Gutiérrez de Mesa, 2004). Some examples in the case of the biopharmaceutical industry are the new drugs placed into the market, meaning not only absolutely novel drugs (absolute innovation), but also better medicines, for example, with less contraindications, fewer side effects, reductions in the dailies, etc., (relative innovation).

However, the discovery of a new substance is the first part of this process of scientific research and technological development. Then, a series of tests and trials have to be carried out, in order to ensure the effectiveness and safety of the drug to be marketed. In this sense, we found a set of stages that the new drug passes from the initial discovery (Lobato et al., 1997):

1. Preclinical phase: where the substance is subjected to a complex battery of *in vitro* tests and animal testing in order to identify possible toxic effects and establish its pharmacological characteristics.
2. Clinical phase: after passing the aforementioned tests, promising products are brought to a second process, generally called clinical trials.

a. Phase I: trials in healthy people (volunteers). If the drug is tolerated and produces the desired effect, it enters the second phase.
b. Phase II: the product is supplied to a number of patients suffering from the disease, which is expected to deal with the drug subject of the experiment (between 3.000 and 4.000 patients). If the product is still promising, it would go to the next stage.
c. Phase III: the product is supplied to a large number of patients. Large-scale tests are used to determine the ideal dosage and refine the levels of safety and efficacy estimates.

In view of this, it can be concluded that innovation in drugs is a process characterized by uncertainty, resource

consumption and time consumption. The complexity of this process is clear in the early stages of obtaining a chemical compound that will form the basis for the subsequent drug.

The subsequent testing phases leading to the production of a new drug from discovery of a new active substance (NPA) are extended over several years. It is common, a length of 12/13 years from the moment the new active substance is obtained until the new drug appears into the market. Obviously, for the company, the economically significant moment is the marketing of the product.

This research process requires a lot of resources, both financial and human, which have increased considerably in the past twenty years. In 1999, for example, the cost of research and development of a new chemical or biological entity was estimated at 560 million euros (around U.S. $ 460 million, considering an exchange rate of 1.20 € per U.S. $) and in 2001 this cost was estimated around 900 million euros, about U.S. $ 750 million (EFPIA, 2003).

Non-financial proposals for the identification and measurement of intangible assets

There have been several previous proposals to complete the information provided in traditional financial statements on intangible assets, but none of them has been shown to be suitable for this type of business. Among the existing international proposals, the most famous are those of Kaplan and Norton (1992) with their balanced scorecard; Edvinsson (1997) with his Skandia navigator, Lev (2000, 2001) with the value chain score board and Sveiby (1997) with the intangible monitor assets. Among the Spanish proposals, the view held by the group of experts who drafted the White Paper on Reform of Accounting in Spain (ICAC, 2002), the Intelec Model (Euroforum, 1998) and the Intellectus Documents Center for the Knowledge Society of Madrid (UAM).

In 1992, Kaplan and Norton questioned whether the measures provided by accounting, based in short-term financial indicators, enabled managers to evaluate new strategies and innovation processes. Deficiencies in traditional accounting have been widely discussed in recent years of the twentieth century and early twenty-first century, as the need for more information about intangible assets has become a highly important and decisive factor for the future of a company. Financial statements, in their traditional conception, do not take intangibles into account. Kaplan and Norton believe that new valuation methods that reflect the new organizational goals and processes are needed. The “balanced scorecard” is a new architecture valuation based in the company's strategy (Roos et al., 2001). It is an assessment accounting system rather than as a specific instrument, as it is based on the company's organizational strategy. It requires a company that has its own rational system of creation and intellectual capital flow. To be effective as a decision tool, it is necessary that the company meets its own objectives, the context in which it operates and is dynamic enough to reflect the temporal dimension (Bontis, 2000).

Sveiby (1997) stated that the book value must equal the value of tangible assets minus debt. He considered that the prevailing traditional accounting system for over 500 years had to make way for a new system which takes into account non-financial flows of knowledge. He proposed a new conceptual framework based on three types of intangible assets:

1. Intangible assets arising from the external structure, such as branding, customer or supplier relationships;
2. Intangible assets arising from the internal structure, this is the organization, management systems, legal structure, operating systems, attitudes, R&D and software;
3. Intangible assets derived from individual skills such as training and experience.

He recommended, first of all, to replace the frame of traditional accounting by a new framework which takes into account knowledge. Within this new framework, the pooling of financial and nonfinancial indicators provides a more complete economic and financial situation and value creation for shareholders.

According to Sveiby, the purpose of identifying and measuring these three groups of intangible assets is to implement better management control. To achieve this aim, the first step is to determine who will be interested in the results. In an external display, the company needs to describe quality of management as accurately as possible to shareholders, customers, creditors and other social. Outsiders are generally interested in knowing the position of the company, as external accounting occurs only after long intervals of time. He therefore recommends including information about intangible assets that the company owns, including certain key indicators as well as necessary explanation about them, in the information given to external agents.

On the other hand, it is also necessary that an internal valuation of such intangible assets for the company managers, as they need to assess progress and take corrective measures. Ultimately, it results in creating an information system for management. That system focuses on the flows, trends and changes of the different variables under control. Sveiby believes that the measurement of intangible assets should include at least three cycles of measurement to assess the results and repeat them annually. He identifies three indicators for each of the three types of intangibles: growth and renewal, efficiency and stability, recommending the directors, that is, the selection of one or two variables for each indicator.

In essence, the “Intangible Assets Monitor” showed in a simple way a series of indicators relevant to each category (Sveiby, 1997). Obviously, the choice of these depends on the strategy of the company. Edvinsson, meanwhile, proposed the famous Skandia Navigator (1997). Skandia is considered the first company that has made real efforts to measure their intellectual capital. This company developed in 1985 an “Intellectual Capital Report” and became the first one to complement their traditional financial reports submitted to its shareholders in 1994 with a report on their intellectual capital. Other companies like Dow Chemical have developed several initiatives to valuate their R&D and patent development process based on the multidimensional concept of value creation for Skandia (Bontis, 2000).

Leif Edvinsson was the “architect” and Skandia executive who developed the report model called Navigator (Skandia Navigator), which focused on five areas, such as, financial, customers, processes, renewal and development and human capital. This new taxonomy of accounting tried to identify the roots of value creation in a company measuring and valuating a set of dynamic and hidden factors that underlie the “visible” part of the company (Edvinsson and Malone, 1997). According to Skandia's model, the basis of intellectual capital is the union of two hidden factors, human capital and structural capital (Bontis, 2000):

1. Human capital is defined as the combination of knowledge, skills and innovative capabilities of the employees of a company, including the values, culture and philosophy prevailing in the organization;
2. Structural capital refers to the hardware, software, databases, organizational structure, patents, trademarks and all other organizational capabilities that underpin the productivity of human resources, including the so called “client capital”, this is the relationships developed with key customers of the organization. Unlike human capital, structural capital does belong to the company and may be subjected to “transaction”;
3. Intellectual capital is defined as the sum of human capital and structural capital. According to Edvinsson and Malone (1997, 1999), intellectual capital includes the applied experience, organizational technology, customer relationships and professional skills that enable the company to achieve a competitive advantage in the market.

The Skandia “CI Report” for the measurement of the five core areas of the Navigator model, used up to 91 new indicators of intellectual capital in addition to traditional 73. Of all these new indicators, Edvinsson and Malone suggested 112 to create what they call a “Universal Intellectual Capital Report”.

Lev (2000) proposed a new accounting paradigm based on three axes (financial assets, nonfinancial assets and accounting improvement), integrated under control loops into a coherent information structure. This author considers that the traditional financial reporting system does not articulate links between capabilities and results, and these are the determinants of success in the new economy. The information system proposed focuses on four innovative capabilities:

a. Ability to innovate/marketing
b. Human resources
c. Customers
d. Networks

His new accounting proposal is a really ambitious system that means expanding the traditional accounting to nonfinancial and nontransactions derivative domains, providing necessary information for decision makers and investors in today's changing global economic environment. The proposed system incorporates better information on certain elements (intangible investments) and a system of “balanced scorecard” focusing on non-financial indicators. It also adds coherence and structure to the information provided.

As regards the existing Spanish main proposals, that take into account the contributions previously analyzed (Edvinsson, 1997; Kaplan and Norton, 1992; Lev, 2000; Sveiby, 1997), it is necessary to mention the view held by the group of experts who drafted the white book on reform of accounting in Spain (ICAC, 2002), the Intelec model (Euroforum, 1998) and the Intellectus Documents of the Center for Knowledge Society of Madrid (UAM).

Intelec model responded to the need to collect in an easily understandable system all the intangible elements that create value in organizations, providing managers with relevant information to decision-making. The model aimed to bring the explicit value of the company to its market value, and report on the organization's ability to generate sustainable results, continuous improvement and long-term growth. The model was structured, following major international contributions, establishing a tripartite classification of intellectual capital.

The Intelec model incorporated present and future dimensions when structuring and measuring intangibles based on its potential and the efforts being made in its development. In addition, it also incorporated the internal and external dimensions, identifying the intangible elements that create value from the consideration of the organization as an open system. It is also important to highlight the dynamic nature of the model, which not only provided the intellectual capital at a particular point in time but also close to the flow of conversion between the different blocks of intellectual capital. Finally, the model did not only consider the explicit (transmittable) knowledge, but also contemplated the more personal, subjective and difficult to share knowledge (Gutiérrez de Mesa, 2004).

Subsequently, the Centre for Research on the Knowledge Society of Madrid (CIC) developed the Intellectus model (2002) based on the Euroforum group

model in 1998. This model also builds on the model of Kaplan and Norton, Edvinsson, Sveiby and Brooking. The objectives were to identify and assess the elements that define the human capital and select the most appropriate indicators for each of them. The model made a restatement of the elements of human capital, establishing a total of five items to be included in the measurement of human capital of an organization. These elements were motivation, satisfaction and commitment of staff, skills, learning ability and capacity to integrate new people. The

"*elements identified gather the essentials of what is meant by valuable human capital or talent [...] one that is motivated and prepared, with adequate diversity of people and can be managed over time, through the incorporation of new people and development of their capacities*" (CIC, 2002).

In addition to the Intelec and Intellectus models, outlined earlier, the "report on the state of accounting in Spain and basic guidelines for its reform" (ICAC, 2002) also refers, in Chapter 7, the financial information that is still relevant. That is why it made a series of recommendations at the time of incorporating this type of voluntary information in the report. The practices followed by companies in Spain are far from providing relevant management reports to users of the information, and the disparity manifests itself in the preparation and submission of the report. Therefore and in order to standardize the information, the white book proposed the information to be included, although its position is that such standardization was considered as a recommendation and not a coercive measure.

There are three factors that should guide the choice of indicators in a company:

1. The strategy
2. The characteristics of the company itself
3. The characteristics of the industry in which it operates.

Proposed report of intellectual capital for biopharmaceutical companies: Model 3C 2P 2R

After the review of the most prominent national and international literature on intellectual capital, and after analyzing the main intangible asset of these businesses, our model proposal supplements the traditional annual financial statements with an intellectual capital report, especially appropriate to this biopharmaceutical sector. The aim is that analysts, investors and managers of these companies have additional evidence to assess more successful at these businesses. It also aims to create a common tool for all which allows, over time, inter-company comparability.

The proposed model is structured graphically as shown in Figure 2. To define the different kinds of indicators, targets must be defined. While in the traditional balance sheet, objectives are implicitly defined. Developing an intellectual capital report (ICR) requires the explicit formulation of organizational objectives. The discussion of goals and strategies forces the organization to focus on the essential process of value creation, which can then be measured, documented and communicated. The ICR is structured on the basis of three pillars:

1. The potential added value of the company.
2. The company's key processes.
3. The results obtained by the company.

Regarding the study of the potential of this added value, it will be conditioned by human, structural and relational capital of the company (3C). The key processes of these companies rely on R&D, distinguishing research projects of development projects (2P). Finally, the results of the company shall be measured from two different perspectives, on one hand the financial and on the other hand the nonfinancial results (2R).

After defining the organization's strategic objectives, once the pillars of ICR have been set, and taking into account the existing data at company level, indicators are formulated for each of the proposed categories. One of the biggest risks in the development of an intellectual capital report is the definition of too many goals or too many indicators; that is why the actual strategic thinking of the company is clarifying priorities (Gutiérrez de Mesa, 2004). When selecting indicators, the priority should be to define them in the most clear and transparent as possible way.

This intellectual capital report, especially suitable for biopharmaceutical companies, is built on the tripartite classification of intellectual capital (3C), generally accepted, by which it is divided into human capital, structural capital and relational capital. Adding to it the R+D+I (2P) of the company and the results (2R), not only financial statements are also scientific, proposing a set of indicators within each of the categories of intellectual capital.

In each years´ ICR shall appear as the indicators of the year for which it is being done, as well as at least indicators of last year, in order to facilitate annual intracompany comparison and to have a tool that allows seeing the company's intellectual capital evolution. The model 3C 2P 2R of ICR is reflected in Figure 3.

DISCUSSION

The balanced scorecard of Kaplan and Norton, through the analysis of the four main perspectives of the business (financial perspective, customer perspective, internal perspective and growth perspective) is a useful tool to demonstrate to the investors the true value of the company.

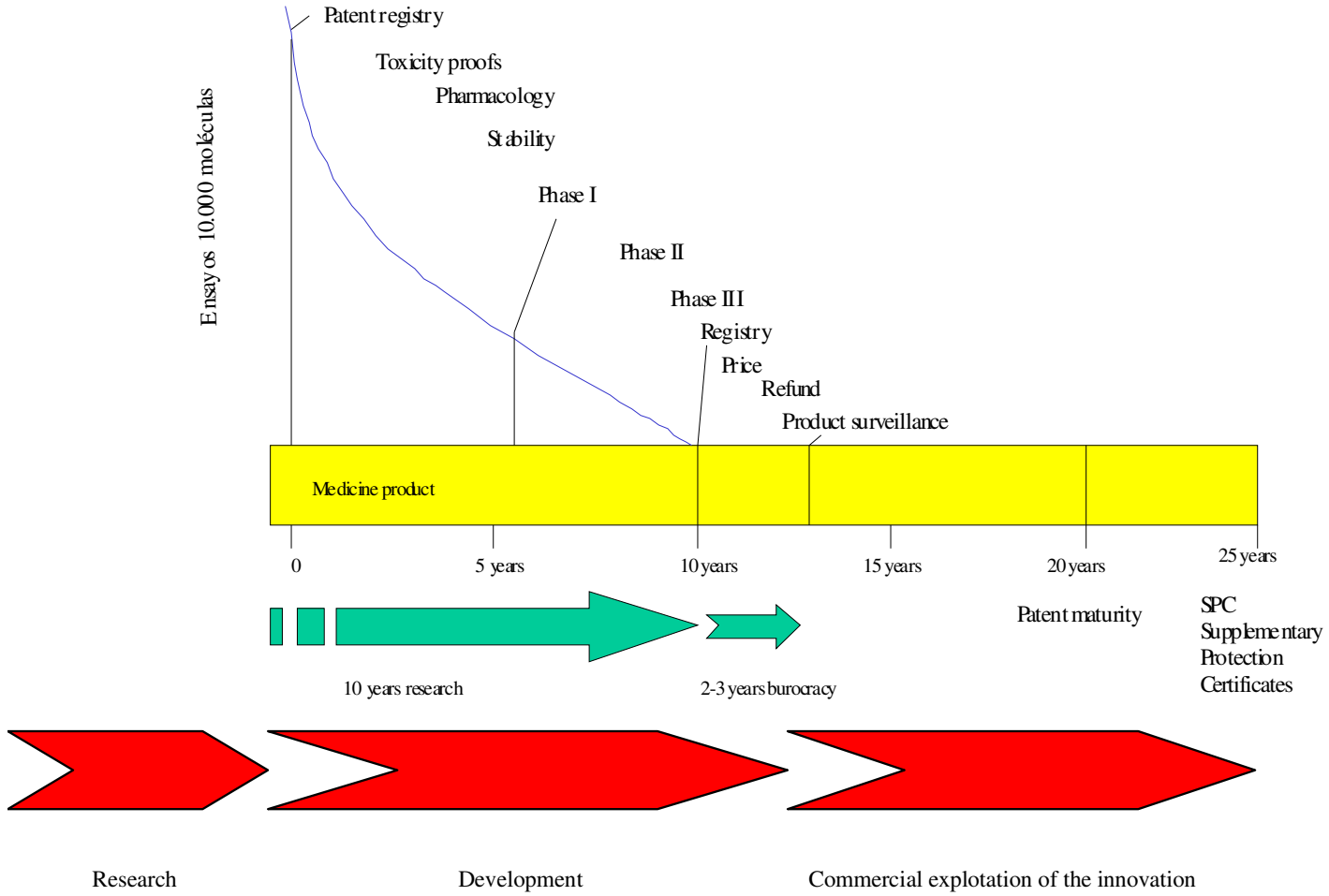

Figure 1. R&D process phases.
Source: AGIM, Recherche et Vie in EFPIA (2003) and authors.

However, some authors, such as Roos et al. (2001) consider that the scorecard presented by Kaplan and Norton reveals too much information about the company's strategy.

The scorecard has another clear disadvantage: its relative stiffness in the identification of key success factors in each of the perspectives. Almost certainly, one of the key factors has an impact on several dimensions of the intangible assets considered by Kaplan and Norton.

The Skandia value scenario included both financial and nonfinancial aspects, allow the estimation of the market value of the company. Edvinsson and Malone (1997; 1999) consider that the intellectual capital represents a new way of seeing the value of the organization, and that the identification and valuation of these intangible assets is possible thanks to accounting.

Many authors acknowledge the considerable efforts made by Skandia for the identification and measurement of intangible assets that served as a basis to encourage academics, researchers, analysts and managers to continue studying the process of value creation in companies. Skandia's model has been particularly important because it acknowledges the crucial role customer relations play in creating value for the organization.

But among the weaknesses of the model, Lynn (1998) stated that Skandia navigator provides only an approximation of what the intellectual capital may be. As it assigns no monetary value, its usefulness is limited. Other authors, such as Johan et al. (2001) argue that because the base model is basically the company's balance sheet, the measure it makes of the intangible is only a static X-ray in time, as it does not capture the dynamic flows of the organization.

The variety of methods to measure intellectual capital means the practical impossibility of carrying out comparisons in the values of the intellectual capital of firms operating in different industries. Even firms that operate in the same industry may have substantially different results when using different methods (Gutiérrez de Mesa,

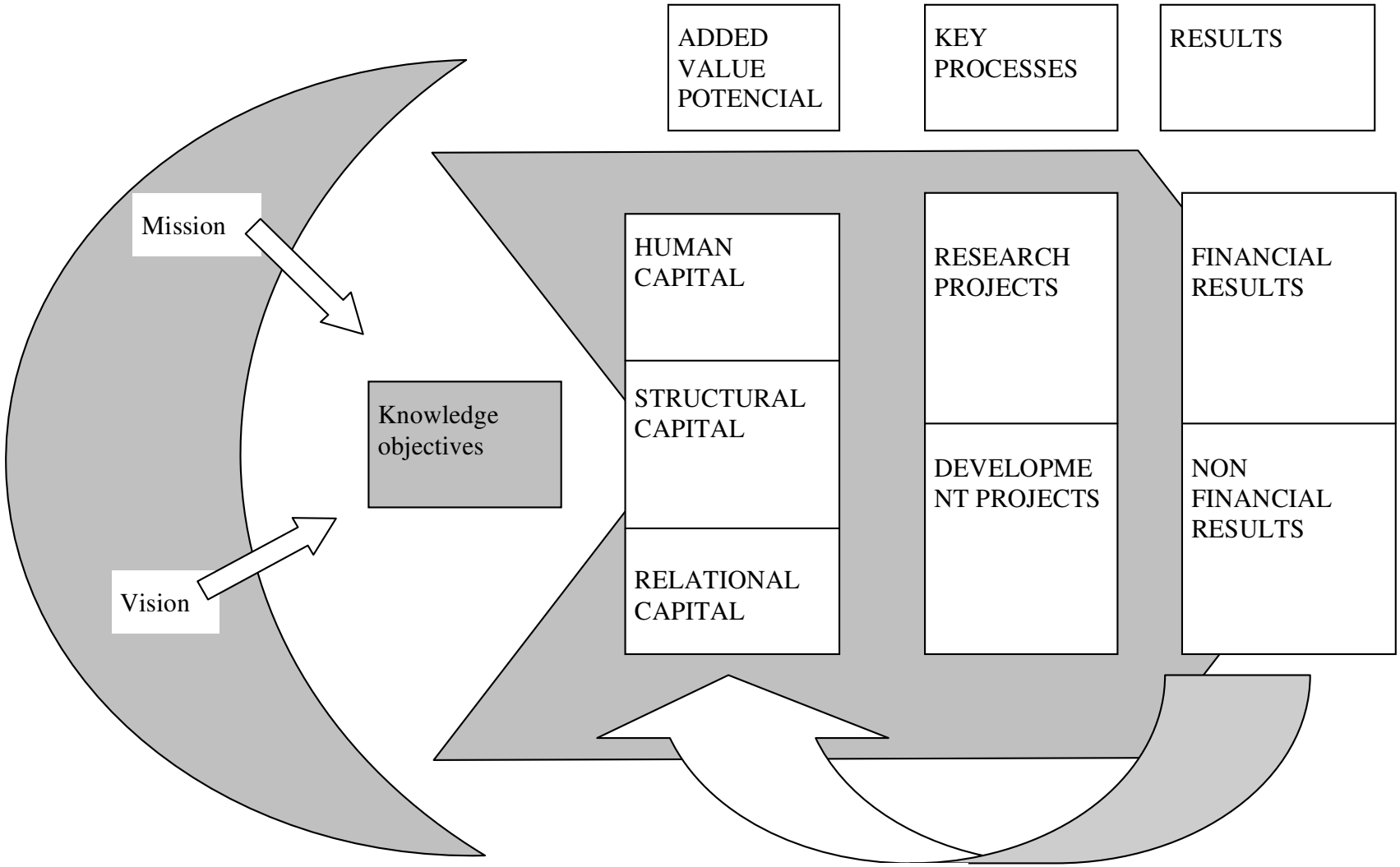

Figure 2. Proposed model 2P 3C 2R.
Source: Gutierrez de Mesa Vazquez, E. (2004). Adapted from Koch, Leitner and Bornemann (2000).

1. HUMAN CAPITAL:

1.1 HUMAN RESOURCES

- ▶Total new hires
- ▶Total new research staff on new additions
- ▶Staff Turnover rate
- ▶Total Staff Departures
- ▶Total Retirements and early retirements
- ▶Average Age Staff
- ▶Average Age of researchers
- ▶Percentage of research staff on total Staff
- ▶Percentage of staff with university training on total staff
- ▶Percentage of PhD researchers on total researchers

1.2 TRAINING

- ▶Total training days per employee
- ▶Total technology training days per employee
- ▶Total R+D training days per researcher
- ▶Total training expenses on wage cost per employee
- ▶Total training expenses on total administrative expenses

2. STRUCTURAL CAPITAL:

2.1 ICT

- ▶Computer rate per employee
- ▶ICT expenses per employee

2.2 KNOWLEDGE BASED INFRASTRUCTURES

- ▶Total data bases the company uses

3. RELATIONAL CAPITAL:

3.1 PROJECTS IN COLLABORATION

- ▶Total new national projects in collaboration with other institutions over new projects
- ▶Total new European projects over new projects
- ▶International research activities
- ▶Total international researchers on the total company's researchers
- ▶Total formalizad strategic alliances
- ▶Total exploitation strategic alliances on total strategic alliances formalized
- ▶ Total exploration strategic alliances on total strategic alliances formalized

3.2 KNOWLEDGE DIFUSSION

- ▶Total conferences and seminars attended by research staff per researcher
- ▶Total lectures given by each researcher
- ▶Total papers published in refereed scientific journals per researcher

3.3 CLIENTS, IMAGE AND STAKEHOLDERS

- ▶Total expenditure on corporate advertising on total expenditure on advertising and promotion
- ▶Total new clients
- ▶Total news stakeholders of the company

4. R+D+I+T PROJECTS:

4.1 RESEARCH PROJECTS

- ▶Total new research projects initiated
- ▶Total new research projects externally financed on new projects total
- ▶Total new international projects on new projects

4.2 DEVELOPMENT PROJECTS

- ▶Total new active substances discovered
- ▶Total projects in preclinical research phase on total development projects
- ▶Total products in clinical research phase I on total development products
- ▶Total products in clinical research phase II on total development products
- ▶Total products in clinical research phase III on total development products
- ▶Total products pending approval by the competent authorities on total development products
- ▶Total outstanding products on total development products
- ▶Total news products on total development products

4.3 INNOVATION EXPENSES (I.E.)

- ▶Total Internal expenses in R+D+I+T over Total I.E.
- ▶Total External expenses in R+D+I+T over Total I.E.
- ▶Total machinery and equipment acquisition on total I.E.
- ▶Total intangible technologies acquisition on total I.E.
- ▶Total Design for production and distribution on total I.E.
- ▶Total Marketing expenditure on Total I.E.
- ▶Total Training expenditure on Total I.E.

5. RESULTS:

5.1 FINANCIAL

- ▶Total Net income (N.I.)
- ▶N.I. increase over previous year
- ▶Total product sales N.I. /Total N.I.
- ▶Servicing N.I./ Total N.I.
- ▶Grants N.I./Total N.I.
- ▶Royalties income/ Total N.I.
- ▶Other income/ Total N.I.
- ▶Total external financial resources on equity
- ▶RONA = Net Profit/Total Net Assets
- ▶Profitability variation over previous year

5.2 SCIENTIFIC

- ▶Total New active substances (N.A.S.) discovered
- ▶New index A* = NAS Type A* / Total NAS
- ▶New index A = NAS Type A / Total NAS
- ▶New index B = NAS Type B/ Total NAS
- ▶New index C = NAS Type C/ Total NAS
- ▶Total bio-pharmaceutical patents registered
- ▶Total Co-Inventions
- ▶Scientific production index = Total bio-pharmaceutical scientific publications/ Nº researchers
- ▶Total licences issued on the company's product to third-party
- ▶Total licences achieved on third-party products
- ▶Average duration of the R+D+I+T processes = Average number of years since the NAS is discovered until the new product is launched
- ▶Scientific success = Number of products launched / Number of Projects in R+D phase

Figure 3. Intellectual capital report for bio-pharmaceutical companies: model 2P 3C 2R.

2004). In addition, for creating intellectual capital, financial capital must be consumed, and in this regard it is essential to determine the profitability of investments in intellectual and financial capital (increased or decreased shareholder value).

These arguments highlight the need for a measure that is generally accepted of the effectiveness of intellectual capital. Furthermore, this measure should be able to "connect" the intellectual capital with financial capital to measure the effectiveness of the company in transforming intellectual capital into financial capital and vice versa. All these factors lead to the development of "second generation" models for measuring intellectual capital, consolidating the various methods into a single index, or at least a small amount of them. The information of the R&D process provided by traditional financial statements is clearly not enough to make a proper valuation of these companies (Gutiérrez de Mesa, 2004).

The proposal made here may become a fundamental tool to improve the assessment of these, especially as from January 1, 2005, some companies in this sector (large pharmaceutical companies listed on certain markets European equity) must submit their accounts in accordance with International Financial Reporting Standards while others (small new biotech companies oriented to pharmaceutical activities) continue presenting its financial information in accordance with the rules laid by the Spanish general accounting plan, so that comparability between the two groups is impossible. Through this report, which orders and standardizes the information provided on this type of intangible assets consistently and systematically, a truer picture of the economic and financial reality of such companies can be achieved (Gutiérrez de Mesa, 2004).

Conclusions

Intangible assets have become a key factor for growth at both the micro (enterprise) and macro (country) level. The change that has occurred in the production process shows, in addition to their growing importance, the inadequacy of traditional economic, financial and management concepts in the reality of the new economy. The problem that arises is the identification and measurement of intangible assets for managers, researchers and analysts. In this regard, intellectual capital report proposals based on nonfinancial aspects have proliferated, in addition to traditional financial statements. It is necessary to highlight two key outcomes of the study:

1. The growing importance of intangibles as a new way to create value and new way of organizing economic activities.
2. The need for new forms and quality of information provided since no proper assessment of this asset has high costs.

Therefore, we must stress the need to improve the process of identifying and measuring intangible assets at both micro and macro level, since a better valuation on a company allows a better measurement at the country level.

This need, in the case of technology-based innovating companies in general and in biopharmaceutical business, in particular, is determinant. It is clear that the main drivers of value creation in these companies are intangible and therefore not directly observable, so their identification and measurement are crucial. It is also a fact that although these assets may generate competitive advantages, information about them and spread abroad is scarce. The result is that in technology-based innovating companies in general and in biopharmaceutical companies, in particular, financial statements do not properly reflect the economic and financial situation of it. Investors, analysts and managers defend that traditional financial statements should include non financial information relevant to a best valuation of those companies.

The proposal made may become a fundamental tool to improve the valuation of these kind of companies, especially as from 2005, some companies in this sector (large pharmaceutical companies listed on certain markets European equity) submit their accounts in accordance with International Financial Reporting Standards while others (small new biotech companies oriented pharmaceuticals) continue presenting its financial information in accordance with the rules laid by the Spanish general accounting plan, so that comparability between the two groups will be impossible. This report, which orders and standardizes the information provided on this type of intangible assets consistently and systematically, allows getting a truer picture of the economic and financial situation of biopharmaceutical companies.

ACKNOWLEDGEMENT

In memoriam of Emma Gutiérrez de Mesa Vázquez, who passed away during the elaboration of this work.

REFERENCES

Alexander D, Pucci A, Smith M, Bloomfield K (2011). Life Science Trends 2011. Carlyle Conlan, Morrisville, NC.

Bontis N (2000). Assessing Knowledge Assets: A Review of the Models Used to measure Intellectual Capital. Framework Paper, Queen's University, Kingston.

Cañibano L, García AM, Sánchez P (1999). The Relevance of Intangibles for the Assessment and Management of Enterprises: Spanish Literatura. Rev. Finan. Account 100:17-88.

CIC (2002). Identificación y Medición del Capital Humano. Taller de Trabajo sobre Capital Humano, Documento Intellectus, Madrid.

DTIDC (1996). Capital and Growth, Danish Trade and Industry Development Council. In www.ll-a.fr/intangibles/denmark.

Edvinsson L, Malone MS (1997). Intellectual Capital: Realizing your Company's True Value by Finding its Hidden Brainpower. Harper Business, New York.

Edvinsson L, Malone MS (1999). Cómo identificar y calcular el valor de los recursos intangibles de su empresa. Ediciones Gestión 2000, 1999. Barcelona.
Edvinsson L (2000). Some Perspectives on Intangible and Intellectual Capital. UNIC, Stockholm.
EFPIA (2003). The Pharmaceutical Industry in Figures. European Federation of Pharmaceutical Industries Associations. Brussels.
Euroforum (1998). Modelo de Medición del Capital Intelectual. Madrid: I.U. Euroforum.
European Commission (2003). Study on the Measurement of Intangible Assets and Associated Reporting Practices. DG Entreprise Workshop on “Intangible Economy”. Brussels.
FASB (1985). Statement of Financial Accounting Concepts No. 6, “Elements of Financial Statements”. Stamford, Connecticut.
FASB (2001a). Business Combinations. FASB Statement No. 141. Stamford, Connecticut.
FASB (2001b). Goodwill and other Intangibles Assets. FASB Statement No. 142. Stamford, Connecticut.
Fidalgo Cerviño E (2003). Financial Information in the Future II: New accounting information and new scenarios. In: Moreno-Luque Abogados, Present and Future of Financial Information. Gestión, Barcelona.
Gutiérrez de mesa Vázquez E (2004). From creative destruction to creative cooperation in biopharmaceutical industry: an economic and accounting analysis, Doctoral Thesis. Universidad Complutense de Madrid.
Holland J (2004). Corporate intangibles, value relevante and disclosure content. The Institute of Chartered Accountants of Scotland, Edinburgh.
ICAC (2002). Report on the current situation of accounting in Spain and Basic Lines for its reform. Instituto de Contabilidad y Auditoría de Cuentas, Madrid.
Kaplan RS, Norton DP (1992). The Balanced Scorecard Measures that Drive Performance. Harv. Bus. Rev. 70(1):71-9.
Koch GR, Leitner KH, Bornemann M (2000). Measuring and Reporting Intangible Assets and Results in a European Contract Research Organization. Paper prepared for the Joint German-OECD Conference Benchmarking Industry-Science Relationship. Berlin, Germany.
Kulkarni U, Deshmukh YA, Moghe VV, Rege N, Madhuri K (2010). Introducing pharmaeconomics (PE) in medical undergraduate curriculum. Afr. J. Pharm. Pharmacol. 4(1):27-30.
Lev B (2000). New Accounting for the New Economy. pp. 1-28.
Lev B (2001). Management, Measurement and Reporting. Brookings Institution Press, Washington.
Lev B, Gu F (2001). Intangible Assets: Measurement, Drivers, Usefulness.
Lobato P, Lobo F, Rovira J (1997). La Industria Farmacéutica en España tras la Unificación del Mercado Europeo. Política Industrial y Estrategia Empresarial. Farmaindustria, Madrid. pp. 49-51.
Lynn LE (1998). The Management of Intellectual Capital: The Issues and the Practice. Management Accounting Issues, Management Accounting Practices Handbook. Society of Management Accountants of Canada, Hamilton. P 16.
Moreno-Luque A (2003). Present and Future of Financial Reporting. Ediciones2000.com Management. EC Regulation No 1725/2003 of 29 September, by adopting certain international accounting standards, OJ October 13, 2003. Barcelona:
Rydzewsky RM (2008). Real World Drug Discovery. A Chemist´s Guide to Biotechnology and Pharmaceutical Research. Elsevier, Amsterdam.
Roche (2006). Biotechnology-new directions in medicine. Hoffman-La Roche Ltd., Basel, Switzerland.
Roos J, Roos G, Dragonetti NC, Edvinsson L (2001). Intellectual Capital: The intangible value of the company. Piados Empresa, Barcelona.
Sveiby KE (1997). The New Organizational Wealth: Managing and Measuring Knowledge-Based Assets. Barret-Kohler Publishers, Inc., San Francisco.
Walsh G (2007). Pharmaceutical biotechnology. Concepts and applications. John Wiley & Sons, Hoboken, USA.

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Vol. 7(17), pp. 1009-1014, 8 May, 2013
DOI 10.5897/AJPP12.149
ISSN 1996-0816 © 2013 Academic Journals
http://www.academicjournals.org/AJPP

African Journal of Pharmacy and Pharmacology

Full Length Research Paper

Identification of errors in antibiotics' prescriptions and prescription writing trends in areas of Hyderabad Sindh, Pakistan

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Accepted 29 October, 2012

Prescribing errors are common in hospitals and outpatient clinical settings. A number of studies have been performed regarding antibiotic prescription errors throughout the world but it is not yet enough in Hyderabad, Pakistan. This study was performed to identify the errors in antibiotics' prescriptions and to propose the ways to minimize such errors. A total of 286 antibiotic containing prescriptions were randomly sampled from a government teaching hospital, three private hospitals and ten outpatient clinical settings. These prescriptions were categorized according to the antibiotic classes, and errors were identified according to various drug references and World Health Organization guidelines. The extent of errors was calculated; the highest proportion of the prescriptions (n = 257, 89.86%) failed to demonstrate the patient's weight and the least number of prescriptions (n = 07, 2.44%) contained the dosage form errors. The mean error per prescription was observed as 6.35 with standard deviation (SD) = 3.138 and 95% confidence interval for population mean is (5.98, 6.71). The major reasons of prescription errors were heavy patients' influx, insufficient knowledge regarding prescription writing guidelines to prescribers, and the lack of pharmacy services. Continuous educational training programs regarding prescription writing skills, introduction of computerized prescription order entry system and by recognizing and appreciating the role of pharmacist in evaluating the prescriptions, can substantially reduce these widespread errors.

Key words: Antibiotics, prescription, errors, Hyderabad.

INTRODUCTION

Prescription is an order written by a physician, dentist or any other registered medical practitioner to a pharmacist to compound and dispense a specific medication for the patient. A prescription order contains the directions for both the pharmacist and patient (Gupta and Basai 2007). Although different countries may have different standards for prescription writing but the prescription should contain the information such as; (a) Name, address, contact number and signature of the prescriber, (b) Name, address, contact number, age and gender of the patient, (c) Date of prescribing the medicine, name, quantity and dosage form of the drug, (d) Directions, instructions and warnings

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for patient (Gupta and Basai 2007; De varies et al., 1994). The prescriber should follow the proper guidelines for writing a prescription in order to minimize prescribing errors. Dean et al. (2000) describes prescribing error as error which occurs as a result of a prescribing decision or in prescription writing process. As a result, there is an unintentional significant reduction in the probability of treatment being timely and effective and increase in the risk of harm.

Several studies have been performed on the prescriptions containing antibiotics and others drugs for identification of the errors. Kaushal et al. (2001) stated that in hospitalized patients, medication errors are the most important problems. They performed a prospective cohort study in two academic institutions on 1120 pediatric inpatients. A total of 10,778 medication orders were analyzed and 5.7% (616) medication errors were identified. Out of these, 28% of errors were found in the prescriptions containing anti infective agents. Simultaneously, in another retrospective study of medication errors, it has been demonstrated that the most common type of serious error was found to be the wrong dose administration (Philips J et al., 2001).

Ridley et al. (2004) worked on prescription errors in 24 critical care units of United Kingdom. A total of 21,589 prescriptions were evaluated and errors were found in 3,141 (15%) of the prescriptions; out of 3,141 errors, 916 (19.6%) were reported as potentially life threatening errors. Another study was performed by Jayawardane et al. (2007) from January, 2004 to January, 2005 in a teaching hospital of South Brooklyn. This study reported 3,513 errors in 466,311 prescriptions, and it was noticed that 53.9% errors were in prescriptions containing antibiotic drugs.

As no study on antibiotic prescription errors has been carried out in Hyderabad, Pakistan, therefore, this study has been conducted to report the trend of prescription errors in antibiotic prescription, to find the extent of these errors and also to propose the ways to minimize such errors.

MATERIALS AND METHODS

This study was carried out after collecting the prescriptions from a government teaching hospital, three private hospitals and 10 outpatient clinical settings in various areas of Hyderabad, Sindh, Pakistan. A total of 286 prescriptions, containing at least one antibiotic, were randomly collected by simple random sampling technique over the period of 6 months, from February, 2011 to July, 2011. The prescriptions were grouped according to the particular class of antibiotics such as penicillin, cephalosporin, tetracycline, quinolone, macrolide and aminoglycoside class (Table 1). The prescriptions were then analyzed to identify prescription errors as per World Health Organization (De Vries et al., 1994) parameters for prescription writing, British National Formulary (2010) and Drug information hand book (Lacy et al., 2010) criteria.

Depending on the requirements for prescriber information, patient information and drug information in each prescription, 16 error categories were designed and then every prescription from each antibiotic class was evaluated by for the presence of errors. The identified errors were placed under specific error category and the extent of errors in percentage was calculated in a predesigned analysis sheet. Finally, as a whole, the total extent of error was calculated for all the 286 prescriptions and a separate sheet was made.

Data analysis

Microsoft office and descriptive statistics were used for analyzing the collected data. Tool of 95% confidence interval was used for population mean by computational software statistical package for social sciences (SPSS) 17.0 version.

RESULTS

In the present study, a total of 286 antibiotic containing prescriptions were collected. These prescriptions were grouped according to the specific classes of antibiotics such as: penicillin group, cephalosporin group, tetracycline group, quinolone group, macrolide group, and aminoglycoside group (Table 1), and then analyzed and evaluated for the presence of errors. Out of 286 collected prescriptions, 257 (89.86%) prescriptions were missing the weight of the patients, 72 (25.17%) prescriptions where no age was mentioned, and 199 (69.58%) prescriptions were missing the patient's diagnosis. We found 155 (54.19%) prescriptions without using metric system, 141 (49.30%) prescriptions having strength error, 126 (44.05%) with patient's gender missing, 124 (43.35%) had dose error, 107 (37.14%) were missing directions for use, 96 (33.56%) were lacking date and patient's name, 92 (32.16%) were with potential for drug interaction, 84 (29.37%) with incorrect frequency, and 82 (28.67%) with incorrect administration route (Table 2). It was further found that omission of prescriber's signature in 79 (27.62%), unclear writing in 77 (26.92%) prescriptions and the least number of errors were identified in case of missing or writing the wrong dosage form as 7 (2.44%) (Figure 1). A total of 1,815 errors were noticed in all antibiotics' prescription, with an average of 6.35 errors per prescription with standard deviation of 3.138. A 95% confidence interval computed for population mean was between 5.98 and 6.71.

DISCUSSION

In our present investigation, we identified the extent of errors in antibiotics containing prescriptions. A total of 286 prescriptions were collected and evaluated for the presence of errors. The error category of dose omission or wrong dose represented 43.35% of the prescriptions. This data is at par with the findings of Costa et al. (2008)

Table 1. Distribution of antibiotic prescriptions according to specific drug classes

Antibiotic class	Number of prescription	Percentage (%)
Penicillin	60	20.97
Cephalosporin	60	20.97
Tetracycline	52	18.18
Quinolone	60	20.97
Macrolide	18	6.29
Amino glycoside	36	12.58
Total Antibiotics	286	100

Table 2. Analysis error categories sheet as per WHO parameters for prescription writing, British national formulary and Drug information hand book parameters/standards (Total prescription = 286).

No.	Error categories/parameters/standards	Errors/not followed (%)	Not error/followed (%)	Total prescription
01	Date and patient's name not mentioned	96 (33.56)	190 (66.43)	286
02	Writing ambiguous medication order	77 (26.92)	209 (73.07)	286
03	Patient's age not mentioned	72 (25.17)	214 (74.82)	286
04	Patient's weight not mentioned	257 (89.86)	29 (10.13)	286
05	Patient's gender not mentioned	126 (44.05)	160 (55.94)	286
06	Patient's diagnosis not mentioned	199 (69.58)	87 (30.41)	286
07	Misspelling of medications	66 (23.07)	220 (76.920)	286
08	Missed directions of use	107 (37.14)	179 (62.58)	286
09	Dose omission or writing incorrect dose	124 (43.35)	162 (56.64)	286
10	Missed or incorrect dosage form	07 (2.44)	279 (97.55)	286
11	Missed or incorrect Strength of medicine	141 (49.30)	145 (50.69)	286
12	Missed or incorrect administration route	82 (28.67)	204 (71.32)	286
13	Missed or incorrect frequency	84 (29.37)	202 (70.62)	286
14	Prescribing without using metric system	155 (54.19)	131 (45.80)	286
15	Omission of prescriber's signature	79 (27.62)	207 (72.37)	286
16	Presence of potential drug interaction	92 (32.16)	194 (67.83)	286

$C = \frac{A}{AB} \times 100$ C = percentage of prescriptions containing errors, A = number of errors containing prescriptions, AB = total number of prescriptions.

who reported that 49.6 and 28.6% of the prescriptions had high dose (wrong dose) and missing dose errors, respectively. The study by Vaishali et al. (2011) revealed similar results, they identified that 54.3% of prescriptions did not have the correct and calculated doses and in 35.1% of prescriptions, the doses were not mentioned clearly.

In a study by Folli et al. (1987), in terms of wrong dose, they found that 55.1% of the medication orders contained the overdose and 26.9% contained the under dose errors. It also stated that most of the serious or potentially lethal errant medication orders include the antibiotics. In contrast to our results, Balbaid and Al-Dawood, (1998) revealed that 7.6% of prescriptions did not contain the dose at all. Another study by Irshaid et al. (2005) revealed that 19.4% of the prescriptions were deficient in dose units. A study by Gandhi et al. (2005) reported 54% of the dose errors, it also revealed that antibiotic was the most common class of medications and contained 25% of the prescribing errors.

In our finding in most of the prescriptions (89.86%), the weight of the patient was not mentioned and also corresponded to the findings of Vaishali et al. (2011) and Irshaid et al. (2005), who found that none of the prescriptions contained the patient's weight. In case of patient's diagnosis, our data revealed that 69.58% of the prescriptions were missing the diagnosis. This is in contrast to the studies of Irshaid et al. (2005) who identified this error only in 15.1% prescriptions, whereas Bawazir (1993) had reported in 9.8% prescriptions and Balbaid and Al-Dawood, (1998) had found in only 6.8% of the prescriptions. This shows that as patient diagnosis factor

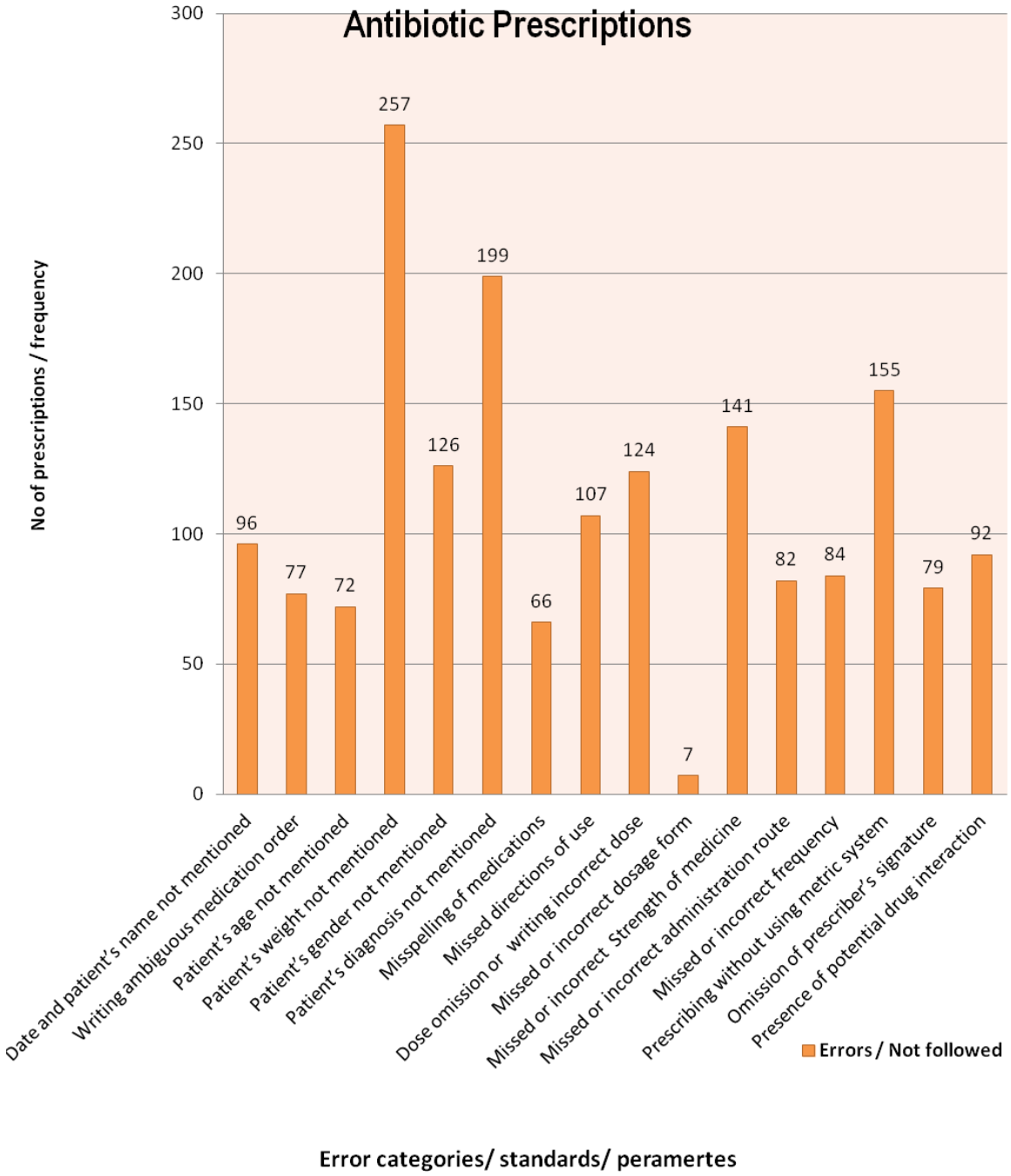

Figure 1. Errors (total prescription = 286)

factor is considered, the conditions of prescription writing in Hyderabad, Pakistan are worse.

Concerning the strength of medications, it is the most important factor especially when a drug is available in market, in more than one strength. We found that 49.30% of the prescriptions are with wrong strength of medication or the strength has not been mentioned. This result is similar to the report of Irshaid et al. (2005) who stated that 52.8% of prescriptions were missing the strength of medications. On the other hand, our results are dissimilar to those reported by Vaishali et al. (2011) who identified that 26.8% of prescriptions did not contain the strength.

A large number of deficiencies also have been found regarding the gender and age of the patient. Our study

investigated that in 44.05 and 25.17% of the prescriptions, the prescriber had not mentioned the gender and age, respectively, of the patients. However, a study by Vaishali et al. (2011) found 10 and 11% of prescriptions in which the gender and age, respectively, of the patient were not written. Balbaid and Al-Dawood, (1998) identified that only 10 and 4.1% of prescriptions were missing the patients age and sex, respectively. Furthermore, Irshaid et al. (2005) found that 22.7 and 48.7% of prescriptions did not contain the age and gender of the patient. We identified a significant number of prescriptions, 37.14%, which did not contain the directions for patients. Our result is comparable to the findings of Vaishali et al. (2011) who recognized that 45.9% of prescriptions were missing the patient's instructions. Irshaid et al. (2005) revealed that 7.1% of the prescriptions were missing the patient's instructions and majority of the prescriptions, 90.7%, had only partial patient's instructions. On the other hand, Bawazir (1993) noticed that only 4% of the prescriptions were lacking in instructions for use to patient.

Our findings revealed that in 33.56% prescription, the name of the patient was not mentioned. It was further found that the same percentage (33.56) of the prescriptions were without the date of generation of the prescription. This number is much higher than the findings by Balbaid and Al-Dawood, (1998), who reported that in only 8.7% of prescriptions, the dates were not mentioned. Francois et al. (1997) reviewed 866 prescriptions and found only 4.5% of prescriptions with missing dates. In case of the drugs which can be administered by more than one route, it is necessary to mention their routes. We evaluated that 28.67% of prescriptions were deficient in mentioning routes of drug administration. These results are somewhat similar to the findings of Phalke et al. (2011), who reported 24.7% prescriptions that did not contain the routes of drug administration. But our results are in conflict with those reported by Gandhi et al. (2005) and Bawazir (1993), where only 13% and 0.1%, respectively cases were found with this deficiency.

Regarding the error category of writing an ambiguous medication order, we explored that 26.92% of prescriptions were not written clearly. Our result is dissimilar to the other findings reported, as Balbaid and Al-Dawood, (1998), Irshaid et al. (2005), Meyer (2000) and Makonnen et al (2002) reported that 7.2%, 64.3%, 15% and 15% of prescriptions, respectively, had poor and incomprehensible hand writing.

There are a number of studies which suggested implementing computer based system for prescribing the drugs (Javier Rodrı´guez-Vera et al., 2002; Ruud et al., 1991). Nightingale et al. (2000) and Meyer (2000) suggested that electronic prescription system can be used to improve the prescription writing by removing the illegible prescriptions. The studies by Bates et al. (1998) and Anton et al. (2004) have shown that it is possible to reduce the medication and prescribing errors by using computer based system of prescribing medications. De Vries et al. (1995) reported that educational training programs can also lead to improve the prescription writing. Obehi et al. (2008) studied the effect of educational intervention on prescription writing and reported the improvement. These data clearly show that there is a need for introducing computerized physician order entry system (CPOE) to improve the prescription writing and reduce the errors. There have been many studies conducted on drug-drug interaction, which is a critical issue in health care system. In our study, potential drug interactions were observed in 32.16% of the prescriptions. In a study carried by Lars et al. (2003), it is reported that 62% persons were exposed to potential drug interaction with single drug, and 38% with two or more different drugs.

Conclusion

It is concluded from this study and literature, that there is high percentage of prescription errors in practice. It was found that majority of the prescription errors are related to the incomplete or wrong information of prescriber, patient and drugs on the prescriptions, and poor or incomprehensible hand writing. This leads to various problems of dispensing, incorrect doses and administration of medicines, drug misuse, and drug interactions. The physicians should pay proper attention to the prescription writing and patient's counseling. The physicians should be provided with the educational training to improve their prescription writing skills according to World Health Organization Guidelines for Prescription Writing or other recognized and published standards. The computerized physician order entry system should be introduced. Pharmacist can also play an important role in preventing the errors by reviewing the prescriptions.

REFERENCES

Anton C, Nightingale PG, Adu D, Lipkin G, Ferner RE (2004). Improving prescribing using a rule based prescribing system. Qual. Saf. Health Care 13:186–190.

Balbaid OM, Al-Dawood KM (1998). Assessment of physician's prescribing practices at Ministry of Health Hospitals in Jeddah City, Saudi Arabia. Saudi Med. J. 19:28–35.

Bates DW, Leape LL, Cullen DJ, Laird N, Petersen LA, Teich JM, Burdick E, Hickey M, Kleefield S, Shea B, Vander Vliet M, Seger DL (1998). Effect of computerized physician order entry and a team intervention on prevention of serious medication errors. JAMA: 280(15):1311–1316.

Bawazir S (1993). Prescribing pattern of ambulatory care physicians in Saudi Arabia. Annals Saudi Med. 13(2):172–177.

British National Formulary, edition 59th (March 2010). British Medical Association and Royal Pharmaceutical Society of Great Britain, London.

Costa LA, Valli C, Alvarenga AP (2008). Medication dispensing errors at

a public pediatric hospital. Rev. Latino-am Enfermagem. 16(5):812-817.
De vries TP, Henning RH, Hogerzeil HV, Fresle DA (1994). Guide to good prescribing: a practical manual, Geneva world health organization eds 1995:52-55 (WHO/DAP/94.11).
De Vries TP, Henning RH, Hogerzeil HV, Bapna JS, Bero L, Kafle KK, Mabadeje A, Santoso B, Smith AJ (1995). Impact of a short course in pharmacotherapy for undergraduate medical students: an international randomized controlled study. Lancet 346(8988):1454-1457.
Dean B, Barber N, Schachter M (2000). What is a prescribing error? Qual. Health Care 2000, 9:232-237.
Folli HL, Poole RL, Benitz WE, Russo JC (1987). Medication Error Prevention by Clinical Pharmacists in Two Children's Hospitals. Pediatrics 79:718-722.
Francois P, Chirpaz E, Bontemps H, Labarère J, Bosson JL, Calop J (1997). Evaluation of prescription-writing quality in a French university hospital. Clin. Perform. qual. health care 5:111–115.
Gandhi TK, Weingart SN, Seger AC, Borus J, Burdick E, Poon EG, Leape LL, Bates DW (2005). Outpatient Prescribing Errors and the Impact of Computerized Prescribing. J. Gen. Int. Med. 20(9):837–841.
Gupta AK, Basaj SS (2007). Introduction to pharmaceutics II, 4th edition. CBS Publisher and Distributors, DehlI, India.
Irshaid YM, Al Homrany M, Hamdi AA, Adjepon-Yamoah KK, Mahfouz AA (2005). Compliance with good practice in prescription writing at outpatient clinics in Saudia Arabia. East Mediterr. Health J. 11(5-6):922-928.
Javier Rodrı´guez-Vera F, Marı´n Y, Sa´ nchez A, Borrachero C, Pujol E (2002). Illegible handwriting in medical records. J. R. Soc. Med. 95:545–546.
Kaushal R, Bates DW, Landrigan C, Mckenna KJ, Clapp MD, Federico F, Goldmann DA (2001). Medication errors and adverse drug events in pediatric inpatients. JAMA 285(16):2114-2120.
Lacy CF, Armstrong LL, Goldman MP, Lance LL (2010). Drug Information Hand book, 19th Edition. Lexi-comp, Inc. Hudson, ohio.
Lars B, Morten A, Gert P, Kragstrup J (2003). Exposure to potential drug interaction in primary health care system. Scand J. Prim. Health Care 21:153.
Makonnen E, Yoseph M, Berhane Y (2002). Quality of prescription at a tertiary care pharmacy in Addis Ababa. Ethiop. Med. J. 40:233–239.
Meyer TA (2000). Improving the quality of the order writing process for in patient order and outpatient prescriptions. Am.J. Health syst. Pharm. SI8-22.
Nightingale PG, Adu D, Richards NT, Peters M (2000). Implementation of rules based computerized. Bed side prescribing and administration: intervention study. BMJ 320:750–753.
Obehi A Akoria, Ambrose O Isah (2008). Prescription writing in public and private hospitals in Benin City, Nigeria: the effects of an educational intervention. Can. J. Clin. Pharmacol. 15(2):e295-305.
Phillips J, Beam S, Brinker A, Holquist C (2001). Retrospective analysis of mortalities associated with medication errors. Am. J. Health syst. Pharm. 58(19):1835-1841.
Ridley SA, Booth SA, Thompson CM (2004). The Intensive Care Society's Working Group on Adverse Incidents. Prescription errors in UK critical care units. Anaesthesia 59:1193–1200.
Ter Wee Ruud JM, Kleijn Eppo Van Der, Brenninkmeijer Rob F, Holmberg Niklas (1991). Development of an electronic prescription processing option: an aid for general practice. Br. J. Gen. Pract. 41:151-154.
Vaishali DP, Deepak BP, Syed MM Aarif, Anjeney Mishra, Saudamini Sikchi, Piyush Kalakoti (2011). Prescription writing practices in a rural tertiary care hospital in Western Maharashtra, India. Austr. Med. J. 4:4-8.

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Vol. 7(17), pp. 1015-1019, 8 May, 2013
DOI 10.5897/AJPP12.412
ISSN 1996-0816 © 2013 Academic Journals
http://www.academicjournals.org/AJPP

African Journal of Pharmacy and Pharmacology

Full Length Research Paper

Antioxidant and anticancer activities of methanolic extract of *Trollius chinensis* Bunge

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Accepted 15 April, 2013

The flower of *Trollius chinensis* Bunge (*Flos trollii*) is used for treating upper respiratory infections, pharyngitis, tonsillitis and bronchitis as a Chinese folk medicine since ancient times. This study aimed to investigate possible antioxidant and anticancer activity of methanolic extract of *F. trollii* (FTME). The antioxidant activity of FTME was determined by 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radical scavenging assay, hydroxyl radical scavenging assay and metal chelating assay. FTME exhibited a powerful free radical scavenging activity against DPPH and acted as a strong hydroxyl radical scavenger to prevent deoxyribose degradation in Fe^{3+}/ascorbate/ethylenediaminetetraacetic acid (EDTA)/H_2O_2 system. In addition, FTME also showed a weak metal chelating activity. Anticancer activity of FTME was determined by 3-[4,5-Dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) assay. FTME showed strong anticancer activities in antiproliferative activities against human gastric (AGS), A375SM, MCF-7, and MDA-MB-231 cancer cell lines. The half-inhibitory concentrations (IC_{50}) for these cancer cells were 143.72, 62.23, 244.50 and 279.06 µg/ml, respectively. All results suggest that *F. trollii* is a powerful natural antioxidant, and also exert stronger anticancer activities for four kinds of human cancer cell lines. It could be a potential source of natural antioxidant and also as a health food.

Key words: *Trollius chinensis* Bunge, antioxidant, anticancer activity.

INTRODUCTION

Free radicals were associated with serious disease such as diabetes, cirrhosis, cancer and cardiovascular diseases (Hertog et al., 1993; Zia-Ul-Haq et al., 2012a). Hence, a considerable number of investigations have been focused on the prevention of oxidative damage initiated by free radicals. In fact, much attention has been focused on the antioxidative compounds present in edible plants, because of some synthetic antioxidants, such as 2,3-*tert*-butyl-4-methoxy phenol (BHA) and 2, 6-di-*tert*-butyl-4-methyl phenol (BHT) which are widely used in food industry (Imadia et al., 1983; Zia-Ul-Haq et al., 2008, 2011a). Those synthetic antioxidants were deemed to have carcinogenic potential (Branen, 1975; Zia-Ul-Haq et al., 2011b). Some investigations showed that antioxidants from plant tissues are correlated with oxidative stress defense and serious different human disease, including cancer, arteriosclerosis and aging processes (Manosroi et al., 1995; Stajner et al., 1995; Zia-Ul-Haq et al., 2012b). The epidemiological investigations showed that more than 80% of cancers are connected to lifestyle. In addition, diet rich in fruits and vegetables are associated with a lower risk of several degenerative diseases (Franceschi et al., 1998). Therefore, investigations of natural plant antioxidants and

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related bioactive compounds for food preservation and certain human diseases have received much attention. *Trollius chinenesis* Bunge, a member of Ranunculaceae's family, is a perennial herb widely distributed in north of China and Mongolia (Jiangsu New College of Medicine, 1977; Bai, 1994). Its flowers (*Flos trollii*), also called Jin Lianhua in China, as a traditional folk medicine since ancient times, have been used to treat colds, high fevers, aphthae, respiratory infections, chronic tonsillitis, pharyngitis, bronchitis and acute tympanitis in China (Jiangsu New College of Medicine, 1977; Kang et al., 1984). Presently, some studies have reported that the aqueous extracts of *F. trollii* exhibited the antimicrobial activities against the *Aurens, Pseudo-monasaeruginosa, Shigella dysenteriae, Esclierichia coli, Streptococcus hemolylieus* and *Diplococcus pneumonia*, and also showed antiviral activities against the coxsackie B3 (Wen et al., 1999), parainfluenza type 3 (Para 3) (Li et al., 2002) and influenza virus A (Cai et al., 2006). In addition, *T. chinenesis* Bunge also showed a protective effect in D-galactose-aged mice (Fang et al., 2012). In this study, we investigated the possible antioxidative effects of *F. trollii* (FTME) and its anticancer activities against four kinds of human cancer cell lines *in vitro*.

MATERIALS AND METHODS

Reagents

Flower of *T. chinenesis* Bunge (*F. trollii*) were purchased from a local market in Chongqing, China in August, 2011. 1,1-Diphenyl-2-picrylhydrazyl (DPPH), 2,3-*tert*-butyl-4-methoxy phenol (BHA), L-ascorbic acid, deoxyribose, trichloroacetic acid (TCA), ferrozine reagent, dimethyl sulfoxide (DMSO) and 3-[4,5-Dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Roswell Park Memorial Institute (RPMI) 1640, Dulbecco's modified eagle's medium (DMEM), fetal bovine serum (FBS) and penicillin/streptomycin were obtained from Gibco BRL (Grand Island, NY, USA). All other reagents were of analytical grade.

Preparation of extracts

The flowers of *T. chinenesis* Bunge (*F. trollii*) were shade dried initially, freeze dried and then ground to a fine powder. Ten grams of the powdered flower sample was then extracted for 24 h with methanol (200 ml) under continuous stirring at room temperature (28°C). Finally, all the methanolic extracts were evaporated to dryness at 50°C and redissolved in DMSO at a concentration of 50 mg/ml, and stored at -20°C prior to further use.

DPPH radical scavenging assay

The DPPH radical scavenging activity of FTME was estimated according to the method of Hatano et al. (1988). The FTME (50, 100, 250, 500 µg/ml) were added to a methanolic solution (0.1 ml) of DPPH radical (final concentration of DPPH was 0.15 mM). The mixture was shaken vigorously and allowed to stand at room temperature for 30 min; the absorbance of the resulting solution was then measured spectrophotometrically at 517 nm using a Shimadzu UV-3600 UV-VIS-NIR spectrophotometer (Kyoto, Japan).

Hydroxyl radical scavenging assay

Hydroxyl radical scavenging activity was carried out as described by Halliwell et al. (1987). The reaction mixture (1.4 ml) which contained FTME (0.2 m, 50 to 500 µg/ml), deoxyribose (6 mM), H_2O_2 (3 mM), KH_2PO_4-K_2HPO_4 buffer (20 mM, pH 7.4), $FeCl_3$ (400 µM), ethylenediaminetetraacetic acid (EDTA, 400 µM), and ascorbic acid (400 µM), was incubated at 37°C for 1 h. The extent of deoxyribose degradation was tested by using the thiobarbituric acid (TBA) method. One milliliter of 1% TBA and 1 ml of 2.8% trichloroacetic acid (TCA) were added to the mixture, which was then heated in a water bath at 90°C for 20 min. The absorbance of the mixture was read spectrophotometrically at 532 nm using a Shimadzu UV-3600 UV-VIS-NIR spectrophotometer (Kyoto, Japan).

Metal chelating activity assay

The metal chelating activity of FTME on Fe^{2+} was measured according to the method of Carter (1971). Briefly, FTME (50 to 500 µg/ml) were incubated with 0.05 ml of $FeCl_2 \cdot 4H_2O$ (2.0 mM). The reaction was initiated by the addition of 0.2 ml of ferrozine (5.0 mM) and finally quantified to 0.8 ml with methanol. After the mixture had reached equilibrium (10 min), the absorbance was read at 562 nm using a Shimadzu UV-3600 UV-VIS-NIR spectrophotometer (Kyoto, Japan).

Cell culture

AGS, MCF-7, MDA-MB-231 and A375SM cancer cell lines were obtained from the American Type Culture Collection (Manassas, VA, USA). The human gastric carcinoma cell lines AGS were grown in RPMI 1640 medium, and the human breast adenocarcinoma cell lines MCF-7, MDA-MB-231, and human melanoma cell lines A375SM were grown in Dulbecco's modified eagle's medium with L-glutamine; medium was supplemented with 10% FBS, 1% antibiotics in a humid atmosphere incubator (Model 3546, Forma Scientific Inc, Marietta, OH, USA) with 5% CO_2 at 37°C.

Cell proliferation inhibitory assay

Cell proliferation inhibitory activity was measured using the MTT assay. Briefly, cancer cells were cultured in 96 well plates (Nunc, Rochester, NY, USA) at a density of 1×10^4 cells/ml and incubated with the FTME (50 ~ 500 ug/ml) for 48 h. After 48 h incubation, 100 µl MTT reagent (final concentration, 0.5 mg/mL) was added to each well, and cells was incubated in a humidified incubator at 37°C to allow the MTT to be metabolized. After 4 h, the media was removed and cells were resuspended in formazan in 100 µl of DMSO. The absorbance of the samples was measured at a wavelength of 490 nm by microplate reader (EL311, BIO-TEK Instruments, Inc, USA).

Statistical analyses

Data were presented as mean ± standard deviation (SD). Differences between the mean values for individual groups were assessed by a one-way analysis of variance (ANOVA) with Duncan's multiple range tests. Differences were considered

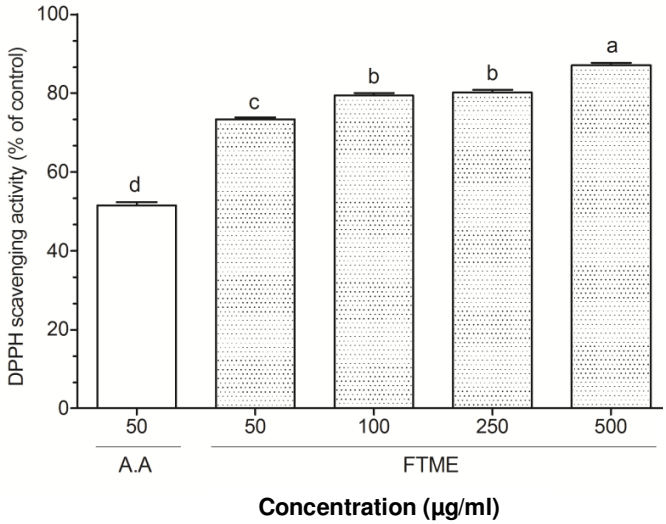

Figure 1. DPPH free radical scavenging activity of *Flos trollii* methanolic extract (FTME).
Each value is expressed as mean ± SD in triplicate. [a~d]Means with the different letters in the same column are significantly different ($p < 0.05$) by Duncan's multiple range test. AA: ascorbic acid.

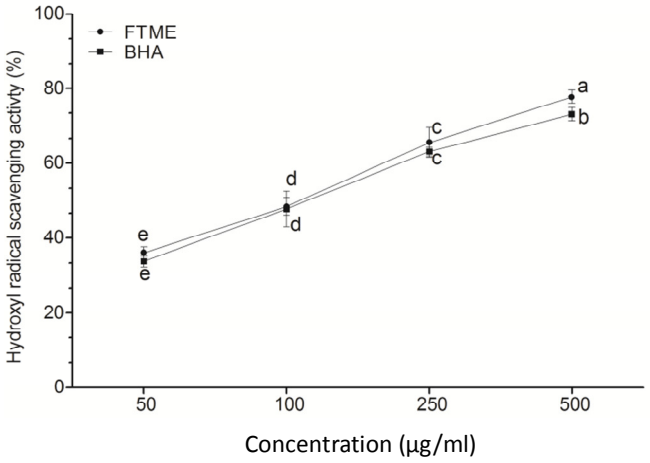

Figure 2. Hydroxyl radical scavenging activity of *Flos trollii* methanolic extract (FTME). Each value is expressed as mean ± SD in triplicate. [a~e] Means with the different letters in the same column are significantly different ($p < 0.05$) by Duncan's multiple range test.

significant when $p < 0.05$. The SAS v9.1 statistical software package (SAS Institute Inc., Cary, NC, USA) was used for these analyses.

RESULTS AND DISCUSSION

DPPH free radical scavenging activity

Free radicals play a very important role in several physiological and pathological events, such as aging, inflammation, immunization, mutagenicity and carcinogenicity (Namiki, 1990). DPPH is a stable free radical (purple in colour) and accepts an electron or hydrogen radical to become a stable yellow diamagnetic molecule (Soares et al., 1997). As shown in Figure 1, the free radical scavenging activities of FTME were 73.29, 79.42, 80.19 and 87.05% at concentration of 50, 100, 250 and 500 µg/ml, respectively. DPPH radical scavenging activities of FTME were increased in a manner dependent on concentration, which is comparable to the standard antioxidant ascorbic acid (51.49%) at 50 µg/ml. The results suggested that FTME was a powerful antioxidant than ascorbic acid.

Hydroxyl radical scavenging activity

The highly reactive hydroxyl ($^{\cdot}OH$) radicals can cause oxidative damage to DNA, proteins and lipids (Spencer et al., 1994), which contributes to carcinogenesis, mutagenesis and cytotoxicty. The effect of FTME on hydroxyl radical scavenging activity was determined by deoxyribose damage induced by Fe^{3+}/ascorbate/EDTA/H_2O_2 system, and measured by the TBA method. Damaged dexoyribose degrades into fragments that react with TBA upon heating at a low pH to form a pink color. As shown in Figure 2, $^{\cdot}OH$ radical scavenging activities of FTME were 30.64, 48.42, 65.64 and 77.69 at concentration of 50, 100, 250 and 500 µg/ml, respectively. The $^{\cdot}OH$ radical scavenging activities of BHA (positive antioxidant) were 33.71, 47.75, 63.15 and 73.05 at concentration of 50, 100, 250 and 500 µg/ml, respectively. The $^{\cdot}OH$ radical scavenging activities of FTME were increasing in a concentration-dependent manner than that of BHA. These results clearly shows FTME can act as effective scavengers against hydroxyl free radical *in vitro*.

Metal chelating activity

Lipid peroxidation is a very important biological consequence of oxidation cellular and aging in living organisms. Some metal ions, especially, ferrous iron can induce lipid peroxidation by the Fenton reaction as well as accelerating peroxidation by decomposing lipid hydroperoxides into peroxyl and alkoxyl radicals (Fridovich, 1995; Halliweill, 1991). Antioxidants inhibit interaction between metal and lipid through formation of insoluble metal complexes with ferrous ion, and reduce the catalyzing transition metal in lipid peroxidation (Hsu et al., 2003; Duh et al., 1999). Ferrozine can quantitatively form complex with ferrous ion. In the presence of chelating agents, the complex formation is disrupted with the result that the red color in the complex is decreased.

As shown in Figure 3, the metal chelating activities of FTME were18.55, 24.13, 27.53 and 36.36% at concentration of 50, 100, 250 and 500 µg/ml, respectively. However, the chelating effect activities of FTME were lower than that of EDTA (positive control) at 0.2 mM (chelating activity is 77.64%). FTME showed a weakly chelating ability of ferrous ions, which caused the lipid oxidation via Fenton reaction.

Cell proliferation inhibitory effects

The cell proliferation inhibitory effects of FTME on different cancer cells, including AGS human gastric carcinoma cells, A375SM human melanoma cells, MCF-7 and MDA-MB-231 human breast adenocarcinoma cells were determined by MTT assay. Cancer cells were exposed for 48 h to various concentration of FTME (50 to 500 µg/ml). As shown in Figure 4, the cell proliferation inhibitory activities of FTME were increasing in a concentration-dependent manner. The half maximal inhibitory concentration (IC_{50}) of FTME were 143.72, 62.23, 244.50 and 279.06 µg/ml to against AGS, A375SM, MCF-7 and MDA-MB-231 cancer cells, respectively (Figure 4). In particular, FTME showed the strongest cell proliferation inhibitory activities than that of other cancer cells.

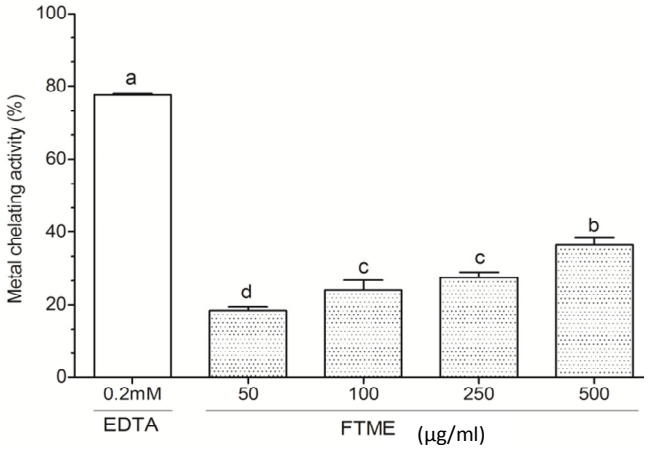

Figure 3. Metal chelating activity of *Flos trollii* methanolic extract (FTME).
Each value is expressed as mean ± SD in triplicate. [a~d]Means with the different letters in the same column are significantly different ($p < 0.05$) by Duncan's multiple range test.

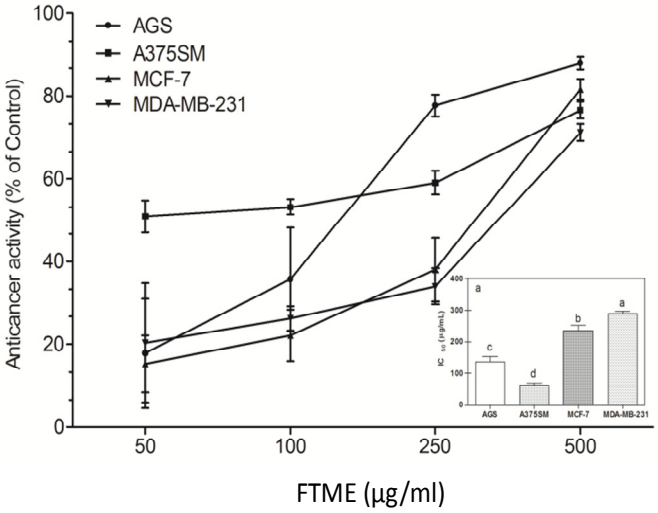

Figure 4. Cell proliferation inhibitory effects of *Flos trollii* methanolic extract (FTME) on AGS, A375SM, MCF-7 and MDA-MB-231 cancer cells.
Each value is expressed as mean ± SD in triplicate. [a~d]Means with the different letters in the same column are significantly different ($p < 0.05$) by Duncan's multiple range test.

REFERENCES

Bai YM (1994). The Chinese medicine material *Trollius chinensis* Bunge. Zhong Cao Yao 25:291.

Branen AL (1975). Toxicology and biochemistry of butylated hydroxyanisole and butylated hydroxytoluene, J. Am. Oil. Chem. Soc. 52:59-63.

Cai SQ, Wang R, Yang X, Shang M, Ma C, Shoyama Y (2006). Antiviral flavonoid-type C-glycosides from the flowers of *Trollius chinensis*. Chem. Biodivers. 3(3):343-348.

Carter P (1971). Spectrophotometric determination of serum iron at the submicrogram level with a new reagent (Ferrozine). Anal. Biochem. 10:450-458.

Duh PD, Tu YY, Yen GC (1999). Antioxidant Activity of Water Extract of Harng Jyur (*Chrysanthemum morifolium* Ramat). Lebensmittel-Wissenschaft. Technol. 32:269-277.

Fang A, Guodong Y, Jiaming T, Shuhua W (2012). Antioxidant effects of the orientin and vitexin in *Trollius chinensis* Bunge in D-galactose-aged mice. Neural Regen. Res. 7:2565-2575.

Franceschi S, Parpinel M, La Vecchia C, Favero A, Talamini R, Negri E (1998). Role and different types of vegetables and fruit in the prevention of cancer of the colon, rectum and breast. Epidemiology 9:338-341.

Fridovich I (1995). Superoxide Radical and Superoxide Dismutases. Annual Rev. Biochem. 64:97-112.

Halliweill B (1991). Reactive oxygen species in living systems: Source, biochemistry, and role in human disease. Am. J. Med. 91:14-19.

Halliwell B, Gutteridge JMC. Aruoma OI (1987). The deoxyribose method: a simple "test tube" assay for determination of rate constants for reactions of hydroxyl radicals. Anal. Biochem. 165:215-219.

Hatano T, Kagawa H, Yasahara T, Okuda T (1988). Two new flavonoids and other constituents in licorice root: their relative astringency and radical scavenging effects. Chem. Pharm. Bull. 36:2090-2097.

Hertog MGL, Feskens EJM, Kromhout D, Hertog MGL, Hollman PCH, Hertog MGL, Katan MB (1993). Dietary antioxidant flavonoids and risk of coronary heart disease: The Zutphen Elderly Study. Lancet 342:1007-1011.

Hsu CL, Chen WL, Weng YM, Tseng CY (2003). Chemical composition, physical properties and antioxidant activities of yam flours as affected by different drying methods. Food Chem. 83:85-92.

Imadia K, Fukushima S, Shirai T, Ohtani M, Nakanish K, Ito N (1983). Promoting activities of butylated hydroxytoluene on 2-stage urinary bladder carcinogenesis and inhibition of γ-glutamyl transpeptidase-positive foci development in the liver of rats. Carcinogenesis 4:895-899.

Jiangsu New College of Medicine (1977). Chinese Matera Medica Dictionary; Shanghai People's Press, Shanghai, China. P 1398.
Kang SW, Yu YF, Wang P (1984). Investigation on the constituents of *Trollius chinensis.* Zhong Cao Yao 15:7-9.
Li YL, Ma SC, Yang YT (2002). Antiviral activities of flavonoids and organic acid from *Trollius chinensis* Bunge. J. Ethnopharmacol. 79:365-368.
Manosroi A, Miller NJ, Bolwell PG, Bramley PM, Pridham JB (1995). The relative antioxidant activity of plant derived polyphenolic flavonoids. Free Radic. Res. 22:375-383.
Namiki M (1990). Antioxidant/antimutagens in food. Crit. Rev. Food Sci. Nutr. 29:273-300.
Soares JR, Dinis TCP, Cunha AP, Ameida LM (1997). Antioxidant activity of some extract of *Thymus zygis*. Free Radic. Res. 26:469-478.
Spencer JP, Jenner A, Aruoma OI, Evans PJ, Kaur H, Dexter DT, Jenner P, Lees AJ, Marsden DC, Halliwell B (1994). Intense oxidative DNA damage promoted by L-DOPA and its metabolites. FEBS Lett. 353:246-550.
Stajner D, Miliç N, Mimica-Dukic N, Lazic B, Igic R (1995). Antioxidant abilities of cultivated and wild species of garlic. Phytother. Res. 12:513-514.
Wen YH, Lin YS, Huang H, Liu XP, Wei GZ, Fu FL, Wu PH, Yang CS (1999). Antiviral activity of crude aqueous extract on *Trollius chinensis* Bunge. Chin. J. Microbiol. Immunol. 19:21.
Zia-Ul-Haq M, Iqbal S, Ahmad S, Bhanger MI, Wiczkowski W, Amarowicz R (2008). Antioxidant Potential of Desi Chickpea varieties commonly consumed in Pakistan. J. Food Lipid 15:26-342.
Zia-Ul-Haq M, Ćavar S, Qayum M, Imran I, Feo V (2011a). Compositional studies: antioxidant and antidiabetic activities of *Capparis decidua* (Forsk.) Edgew. Int. J. Mol. Sci. 12(12):8846-8861.
Zia-Ul-Haq M, Ahmad S, Iqbal S, Luthria DL, Amarowicz R (2011b). Antioxidant potential of lentil cultivars. Oxid. Commun. 34:819-831.
Zia-Ul-Haq M, Shahid SA, Ahmad S, Qayum M, Khan I (2012 a). Antioxidant potential of various parts of *Ferula assafoetida* L. J. Med. Plant. Res. 6:3254-3258.
Zia-Ul-Haq M, Shahid SA, Ahmad S, Qayum M, Rasool N (2012 b). Mineral contents and antioxidant potential of selected legumes of Pakistan. J. Med. Plant Res. 6:2256-260.

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Vol. 7(17), pp. 1020-1025, 8 May, 2013
DOI 10.5897/AJPP12.544
ISSN 1996-0816 © 2013 Academic Journals
http://www.academicjournals.org/AJPP

African Journal of Pharmacy and Pharmacology

Full Length Research Paper

The activity against methicillin-resistant *Staphylococcus aureus* and quantitative structure-activity relationship (QSAR) study of aza-naphthindolizinedione derivatives

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Accepted 18 April, 2013

The antibacterial activity of aza-naphthindolizinedione derivatives has been determined. Three compounds, 15, 21 and 40 exhibited strong inhibitory activity against Gram-positive bacterial strains and especially, showed 16 times activity better than vancomycin against methicillin-resistant *Staphylococcus aureus* (MRSA). Quantitative structure-activity relationship (QSAR) study indicated that the proposed method was a useful computational tool for prediction of antibacterial activity of aza-naphthindolizinedione derivatives. The molecular descriptors contained in the optimized descriptor subsets encoded information about the van der Waals volumes and AlogP of these derivatives and thus implied the relationship between these factors and antibacterial activity.

Key words: Aza-naphthindolizinedione derivative, antibacterial agent, methicillin-resistant *Staphylococcus aureus*, quantitative structure-activity relationship.

INTRODUCTION

Methicillin-resistant *Staphylococcus aureus* (MRSA) is a prominent pathogen, which cause a public health concern worldwide and is associated with a high mortality (Ayliffe, 1997; Oliveira et al., 2001). Novel antimicrobial agents against MRSA have been introduced recently. However, the emergency of resistance and side effects for those agents raise the need for novel antimicrobial agents (Cosgrove et al., 2003; Asgarpanah and Ramezanloo, 2012). In our previous work, we found 9-bromo-substituted indolizinoquinoline-5,12-dione derivatives were inhibitors of *Escherichia coli* DNA gyrase, and showed significant activity against Gram-positive bacteria, especially especially against MRSA (Wu et al., 2011). In this study, we studied the antimicrobial activity of aza-naphthindolizinedione derivatives and Quantitative structure-activity relationship (QSAR) of the aza-naphthindolizinedione derivatives was also studied. In the investigation of QSAR, the improved hybrid particle swarm algorithm was used as a descriptor selection and parameter optimization of model. The support vector machine was then utilized to construct a nonlinear QSAR model. The results indicated that van der Waals volumes and AlogP were important properties for bioactivity of these derivatives.

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MATERIALS AND METHODS

General experiments

The aza-naphthindolizinedione derivatives were synthesized by our lab according to our previous reported methods (Shen et al., 2010a, b; Cheng et al., 2008). The purities of the tested compounds were more than 95%, analyzed by High Performance Liquid Chromatography (HPLC) on SHIMADZU LC-20A equipped with a reverse-phase Waters Sunfire C18 column, 5 µm, 4.6 × 250 mm. Gram-negative bacteria strain (E. coli ATCC 43895) and Gram-positive bacterial strains (*S. aureus* ATCC 25923 and MRSA ATCC 43300) were purchased from ATCC.

Antibacterial assay

Representative microbes, including *E. coli*, *S. aureus* and MRSA were used as test organisms. The antimicrobial activity of aza-naphthindolizinedione derivatives was measured using the standard microdilution two-fold assay in Mueller-Hinton broth (Mladenovic et al., 2010; Bommineni et al., 2010). Briefly, the mid-log phase bacterial culture at 5 ×105 CFU/ml in Mueller Hinton broth was transferred to a 96-well cell culture plate with 300 µl of culture in each well. The compounds ranging from 64 to 0.031 µg/ml in serial 2-fold dilution were added to the well and the culture without compound was used as a negative control. The cultures were incubated at 37°C for 24 h. The bacterial growth was measured by optical density at 600 nm with a microplate reader.

Descriptor calculation and preprocess

A total of 3778 molecule descriptors were calculated by using the computer program MODEL (Molecular Descriptor Lab), which is accessible free of charge for academic use (Yap et al., 2006). These descriptors could be divided into six classes: constitutional descriptors, electronic descriptors, physicochemical properties, topological indices, geometrical molecular descriptors and quantum-mechanical descriptors according to the reference manual provided by MODEL. In order to get the set of informative descriptor, the following two preprocess steps were taken: (i) the descriptors with constant values for all molecules were discarded; (ii) the descriptors that had high correlation coefficients (R) (> 0.8) with others were removed. Before applied to modeling, these descriptor values were subjected to the equation (1):

$$x(i)_{\text{new}} = \frac{x(i) - x_{\min}}{x_{\max} - x_{\min}} \quad (1)$$

Where, x(i) is descriptor value for the compound of i, x(i)new is the conversion value, xmax and xmin is the maximum and minimum value of a feature x, respectively. Therefore, each new descriptor value is in the range from 0 to 1.

Coupling support vector machine with improved hybrid particle swarm optimization and its adaptation to QSAR modeling

The support vector machine (SVM) was utilized to solve nonlinear regression estimation by the introduction of ε-insensitive loss function. In order to implement QSAR study, the SVM regression was processed by using the computer program LIBSVM which could be downloaded freely from the website (Chang and Lin, 2001). The radial basis function was selected as the kernel function due to its effectiveness and speed in training process. In the particle swarm optimization (PSO) algorithm, each individual as a particle represents a potential solution in the multidimensional search space. Subsequently, the binary version of PSO algorithm developed by Kennedy and Eberhart (Kennedy and Eberhart, 1997) was used to solve discrete combination optimization problem. In the PSO algorithm, the position of every particle was restricted to 0 and 1 binary search space and the velocity represented the probability that the position of each dimension took the value 1 or 0.

In the continuous PSO algorithm, the diversity of particle could be quickly reduced and the search might lead to premature convergence after some iteration. Similarly, in the binary PSO algorithm, the velocity of particle converged near the maximum velocity (Vmax) or minimum velocity (–Vmax) and the search did not escape from local optima. The novel method was executed through the personal best position (Pi) and the global best position (Pg) crossover operator to replace the worst particles in the current generation.

An improved hybrid PSO was proposed to overcome the drawbacks for QSAR study. In the improved algorithm, the modified binary and continuous PSO was invoked to simultaneously optimize features subsets and kernel parameters. The optimal feature subsets and kernel parameters were decided by the 5-fold cross-validation. To implement proposed method, each particle was encoded to a string that was composed of two parts: binary and decimal coding system. To the two parts, the velocity and position of each particle were updated according to the modified binary and continuous PSO, respectively. The binary coding system consisted of some binary bits for the selection of descriptors. A bit "0" implied that the corresponding descriptor was excluded from the feature subset. Otherwise, the descriptor was included. The decimal coding system included three real numbers denoting kernel parameters (C, σ, and ε). The fitness of the particle was judged by the root-mean-squared error (RMSE) and the number of descriptor chosen. Therefore, the fitness function was defined as the equation (2):

$$fitness = p \times \text{RMSE} + (1\text{-}p) \times Nc / N \quad (2)$$

Where RMSE represent the regression accuracy of SVM based on 5-fold cross-validation, Nc is the number of descriptor chosen, N is the total number of descriptor and p is the weighting coefficient controlling the tradeoff between the precision and the number of selected feature. In this study, the population size of improved hybrid PSO was 30. The termination condition was the iteration number of 10,000.

RESULTS AND DISCUSSION

Antibacterial activity

The minimum inhibitory concentrations (MIC) of aza-naphthindolizinedione derivatives (Figure 1) were assessed according to the standard microdilution two-fold assay in Mueller-Hinton broth (Mladenovic et al., 2010; Bommineni et al., 2010). The antibacterial activity was summarized in Tables 1 and 2. The results showed that these compounds exhibited low activity against Gram-negative bacteria strain, such as *E. coli*, and strong activity against Gram-positive bacterial strains including *S. aureus* (SA) and MRSA (ATCC 43300). Some compounds, such as compound 15 and 40, showed significant activity against SA and MRSA except for 9-bromo-substituted indolizinoquinoline-5,12-dione derivative

Table 1. Training set. The antibacterial activity and predicted value based on the constructed model.

Compound	Structural feature							Antibacterial activity[a] (MIC μg/ml)			-log$_{10}$MIC[b]	Predicted value
	V	X	Y	Z	R^1	R^2	R^3	*E. coli* ATCC 25922	*S. aureus* ATCC 25923	MRSA ATCC 43300		
1	CH	CH	CH	CH	CO_2Et	H	H	>64.00	0.25	8.00	-0.9031	-0.8514
2	N	CH	CH	CH	CO_2Et	H	H	64.00	8.00	16.00	-1.2041	-1.2085
3	N	CH	CH	CH	COMe	H	H	64.00	32.00	32.00	-1.5051	-1.5577
4	N	CH	CH	CH	H	H	H	>64.00	8.00	4.00	-0.6021	-0.6408
5	N	CH	CH	CH	CO_2Et	F	H	64.00	8.00	16.00	-1.2041	-0.3014
6	N	CH	CH	CH	CO_2Et	Cl	H	>64.00	4.00	2.00	-0.301	-0.3007
7	N	CH	CH	CH	CO_2Et	OH	H	>64.00	8.00	16.00	-1.2041	-1.0337
8	N	CH	CH	CH	COMe	F	H	64.00	4.00	8.00	-0.9031	-0.8978
9	N	CH	CH	CH	COMe	Cl	H	64.00	0.50	8.00	-0.9031	-0.724
10	N	CH	CH	CH	COMe	NH_2	H	64.00	16.00	16.00	-1.2041	-1.0271
11	CH	CH	CH	N	CO_2Et	H	H	8.00	0.13	0.13	0.8861	0.7658
12	CH	CH	CH	N	H	H	H	64.00	16.00	16.00	-1.2041	-1.1662
13	CH	CH	CH	N	CO_2Et	Cl	H	>64.00	2.00	2.00	-0.301	-0.3008
14	CH	CH	CH	N	CO_2Et	Br	H	64.00	0.031	0.25	0.6021	0.6796
15	CH	CH	CH	N	CO_2Et	Me	H	64.00	0.063	0.063	1.2007	1.2233
16	CH	CH	CH	N	CO_2Et	OH	H	>64.00	4.00	2.00	-0.301	-0.2809
17	CH	CH	CH	N	CO_2Et	NH_2	H	32.00	4.00	8.00	-0.9031	-0.9407
18	CH	CH	CH	N	COMe	F	H	32.00	4.00	4.00	-0.6021	-0.5643
19	CH	CH	CH	N	COMe	Cl	H	64.00	0.063	1.00	0	-0.1365
20	CH	CH	CH	N	COMe	Br	H	32.00	0.063	0.13	0.8861	0.7678
21	CH	CH	CH	N	CO_2Et	H	Br	16.00	0.063	0.063	1.2007	1.2032
22	N	CH	CH	N	CO_2Me	H	H	64.00	8.00	8.00	-0.9031	-0.9621
23	N	CH	CH	N	COMe	H	H	64.00	32.00	32.00	-1.5051	-1.4589
24	N	CH	CH	N	CN	H	H	32.00	4.00	4.00	-0.6021	-0.5271
25	N	CH	CH	N	CO_2Et	F	H	64.00	1.00	2.00	-0.301	-0.2142
26	N	CH	CH	N	CO_2H	H	H	>64.00	>64	64.00	-1.8062	-1.6531
27	CH	N	N	CH	CO_2Et	H	H	64.00	4.00	8.00	-0.9031	-0.8138
28	CH	N	N	CH	CO_2Me	H	H	>64.00	4.00	2.00	-0.301	-0.3477
29	CH	N	N	CH	CN	H	H	64.00	0.13	0.13	0.8861	0.9075
30	CH	N	N	CH	Me	H	H	>64.00	0.50	0.50	0.3010	0.3694
31	CH	N	N	CH	CO_2Et	F	H	>64.00	4.00	4.00	-0.6021	-0.6884
32	CH	N	N	CH	CO_2Et	Cl	H	>64.00	16.00	16.00	-1.2041	-1.3277
33	CH	N	N	CH	COMe	F	H	>64.00	2.00	2.00	-0.3010	-0.2615
Van										1.00		

[a]The antibacterial activity is expressed by the minimum inhibitory concentration.[b]The MIC value is against MRSA.[c]"--" means " not determined".

Table 2. Testing set. The antibacterial activity and predicted value based on the constructed model.

Compound	Structural feature							Antibacterial activity[a] (MIC μg/ml)			-log$_{10}$MIC[b]	Predicted value
	V	X	Y	Z	R^1	R^2	R^3	*E. coli* ATCC 25922	*S. aureus* ATCC 25923	MRSA ATCC 43300		
34	N	CH	CH	CH	Me	H	H	64.00	64.00	>64.00	-	-2.0005
35	N	CH	CH	CH	CO_2Et	Br	H	64.00	1.00	1.00	0	0.0002
36	N	CH	CH	CH	CO_2Et	NH_2	H	>64.00	64.00	64.00	-1.8062	-1.806
37	N	CH	CH	CH	COMe	Br	H	64.00	0.13	0.25	0.6021	0.6019
38	CH	CH	CH	N	COMe	H	H	16.00	8.00	8.00	-0.9031	-0.9033
39	CH	CH	CH	N	Me	H	H	>64.00	64.00	>64.00	-	-1.9996
40	CH	CH	CH	N	CO_2Et	F	H	64.00	0.063	0.063	1.2007	1.2009
41	CH	CH	CH	N	COMe	OH	H	>64.00	32.00	>64.00	-	-1.9998
42	CH	CH	CH	N	COMe	NH_2	H	>64.00	64.00	>64.00	-	-2.0002
43	N	CH	CH	N	CO_2Et	H	H	>64.00	4.00	8.00	-0.9031	-0.9031
44	N	CH	CH	N	Me	H	H	>64.00	32.00	>64.00	-	-2.0001
45	N	CH	CH	N	CO_2Et	OH	H	>64.00	>64.00	>64.00	-	-1.9997
46	N	CH	CH	N	COMe	F	H	32.00	8.00	8.00	-0.9031	-0.9027
47	CH	N	N	CH	COMe	H	H	>64.00	1.00	2.00	-0.301	-0.3012
48	CH	N	N	CH	H	H	H	>64.00	32.00	>64.00	-	-1.9999
49	CH	N	N	CH	CO_2Et	OH	H	>64.00	8.00	16.00	-1.2041	-2.0005
50	CH	N	N	CH	CO_2Et	Me	H	>64.00	>64.00	>64.00	-	-2.0005
51	CH	N	N	CH	CO_2Et	H	Me	64.00	32.00	>64.00	-	-2

[a]The antibacterial activity is expressed by the minimum inhibitory concentration. [b]The MIC value is against MRSA.

derivative 21 (Wu et al., 2011). The MIC values of compound 15 and 40 against MRSA were all 0.063 μg/ml. They showed 16 times antibacterial activity better than Vancomycin (Van, MIC = 1.00 μg/ml) against MRSA. These results indicated that aza-naphthindolizinedione derivatives were potential agents against MRSA.

QSAR study

In order to study the structure-activity relationship Of aza-naphthindolizinedione derivatives, QSAR was studied. When the SVM was utilized to analyze QSAR modeling, the key step was the generation and selection of molecular descriptors as well as the setting of the kernel function and corresponding parameter. For descriptor generation and selection, thousands of various type of molecular descriptor was applied to QSAR study.

However, the accuracy of model does not monotonically depend on the number of descriptor employed. It is well-known that many descriptor fed to SVM cannot only increase computational complexity but also suffer from the risk of overfitting. Whereas, few descriptor that is not relevant to bioactivity can result in low accuracy and bad generalization performance. Consequently, the selection of descriptor is necessary to speed up computation and obtain high performance model. In addition to the descriptor selection, the selection of the kernel function as well as the setting of the corresponding parameter are also every important for building model of QSAR because they define the distribution of the training set samples in the high dimensional feature space (Luan et al., 2006).To overcome the problems, an improved hybrid PSO algorithm was presented to the study of QSAR. In the method, the

method, the binary and continuous PSO was utilized to simultaneously optimize descriptor subset and kernel parameter.

In order to evaluate the performance of the current method, the entire dataset was divided into two groups: training set and testing set. The training set was composed of 33 compounds for developing regression model, while the remaining 18 compounds (34 ~ 51) were used as the testing set. The activity of these compounds was expressed as –log10MIC. A good fitness function is the key to assess the performance of each particle and to obtain high regression procession. In the study, the fitness function must be considered two objectives: (1) maximize the regression precision of 5-fold cross-validation; (2) minimize the number of selected descriptor. Based on the experience, p was set as 0.02 to balance the accuracy and the number of selected descriptor.

The training set was carried out for the QSAR study. As shown in Figure 2, the good relationship between the experimental value and the calculated value of –log10MIC was observed and the experimental and calculated activity values were listed in Table 1. The correlation coefficient (R) and RMSE of the modeling was 0.9769 and 0.1787, respectively. The results indicated the validity of the constructed model. In order to evaluate the predictive ability of the model for the external samples, the model was used to the testing set, shown in Table 2. It could be seen that the predicted activity value was very close to the experimental value for the compound with precise experimental activity. The R and RMSE of the compound was 0.9703 and 0.2655, respectively. The results suggested that the model was stabilized and had high predictive ability for the external samples.

The optimized descriptor subset contained 9 topological indices, 6 geometrical molecular and 7 quantum chemistry descriptors, respectively. In the 9 topological indices, the first one was the sum of E-state of atom type dssC, which means the C atom with a double bond and two single bonds. The second and the third were Moreau-Broto topological autocorrelation descriptors with the AlogP of the atomic as the weight with lag 4 and with the charge of the atomic as the weight with lag 7. Subsequent three topological indices were Moran topological autocorrelation descriptors with the van der Waals radius of the atom as the weight, with lag 10 as well as with the AlogP of the atom as the weight with lag 4 and 5. The seventh was the Geary topological autocorrelation descriptors with atomic polarizability as the weight with lag 5. The eighth and the ninth were 5th highest eigenvalues of the matrix with the atomic masses or polarizability of the atoms as the diagonal elements.

In the 6 geometrical molecular descriptors, the first two were the radial distribution function (RDF) descriptors with the AlogP of the atoms as the weight with lag 5 and

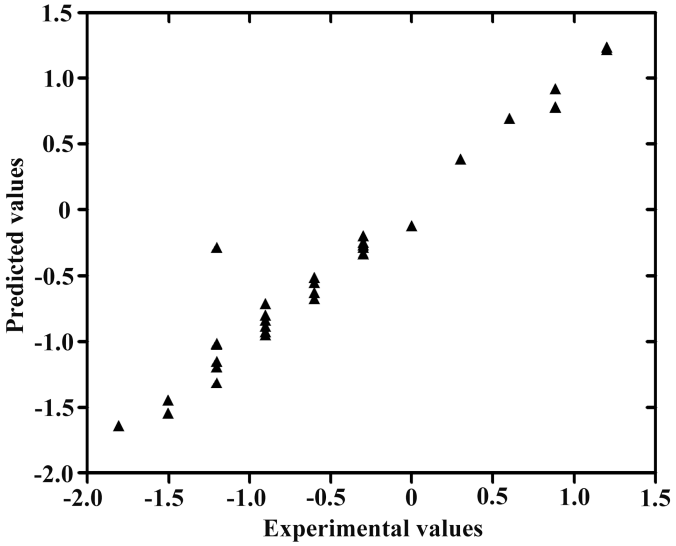

Figure 1. Calculated values versus experimental values of the activity.

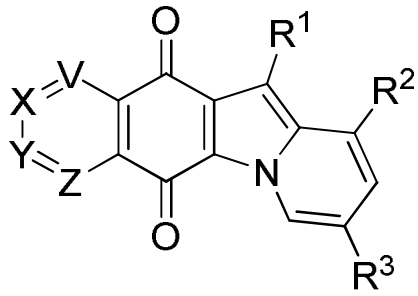

Figure 2. The structure of aza-naphthindolizinedione derivatives.

6, the third was the 1st directional weighted holistic invariant molecular (WHIM) density descriptors, and the final three were unweighted 3D-MoRSE descriptors with s = 15 and 23A0 and 3D-MoRSE descriptor with the atomic mass as the weight with s = 23 A0. In the 7 quantum chemistry descriptors, the first two were the electronic eigenvalue descriptors with x = -34.75 and -15.00 eV, the final five were the eigenvalue descriptors with x = 662.00, 1564.00, 2054.00, 3086.00 and 3448.00.

It was very difficult for interpretation of regression model because the SVM was a black box modeling technique and the relationship between the descriptors and bioactivity was embedded in the model. However, some information could still be obtained from the scrutiny of the descriptor. For example, descriptor about dssC encoded not only the information about the topological environment of the type of carbon atom but also its electronic interactions to all other atoms in molecular

level. Two Moreau-Broto descriptors described how AlogP and charge of the atom were distributed along the topological structure. Three Moran descriptors described how van der Waals radius and AlogP of the atom were distributed along the topological structure. One Geary descriptor was computed by using the atomic polarizability as the weight. Two burden eigenvalues (BCUT) descriptors described the surface distributions of atomic mass and polarizability of the atoms.

RDF descriptors provided the information about interatomic distances in the entire molecule and also other useful information such as bond distances, ring types, planar and non-planar systems, atom types and molecular weight (Yap et al., 2006). In current model of QSAR, the descriptors were computed based on the distance distribution in the three-dimension geometry of the molecule weighted by the AlogP of the atom. WHIM descriptors were derived from the 3D-information of a molecule with different weighting schemes, and contained the information about the whole molecular structure in terms of size, shape, symmetry and atom distribution. In the current study, the descriptor was related to density. Three 3D-MoRSE descriptors described the distribution of the atoms in molecule. Electronic eigenvalue descriptors and eigenvalue descriptors, which were related to the molecular orbital energy and vibration of molecular, belonged to the quantum chemistry descriptors. The analysis showed that most descriptors contained in the optimized descriptor subset were related to the van der Waals volumes and AlogP, which in general was the most important key for molecular affinity. Therefore, it could infer that these factors played the key roles to the activity of these compounds.

Conclusion

The antibacterial activity of aza-naphthindolizinedione derivatives has been determined against three bacterial strains including *E. coli*, *S. aureus* and MRSA. The results showed that some compounds exhibited stronger inhibitory activity against gram-positive bacterial strains and MRSA, which indicated that aza-naphthindolizinedione skeleton might be a potential antibacterial pharmacophore. The QSAR study exhibited that the molecular descriptors were contained in the optimized descriptor subsets encode information about the van der Waals volumes and AlogP of these derivatives, and thus implied the relationship between these factors and antibacterial activity.

ACKNOWLEDGMENTS

We gratefully acknowledge the financial support of the National Natural Science Foundation of China (No. 30801425) and National S & T Major Project (No. 2009ZX09103-042).

REFERENCES

Asgarpanah J, Ramezanloo F (2012). Chemistry, pharmacology and medicinal properties of Peganum harmala L. Afr. J. Pharm. Pharmacol. 6:1573-1580.

Ayliffe GA (1997). The progressive intercontinental spread of methicillin resistant *Staphylococcus aureus*. Clin. Infect. Dis. 24:S74-79.

Bommineni YR, Achanta M, Alexander J, Sunkara LT, Ritchey JW, Zhang GL (2010). A fowlicidin-1 analog protects mice from lethal infections induced by methicillin-resistant *Staphylococcus aureus*. Peptides 31:1225-1230.

Chang CC, Lin CJ (2001). LIBSVM: a library for support vector machine. Software. Available at http://www.csie.ntu.edu.tw/~cjlin/libsvm.

Cheng Y, An LK, Wu N, Wang XD, Bu XZ, Huang ZS, Gu LQ (2008). Synthesis, cytotoxic activities and structure–activity relationships of topoisomerase I inhibitors: indolizinoquinoline-5,12-dione derivatives. Bioorg. Med. Chem. 16:4617-4625.

Cosgrove SE, Sakoulas G, Perencevich EN, Schwaber MJ, Karchmer AW, Carmeli Y (2003). Comparison of mortality associated with methicillin-resistant and methicillin-susceptible Staphylococcus aureus bacteremia: a meta-analysis. Clin. Infect. Dis. 36:53-59.

Kennedy J, Eberhart R (1997). A discrete binary version of the particle swarm algorithm. In: Proceedings of 1997 IEEE international Conference on Systems, Man, and Cybernetics, Volume 5. Orlando, FL. pp. 4104-4108.

Luan F, Ma WP, Zhang XY, Zhang HX, Liu MC, Hu ZD, Fan BT (2006). QSAR study of polychlorinated dibenzodioxins, dibenzofurans, and biphenyls using the heuristic method and support vector machine. QSAR Comb. Sci. 25:46-55.

Mladenovic M, Vukovic N, Sukdolak S, Solujic S (2010). Design of Novel 4-Hydroxy-chromene-2-one Derivatives as Antimicrobial Agents. Molecules 15:4294-4308.

Oliveira DC, Tomasz A, de Lencastre H (2001). The evolution of pandemic clones of methicillin- resistant *Staphylococcus aureus*, identification of two ancestral backgrounds and the associated mec elements. Microb. Drug Resist. 7:349-361.

Shen DQ, Wu N, Li YP, Wu ZP, Zhang HB, Huang ZS, Gu LQ, An LK (2010a). Design, synthesis, and cytotoxicity of indolizinoquinoxaline-5,12-dione derivatives, novel DNA topoisomerase IB inhibitors. Aust. J. Chem. 63:1116-1121.

Shen DQ, Wu ZP, Wu XW, An ZY, Bu XZ, Gu LQ, Huang ZS, An LK (2010b). Synthesis and Antiproliferative Activity of Indolizinophthalazine-5,12-dione Derivatives, DNA Topoisomerase IB Inhibitors. Eur. J. Med. Chem. 45:3938-3942.

Wu XW, Wu ZP, Wang LX, Zhang HB, Chen JW, Zhang W, Gu LQ, Huang ZS, An LK (2011). Synthesis, antimicrobial activity and possible mechanism of action of 9-bromo-substituted indolizinoquinoline-5,12-dione derivatives. Eur. J. Med. Chem. 46:4625-4633.

Yap CW, Li ZR, Chen YZ (2006). Quantitative structure–pharmacokinetic relationships for drug clearance by using statistical learning methods. J. Mol. Graph. Model. 24:383-395.

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Vol. 7(17), pp. 1026-1032, 8 May, 2013
DOI 10.5897/AJPP12.595
ISSN 1996-0816 © 2013 Academic Journals
http://www.academicjournals.org/AJPP

African Journal of Pharmacy and Pharmacology

Full Length Research Paper

Inhibition of α-amylase and α-glucosidase activities by ethanolic extract of *Amaranthus cruentus* leaf as affected by blanching

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Accepted 18 April, 2013

This study investigated the inhibitory effect of *Amaranthus cruentus* leaf on key enzyme linked to type-2 diabetes (α-amylase and α-glucosidase) as well as assessing the effect of blanching (a commonly practiced food processing technique) of the vegetable on these key enzymes. Fresh leaves of *A. cruentus* were blanched in hot water for 10 min, and the ethanolic extracts of both the fresh and blanched vegetables were prepared and used for subsequent analysis. The inhibitory effect of the extract on α-amylase and α-glucosidase activities as well as some antioxidant parameter was determined *in vitro*. The result revealed that extract of unprocessed *A. cruentus* leaf reduce Fe^{3+} to Fe^{2+} and also inhibited α-amylase and α-glucosidase activities in a dose dependent manner. However, blanching of the leafy vegetables caused a significant ($P < 0.05$) increase in the antioxidant properties but decreased their ability to inhibit α-amylase and α-glucosidase activities. This antioxidant properties and enzyme inhibition could be part of the mechanism by which they are used in the treatment/prevention of type-2 diabetes. However, the blanched vegetable reduced their ability to inhibit both α-amylase and α-glucosidase activity *in vitro*.

Key words: *Amaranthus cruentus*, blanching, antioxidants, α-amylase, α-glucosidase.

INTRODUCTION

Diabetes mellitus is a chronic metabolic disorder characterized by hyperglycemia due to insulin deficiency and/or insulin resistance resulting in excess blood sugar (Beverley and Eschwège, 2003). Plants and herbal preparations have been used from ancient times for the treatment of diabetes mellitus and are still used in traditional medicine. There are several reports of wide range of plants and plants constituents that are active hypoglycemic agents (Hilary et al., 1998; Jalalpure et al., 2004; Onyeche and Kolawole, 2005). A sudden rise in blood glucose levels, causing hyperglycemia in type 2 diabetes patients happens due to hydrolysis of starch by pancreatic α-amylase and uptake of glucose by intestinal α-glucosidases (Kwon et al., 2007). The inhibition of enzymes involved in the breakdown of starch (α-amylase) and uptake of glucose (α–glucosidase) has been suggested to be a useful approach to the management and prevention of type 2 diabetes and dietary phyto-chemicals, have promising potential (Kwon et al., 2007). Amylase inhibitors are also known as starch blockers because they contain substances that prevent dietary starch from being absorbed by the body. Starches are

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complex carbohydrates that cannot be absorbed unless they are first broken down by the digestive enzyme amylase and other secondary enzymes (Ranilla et al., 2010; El-kaissi and Sherbeeni, 2011). In recent years, secondary plant metabolites previously with unknown pharmacological activities have been extensively investigated as sources of medicinal agents (Asgarpanah and Ramezanloo, 2012; Nasri et al., 2012). Vegetables contain compounds that are valuable antioxidants and protectants; the main protective action of vegetables has been attributed to the presence of antioxidants, especially antioxidant vitamins including ascorbic acid, α-tocopherol, β-carotene and phenolics (Oboh and Rocha, 2007).

However, numerous studies have conclusively shown that the majority of the antioxidant activity may be from compound such as flavonoids, isoflavone, flavones, anthocyanin, catechin and isocatechin, rather than vitamins C, E and β-carotene (Oboh and Rocha, 2007). Several green leafy vegetables with high phenolic contents abound in tropical Africa, they are utilized either as condiments or spices in human diets (Akindahunsi and Oboh, 1999); these vegetables could be harvested at all stages in the process of growth, and could be fed upon in fresh, processed, or semiprocessed forms (Oboh and Akindahunsi, 2004). They are very rich sources of β-carotene, ascorbic acid, minerals and dietary fiber (Makobo et al., 2010). Epidemiological analyses in a large Chinese population have revealed that consumption of vegetables is inversely associated with the risk of type 2 diabetes (Tang et al., 2008).

In Nigeria, unlike fruits, green leafy vegetables are not usually consumed in their fresh form; however, they are usually blanched before consumption or in soup preparation (Akindahunsi and Oboh, 1999). Blanching inactivates the enzyme action, sets the colour, and shortens the drying and dehydration time (Oboh and Akindahunsi, 2004). *Amaranthus* spp. leaves are an excellent source of protein (Kadoshnikov et al., 2005), they have also been reported to contain considerable high calcium, iron and phosphorus (Makobo et al., 2010). The vegetable has been reported to have a high concentration of antioxidant components (Hunter and Fletcher, 2002). Losses of antioxidant components from vegetables during cooking have been reported elsewhere (Chu et al., 2000; Yadav and Sehgal, 1995).

Amaranthus extract has been shown to possess antidiabetic activity in both alloxan and streptozotocin diabetic animals (Tang et al., 2008; Nwozo et al., 2004). Although a lot had been reported on the chemical characterization of phytoconstituents and antidiabetic properties of *Amaranthus* spp., limited information is available on the possible mechanism by which they render their antidiabetic properties. Hence, this study sought to investigate the inhibitory effect of *A. cruentus* on key enzyme linked to type-2 diabetes (α-amylase and α-glucosidase) as well as assessing the effect of blanching (a commonly practiced food processing technique) on these key enzymes.

MATERIALS AND METHODS

Sample collection

Fresh samples of *A. cruentus* were sourced from the University garden of The Federal University of Technology, Akure. Authentication of the vegetables was carried in the Department of Biology, Federal University of Technology, Akure, Nigeria.

Chemicals

Chemicals and reagents used such as Hog pancreatic α-amylase, gallic acid, Folin-Ciocalteau's reagent, dinitrosalicylic acid, α-glucosidase, and *p*-nitrophenyl-α-D-glucopyranoside were procured from Sigma-Aldrich, Inc., (St Louis, MO), trichloroacetic acid (TCA), quercetin, DPPH (1,1-diphenyl–2 picrylhydrazyl) were sourced from Sigma-Aldrich, Chemie GmbH (Steinheim, Germany), sodium carbonate, methanol, $AlCl_3$ (aluminium chloride), potassium acetate, potassium ferricyanide, ferric chloride and starch were of analytical grade while the water was glass distilled.

Preparation of 70% ethanol extract

The inedible parts of the vegetables were removed from the edible parts by hand picking. The edible parts were thoroughly washed in tap water to remove any dirt, and chopped into small pieces by table knife. A portion of the chopped vegetables was then blanched for 10 min at 80°C, while the other portion was not. The blanched portion was then drained of water. Both portions were then sun dried and milled to be obtained in a powder form. The powder was extracted with 70% ethanol then, the extract was filtered with Whatman filter paper and the filtrate was concentrated under reduced pressure using a freeze dryer to give a solid extract. The concentrated extract was further lyophilized. Then, the vegetable extract was reconstituted in distilled water and used for subsequent analysis.

α-Amylase inhibition assay

The α-amylase inhibitory activity was determined according to the method of Bernfield (1951). Appropriate dilutions of the vegetable extracts (500 µl) and 500 µl of 0.02 mol/L sodium phosphate buffer (pH 6.9 with 0.006 mol/L NaCl) containing Hog pancreatic α-amylase (EC 3.2.1.1) (0.5 mg/ml) were incubated at 25°C for 10 min. Then, 500 µl of 1% starch solution in 0.02 mol/L sodium phosphate buffer (pH 6.9 with 0.006 mol/L NaCl) was added to the reacting mixture. Thereafter, the reaction mixture was incubated at 25°C for 10 min and stopped with 1.0 ml of dinitrosalicylic acid (DNSA). The mixture was then incubated in a boiling water bath for 5 min and cooled to room temperature. The reaction mixture was then diluted by adding 10 ml of distilled water, and absorbance measured at 540 nm in the UV-Visible spectrophotometer (Model 6305; Jenway, Barlo world Scientific, Dunmow, United Kingdom). Then, the α-amylase inhibitory activity was calculated as percentage inhibition.

$$\% \text{ Inhibition} = [(Abs_{Ref} - Abs_{Samples}) / Abs_{Ref}] \times 100$$

α-Glucosidase inhibition assay

The α-glucosidase inhibitory activity was determined according to

the method of Apostolidis et al. (2007). Appropriate dilution of the vegetable extracts (50 µl) and 100 µl of α-glucosidase solution was incubated at 25°C for 10 min. Thereafter, 50 µl of 5 mmol/L *p*-nitrophenyl-α-D-glucopyranoside solution in 0.1 mol/l phosphate buffer (pH 6.9) was added. The reacting mixture was then incubated at 25°C for 5 min before reading the absorbance at 405 nm in the UV-Visible spectrophotometer (Model 6305; Jenway, Barlo world Scientific, Dunmow, United Kingdom). Then, the percentage of α-glucosidase inhibitory activity was calculated from inhibition.

$$\% \text{ Inhibition} = [(Abs_{Ref} - Abs_{Samples}) / Abs_{Ref}] \times 100$$

Determination of total phenol content

The total phenol content was determined according to the method of Singleton et al. (1999). Briefly, appropriate dilution of the vegetable extracts were oxidized with 2.5 ml 10% Folin-Ciocalteau's reagent (v/v) and neutralized by 2.0 ml of 7.5% sodium carbonate. The reaction mixture was incubated for 40 min at 45°C and the absorbance was measured at 765 nm in the UV-Visible spectrophotometer (Model 6305; Jenway, Bar loworld Scientific, Dunmow, United Kingdom). Gallic acid solution (0.01 to 0.1 mg/ml) was used as standard curve and then, the total phenol content was subsequently calculated as gallic acid equivalent.

Determination of total flavonoid content

The total flavonoid content was determined using a slightly modified method reported by Meda et al. (2005). Briefly, 0.5 ml of appropriately diluted sample was mixed with 0.5 ml methanol, 50 µl 10% $AlCl_3$, 50 µl 1 M potassium acetate and 1.4 ml water, and allowed to incubate at room temperature for 30 min. The absorbance of the reaction mixture was subsequently measured at 415 nm in the UV-Visible spectrophotometer (Model 6305; Jenway, Barloworld Scientific, Dunmow, United Kingdom). Quercetin solution (0.01 to 0.1 mg/ml) was used as standard curve and then, the total flavonoid content was subsequently calculated as quercetin equivalent.

Determination of reducing property

The reducing property of the vegetable extracts was determined by assessing the ability of the extract to reduce $FeCl_3$ solution as described by Oyaizu (1996). 2.5 ml aliquot was mixed with 2.5 ml 200 mM sodium phosphate buffer (pH 6.6) and 2.5 ml 1% potassium ferricyanide. The mixture was incubated at 50°C for 20 min and then 2.5 ml 10% trichloroacetic acid was added. This mixture was centrifuged at 650 rpm for 10 min. 5 ml of the supernatant was mixed with an equal volume of water and 1 ml 0.1% ferric chloride. The absorbance was measured at 700 nm in the UV-Visible spectrophotometer (Model 6305; Jenway, Barloworld Scientific, Dunmow, United Kingdom). Then, the ferric reducing antioxidant property was subsequently calculated as ascorbic acid equivalent.

Statistical analysis

The result of three replicate experiments were pooled and expressed as mean ± standard deviation. A one-way analysis of variance (ANOVA) and positive analysis was done using Duncan multiple test. Significance was accepted at $P \leq 0.05$ (Zar, 1984).

RESULTS

First, the ability of *A. cruentus* leaf extract to inhibit α-amylase activity *in vitro* was investigated and the result presented in Figure 1. The results revealed that *A. cruentus* leaf extracts inhibited α-amylase in a dose-dependent manner (0 to 0.2 mg/ml). However, as revealed by the EC_{50} (extract concentration causing 50% enzyme inhibition) values (Table 1), unprocessed *A. cruentus* (0.32 mg/ml) had a significantly ($P < 0.05$) higher α-amylase inhibitory activity than blanched *A. cruentus* (0.72 mg/ml). Furthermore, the ability of the vegetable extracts to inhibit α-glucosidase activity *in vitro* was also investigated and the result is presented in Figure 2. The results revealed that *A. cruentus* leaf extracts inhibited α-glucosidase in a dose-dependent manner (0 to 0.2 mg/ml). However, as revealed by the EC_{50} (extract concentration causing 50% enzyme inhibition) values (Table 1), unprocessed *A. cruentus* (0.21 mg/ml) had a significantly ($P < 0.05$) higher α-glucosidase inhibitory activity than blanched *A. cruentus* (0.29 mg/ml).

The result of the total phenol and flavonoid content of *A. cruentus* leaf is presented in Table 2. The result revealed that there was a significant ($P < 0.05$) difference between the total phenol content of unprocessed *A. cruentus* leaf (9.3 mg/100 g) and blanched *A. cruentus* leaf (7.0 mg/100 g). Also, unprocessed *A. cruentus* leaf (3.6 mg/100 g) had a significantly ($P < 0.05$) higher total flavonoid content than blanched *A. cruentus* leaf (1.4 mg/100 g). The reducing power of *A. cruentus* leaf is presented as ascorbic acid equivalent in Figure 3. The result revealed that *A. cruentus* leaf was able to reduce Fe (III) to Fe (II). However, blanched *A. cruentus* (36.2 mg AAE/100 g) had a significantly ($P < 0.05$) higher reducing power than unprocessed *A. cruentus* leaf (29.2 mg AAE/100 g).

DISCUSSION

Management of the blood glucose level is a critical strategy in the control of diabetes complications. Inhibitors of saccharide hydrolysing enzymes (α-amylase and α-glucosidase) have been useful as oral hypoglycemic drugs for the control of hyperglycemia especially in patients with type-2 diabetes mellitus. Inhibition of these enzymes delay carbohydrate digestion and prolong overall carbohydrate digestion time, causing a reduction in the rate of glucose absorption and consequently reducing the postprandial plasma glucose rise (Kwon et al., 2007). The results as presented in Figure 1 revealed that unprocessed *A. cruentus* had a significantly ($P < 0.05$) higher α-amylase inhibitory activity than blanched *A. cruentus*. This significant ($P < 0.05$) decrease in the inhibition of α-amylase activity as a result of blanching of the vegetable could be attributed to the damage/loss of physiologically active phytochemicals having α-amylase inhibitory activities during the heat processes involved in blanching such as observed in phenol content (Table 2). Nevertheless, the determined α-amylase inhibitory activity of the vegetable agreed with some earlier reports

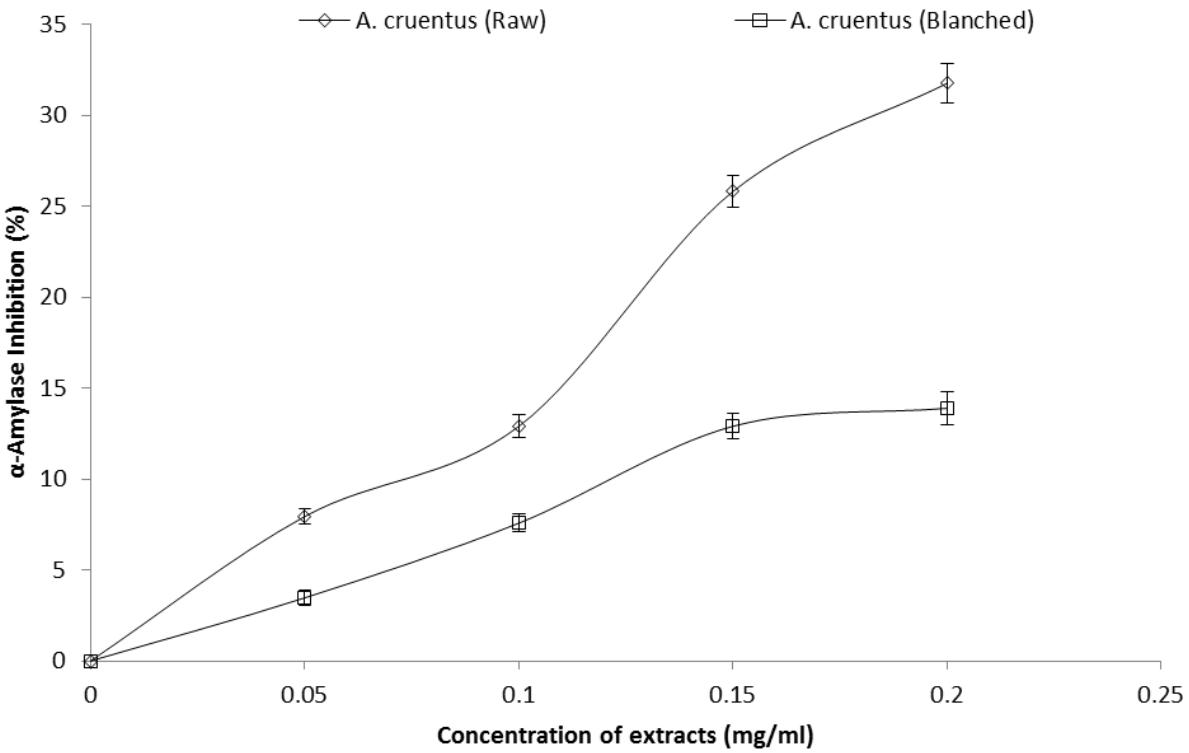

Figure 1. α-Amylase inhibitory activity of *Amaranthus cruentus* leaf extract. Values represent mean ± standard deviation, n = 3.

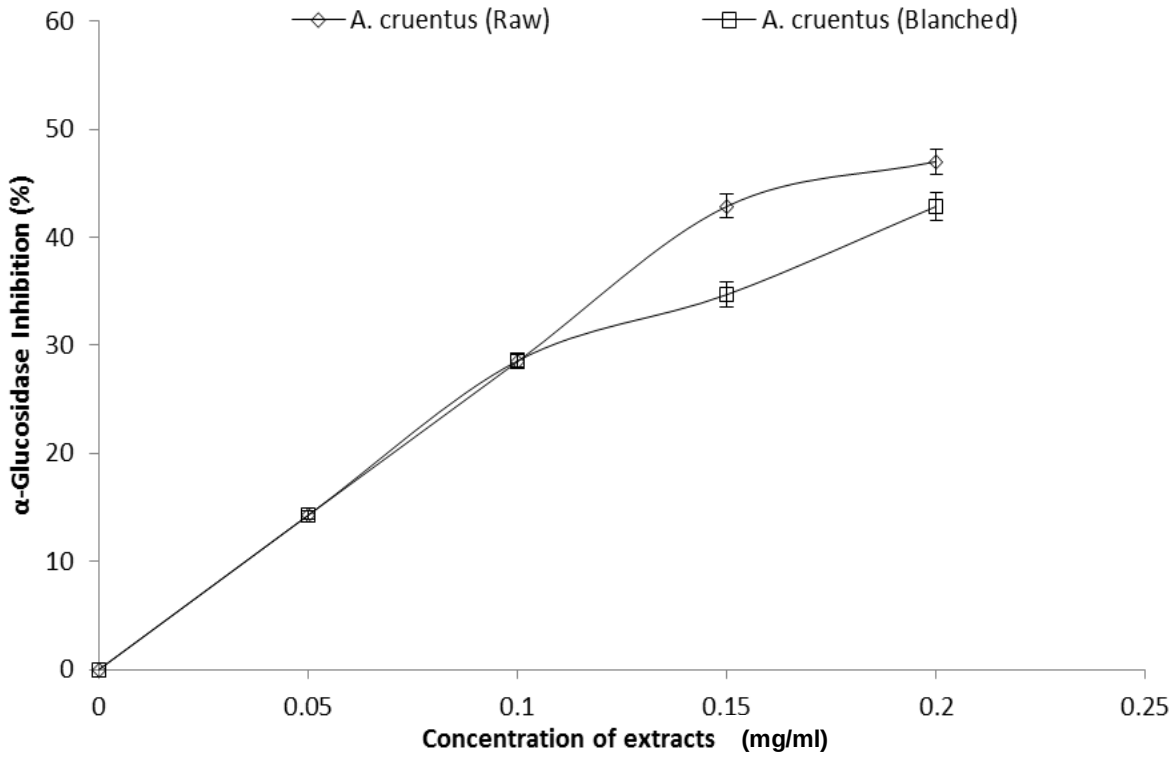

Figure 2. α-Glucosidase inhibitory activity of *Amaranthus cruentus* leaf extract**.** Values represent mean ± standard deviation, n = 3.

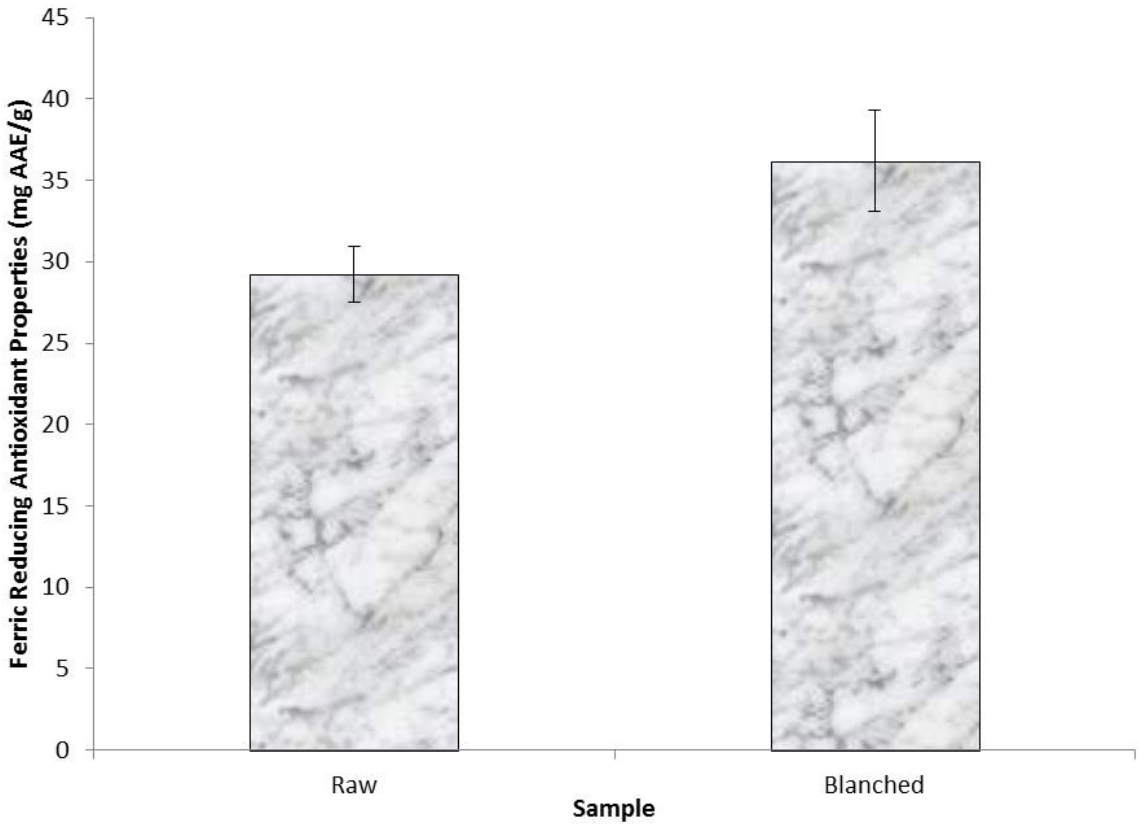

Figure 3. Ferric reducing antioxidant properties (FRAP) of *Amaranthus cruentus* leaf**.** Values represent mean ± standard deviation, n = 3.

where plant phytochemicals from pepper inhibited saliva α-amylase activity (Kwon et al., 2007) and inhibitory effects of *Allium* spp. on α-amylase activity (Nickavar and Yousefian, 2009). This also agreed with a recent worked where with bitter leaf inhibited α-amylase activity *in vitro* (Saliu et al., 2012).

Furthermore, the vegetable extracts inhibited α-glucosidase activity *in vitro* as presented in Figure 2. The results revealed that unprocessed *A. cruentus* had a significantly ($P < 0.05$) higher α-glucosidase inhibitory activity than blanched *A. cruentus*. This significant ($P < 0.05$) decrease in the inhibition of α-glucosidase activity as a result of blanching of the vegetable could not be categorically stated, however, it could be attributed to the excessive loss of physiologically active phytochemicals as a result of blanching such as observed in Table 2. The determined α-glucosidase inhibitory activity follows the same pattern as observed in Figure 1. This result is in agreement with a recent work reported by Saliu et al. (2012) where bitter leaf inhibited α-glucosidase activity *in vitro*.

The results of the enzyme (α-amylase and α-glucosidase) inhibitory assays showed that ethanolic extract of the *A. cruentus* leaves were strong inhibitors of α-glucosidase but mild inhibitors of α-amylase as shown in Figures 1 and 2. This however, is in agreement with earlier reports that showed that plant phytochemicals are mild inhibitors of α-amylase and strong inhibitors of α-glucosidase activity (Kwon et al., 2007), a property that confers advantage over synthetic drugs such as acarbose; used by diabetics in the management of postprandial blood glucose, which strongly inhibit α-amylase. Stronger inhibition of α-glucosidase activity and mild inhibition of α-amylase activity of the ginger extracts could address the major drawback of currently used α-glucosidase and α-amylase inhibitor drugs with side effects such as abdominal distention, flatulence, meteorism and possibly diarrhea (Pinto et al., 2009).

It has been suggested that such adverse effects might be caused by the excessive pancreatic α-amylase inhibition resulting in the abnormal bacterial fermentation of undigested carbohydrates in the colon (Kwon et al., 2007). Therefore, this study buttress the claim that natural inhibitors from dietary plants have mild inhibitory effect on α-amylase activity but strong α-glucosidase inhibitory activity, and could be used as effective therapy for the management of postprandial hyperglycemia with minimal side effects (Kwon et al., 2007). This agrees with the finding on eggplant phenolics, which have been recommended as a choice diet for the management of

Table 1. EC_{50} values (mg/ml) of α–amylase inhibitory and α–glucosidase activity of *Amaranthus cruentus* leaf as affected by blanching.

Sample	α-Amylase (mg/ml)	α-Glucosidase (mg/ml)
Fresh	0.32^{b}±0.03	0.19^{a}±0.01
Blanched	0.72^{c}±0.05	0.29^{b}±0.01

Values represent mean ± standard deviation of triplicate experiments. Values with the same superscript letter along the same column are not significantly different ($P < 0.05$).

Table 2. Total phenol and flavonoid content of *Amaranthus cruentus* leaf (mg/100g) as affected by blanching.

Sample	Total phenol (mg/100 g)	Total flavonoid (mg/100 g)
Fresh	9.3^{a}±0.05	3.6^{a}±0.30
Blanched	7.0^{b}±0.30	1.4^{b}±0.00

Values represent mean ± standard deviation of triplicate experiments. Values with the same superscript letter along the same column are not significantly different ($P < 0.05$).

type 2 diabetes (Pinto et al., 2009). Also, this agrees with Saliu et al. (2012) for bitter leaf extract.

The result of the total phenol and flavonoid content of *A. cruentus* leaf revealed that there was a significant ($P < 0.05$) difference between the total phenol and flavonoid contents of unprocessed *A. cruentus* leaf and blanched *A. cruentus* leaf. The values obtained were lower than what Oboh and Akindahunsi (2004) reported for some tropical green leafy vegetables (1 to 3 mg/g). The difference in phenolic value is as a result of the extraction medium used in the study. However, there was a decrease in the flavonoid content due to blanching. The basis of the decrease could not be categorically stated, however, it could be that during blanching, some of the flavonoids would have been leached into the water. However, the result was in agreement with Chen and Lin (2007) that phenolics content in cooked yams prepared at different temperatures (50 to 100°C) was lower compared to the raw ones. Also, this result was in line with Chung et al. (2008) that more than 40% of phenolic content in yam peels were lost after blanching at 85°C for 30 s.

Phenolic compounds can protect the human body from free radicals whose formation is associated with the normal metabolism of aerobic cells. They are strong antioxidants capable of removing free radicals, chelate metal catalysts, activate antioxidant enzymes, reduce α-tocophenol radicals and inhibit oxidases (Oboh and Rocha, 2007). The presence of derivatives of flavonoids has been found in many fruits and vegetables; moreover, numerous studies have conclusively shown that the majority of the antioxidant activity maybe from compounds such as flavonoids, isoflavones, flavones, anthocyanins, catechin and isocatechin rather than from vitamins C, E and β-carotene (Rong, 2010). Flavonoids have antioxidant activity and could therefore lower cellular oxidative stress (Rong, 2010).

Polyphenols are considered to be strong antioxidants due to the redox properties of their hydroxyl groups (Rong, 2010). Reducing power is a novel antioxidation defence mechanism; the mechanisms available to affect this property are by electron transfer and hydrogen atom transfer (Dastmalchi et al., 2007). This is because the ferric-to-ferrous ion reduction occurs rapidly with all reductants, with half reaction reduction potentials above that of Fe^{3+}/Fe^{2+}, the values in the ferric reducing antioxidant property (FRAP) assay will express the corresponding concentration of electron-donating antioxidants (Dastmalchi et al., 2007). The reducing power of *A. cruentus* leaf revealed that blanched *A. cruentus* had a significantly ($P < 0.05$) higher reducing power than unprocessed *A. cruentus* leaf. The basis for the significant increase in the reducing power could not be categorically stated, however, it could be reasoned out that the temperature at which blanching is carried out would have enhance the activity of the phenolic compound or other Fe^{3+} reducing agents in the blanched vegetable to the extent that the high phenol content observed in the unprocessed vegetable could not shield their effect.

Conclusion

A. cruentus leaf exhibited antioxidant properties and inhibited α-amylase and α-glucosidase (key enzyme linked to type-2 diabetes) activities. This antioxidant properties and enzyme inhibition could be part of the possible mechanism by which *A. cruentus* leaf is used in the management/prevention of type-2 diabetes. However, blanching of the vegetable could reduce their ability to inhibit both α-amylase and α-glucosidase activity, but could enhance their antioxidant properties *in vitro*.

REFERENCES

Akindahunsi AA, Oboh G (1999). Effect of some post-harvest treatments on the bio-vailability of zinc from some selected tropical vegetables. La Riv Ital Delle Sost Grasse 76:285-287.

Apostolidis E, Kwon YI, Shetty K (2007). Inhibitory potential of herb, fruit, and fungal-enriched cheese against key enzymes linked to type 2 diabetes and hypertension. Innov. Food Sci. Emerg. Technol. 8:46-54.

Asgarpanah J, Ramezanloo F (2012). Chemistry, pharmacology and medicinal properties of Peganum harmala L. Afr. Pharm. Pharmacol. 6(22):1573-1580.

Bernfield P (1951). Enzymes of starch degradation and synthesis. Adv. Enzymol. 12:379-380.

Beverley B, Eschwège E (2003). The diagnosis and classification of diabetes and impaired glucose tolerance. In: John C Pickup, Gareth Williams (eds.), Textbook of Diabetes 1, Third edition. Chapter 2, pp. 21-211.

Chen YT, Lin KW (2007). Effects of heating temperature on the total phenolic compound, antioxidative ability and the stability of dioscorin of various yam cultivars. Food Chem. 101(3):955-963.

Chu YH, Chang CL, Hsu HF (2000). Flavonoid content of several vegetables and their antioxidant activity. J. Sci. Food Agric. 80:561-566.

Chung YC, Chiang BH, Wei JH, Wang CK, Chen PC, Hsu CK (2008). Effects of blanching, drying and extraction processes on the antioxidant activity of yam (*Dioscorea alata*). Int. J. Food Sci. Technol. 43(5):859-864.

Dastmalchi K, Dorman HJD, Kosar M, Hiltunen R (2007). Chemical composition and in vitro antioxidant evaluation of a water soluble Moldavian balm (*Dracocephalum moldavica* L.) extract. Lebensm. Wissen. Und. Technol. 40:239-248.

El-kaissi S, Sherbeeni S (2011). Pharmacological Management of Type 2 Diabetes Mellitus: An Update. Curr. Diabetes Rev. 7(6):392-405.

Hilary K, Ronald EA, William HH (1998). Global burden of diabetes 1995-2005. Prevalence, numerical estimates and projections. Diabetes care 21(9):141-143.

Hunter KJ, Fletcher JM (2002). The antioxidant activity and composition of fresh, frozen, jarred and canned vegetables. Innov. Food Sci. Emerg. Technol. 3:399-406.

Jalapure SS, Patil MB, Pai A, Salahuddin MD (2004). Anti-diabetics activity of *Cassia auriculata* seed in alloxan induced diabetes rats. Nig. J. Nat. Prod. Med. 8:22-23.

Kwon YI, Apostolidis E, Kim YC, Shetty K (2007). Health benefits of traditional corn, beans and pumpkin: In vitro studies for hyper-glycemia and hypertension management. J. Med. Food 10: 266-275.

Makobo ND, Shoko MD, Mtaita TA (2010). Nutrient content of Amaranth (*Amaranthus cruentus* L.) under different processing and preservation methods. World J. Agric. Sci. 6:639-643.

Meda A, Lamien CE, Romito M, Millogo J, Nacoulma OG (2005). Determination of the total phenolic, flavonoid and proline contents in Burkina Fasan honey, as well as their radical scavenging activity. Food Chem. 91:571-577.

Nasri S, Anoush M, Khatami N (2012). Evaluation of analgesic and anti-inflammatory effects of fresh onion juice in experimental animals. Afr. J. Pharm. Pharmacol. 6(23):1679-1684.

Nickavar B, Yousefian N (2009). Inhibitory effects of six *Allium* species on α-amylase enzyme activity. Iran J. Pharm. Res. 8(1):53-57.

Oboh G, Akindahunsi AA (2004). Change in the ascorbic acid, total phenol and antioxidant activity of sun-dried commonly consumed green leafy vegetables in Nigeria. Nutr. Health 18:29-36.

Oboh G, Rocha JBT (2007). Antioxidant in Foods: A New Challenge for Food Processors: Leading Edge Antioxidants Research. Nova Science Publishers Inc. New York USA, pp. 35-64.

Onyeche OC, Kolawole JA (2005). Preliminary screening of aqueous extract of the leaves of *Securidaca longepedunculata* (Linn) for anti-hyperglycemic property. Niger. J. Pharm. Res. 49(2):18-21.

Oyaizu M (1996). Studies on products of browning reaction: antioxidative activity of products of browning reaction prepared from glucosamine. Jpn. J. Nutr. 44:307-315.

Pinto MDS, Ranilla LG, Apostolidis E, Lajolo FM, Genovese MI, Shetty K (2009). Evaluation of anti-hyperglycemia and anti-hypertension potential of native Peruvian fruits using *in vitro* models. J. Med. Food 12:278-291.

Ranilla LG, Kwon YI, Apostolidis E, Shetty K (2010). Phenolic compounds, antioxidant activity and *in vitro* inhibitory potential against key enzymes relevant for hyperglycemia and hypertension of commonly used medicinal plants, herbs and spices in Latin America. Bioresour. Technol. 101(12):4676-89.

Rong T (2010). Chemistry and Biochemistry of Dietary Polyphenol; A Review. Nutrients 2:1231-1246.

Saliu JA, Ademiluyi AO, Akinyemi AJ, Oboh G (2012). In vitro antidiabetes and antihypertension properties of phenolic extracts from bitter leaf (*Vernonia amygdalina* Del.). J. Food Biochem. 36:569-575.

Singleton VL, Orthofer R, Lamuela-Raventos RM (1999). Analysis of total phenols and other oxidation substrates and antioxidants by means of Folin–Cioalteau reagent. Meth. Enzymol. 299:152-178.

Tang GY, Li XJ, Zhang HY (2008). Antidiabetic components contained in Vegetables and Legumes. Molecules 13:1189-1194.

Yadav SK, Sehgal S (1995). Effect of home processing on ascorbic acid and b-carotene content of spinach and amaranth leaves. Plant Foods Hum. Nutr. 47:125-131.

Zar JH (1984). Biostatistical analysis, USA: Prentice-Hall Inc. P 620.

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Vol. 7(17), pp. 1033-1037, 8 May, 2013
DOI 10.5897/AJPP12.709
ISSN 1996-0816 © 2013 Academic Journals
http://www.academicjournals.org/AJPP

African Journal of Pharmacy and Pharmacology

Full Length Research Paper

Effect of dexmedetomidine on myocardial oxygen consumption during extubation for old patients: A bispectral index-guided observation study

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Accepted 18 October, 2012

The aim of this study was to investigate the effect of dexmedetomidine (DEX) maintenance on myocardial oxygen consumption during extubation for generally-anesthetized old patients under bispectral index (BIS) monitoring. A total of 40 patients who were subjected to thyroid operation and laparoscopic cholecystectomy under general anesthesia (ASA I or II) were randomized into the experimental (n = 20) and control (n = 20) groups. General anesthesia was induced using midazolam, etomidate, sufentanil, and vecuronium bromide and was maintained using propofol, remifentanil, and atracurium besilate. The experimental group received micropump infusion of DEX at 0.2 ug kg^{-1} h^{-1} from 30 min before the end of operation to the end of extubation. The control group was given physiological saline with the same volume during the same period. BIS monitors were connected. Hemodynamic indexes [systolic blood pressure (SBP), diastolic arterial blood pressure (DBP), and heart rate (HR)] were recorded, and myocardial oxygen consumption index and the recovery time of consciousness were determined. HR of the experimental group decreased from 65 ± 8 to 60 ± 5 times/min at 10 min after micropump infusion, whereas that of the control group increased from 73 ± 10 to 85 ± 12 times/min, showing a significant difference ($P < 0.01$). Both groups did not show significant changes in HR during the following maintenance period. The two groups showed significant differences in SBP, DBP, HR, and BIS at 1, 5, and 10 min during extubation period ($P < 0.05$). They did not show any significant difference in extubation score, the recovery time of consciousness, or extubation time ($P > 0.05$). BIS-guided DEX has a stable effect on myocardial oxygen consumption in generally-anesthetized old patients during extubation period. It has no obvious influences on extubation score and the recovery time of consciousness. Thus, 0.2 ug kg^{-1} h^{-1} is a proper DEX micropump infusion rate.

Key words: Bispectral index, dexmedetomidine, myocardial oxygen consumption.

INTRODUCTION

Post-operative endotracheal catheter extraction can easily cause restlessness, bucking, hypertension, and increased heart rate (HR) to patients; to the elderly undergoing generally anesthesia, it easily causes a change in myocardial oxygen consumption during the recovery time of consciousness (Basali et al., 2000; Tanskanen et al., 2006). Particularly, due to vascular elastic change, the extubation is more likely to cause hemodynamic changes in old patients or even an increase in myocardial oxygen consumption in them which leads to myocardial ischemia and arrhythmia, and increases their post-operative risks. Rate-pressure product (RPP) is an

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index which is primarily used for myocardial oxygen consumption observance. RPP increases when myocardial oxygen requirement or consumption exceeds myocardial oxygen supply. As an increase in RPP can ultimately lead to pathological changes in myocardial cells and tissues, which can further induce risk factors of angina pectoris and myocardial infarction, reducing myocardial oxygen consumption during operation plays an important role in decreasing the risk of coronary artery blood-supply insufficiency.

Dexmedetomidine (DEX) is a highly selective adrenoceptor α2 agonist. DEX has sedative and analgesic effects which can inhibit sympathetic tone; it can decrease blood pressure (BP) and slow HR down in a dose-dependent manner to enhance hemodynamic stability; in addition, it has little influence on respiration (Souter et al., 2007). The application of DEX in anesthesia induction can relieve patients' reactions to tracheal cannula (Nenmann et al., 2009). DEX in total intravenous anesthesia for medium and minor operations can ease hemodynamic reactions and effectively reduce myocardial oxygen consumption during extubation (Han et al., 2011). Yet, to the best of our knowledge, there is no study on the effect of DEX on myocardial oxygen consumption during extubation under bispectral index (BIS) monitoring reported in literature.

In the current study, old patients undergoing general operations were selected and divided into two groups. They were respectively given DEX and physiological saline from the time close to the end of the operations. The effect of DEX on myocardial oxygen consumption index was then BIS-monitored.

MATERIALS AND METHODS

General data

A total of 40 patients, including 12 undergoing thyroid operation and 28 undergoing laparoscopic cholecystectomy under general anesthesia (ASA I or II) in Dongyang Peoples' Hospital between January and November, 2011 were involved in this study. This study was conducted in accordance with the declaration of Helsinki. This study was conducted with approval from the Ethics Committee of Dongyang People's Hospital. Written informed consent was obtained from all participants. Their ages ranged from 60 to 75 years. Among them, 18 were males and 22 were females. Patients with uncontrolled endocrine diseases, hyperthyroidism, and serious cardiovascular diseases were excluded from the current study. The enrolled patients were randomized into the experimental and control groups with 20 in each. The experimental group was given DEX micropump infusion from 30 min before the end of operation, whereas the control group was given physiological saline with the same volume from the same time point. The double-blind method was adopted.

Anesthesia

Electrocardiogram (ECG), pulse blood oxygen saturation (SpO_2), invasive blood pressure (ABP), and BIS index were monitored in the operating room. Peripherally intravenous administration route was opened. Anesthesia was induced with midazolam (Jiangsu Nhwa, China; Batch No.: 20020512) at 0.02 mg/kg and sufentanil citrate (Yichang Humanwell, China; Batch No.: 2101020) at 0.5 ug/kg through a Murphy's dropper, etomidate fat emulsion (Yichang Humanwell, China; Batch No.: 20010512) at 0.08 mg/kg, and vecuronium bromide (Zhejiang Xianju, China; Batch No.: 11011071. 1) at 0.1 mg/kg. Five minutes later, endotracheal intubation was performed under direct vision. Afterwards, the patient received micropump infusion of propofol (Sichuan Guorui, China; Batch No.: 1101056) and remifentanil hydrochloride (Yichang Humanwell; Batch No.: 2101020), injection of atracurium besilate (Shanghai Hengrui, China; Batch No.: 11020221), and oral administration of sevoflurane (Shanghai Hengrui; Batch No.: 11010222). Sevoflurane and atracurium besilate were stopped at 30 min before the end of operation, but the micropump infusion of propofol and remifentanil was continued till the end of the operation. Micropump infusion of DEX (Jiangsu Hengrui) at 0.2 ug kg^{-1} h^{-1} was given from 30 min before the end of operation to the end of extubation.

Observational indexes

Blood pressure (BP), HR, BIS, and myocardial oxygen consumption index before operation (T_0), 30 min before the end of the operation (T_1), 1 (T_3) during extubation, 5 (T_4), and 10 min (T_5) after extubation, as well as bucking and restlessness during extubation were recorded. Myocardial oxygen consumption was evaluated based on RPP. A value < 12,000 is counted normal, whereas a value beyond that threshold reflects an increase in myocardial oxygen consumption, which suggests possible myocardial ischemia. The reaction severity of bucking during extubation was graded into: (1) no bucking with stable respiration; (2) non-continuous mild bucking; (3) moderate bucking with continuous time < 30 s; and (4) severe bucking with continuous time ≥ 30 s. The severity of restlessness during extubation was scored into: (1) 0: patients were quiet and cooperative; (2) 1: there was limb restlessness at the time of sputum aspiration; (3) 2: limbs struggled even without stimuli, but such restlessness did not need to be stopped externally; and (4) 3: patients struggled violently, and they had to be held down.

Statistical analysis

Data were presented as means ± standard error and analyzed by the statistical package for social sciences (SPSS 11. 5 software). Normality test was used to determine the Shapiro-Wilk test. Paired t-test was performed for comparison between groups on the basis of analysis of variance, and χ^2-test was performed for enumeration data. $P < 0.05$ was considered significant.

RESULTS

General data

The two groups did not show any significant difference in age, sex, body weight, or operating time (Table 1).

The recovery time of consciousness

The two groups did not show any significant difference in the recovery time of spontaneous breathing or extubation time; although the experimental group showed a delay in eye opening time when compared with the control group,

Table 1. Comparisons of the general data between groups (mean ± SD).

Group	n	Sex (male/female)	Age (years)	Height (cm)	Weight (kg)	Operating time (min)
Experimental	20	11/9	44. 67±8.56 67. 67±6.56	155.65±11.78	54.78±6.53	156±35.35
Control	20	10/10	45. 68±5.86 68. 68±5.86	155.86±9.67	48.48±7.59	158±32.65

Table 2. Comparisons of the recovery time of consciousness between groups (mean ± SD).

Group	n	Spontaneous respiration (min)	Eye opening (min)	Extubation time (min)
Experimental	20	11±03	12±03	13±04
Control	20	10±04	10±02	13±03

such a difference was not significant. The results are shown in Table 2.

BP, HR, myocardial oxygen consumption, and BIS at different time points

The two groups did not show any significant difference in systolic blood pressure (SBP), diastolic arterial blood pressure (DBP), HR, myocardial oxygen consumption, or BIS before extubation. At 3 and 5 min after micropump infusion, these indexes in the experimental group decreased when compared with the control group, but no significant differences were observed. At the post-extubation instant time point, 1, 3, 5, and 10 min, they significantly decreased in the experimental group, compared with the control group ($P < 0.05$). The results are shown in Table 3.

Restlessness scores

The restlessness scores in the experimental group were significantly lower than those in the control group ($P < 0.05$) (Table 4).

DISCUSSION

Generally-anesthetized patients enter into the state of light anesthesia before extubation in which the catheter can cause stimulation to the respiratory tract. The stimulation transmits injurious nerves to the medulla oblongata cardiovascular center through airway circulation to cause the release of catecholamine, leading to great changes in BP and HR as well as an increase in myocardial oxygen consumption. This condition, plus the existence of a more or less certain degree of cardiovascular disease in old patients can pose a greater risk of a cardiovascular accident. Apart from the release of catecholamines, the stimulation can also cause the organism to produce inflammatory reactions which can further result in adverse effects to the patient (Groeneweg et al., 2009). Although lidocaine and fentanyl can alleviate severe coughing and cardiovascular reactions, they cannot achieve a satisfactory effect (Zamora et al., 2007). Based on this finding, anesthetics with a short-term and rapid effect are clinically recommended during generally-anesthetic extubation period nowadays; these drugs enable patients to establish protective responses on the one hand, and allow them a sufficient ventilation drive and a stable cardiovascular system on the other hand (Su et al., 2010).

Propofol has a good and complete sedative effect and short recovery time of consciousness. Meanwhile, it has the effects of dilating peripheral blood vessels, inhibiting the vasomotor center, blocking sympathetic terminals to release noradrenaline, and anti-vomiting (Lee et al., 2009). DEX is another effective anesthetic. Its action sites are mainly located at the sympathetic nerve endings of the central nervous system (the pallium and medulla) and peripheral nervous system, where the excited receptors can inhibit adenylate cyclase to deactivate potassium and calcium channels. This condition further reduces the release of noradrenaline, bringing about the effects of low BP, sinus bradycardia, sedation, and analgesis (Sanders and Maze, 2007; Schlichter, 2010). Rui fentanyl is a fentanyl μ-type opiate receptor agonist. It has the properties of fast blood-brain balance, which can be reached within 1 min, and high susceptibility to metabolism in blood and other tissues, which endow rui fentanyl with the virtues of rapid action onset time and short lasting time (only 5 to 10 min). Further, long time infusion or repeated injection of rui fentanyl has no influence on its metabolic rate, nor results in internal accumulation.

Table 3. Comparisons of SBP, DBP, HR, myocardial oxygen consumption, and BIS between groups (mean ± SD).

Index group	n	T_0	T_1	T_2	T_3	T_4	T_5
SBP experimental	20	125±15	118±12	122 ±14	129 ±14[b]	124±15	122±16
(mmHg) Control	20	128±11	114±80	123±11	138±16	133±14	130±13
DBP experimental	20	75±08	72±07	76±08	76±09	73±07	73±06
(mmHg) Control	20	78±09	73±09	78±12	87±13	82±11	84±10
HR experimental	20	72±06	62±08	65±07	66±08[b]	65±07[b]	64±05[b]
(Times/min) Control	20	76±10	67±08	73±10	79±14[a]	81±13[a]	85±12[a]
RPP experimental	20	93±11	69±09	68±12	70±15[b]	72±13[b]	70±13[b]
(%) Control	20	96±14	70±10	80±20	110±20	102±18	104±20
BIS experimental	20	94±03	70±06	85±03	87±03	93±02	94±03
Control	20	94±04	71±05	87±02	88±02	94±02	95±02

[a] $P < 0.05$, compared with the reference value, and [b] $P < 0.05$, compared with the control group.

Table 4. Comparisons of restlessness score between groups (mean ± SD).

Group	n	T_0	T_1	T_2	T_3	T_4	T_5
Experimental	20	0.82±0.21[a]	0.75±0.12[a]	0.55±0.16[a]	0.58±0.08[a]	0.33±0.12[a]	0.35±0.14[a]
Control	20	1.65±0.46	1.50±0.31	1.53±0.35	1.48±0.45	1.26±0.23	1.16±0.43

[a] $P < 0.05$, compared with the control group.

Nowadays, the sympatholytic activity of DEX is presumed to be primarily manifested by a decrease in HR because its application for patients who have received beta-blockers cannot bring about such a decrease (Jalonen et al., 1997). Some scholars also propose a second mechanism for DEX-induced HR decrease, that is, the application of DEX may increase cardiac vagal tone (Laubie et al., 1979). According to the previous studies, the application of DEX effectively avoids BP volatile fluctuation (Bekker et al., 2008; Abdullah et al., 2012). In the present study, SBP, DBP, HR, and myocardial oxygen consumption index in the experimental group kept stable, compared with the pre-operative reference values, whereas those in the control group showed significant increases. These findings seem to suggest that DEX can excite medullispinal and peripheral α_2A and α_2C adrenergic receptors to perform an analgesic effect (Hofer et al., 2009).

DEX can reduce the administered dose of analgesics, and can prevent the occurrence of nausea and emesis (Al-Zaben et al., 2010; Massad et al., 2009). A plasma propofol concentration at 1 mg/L in target controlled infusion during extubation can achieve a good sedative effect (Wang and Chen, 2008). Compared to propofol, although DEX shows a delay in action onset time, it can achieve a similar effect 25 min later (Arain and Ebert, 2002). BIS is an index used to reflect the electrical activity of the brain (Muhammad et al., 2012).

In the present study, the result showed that the experimental and control groups had no significant difference in this index, indicating that DEX combined with propofol does not result in a change in the recovery time of consciousness (Ohtani et al., 2008). A study has reported that DEX combined with sevoflurane does not prolong the recovery time of consciousness, but its combination with propofol does (Hofer et al., 2009). But in two other studies, a different result was obtained. The two different combination manners do not result in a significant difference in consciousness recovery time (Salman et al., 2009; Turgut et al., 2008). This study showed that the experimental and control groups had no significant differences in the recovery time of spontaneous respiration, extubation time, and eye opening time even though the experimental group had a delay in eye opening time when compared with the control group.

The application of DEX can reduce the catecholamine concentration in plasma (Jaakola et al., 1992; Talke et al., 2000; Zhou et al., 2012). The results in this study showed that the experimental group had obviously reduced restlessness reactions after extubation and lessened pain after operation; the restless scores in the experimental group at different time points were significantly lower than those in the control group. These findings indicate that the combination of DEX on the basis of the maintained administration of propofol and remifentanil before the end of operation can effectively inhibit reactions to extubation. Further, this study showed that the patients in the experimental group were more cooperative without restlessness and gastrointestinal reactions before and

after extubation.

Conclusion

Micropump infusion of DEX at 0.2 ug kg^{-1} h^{-1} from 30 min before operation to the end of extubation can ease old patients' reactions to extubation; it reduces myocardial oxygen consumption to decrease the risk of cyclic fluctuation caused by coronary artery blood-supply insufficiency; furthermore, it does not delay consciousness recovery time. Therefore, DEX at such a dosage has a reliable and safe effect on extubation-induced reactions, and it is worth populating in clinical practice.

REFERENCES

Abdullah SS, Fahaid A, Abbas E, Hussein S, Mahmoud A (2012). EC50 of adrenaline-atenolol: Functional agonist assay using Langendorff isolated rabbit heart tethered to powerLab data acquisition system. Afr. J. Pharm. Pharmacol. 6:1092-1098.

Al-Zaben KR, Qudaisat IT, Al-Ghanem SM, Massad IM, Al-Mustafa MM, Al-Oweidi AS, Abu-Halaweh SA, Abu-Ali HM, Saleem MM (2010). Intraoperative administration of dexmedetomidine reduces the analgesic requirements for children undergoing hypospadius surgery. Eur. J. Anaesthesiol. 27:247-252.

Arain SR, Ebert TJ (2002). The efficacy, side effects, and recovery characteristics of dexmedetomidine versus propofol when used for intraoperative sedation. Anesth. Analg. 95:461-466.

Basali A, Mascha E, Kalfas I, Schubert A (2000). Relation between perioperative hypertension and intracranial hemorrhage after craniotomy. Anesthesiology 93:48-54.

Bekker A, Sturaitis M, Bloon M, Moric M, Golfinos J, Parker E, Babu R, Pitti A (2008). The effect of dexmedetomidine on perioperative hemodynamics in patients undergoing craniotomy. Anesth. Analg. 107:1340-1347.

Groeneweg G, Huyren FJ, Coderre TJ, Zijlstra FJ (2009). Regulation of peripheral blood flow in complex regional pain syndrome: clinical implication for symptomatic relief and pin management. BMC Musculoskelet. Disord. 23:116.

Han M, Nie Y, Xiong JY (2011). Effect of of dexmedetomodine in total intravenous anesthesia: an observation on hemodynamics. Int. J. Anesthesiol. Resusc. 32:257-276.

Hofer S, Steppan J, Wagner T, Funke B, Lichtenstern C, Martin E, Graf BM, Bierhaus A, Weigand MA (2009). Central sympatholytics prolong survival in experimental sepsis. Care 13:R11.

Jaakola ML, Melkkila TA, Kanto J, Kallio A, Scheinin H, Scheinin M (1992). Dexmedetomidine reduces intraocular pressure, intubation responses and anaesthetic requirements in patients undergoing ophthalmic surgery. Br. J. Anaesth. 68:570-575.

Jalonen M, Hynynen M, Kuitunen A, Heikkilä H, Perttilä J, Salmenperä M, Valtonen M, Aantaa R, Kallio A (1997). Dexmedetomidine as an anesthetic adjunct in coronary artery bypass grafting. Anesthesiology 86:331-345.

Laubie M, Schmitt H, Vincent M (1979). Vagal bradycardia produced by microinjections of morphine-like drugs into the nucleus ambiguous in anaesthetized dogs. Eur. J. Pharmacol. 59:287-291.

Muhammad A, Ghiasuddin AS, Naveed M, Ashfaq AK, Bina SS (2102). Evaluation of Viburnum grandiflorum for its *in-vitro* pharmacological screening. Afr. J. Pharm. Pharmacol. 6:1606-1610.

Lee SH, Chim JL, Song MH, Choi HG, Choi BM, Lee HM, Lee EK, Roh YJ, Noh GJ (2009). Pharmacokinetics and pharmacodynamics of a new reformulated microemulsion and the long chain trglyceride emulsion of propofol in beagle dogs. Br. J. Pharmacol. 158:1982-1995.

Massad IM, Mohsen WA, Basha AS, Al-Zaben KR, Al-Mustafa MM, Alghanem SM (2009). A balanced anesthesia with dexmedetomidine decreases postoperative nausea and vomiting after laparoscopic surgery. Saudi Med. J. 30:1537-1541.

Nenmann MM, Davio MB, Macknet MR, Applegate RL 2nd (2009). Dexmedetomidine for awake fiberoptic intubation in a parturient with spinal musculer atrophy type III for cesarean delivery. Int. J. Obstet. Anesth. 18:403-407.

Ohtani N, Kida K, Shoji K, Yasui Y, Masaki E (2008). Recovery profiles from dexmedetomidine as a general anesthetic adjuvant in patients undeegoing lower abdominal suegery. Anesth. Analg. 107:1871-1874.

Salman N, Uzun S, Coskun F, Salman MA, Salman AE, Aypar U (2009). Dexmedetomidine as a substitute for remifentanil in ambulatory gynecologic laparoscopic surgery. Saudi J. 30:77-81.

Sanders RD, Maze M (2007). Alpha2-adrenoceptor agonists. Curr. Opin. Investig. Drugs 8:25-33.

Schlichter RA (2010). Dexmedetomidine is an excellent agent for sedation status-post lung transplant. J. Clin. Anesth. 22:1-2.

Souter MJ, Rozet I, Ojemonn JG, Souter KJ, Holmes MD, Lee L, Lam AM (2007). Dexmedetomidine sedation during awake craniotomy for seizuze resction: effects on electrocorticography. J. Neurosurg. Anesthesiol. 19:38-44.

Su WL, Chen YH, Chen CW, Yang SH, Su CL, Perng WC, Wu CP, Chen JH (2010). Involuntary cough strength and extubation outcomes for patients in an Intensive care unit: involuntary cough and extubation outcomes. Chest 137:2807-2808.

Talke P, Chen R, Thomas B, Aggarwall A, Gottlieb A, Thorborg P, Heard S, Cheung A, Son SL, Kallio A (2000). The hemodynamic and adrenergic effects of perioperative dexmedetomidine infusion after vasculary. Anesth. Analg. 90:834-839.

Tanskanen PE, Kytt JV, Randell TT, Aantaa RE (2006). Dexmedetomidine as an anesthetic adjuvant in patients undergoing intracracranial tumor surgery: a double-blind, randomized and placebo-controlled study. Br. J. Anesth. 97:658-665.

Turgut N, Turkmen A, Gokkaya S, Altan A, Hatiboglu MA (2008). Dexmedetomidine-based versus fentanyl-based total intravenous anesthesia for lumbar laminectomy. Minerva Anestesiol. 74:469-474.

Wang Y, Chen Y (2008). Application of propofol in prevention of the adverse effects during general anesthetic extubation. J. Clin. Anesthesiol. 24:177-178.

Zamora LJ, Cruz VJA, Rodriguez RJ, Sánchez RJP, Briones CG, Gallardo ALA (2007). Comparison of topical, intravenous, and intracuff lidocaine for reducing coughing after extubation during emergence from general anesthesia. Rev. Esp. Anestesiol. Reanim 54:596-601.

Zhou J, Wang J, Meng F(2012). The use of dexmedetomidine in aged patients with total hip replacement surgery under general anesthesia: Changes in hemodynamics, cerebral state index and wakening quality. Afr. J. Pharm. Pharmacol. 6:1833-1836.

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Vol. 7(17), pp. 1038-1045, 8 May, 2013
DOI 10.5897/AJPP12.723
ISSN 1996-0816 © 2013 Academic Journals
http://www.academicjournals.org/AJPP

African Journal of Pharmacy and Pharmacology

Full Length Research Paper

Synthesis and characterization of folic acid-conjugated human serum albumin (HSA) nanoparticles for isoalantolactone cellular uptake in HeLa

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Accepted 18 April, 2013

Folic acid was previously demonstrated to mediate intracellular nanoparticle uptake. Isoalantolactone (IAL) was demonstrated to possess a variety of pharmacological activities *in vivo* and *in vitro*, including cytotoxic, diuretic and immunosuppressive activity. Here, we developed folic acid-conjugated human serum albumin nanoparticles for IAL (FHNs-IAL) encapsulation to improve the targeted activity, water solubility and to reduce untoward effects. Human serum albumin nanoparticles for IAL (HNs-IAL) were prepared by desolvation and stabilized by chemical cross-linking with glutaraldehyde. Folic acid was covalently coupled to amino groups on the surface of HNs-IAL by carbodiimide reaction. The average diameter of spherical FHNs-IAL was 118.7 ± 11.6 nm and the IAL encapsulation efficiency was 36.1 ± 3.3%. The cytotoxic activity *in vitro* and the cellular uptake of FHNs-IAL were examined by HeLa cells. The results suggested that covalent conjugation of folic acid to HNs-IAL increased IAL uptake into cancer cells. Moreover, the cytotoxic effects of IAL as monotherapy on HeLa cells were smaller than those encapsulated with FHNs. The experiments *in vivo* also confirmed a superior anti-tumor effect of FHNs-IAL by human tumor xenograft animals. These data suggested that covalent linkage of folic acid could specifically increase the cellular uptake of FHNs-IAL by cancer cell. The FHNs-IAL exhibited good property to improve the uptake of HeLa cells and could become a potential targeted drug delivery system for the future cancer chemotherapy. Therefore, folic acid-conjugated human serum albumin (HAS) nanoparticles for IAL encapsulation would be highly beneficial for biomedical and pharmaceutical applications.

Key words: Isoalantolactone, nanoparticles, serum albumin, folic acid, drug delivery system.

INTRODUCTION

Traditional Chinese medicines have been used to treat human diseases in China for centuries. The dried roots of *Inula helenium* L. and *Inula racemosa* Hook f. are used commonly as folk medicine under the name of 'Tumuxiang'. Several experimental studies demonstrated that the major active component in *I. helenium* L. and *I. racemosa* Hook f. was isoalantolactone (IAL) (Khvorost and Komissarenko, 1976; Wang et al., 2010; Zhang et al., 2010). Pharmacological investigations showed IAL possessed the effects of anticancer (Cho et al., 2004; Zhang et al., 2005; Pal et al., 2010; Konishi et al., 2002), significant anti-inflammatory and hepatoprotective activity similar to that of silymarin (Wang et al., 2000), anti-dematophytic and antifungal activity (Cantrell et al.,

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1999; Liu et al., 2001). However, the clinical application was restricted by its insolubility and toxicity. To maintain natural drug's high activity against many kinds of cancers and overcome the problems associated with its formulation, some new formulations, including liposomes, micelles, and polymeric nanoparticles were created to develop its local drug delivery methods (Yoshizawa et al., 2011; Watanabe et al., 2008; Saad et al., 2008; Patil et al., 2009). Among these formulations, nanoparticles (NPs) were developed to enhance the therapeutic activity of anticancer agents (Wang et al., 2010, 2010).

To increase the therapeutic effect on tumors, various targeting ligands were investigated as tumor targeted drug carriers. Folic acid was a low-weight vitamin that could selectively bind to folate receptors, which were frequently overexpressed on the surfaces of many human cancer cell types but highly restricted in most normal tissues (Toffoli et al., 1997). Therefore, liposomes functionalized with folic acid could specifically promote their cancer cellular uptake through folate receptor mediated endocytosis (RME), and the nonspecific binding to extracellularplate components was observed as well (Gabizon et al., 1999; Park et al., 2005).

Human serum albumin (HSA) is a natural material, and therefore potentially used as a biocompatible and biodegradable carrier with anticancer drugs (Chen et al., 2010). The anticancer agent, paclitaxel, was conjugated to HSA with further modifications by folic acid via N-(3-dimethylaminopropyl)-N-ethylcarbodiimide (EDC) (Dosio et al., 2009). Folic acid could be covalently coupled to amino groups on the surface of HSA NPs by carbodiimide reaction, specifically increasing NPs uptake into cancer cells but not into normal cells (Ulbrich et al., 2011).

In the present study, folic acid-conjugated human serum albumin nanoparticles via glutaraldehyde were prepared for IAL (FHNs-IAL) encapsulation. The preparation process related characteristics were investigated (Nasri et al., 2012), and these would be used to lay the foundation for further study, including determining the mechanism of the nanoparticles uptake by tumor cells and their cytotoxicity (Alam et al., 2012; Asgarpanah et al., 2012).

MATERIALS AND METHODS

Reagents and chemicals

The IAL was isolated from the dried roots of *I. helenium* (Figure 1). Its structure was characterized by chemical and spectroscopic methods (proton nuclear magnetic resonance [(^{1}H NMR), carbon nuclear magnetic resonance (^{13}C NMR) and mass spectrometry (MS)] (Konishi et al., 2002). Analysis showed that its purity was above 99% [high-performance liquid chromatography (HPLC)]. HSA, glutaraldehyde 8% solution, EDC, 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H- tetrazolium bromide (MTT) and folic acid were purchased from (Sigma-Aldrich, USA). HPLC-grade methanol was purchased from Baker Company (Baker Inc., USA). Ultrapure water was prepared by a Millipore-Q SAS 67120 MOLSHEIM (France).

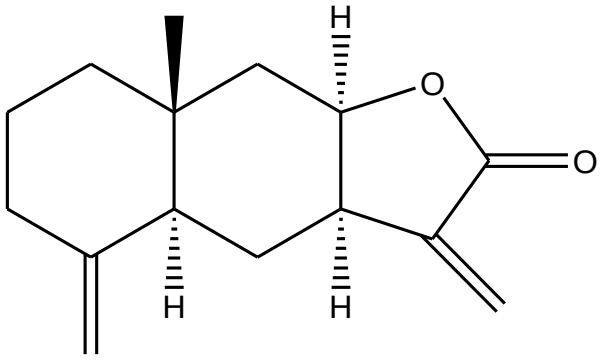

Figure 1. Molecular structure of isoalantolactone.

Perparation of HeLa cells

HeLa (Human cervical carcinoma) cells were obtained as a gift from the Fourth Military Medical University, Xi'an, China. All the cell lines were maintained in Roswell Park Memorial Institute (RPMI) 1640 medium without folate and supplemented with 10% heat-inactivated fetal bovine serum, 100 units/ml penicillin, and 100 mg/ml streptomycin. Cultures were maintained in a humidified incubator at 37°C in an atmosphere of 5% CO_2 and 95% air until they reached confluence.

Perparation of experimental animals

Female BALB/c mice (body weight = 18 to 22 g) were purchased from the Experimental Animal Center of the Fourth Military Medical University, which were used in accordance with the Guidelines on the Care and Use of Laboratory Animals issued by the Chinese Council on Animal Research and the Guidelines of Animal Care. The study was approved by the ethical committee of the Fourth Military Medical University. These mice were maintained in an aseptic environment. Tumor implantation was carried out by injecting 0.1 ml of the HeLa cell suspension (1.0×10^6 cells) into the right limb armpits of each mouse. Subcutaneous tumor growth was monitored daily until 10 to 12 days after implantation. Afterwards, tumor-bearing mice were randomly assigned to groups used for *in vivo* anti-cancer studies.

Preparation and characterization of FHNs-IAL

The folic acid-conjugated human serum albumin nanoparticles for IAL (FHNs-IAL) encapsulation were prepared by an established desolvation process as reported previously (Ulbrich et al., 2009) and modified as follows. Briefly, 200 mg HSA in 2.0 ml 10 mM NaCl solution was adjusted to pH 8.4 and added into 8.0 ml ethanol under constant stirring at room temperature. Subsequently, 250 µl of an 8% glutaraldehyde solution was added to achieve particle cross-linking. 1 ml of folic acid solution (20 mg/ml) in 0.1 M sodium hydroxide was incubated with 200 µl EDC under constant shaking in the dark for 15 min at 20°C. Subsequently,1 ml HSA nanoparticle suspension (content 15 mg/ml) was added, and shaking continued for 1 h. Reaction was stopped by adding 100 µl hydroxylamine (500 mg/ml). The folate-conjugated nanoparticles suspensions were put in pretreated dialysis bags to remove any conjugate unassociated with NPs and dispersed the pellet to the original volume in water again. After the external pH was adjusted to pH 9.0, FHNs were incubated in 150 ml ethanol with IAL (drug: NPs = 1:10) and stirred

for 30 min. 50 µl glutaraldehyde was added slowly with continuous stirring and cured for 12 h. The reaction solution was centrifuged at 7,000 rpm, and the precipitate was washed three times with phosphate buffered saline (PBS) (pH 7.4), and freeze-dried under vacuum. Finally, reserve samples were maintained as described.

Final IAL and folic acid concentration after loading was determined by high-performance liquid chromatography (HPLC, Agilent LC1200) at 230 and 360 nm with mobile phase (V/V, PBS/methanol = 9:1). The elution rate was 1.0 ml/min. The retention time of IAL and folic acid was 1.5 and 3.6 min under these conditions, respectively. The drug encapsulation efficiency (EE) was calculated according to the following equation:

$$EE\% = (IAL_{FHN} \div IAL_{Theoretic}) \times 100$$

Cytotoxicity assay

The cytotoxicity assay was performed by the MTT method modified from the one previously described (Dorn et al., 2006). Cells (2 × 10^3/well) that were seed in 96-well plates were incubated for 12 h. The freeze-dried FHNs-IAL was dissolved into PBS buffer at the concentration of 20, 40 and 60% (w/v). The culture medium was then replaced with an equal volume of fresh medium containing different drug formulations of IAL and the different concentrations of FHNs-IAL. All of the samples were ultra violet (UV) sterilized. After 24 h or 48 h incubation, 20 µl MTT solution was added to each well of 96-well plates, which were incubated for additional 4 h at 37°C. MTT solution in the medium was aspirated off and 150 µl of dimethyl sulphoxide (DMSO) were added to each well and shaked softly for 10 min to solubilize the formazan crystals formed in viable cells. The optical density (OD) was read at a wavelength of 550 nm using a microplate reader (Spectra MAX 340, Molecular Devices Co, Sunnyvale, CA). Data were averaged from the six different wells per condition and plotted as mean ± standard error.

Cellular uptake assay

In vitro cellular uptake assay was performed following the general protocol previously reported (Wang et al., 2010). To visualize cellular uptake of FHNs-IAL, HeLa cells were reseeded in the Lab-Tek chambered slide (Miles Laboratories, U.S.A.) and incubated for 24h at 37°C. The culture media were then replaced with 1 ml of medium with Oregon green labeled FHNs-IAL for 3 h at 37°C. Cells were washed three times with PBS after treatment and then fixed by 4% (w/v) paraformaldehyde solution. The fluorescent images were viewed by fluorescent microscope (Olympus BX51, Japan).

Apoptosis assay

Apoptosis was determined by staining cells with annexin V-fluorescein isothiocyanate (FITC, Sigma-Aldrich, USA) as previously described (Dorn et al., 2006). Cells (1 × 10^5/well) were plated in 24-well plates and incubated with different drug formulations of IAL and various FHNs-IAL (20, 40, and 60% FHNs) at the concentration of 10 µg/ml for 24 h. Thereafter, cells were collected and washed twice with 400 µl cold PBS and resuspended in 100 µl binding buffer with 5 µl of Annexin V-FITC and 1 µl propidium iodide (PI). After 15 min of incubation at room temperature in the dark, the cells were diluted with 400 µl of binding buffer and immediately analyzed by Epics Elite flow cytometer, and the data were analyzed using Expo32 software (Beckman Coulter).

In vivo anticancer activity

Forty tumor-bearing mice were divided into five groups randomly. After the tumor inoculation, IAL solution and various FHNs-IAL (dose of IAL = 10 mg/kg body weight) suspended in PBS were injected intravenously via the tail vein of animals at 5 days intervals. At predetermined time, the width and length of tumors were measured with a caliper and tumor volumes were then determined by the following equation:

$$V_{Tumor} = \frac{length \times Width^2}{2}$$

Therefore, the tumor control rate (TCR) was calculated according to the following equation:

$$TCR(\%) = \left(1 - \left(\frac{V_{ds} - V_{de}}{V_{cs} - V_{ce}}\right)\right) \times 100$$

Where V_{ds} is the tumor volume at the begining of drug adminstration; V_{de} is the tumor volume at the end of drug adminstration; V_{cs} is the tumor volume of the control group at the begining; V_{ce} is the tumor volume of the control group at the end.

Statistical analysis

Data are expressed as mean ± standard deviation (SD). Statistical analyses were assessed using the Student's t-test for two groups and one-way ANOVA for multiple groups. Differences were considered to be significant at $P < 0.05$.

RESULTS

Characterization of FHNs-IAL

The physicochemical properties of IAL-nanoparticles prepared were assessed and the results were listed in Figure 2 and Table 1. The results of the FHNs-IAL prepared in the this work showed that particle sizes were around 100 nm and the zeta potential of surface charges for the FHNs-IAL were negative.

Cytotoxicity assay

IAL and various FHNs-IAL (20, 40, and 60% FHNs) were screened for *in vitro* cytotoxicity against HeLa cells for 24 and 48 h by applying MTT assay (Figure 3). The highest lethality of cancer cells occurred at the highest concentration of FHNs formulations after treatment and for the longest period of time. The orders of all HeLa cells

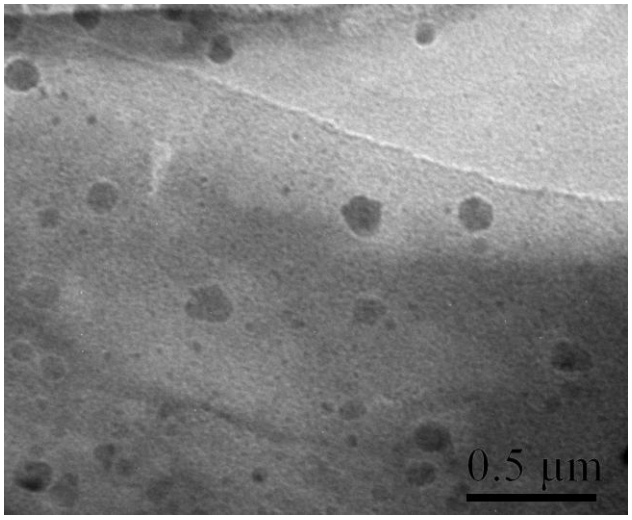

Figure 2. Scanning electron microscope (SEM) image of 40% FHNs.

viabilities examined to different drug-loaded NPs were 60% FHNs -IAL > 40% FHNs -IAL > 20% FHNs -IAL > 0% FHNs.

Cellular uptake assay

Cellular uptakes of FHNs-IAL by HeLa cells were visualized by a fluorescent microscope as shown in Figure 4. Cellular uptake extent of 60% FHNs-IAL was significantly higher than those of free IAL, 20% FHNs-IAL and 40% FHNs-IAL under the same condition. Furthermore, 60% FHNs-IAL could be uptaken fast by endocytosis process and induced apoptosis of cells.

Apoptosis assay

The results of IAL and FHNs-IAL induced apoptosis indicated that the proportion of annexin V/PI-stained cells, signifying the apoptotic cells, increased with the concentration of FHNs-IAL (Figure 5). In addition, the proportion of late apoptotic cells was extremely greater than that of early apoptotic cells (Table 2). The proportion of late apoptosis induced by 60% FHNs-IAL was 80.7% compared to 64.6% of IAL solution at concentration of 10 μg/ml.

In vivo anticancer activity

In vivo anti-tumor activity of FHNs-IAL was evaluated by female BALB/c mice. As shown in Figure 6, the growth rate of tumor were inhibited for all the treated groups after injection of various formulations compared with the PBS control group. The TCRs of IAL, 20, 40 and 60% FHNs-IAL

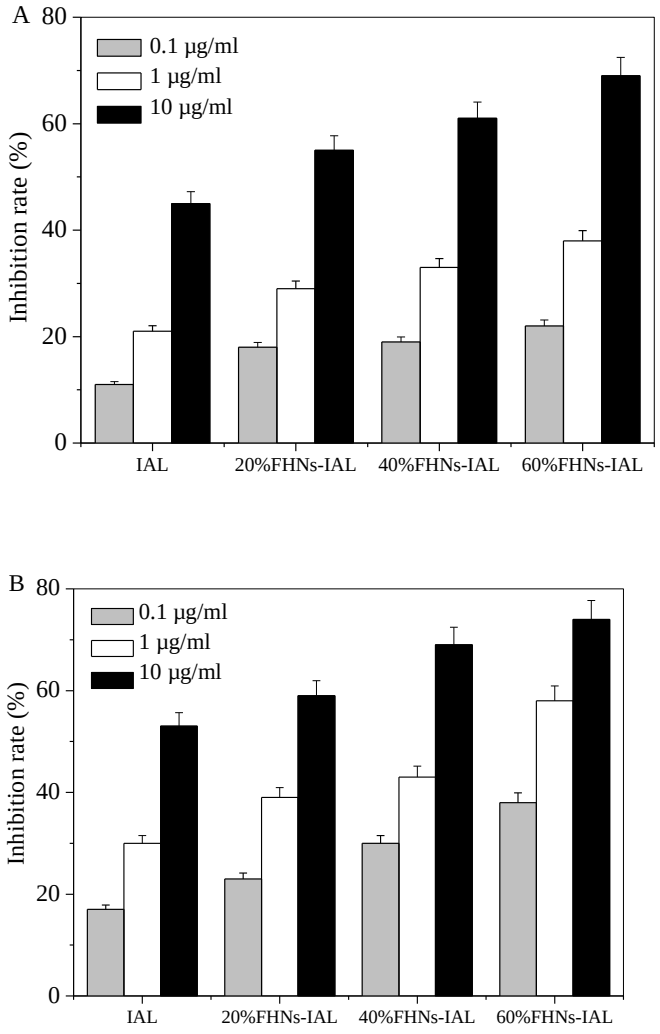

Figure 3. Cell inhibition rate of HeLa cells treated with IAL and various IAL-loaded FHNs (20, 40, and 60% FHNs).Three IAL concentrations (0.1, 1, and 10 μg/mL) were used here. (A) HeLa cells 24 h after treatment. (B) HeLa cells 48 h after treatment.

were 41.4, 47.8, 57 and 63.9%, respectively.

DISCUSSION

The results listed in Figure 2 and Table 1 indicated that the folate binding was 7.9% with the drug encapsulation efficiency (EE) of 36.1%. The EE of FHNs-IAL did not show any significant difference compared with that of HNs-IAL (35.5%). Therefore, the physicochemical properties of IAL formulations prepared in this work would be adequate. Moreover, the mean particle size of HNs-IAL increased from 112.5 to118.7 nm by the coupling of folic acid alone (EDC activated HSA NP). The EE and the folate loading of FHNs-IAL were only reduced to 33.9 and 6.3%, respectively, when it was stored for 3 months

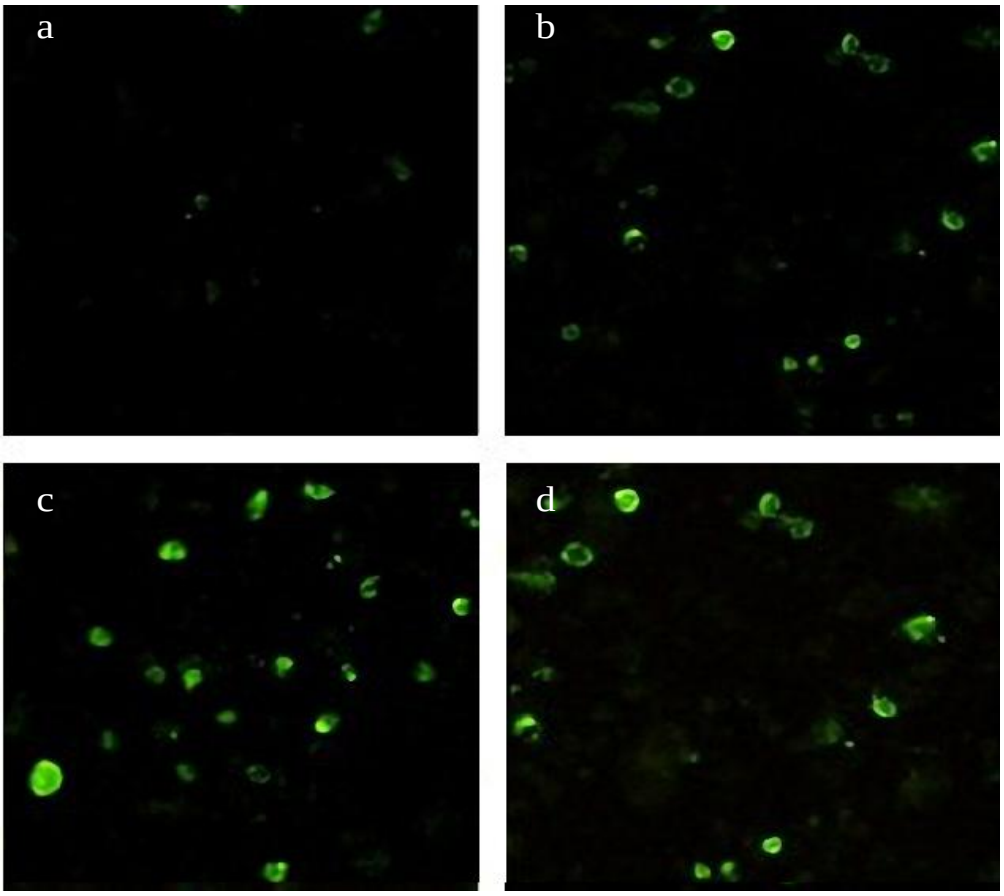

Figure 4. Fluorescent microscopic pictures of HeLa cells incubated with (a) IAL at at concentrations of 10 µg/ml, (b) 20% FHNs-IAL, (c) 40% FHNs-IAL and (d) 60% FHNs-IAL for 3 h at 37°C.

at 4°C. Therefore, these data suggested FHNs-IAL were excellent stability.

The results of Figure 3 demonstrated that the cytotoxicity of FHNs-IAL to cancer cells was improved by the folic acid component, which has been reported to bind to folate receptors with high affinity, thus mediating in cellular uptake via receptor-mediated endocytosis. In addition, it was suggested that the cytotoxic effect of IAL or FHNs-IAL was related to the induction of apoptosis. The results of Figure 4 could be visualized that FHNs-IAL were primarily located on the surface of cell membrane due to their preferential binding to the folate receptors on the membrane. The fluorescent results directly indicated that FHNs-IAL were taken up by a folate-receptor-mediated endocytosis process. In contrast to free IAL, FHNs-IAL were mainly distributed in the cytoplasm for their higher solubility without exhibiting much accumulation in the nucleus after 3 h of incubation. However, most of IAL molecules transported inside the cells were likely to still be in an aggregated state in the cytoplasm region with little chance to be solubilized in the cytoplasmic fluid. It was suggested that IAL probably could induce apoptosis through a mitochondria-dependent pathway in HeLa cells (Zhang et al., 2005).

The results of Figure 5 and Table 2 showed that the apoptosis of HeLa cell induced by FHNs-IAL was obviously dose-dependent, which also indicated that folate component improved the cytotoxicity of IAL. The results obtained in the *in vivo* antitumor studies clearly indicated that FHNs could serve as a novel formulation of IAL for targeting to the tumor site, enhancing cellular uptake, and achieving sustained release within cells. Compared with IAL solution, 60% FHNs-IAL increased the TCR over 20%. Hence, FHNs was one of the efficient anticancer drug carriers.

In this study, IAL-loaded FHNs was successfully synthesized and characterized as a new carrier for tumor-targeted drug delivery. The *in vitro* cytotoxicity assay of FHNs-IAL showed that NPs with folate components had higher cytotoxicity to cancer cells. It revealed that 60% FHNs-IAL had greater cellular uptake compared with IAL as monotherapy applied. *In vivo* experiment employing a human tumor xenograft animal also confirmed a superior anti-tumor effect of FHNs-IAL. Our present study clearly indicated that FHNs would serve as a potent IAL delivery vehicle for the future cancer chemotherapy.

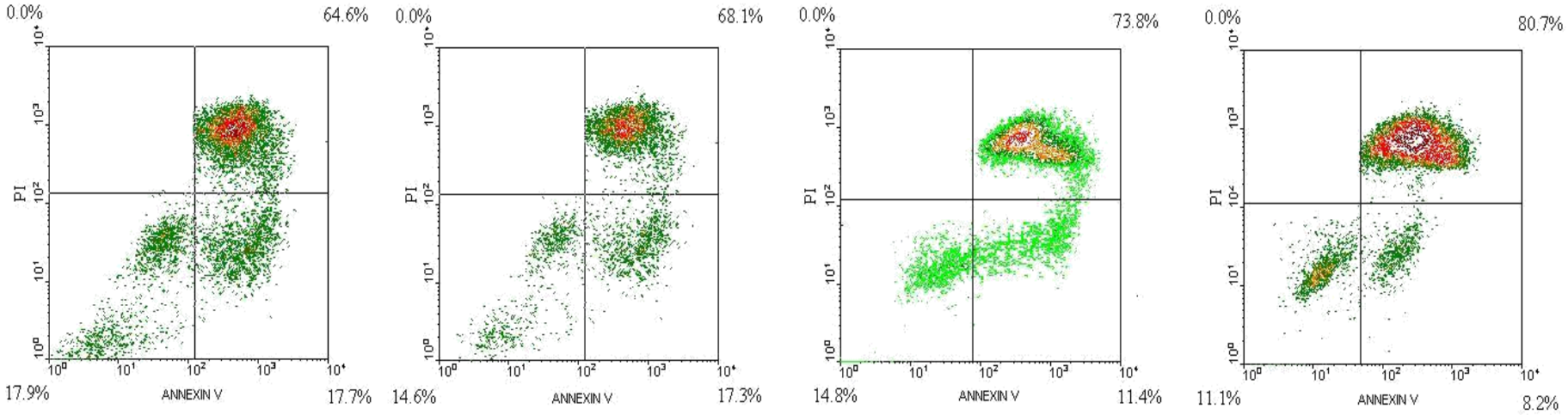

Figure 5. Detection of apoptosis of the HeLa cells induced by IAL at the concentration of 10 μg/ml and various FHNs-IAL (20, 40, and 60% FHNs) for 24 h with annexin V/PI staining method. A: HeLa cells were treated by IAL as monotherapy. B1 area-mechanical damage Annexin V^-/PI^+, B2 area-late apoptosis Annexin V^+/PI^+, B3 area-normal cell AnnexinV^-/PI^-, B4 area-early apoptosis Annexin V^+/PI^-. B-D: HeLa cells were treated by various IAL-loaded FHNs (20, 40, and 60% FHNs), respectively.

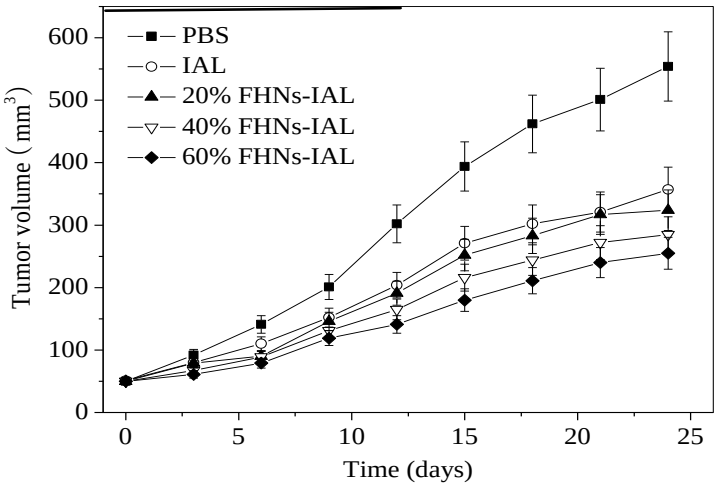

Figure 6. *In vivo* anti-tumor effects of IAL solution and various FHNs-IAL (20, 40, and 60%). BALB/c nude mice bearing HeLa tumors were intravenously injected with various formulations of IAL and with PBS as the control group.

Table 1. Physicochemical properties of FHNs-IAL. Results are expressed as the mean ± SD (n = 3).

Formulation	Property	0 month	3 months
HNs-IAL	Particle size (nm)	112.5±14.3	134.8±24.5
	Zeta potential (mV)	−21.1±5.9	−18.4±7.2
	Folate loading (%)	—	—
	IAL EE (%)	35.5±6.9	31.2±5.1
FHNs-IAL	Particle size (nm)	118.7±11.6	129.1±19.6
	Zeta potential (mV)	−24.1±8.8	−20.1±4.4
	Folate loading (%)	7.9 ±0.8	6.3±0.3
	IAL EE (%)	36.1±3.3	33.9±3.9

Table 2. Apoptosis rate of the HeLa cells was detected by flow cytometer with annexin V/PI staining methods ($P < 0.01$).

Formulation	Annexin V/PI (%)		
	Early apoptosis	Late apoptosis	Total apoptosis
IAL	17.7±3.62	64.6±5.35	82.3±7.70
20% FHNs-IAL	17.3±4.28	68.1±6.36	85.4±8.11
40% FHNs-IAL	11.4±2.85	73.8±8.49	85.2±11.5
60% FHNs-IAL	8.2±2.60	80.7±10.49	88.9±8.6

ACKNOWLEDGEMENTS

This work was supported by Graduate Experimental Research Funds of Northwest University (09YSY27）and the National Scientific Foundation of China (81001622) and As a Major New Drug to Create a Major National Science and Technology Special from Ministry of Science and Technology of the People's Republic of China (No. 2011ZX09401-308-34).

REFERENCES

Alam M, Ghiasuddin, Sadat A, Muhammd N, Khan AA, Siddiqui BS (2012). Evaluation of *Viburnum grandiflorum* for its *in-vitro* pharmacological screening. Afr. J. Pharm. Pharmacol. 6:1606-1610.

Asgarpanah J, Ramezanloo F (2012). Chemistry, pharmacology and medicinal properties of *Peganum harmala* L. Afr. J. Pharm. Pharmacol. 6:1573-1580.

Cantrell CL, Abate L, Fronczek FR, Franzblau SG, Quijano L, Fischer NH (1999). Antimycobacterial eudesmanolides from *Inula helenium* and *Rudbeckia subtomentosa.*Planta Med. 65:351-355.

Chen DZ, Tang QS, Xue WQ, Xiang JY, Zhang L, Wang XR (2010). The preparation and characterization of folate-conjugated human serum albumin magnetic cisplatin nanoparticles. Biomed. Res. 24:26-32

Cho JY, Kim AR, Jung JH, Chun T, Rhee MH, Yoo ES (2004). Cytotoxic and and proapototic activities of *cynaropicrin*,a sesqutiterpene lactione,on the viability of leukocyte cancer cell lines. Eur. J. Pharmacol. 492:85-94.

Dorn DC, Maja Alexenizer M, Hengstler JG, Dorn A (2006). Tumor Cell Specific Toxicity of *Inula helenium* Extracts. Phytother. Res. 20:970–980.

Dosio F, Arpicco S, Stella B, Brusa P, Cattel L (2009). Folate-mediated targeting of albumin conjugates of paclitaxel obtained through a heterogeneous phase system. Int. J. Pharm. 382:117–123.

Gabizon A, Horowitz AT, Goren D, Tzemach D, Mandelbaum-Shavit F, Qazen MM, Zalipsky S(1999). Targeting folate receptor with folate linked to extremities of poly(ethylene glycol)-grafted liposomes: in vitro studies. Bioconjug. Chem. 10:89–298.

Khvorost PP, Komissarenko NF (1976). Lactones of *Inula helenium*. Khimija Prirodnykh Soyedineniy 6:820-821.

Konishi T, Shimada Y, Nagao T, Okabe H, Konshima T (2002). Antiproliferative sesquiterpene lactones from the roots of *Inula helenium*. Biol. Pharm. Bull. 25:1370-1372.

Liu CH, Mishra AK, He B, Tan RX (2001). Antimicrobial activities of isoalantolactone, a major sesquiterpene lactone of *Inula racemosa*. Chin. Sci. Bull. 46:498-500.

Nasri S, Anoush M, Khatami N (2012). Evaluation of analgesic and anti-inflammatory effects of fresh onion juice in experimental animals. Afr. J. Pharm. Pharmacol. 6:1679-1684.

Park EK, Lee SB, Lee YM (2005). Preparation and characterization of methoxy poly(ethylene glycol)/poly(ε-caprolactone) amphiphilic block copolymeric nanospheres for tumor-specific folate-mediated targeting of anticancer drugs. Biomaterials 26:1053–1061.

Pal HC, Sehar I, Bhushan SI, Gupta BD, Saxena AK (2010). Activation of caspases and poly (ADP-ribose) polymerase cleavage to induce apoptosis in leukemia HL-60 cells by *Inula racemosa*. Toxicol. *In Vitro* 24:1599-1609.

Patil YB, Toti US, Khdair A, Ma L, Panyam J (2009). Single-step surface functionalization of polymeric nanoparticles for targeted drug delivery. Biomaterials 30:859-866.

Saad M, Garbuzenko OB, Ber E (2008). Receptor targeted polymers, dendrimers, liposomes: Which nanocarrier is the most efficient for tumor-specific treatment and imaging? J. Control Release 130:107-114.

Toffoli G, Cernigoi C, Russo A, Gallo A, Bagnoli M, Boiocchi M (1997). Overexpression of folate binding protein in ovarian cancers. Int. J. Cancer 74:193-198.

Ulbrich K, Michaelis M, Rothweiler F (2011). Interaction of folate-conjugated human serum albumin (HSA) nanoparticles with tumour cells. Int. J. Pharm. 406:128-134.

Ulbrich K, Hekmatara T, Herbert E, Kreuter J (2009). Transferrin- and

transferrin-receptor-antibody-modified nanoparticles enable drug delivery across the blood–brain barrier (BBB). Eur. J. Pharm. Biopharm. 71:251-256.
Wang JF, Liu WM, Tu Q (2011). Folate-Decorated Hybrid Polymeric Nanoparticles for Chemically and Physically Combined Paclitaxel Loading and Targeted Delivery. Biomacromolecules 12:228–234. Wang KT, Liu HT, Zhao YK (2000). Separation and determination of alantolactone and isoalantolactone in traditional Chinese herbs by capillary electrophoresis. Talanta 52:1001-1005.
Wang Y, Wang YQ, Xian JN, Yao KT (2010). Target-Specific Cellular Uptake of Taxol-Loaded Heparin-PEG-Folate Nanoparticles. Biomacromolecules 11:3531–3538.
Watanabe M, Kawano K, Toma K, Hattori Y, Maitani Y (2008). In vivo antitumor activity of camptothecin incorporated in liposomes formulated with an artificial lipid and human serum albumin. J. Control Release 127:231-238.
Yoshizawa Y, Kono Y, Ogawara K, Kimura T, Higaki K (2011). PEG liposomalization of paclitaxel improved its in vivo disposition and anti-tumor efficacy. Int. J. Pharm. 412:132-141.
Zhang S, Won YK, Ong CN, Shen HM (2005). Anti-cancer potential of sesquiterpene lactones: bioactivity and molecular mechanisms. Current Medicinal Chemistry. Anticancer Agents 5:239-249.
Zhang T, Xiao W, Gong T (2010). Two new eudesmanolides from *Inula racemosa*. J. Asian Nat. Prod. Res. 12:788–792.

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Vol. 7(17), pp. 1046-1053, 8 May, 2013
DOI 10.5897/AJPP12.900
ISSN 1996-0816 © 2013 Academic Journals
http://www.academicjournals.org/AJPP

African Journal of Pharmacy and Pharmacology

Full Length Research Paper

Controlled release formulation of levocetirizine dihydrochloride by casein microparticles

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Accepted 5 March, 2013

The study aims to develop a microparticulate casein based delivery system by the controlled drug delivery approach using Levocetirizine dihydrochloride as the model drug. Fourier transform infrared spectroscopy (FTIR), X-ray and differential scanning calorimetry (DSC) studies substantially indicates the presence of molecularly dispersed drug within the particles with preserved stability during microencapsulation. *In vitro* release studies of levocetirizine dihydrochloride loaded microparticles were performed by simulating the condition of gastrointestinal tract, and showed the minimal drug leakage (less than 5%) at acidic pH (1.2) and significantly higher release at basic pH (7.4). The results were found to be critical in confirming the role of casein microparticles as potential candidate for the controlled and targeted release of levocetirizine dihydrochloride.

Key words: Extended release, casein, levocetirizine dihydrochloride, steric stabilization.

INTRODUCTION

Controlled drug delivery systems are found to be vital in rectifying some of the problems associated with conventional therapy and in enhancing the therapeutic efficacy of a given drug. To obtain maximum therapeutic efficacy, it becomes necessary to deliver the agent to the target tissue in the optimal amount, in the right period of time, to reduce toxicity and side effects. The usage of microspheres as drug carriers to deliver a therapeutic agent in sustained controlled fashion has been reported (Yanhong et al., 2012).

It is really fascinating to find the role of milk protein, casein, as a drug carrier mainly for the sustained delivery of various drugs (Willmott et al., 1992; Chen et al., 2006; Knepp et al., 1993; Yoav, 2010). Casein is an inexpensive, readily available, non-toxic and highly stable milk protein and as a natural food product, this generally recognized as safe (GRAS) protein is biocompatible and biodegradable (Ana et al., 1999; Katz et al., 2009). Casein comprises about 94% protein and 6% low molecular weight compounds collectively called colloidal calcium phosphate. There are mainly four casein phosphoproteins, αS1-, αS2-, β-, and κ-casein, which exist approximately in proportions of 4:1:4:1 by weight, respectively in cow milk. Their molecular weights are between 19 and 25 kDa and average isoelectric point (pI) is between 4.6 and 4.8. All of the four caseins are amphiphilic and have ill-defined structures (Fox et al., 2003), with distinct hydrophobic and hydrophilic domains (Dalgeish, 1998). The usage of casein in various drug delivery studies has been well reported (Latha et al., 1994, 1995, 2000; Elzoghby et al., 2011; Arora et al., 2012).

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Levocetirizine dihydrochloride, the active R enantiomer of cetirizine, is a selective H1 histamine blocker that contains antihistaminic properties and is used to manage intermittent and persistent allergic rhinitis. It is zwitterionic and relatively polar, and thus, does not penetrate the blood brain readily. It is effective in relieving nasal symptoms, improving nasal air flow, reducing leukocyte infiltration, and diminishing the cytokine level, which results in evidence of the effectiveness of levocetirizine in seasonal allergic rhinitis (Ciprandi et al., 2004). Levocetirizine has several pharmacokinetic properties that are desirable for an antihistamine, providing a combination of both potency and safety (Cranswick et al., 2005; Molimard et al., 2004). Its clinical advantages are derived from its rapid and extensive absorption, limited distribution and its very low degree of metabolism (Ferrer et al., 2011). The incorporation of levocetirizine in an extended-release of oral dosage form would have many advantages such as aiding in enhancement of bioavailability and prolonged plasma level drug concentration (Prabakara et al., 2011; Patel et al., 2012).

MATERIALS AND METHODS

Levocetirizine was obtained as a gift sample from Praveen Laboratories Pvt Ltd, Gujarat and Casein (alkali soluble) was purchased from Himedia laboratories pvt. Ltd, Mumbai. All reagents used were of analytical grade.

Preparation of levocetirizine dihydrochloride loaded casein microparticles

A total of 10 ml of casein solution in 0.1 M NaOH was prepared and the calculated amount of levocetirizine dihydrochloride was dissolved in the 10 ml of casein solution. 4 ml of this solution was dropped into a 50 ml ethylcellulose solution. The ethyl cellulose solution was made by adding 1 g of ethylcellulose in 50 ml of chloroform. The resulting solution was homogenized for 30 min at 25,000 rpm. Crosslinking was done by adding glutaraldehyde at the concentration of 3.12% during the homogenization process. Thus, formed particles were stirred for 12 h. Then, the particles were separated by centrifugation at 6,000 rpm for 10 min and washed repeatedly with acetone to remove excess ethyl cellulose, chloroform and glutaraldehyde. The microparticles obtained were dried at room temperature (Jalil et al., 1990). Different concentrations of casein 10 and 15 % w/v were tried. The effect of ethyl cellulose concentration (0.5, 1.0, 2.0 and 3.0% w/v), as well as volume of casein (4 and 5 ml) on the microsphere formation, was investigated. Furthermore, the effect of stirring time on the properties of the prepared microspheres was studied.

Encapsulation efficiency

Encapsulation efficiency was calculated by weighing 50 mg of the loaded microparticles and dispersing them in 50 ml of phosphate buffer saline (pH 7.4). The sample was allowed to stir overnight at 50 rpm and the concentration of levocetirizine dihydrochloride was analyzed in the supernatant at 230 nm using ultra violet (UV)-Vis spectrophotometer (UV – 1700 Pharma spec - Schimadzu) (Pradeep and Inderbir, 2010; Rao et al., 2007; Kim et al., 2005; Kim and Lee, 1992).

$$\text{Encapsulation effciency}(\%) \equiv \frac{\text{Actual Weight (Wa)}}{\text{Theoretical Weight (Wt)}} \times 100$$

Morphological characterization using scanning electron microscopy (SEM)

The drug loaded casein microparticles were analysed for its diameter and surface morphology using SEM (FEI Quanta FEG 200). The particles were sprinkled on adhesive aluminium stub and then surface coating was done with gold to a thickness of ~300 Å using a sputter coater.

Particle size distribution

Particle size distribution was performed using Mastersizer 2000 (Malvern India Pvt ltd). The microparticles loaded with levocetirizine dihydrochloride were dispersed with poly (dimethylsiloxane). The refractive index for the powder samples was set to 1.52, and the poly (dimethylsiloxane) was 1.40.

X-ray powder diffraction studies

The X-ray diffractograms of the microparticles and the levocetirizine dihydrochloride loaded microparticles were obtained in a D8 Advance Model X-Ray Diffractometer (Bruker, Germany) using Ni filtered radiation (λ = 15.4 nm, 40 kV and 30 mA). The measurements were carried out using Poly (methyl methacrylate) (PMMA) sample holder and lynx eye detector.

Fourier transform infra red spectral analysis

The fourier transform infrared (FTIR) spectrum of the title compound was recorded in the range of 400 to 4000 cm^{-1} using KBr pellet with a FTIR spectrophotometer (Nicolet Avatar 330) at room temperature.

Differential scanning calorimetry

The DSC thermograms of placebo and levocetirizine dihydrochloride loaded casein microparticles were carried out using Netzsch DSC 204. The samples were heated from 50 to 230°C at a heating rate of 10°C at a heating rate of 10°C/min in an inert nitrogen atmosphere.

In-vitro release of levocetirizine dihydrochloride

Levocetirizine dihydrochloride loaded casein microparticles were subjected to *in vitro* release in the simulated gastric fluid pH 1.2 (SGF) [as per United States Pharmacopeia (USP)] and simulated intestinal fluid (phosphate buffer Saline (PBS)) pH 7.4 without enzymes. 50 mg of levocetirizine dihydrochloride loaded casein microparticles was taken in a dialysis cassette along with 0.5 ml of simulated intestinal fluid and immersed in the 50 ml of simulated intestinal fluid. The dissolution was done at 50 rpm at 37°C. Aliquots were collected at predetermined points and an equal

Table 1. Optimization of casein microparticles.

Formulation no.	Ethylcellulose concentration (%)	Casein concentration (%)	Casein volume (ml)
1	0.5	15.0	4.0
2	1.0	15.0	4.0
3	2.0	15.0	4.0
4	3.0	15.0	4.0
5	0.5	10.0	4.0
6	1.0	10.0	4.0
7	2.0	10.0	4.0
8	3.0	10.0	4.0
9	0.5	15.0	5.0
10	1.0	15.0	5.0
11	2.0	15.0	5.0
12	3.0	15.0	5.0
13	0.5	10.0	5.0
14	1.0	10.0	5.0
15	2.0	10.0	5.0
16	3.0	10.0	5.0

amount of buffer was replaced to maintain the volume. The amount of levocetirizine dihydrochloride was quantified by UV-Vis spectrophotometer at 230 nm. The release of levocetirizine dihydrochloride in simulated gastric fluid was also analyzed.

RESULTS AND DISCUSSION

Preparation and optimization of levocetirizine dihydrochloride loaded casein microparticles

The casein microparticles were produced by steric stabilization process by optimizing the concentration, volume of casein and ethylcellulose which is used as the stabilization agent as shown in Table 1. The result depicts that formulation No. 15 with 5 ml of 10% casein and 2% ethylcellulose yielded better particles. This shows that casein microparticles concentration and volume of casein solution was observed to have a strong influence on microsphere morphology. Ethyl cellulose used as a steric stabilization agent plays an important role in establishing emulsion between the aqueous (casein solution) and organic (chloroform) phases, hence influence the formation of particles. At decreased ethyl cellulose concentration, particles were formed as large clumps and at increased ethyl cellulose concentration, particles size decreased but particles were clumped and unstable.

Accordingly, the increased solubility of the drug in casein solution indicates strong hydrophobic interactions leading to association of the drug with the protein micelles (Bachar et al., 2012).

Particle size distribution and morphology

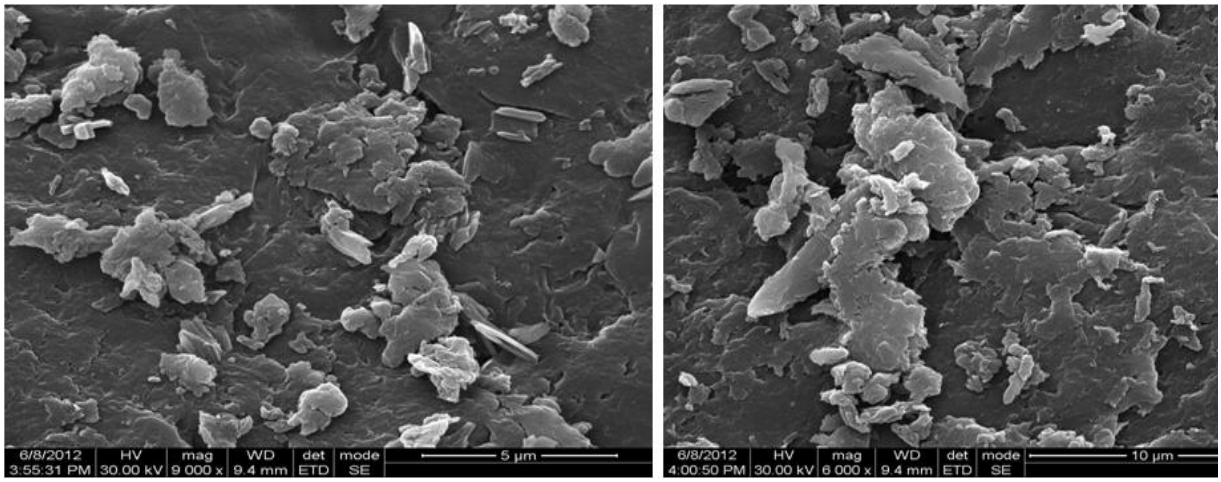
The mean diameter of the microparticles was found to be 50 microns (Figure 2) by the laser particle size analyzer. From the SEM report, the microparticles size was found to be less than 3 µm. Casein microparticles showed macropore surfaces with follicular structure appearance (Figure 1).

X-ray powder diffraction studies

X-ray diffraction (XRD) was implemented to investigate whether levocetirizine dihydrochloride retains its crystalline state in the microparticles obtained. Pure forms of the drug, polymer, placebo microparticles and drug loaded microparticles were scanned as shown in Figure 3. Several sharp and intense peaks can be identified clearly in the levocetirizine dihydrochloride diffractogram at 2θ values. However, the disappearance of levocetirizine dihydrochloride peaks in the drug loaded microparticles indicates that the drug is encapsulated in the polymer effectively.

FTIR spectral analysis

The FTIR spectra of placebo microparticles and levocetirizine dihydrochloride loaded casein microparticles are shown in Figure 4. The FTIR spectrum indicates that there is no modification or interaction of



(A) (B)

Figure 1. Scanning electron micrograph of casein microparticles.

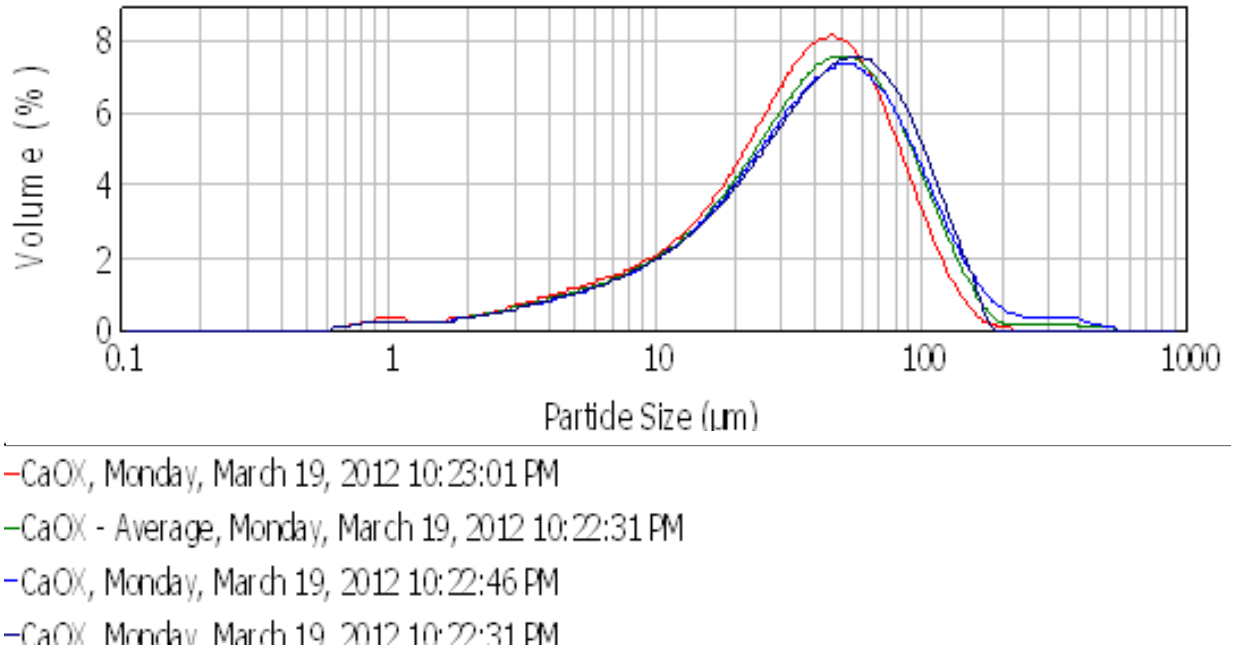


Figure 2. Particle size distribution.

casein and levocetirizine dihydrochloride. The absence of the intense peaks (500 to 1700 cm^{-1}) corresponding to levocetirizine dihydrochloride assures the encapsulation of the drug within the protein matrix. The bands observed in the casein microparticles spectrum did not show any shift, suggesting that no new chemical bond was formed after preparing the formulation, and the results confirmed that the drug is physically encapsulated inside the polymer matrix.

Differential scanning calorimetry

The DSC thermogram showed endothermic peak of levocetirizine at 206.14°C, which corresponded to its melting point. The evaluation of the thermo gram obtained from DSC (Figure 5) shows only exothermic peaks at 95.3 and 96.8°C, respectively which revealed no interaction between the polymer and the drug in the film and the drug is encapsulated in the polymer matrix.

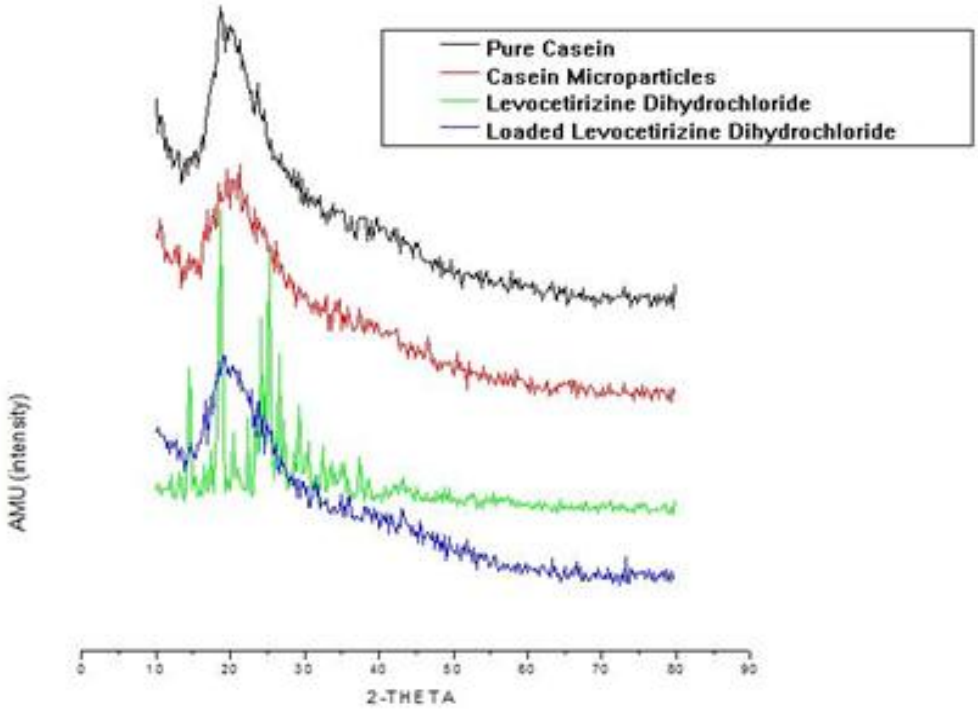


Figure 3. Combined XRD pattern having constant X-axis.

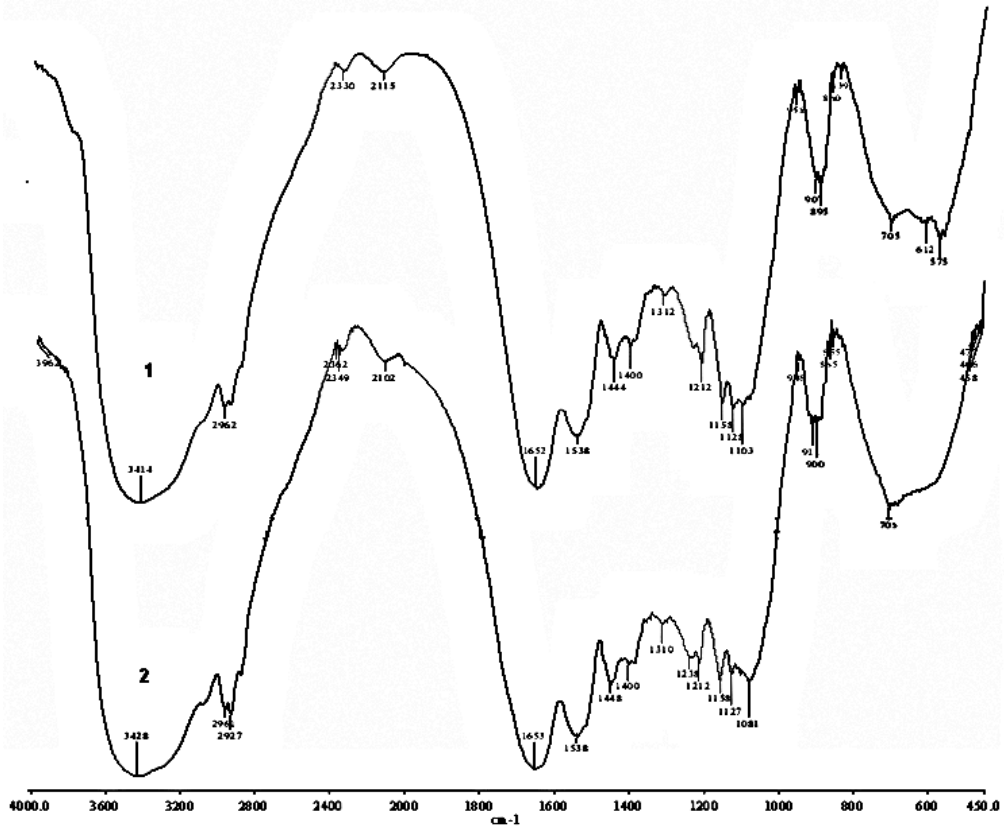


Figure 4. IR spectrum of placebo and levocetirizine dihydrochloride loaded casein microparticles.

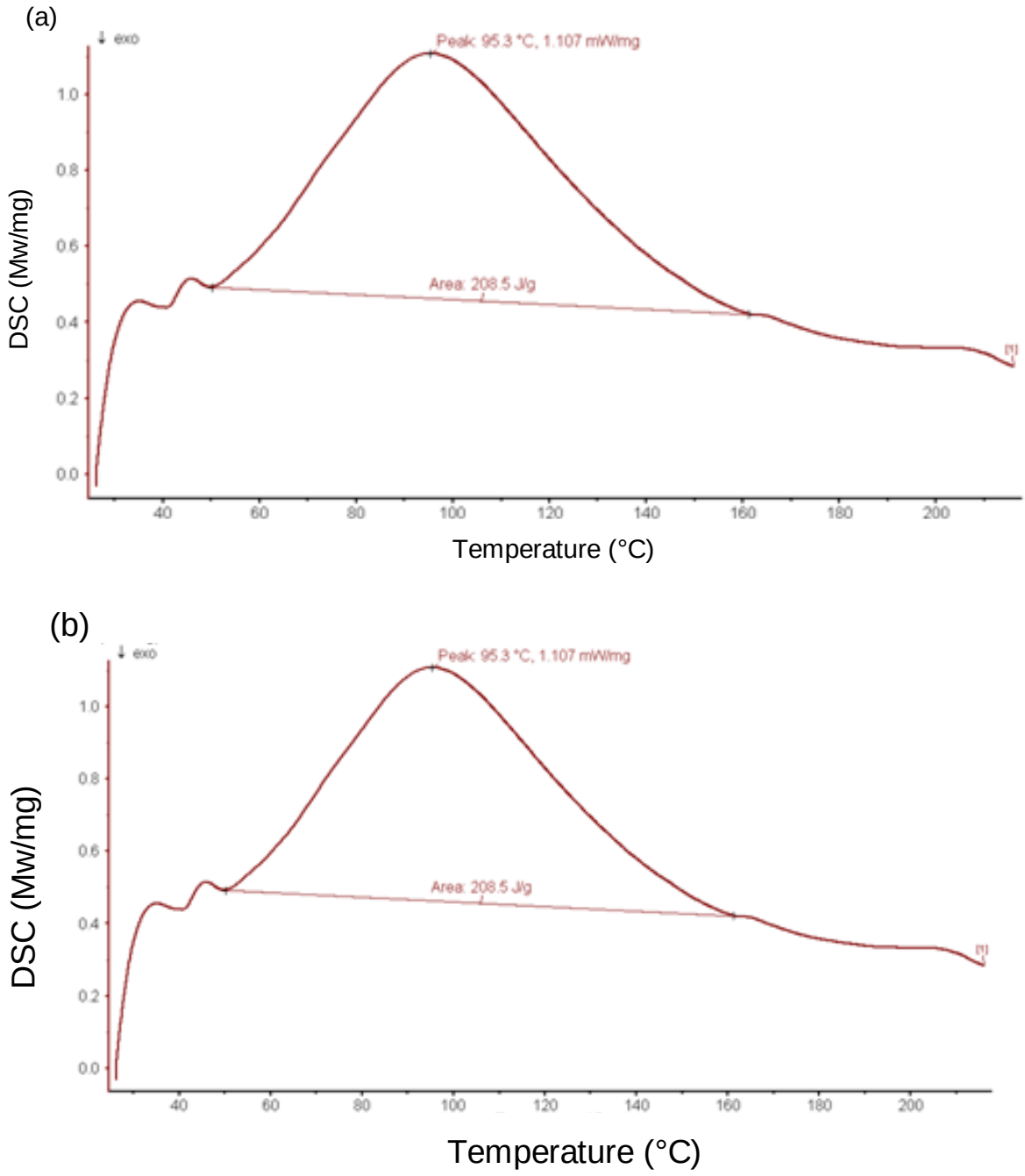


Figure 5. DSC thermograms.

Loading efficiency and *in vitro* release

Levocetirizine dihydrochloride loaded casein microparticles possess a high percentage of encapsulation efficiency which was found to be 76.5%. *In vitro* release studies were performed in simulated intestinal fluid (SIF) and simulated gastric fluid (SGF) in a pH-responsive drug delivery system. In a pH sensitive drug delivery system in which the drug is ionically linked, the release of the drug is controlled firstly by the rate of cleavage of electrostatic bonds and secondly by diffusion. The cumulative percentage drug release profile (Figure 6) reveals that the amount of drug releases in SGF is 2.33 ± 0.13%, which is a significantly negligible fraction, and the drug was found to be released in a controlled manner in SIF (58.14 ± 3.11%) over the period of eight hours.

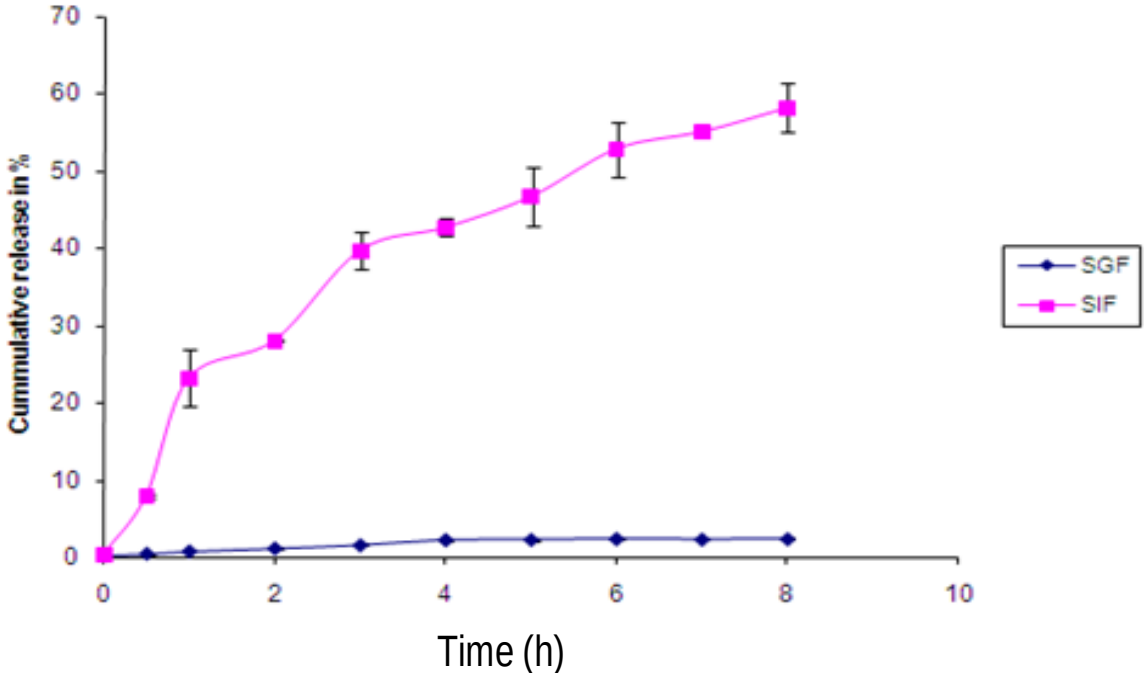


Figure 6. *In vitro* release profile of levocetirizine dihydrochloride.

Conclusion

The main theme of this research is focused on the preparation of casein microparticles which can be loaded with a range of drugs and the effects of processing conditions on particle size, drug loading and release. Casein microparticles were prepared by steric stabilization process. The parameters such as ethyl cellulose concentration, casein concentration and volume of casein solution were optimized. Ethyl cellulose concentration of 2% was found to be effective in obtaining spherical, uniform and evenly distributed microparticles. SEM reports that the microparticles size was found to be less than 3 µm. Casein microparticles showed macropore surfaces with follicular structure appearance. The bands observed in the casein microparticles spectrum suggest that drug is physically dispersed in the polymer. The DSC studies were also performed to investigate the casein-levocetrizine electrostatic interaction which indicates the absence of electrostatic interaction and it proves that the drug is encapsulated within the casein matrix. Release profile suggests that the drug be released till 8 hours in a controlled fashion.

ACKNOWLEDGEMENTS

The authors would like to gratefully acknowledge the financial and technical support of VIT University. Thanks are due to SAIF, IIT-Madras for providing the SEM and Thermal analysis data.

REFERENCES

Ana JPS, Newton LP, Osvaldo F, John HC (1999). Influence of formulation on the physicochemical properties of casein microparticles. Int. J. Pharm. 186(2):191-198.

Arora N, Garg T, Bilandi A (2012). Review on casein production and casein based nano-formulations. Int. Res. J. Pharm. 3(1):41-45.

Bachar M, Mandelbaum A, Portnaya I, Perlstein H, Even-Chen S, Barenholz Y, Danino D (2012). Development and characterization of a novel drug nanocarrier for oral delivery, based on self-assembled β-casein micelles. J. Control Release 160:164-171

Chen L, Remondetto GE (2006). Food protein-based materials as nutraceutical delivery systems. Trends Food Sci. Technol. 17:272-283.

Ciprandi G, Cirillo L, Vizzaccaro A, Tosca MA (2004). Levocetirizine improves nasal obstruction and modulates cytokine pattern in patients with seasonal allergic rhinitis: a pilot study. Clin. Exp. Allergy 34(6):958–964.

Cranswick N, Turzikova J, Fuchs M, Hulhoven R (2005). Levocetirizine in 1-2 year old children: pharmacokinetic and pharmacodynamic profile. Int. J. Clin. Pharmacol. Ther. Toxicol. 43(4):172-177.

Dalgleish DG (1998). Casein micelles as colloids. Surface structures and stabilities. J. Dairy Sci. 81:3013–3018.

Elzoghby AO, El-Fotoh WS, Elgindy NA (2011). Casein-based formulations as promising controlled release drug delivery systems. J. Control Release 153(3):206-216.

Ferrer M (2011). Pharmacokinetic evaluation of Levocetirizine. Expert Opin. Drug Metab. Toxicol. 7:1035-1047.

Jalil R, Nixon JR (1990). Microencapsulation using poly(DL-lactic acid). IV: Effect of storage on the microcapsule characteristics. J. Microenapsul. 7(3):375-383

Katz Y, Rajuan N, Goldberg M, Cohen A, Heyman E, Leshno M (2009). Avoidance of early regular exposure to cow's milk protein is a major risk factor for development of IGe mediated cow's milk allergy (cma).

J. Allergy Clin. Immunol. 123:S211.
Kim CK, Lee EJ (1992). The controlled release of blue dextran from alginate beads. Int. J. Pharm. 92:11-19.
Kim YJ, Park HG, Yang YL. Yoon Y, Kim S, Oh E (2005). Multifunctional drug delivery system using starch – alginate beads for controlled release. Biol. Pharm. Bull. 28:394-397.
Knepp WA, Jayakrishnan A, Quigg JM, Sitren HS, Bagnall JJ, Goldberg EP (1993). Synthesis, properties and intratumoral evaluation of mitoxantrone-loaded casein microspheres in Lewis lung carcinoma. J. Pharm. Pharmacol. 45:887–891.
Latha MS, Jayakrishnan A (1994). Glutaraldehyde cross-linked bovine casein microspheres as a matrix for the controlled release of theophylline: *In-vitro* studies. J. Pharm. Pharmacol. 46(1):8-13.
Latha MS, Jayakrishnan A, Rathinam K, Mohanty M (1994). Casein as a carrier matrix for 5-fluorouracil: drug release from microspheres, drug–protein conjugates and in vivo degradation of microspheres in rat muscle. J. Pharm. Pharmacol. 46:858–862.
Latha MS, Lal AV, Kumary TV, Sreekumar R, Jayakrishnan A (2000). Progesterone release from glutaraldehyde cross-linked casein microspheres: *in vitro* studies and *in vivo* response in rabbits. Contraception 61(5):329-334.
Latha MS, Ranthinam K, Mohanan P, Jayakrishnan A (1995). Bioavailability of theophylline from glutaraldehyde cross-linked casein microspheres in rabbits following oral administration. J. Control Release 34:1–7.
Molimard M, Diquet B, Benedetti MS (2004). Comparison of pharmacokinetics and metabolism of desloratadine, fexofenadine, levocetirizine and mizolastine in humans. Fundam. Clin. Pharmacol. 18(4):399-411.
Patel JG, Modi AD (2012). Formulation, optimization and evaluation of levocetirizine dihydrochloride oral thin strip. J. Pharm. Bioall. Sci. 4:35-36.
Prabakara P, Ravi M, Marina K, Vijaynarayana K, Ullas DS, Harish NM, Shastry CS, Charyulu RN (2011). Formulation and evaluation of fast dissolving films of levocetirizine dihydrochloride. Int. J. Pharm. InvestIG. 1(2):99-104.
Pradeep K, Inderbir S (2010). Formulation and characterization of tramadol – loaded IPN mirogels of alginate and gelatin: Optimization using response surface methodology. Acta Pharm. 60(3):295-310.
Rao VU, Vasudha K, Bindu K, Samanta S, Rajinikanth PS, Mishra B, Balasubramaniam J (2007). Formulation and invitro characterization of sodium alginate-gellan beads of glipizide. Acta Pharm. Sci. 49:13-28.
Willmott N, Magee GA, Cummings J, Halbert GW, Smyth JF (1992). Doxorubicin loaded casein microspheres: protein nature of drug incorporation. J. Pharm. Pharmacol. 44:472–475.
Yanhong W, Monica RG, Lene FN, Lene J, Hanne E, Eva HM, Hanne MN (2011). Biodegradable nanocomposite microparticles as drug delivering injectable cell scaffolds. J. Control Release 156(1):11-20.
Yoav DL (2010). Milk proteins as vehicles for bioactives. Curr. Opin. Colloid Interface Sci. 15(1-2):73-83.

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Vol. 7(17), pp. 1054-1060, 8 May, 2013
DOI 10.5897/AJPP12.1368
ISSN 1996-0816 © 2013 Academic Journals
http://www.academicjournals.org/AJPP

African Journal of Pharmacy and Pharmacology

Full Length Research Paper

Molecular cloning and characterization of a new cDNA encoding a trypsin-like serine protease from the venom gland of *Scolopendra subspinipes* mutilans

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Accepted 24 April, 2013

A cDNA encoding a new putative serine protease, named Ssmase, was cloned and characterized from the venom gland of the centipede *Scolopendra subspinipes* mutilans. The cDNA sequence was 1029 bp length, including a 780 bp open reading frame (ORF), a 105 bp 5′ untranslated region, and a 144 bp 3′ untranslated region. The precursor nucleotide sequence of Ssmase was deduced to encode a prepro-peptide of 19 residues and a mature protein of 240 residues. The 19 amino acid residues prepro-peptide of Ssmase putatively composed of 14 amino acids of pre-peptide and 5 amino acids of propeptide (QGSSA). The mature protein of Ssmase contained the typical domain of a trypsin-like serine protease, where His61, Asp108 and Ser208 were the principal residues of the catalytic center. The cysteine residues at 46 to 62, 141 to 214, 180 to 195 and 204 to 233 possibly formed four pairs of disulfide bridges. Ssmase was found to have five N-glycosylation sites (N-Xaa-T/S). To the best of our knowledge, Ssmase was a trypsin-like serine protease firstly characterized from centipede venoms. Ssmase represented a new family of trypsin-like serine protease with four disulfide bridge motif.

Key words: Centipede, *Scolopendra subspinipes* mutilans, trypsin-like serine protease, cysteine motif.

INTRODUCTION

Centipedes are one major group of venomous arthropods, which nearly occur all over the world. They prey on many other species of arthropods, earthworms, snails, and other small animals, mainly killing them with their toxicognanths (Antoniazzi et al., 2009; Undheim and King, 2011). Envenomations by centipedes are characterized by an instant, local burning pain that ranges in intensity from excruciating to mild, and in some cases radiates or spreads to other parts of the victim's body (Bush et al., 2001; Acosta and Cazorla, 2004). Several centipede species are capable of inflicting severe symptoms in humans, including myocardial ischemia and infarction, hemoglobinuria and hematuria, hemorrhage, and rhabdomyolysis (Gomes et al., 1982; Acosta and Cazorla, 2004; Medeiros et al., 2008). The venom of the centipede is composed of many active ingredients including enzymes (acid/alkaline phosphatases, esterases, hyaluronidases, etc), non-enzymatic proteins (cardiotoxins, myotoxins, etc), other non-peptidic active components (histamine and serotonin) and several neurotoxins, which has been reported to have many biochemical and physiological effects (Mohamed et al., 1983; Stankiewicz et al., 1999; Rates et al., 2007; Undheim and King, 2011; Liu et al., 2012; Yang et al., 2012). Among these enzymes, both *Otostigmus pradoi* and *Scolopendra viridicornis* venoms showed weak

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fibrinogenolytic activity (Malta et al., 2008), while the venom of *Scolopendra subspinipes* has anticoagulant activity. A phospholipase A_2 "Scol/Pla" was purified and cloned from the centipede *Scolopendra viridis* (Gonzalez-Morales et al., 2009). Although several proteases have been recorded from centipede venom, the venom of centipedes is poorly characterized, compared with those of other venomous animals (Rates et al., 2007; Qiu, 2012).

Serine proteases, found in many organisms, are of broad interest because they have diverse physiological functions, affecting processes such as digestion, immune response, complement activation, cellular differentiation, and hemostasis (Qiu, 2012). Trypsin-like serine protease (Tryptase) is a member of serine proteases and plays an important role in some physiological processes including wound healing, inflammatory reaction, blood clotting, regeneration, etc (Jin et al., 2002; D´ora Dienes a and Gunnar Lid´en d, 2007; Yuan et al., 2012). Snake venom is very rich in various types of enzymes such as metalloproteinases, serine proteinases, phospholipases, and hyaluronidases (Jin et al., 2002; Cidade et al., 2006; Rojnuckarin et al., 2006). Serine proteases from snake venom were well-characterized (Serrano and Maroun, 2005). A serine protease gene was cloned from the centipede body tissue (You et al., 2004), but there is few report on serine proteases derived from centipede venom so far. In this study, a new cDNA encoding a serine protease was cloned and characterized from the venom gland of the centipede *S. subspinipes* mutilans. Sequence analysis showed that the encoded protease had a conserved C-terminal domain belonging to the trypsin-like serine protease superfamily. Ssmase shared the principal residues of the catalytic center with most serine proteases (His61, Asp108 and Ser208). However, Ssmase, as the first serine protease from centipede venoms, displays unique features, e.g. no significant similarities to other serine proteases.

MATERIALS AND METHODS

Construction of cDNA library

S. subspinipes mutilans was collected from Xiangfan, Hubei province in China. The venomous glands connected to the first pair forceps of centipedes were stimulated using a 3 V alterative current. After 48 h, the venomous glands were dissected and grinded into liquid nitrogen. RNA was extracted using Trizol Reagent (Gibco) and mRNA was purified by PolyA Tract mRNA kit according to the manual. About 10 µg of mRNA was used for the synthesis of the double strand cDNAs. The cDNA Library was constructed by SMART™ cDNA Library Construction Kit according to the protocols. The ligated products were transformed into *Escherichia coli* DH5a strain. A cDNA library was constructed from the venomous gland of the centipede *S. subspinipes* mutilans.

Screening of cDNA library

Polymerase chain reaction (PCR) method was used to screen the positive clones from the venomous gland cDNA library. The clones of the venomous gland cDNA library were amplified and used as the templates for PCR. The forward and reverse primers used for PCR were 5'-TGT AAA ACG ACG GCC AGT-3' and 5'-CAG GAA ACA GCT ATG ACC-3', respectively. The positive clones were randomly selected for DNA sequencing.

Sequence analysis

The open reading frame (ORF) for the precursor of Ssmase was analyzed using an ORF finder tool from (http://www.ncbi.nlm.nih.gov/gorf/gorf.html). The deduced amino acid sequences of the Ssmase were received using National Center for Biotechnology Information (NCBI) online search tool (BLAST) (http://www.ncbi.nlm.nih.gov/blast/Blast.cgi). The signal peptide was predicted at http://www.cbs.dtu.dk/services/SignalP/. The protein structure and function domain were analyzed using online analysis software ExPASy (http://www.expasy.org/prosite/) and SMART (http://smart.embl-heidelberg.de/), respectively. The theoretical isoelectric point (pI) and molecular weight (Mw) of the protein were predicted using ExPASy Proteomics Server (http://web.expasy.org/compute_pi/). Multiple sequence alignment analysis of proteases from different organisms was performed by ClustalX1.83 program. The gaps were added and adjusted by manuals to acquire the maximum homology.

RESULTS AND DISCUSSION

Using cDNAs obtained from mRNA of *S. subspinipes* mutilans venomous glands, a cDNA library was specific constructed for its venomous gland. Over two hundred clones from the venomous gland cDNA library were amplified and prepared, and the plasmids were used as the templates for PCR screening. The primers used for PCR were universal in the vector of the cDNA library. Thus, the clones from the venomous gland cDNA library facilely characterized their size of inserted fragments by PCR method.

Sixty clones with different sizes from the constructed centipede venom gland cDNA library were randomly selected to sequence and analyze (data not shown). A 1029 bp full-length cDNA sequence was characterized as shown in Figure 1. The ORF of this cDNA was analyzed using an online ORF finder tool. The cDNA sequence contains a 780 bp ORF, a 105 bp 5' untranslated region, and a 144 bp 3' untranslated region. The translation initiation site was assigned to the methionine codon at nucleotides 106 to 108, and the termination codon (TGA) was found at nucleotides 883 to 885. The "AT" content of ORF is 64%, significantly lower than those of 5' untranslated region (77.2%) and 3' untranslated region (81.6%), which suggested that a specific secondary structure was formed for its transcription. Although NCBI Basic Local Alignment Search Tool (BLAST) tool showed that the cDNA molecule had no high homology with known genes, the results of theoretical protein revealed that it had sequence identities with trypsins, tryptases, and peptides belonging to the trypsin family of serine protease (so named as Ssmase). The cDNA of Ssmase was theoretically deduced to encode a precursor protein

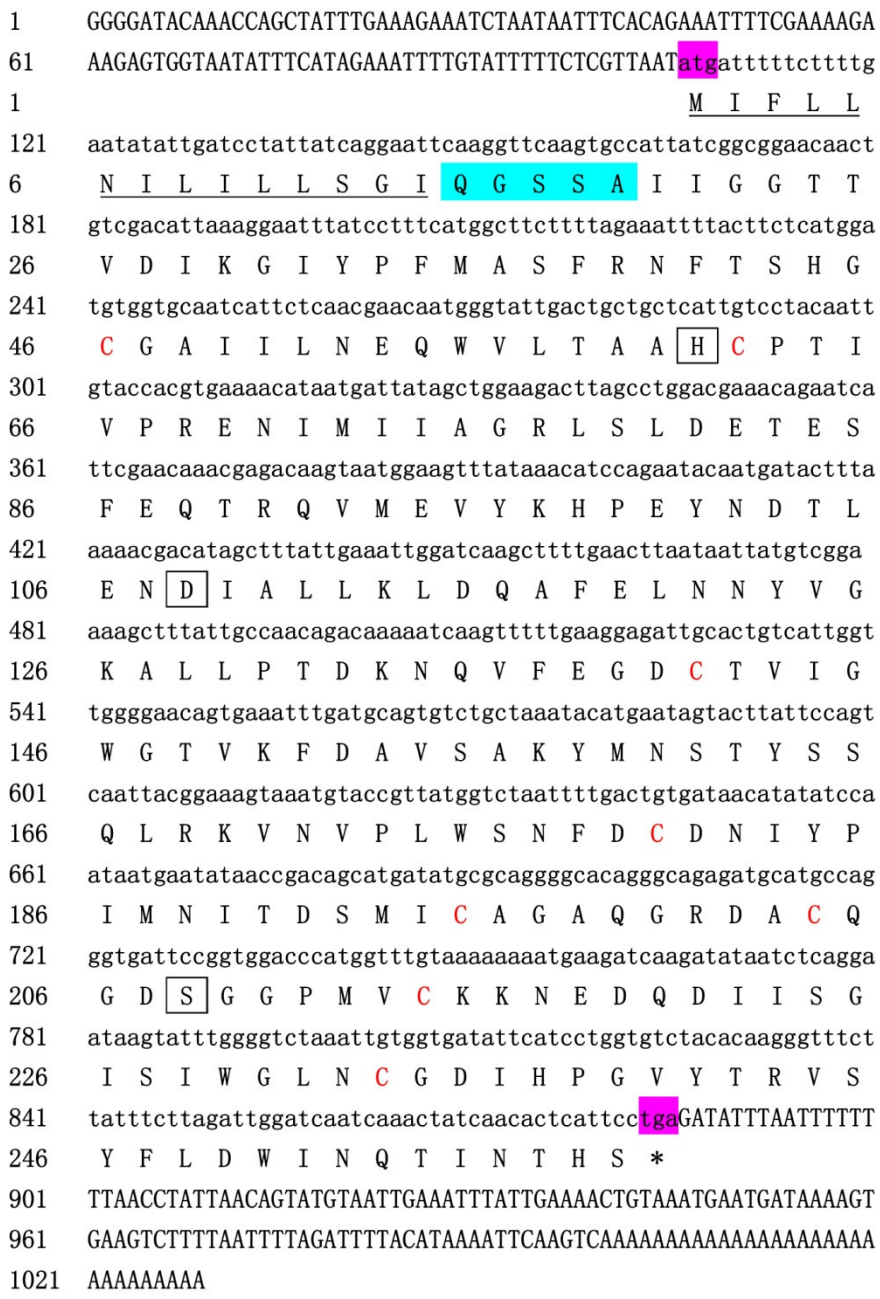

Figure 1. The deduced amino acid sequence of Ssmase was shown below the nucleotide sequence. 5' and 3' untranslated region nucleotide sequences were indicated in capital letters, while ORF nucleotide sequences were indicated in lowercase letters. The initial codon (atg) and end codon (tga) were shaded with purple. The pre-peptide residues were underlined, while the pro-peptide residues were shaded with green. Boxed amino acid residues were the conserved catalytic triad in serine protease. The cysteine residues were highlighted in red color.

with 259 amino acid residues, a calculated molecular mass of 28722.74 Da and a predicted isoelectric point of 4.77. Venom is a key element in the predatory behaviors of centipedes, because centipede venom is a complex mixture of various molecules. It has been proposed that centipede venom also contains digestive enzymes used to soften-up the flesh of the prey which is subsequently sucked-up (Undheim and King, 2011). Numerous studies reported on the venom components and their functions from snake, scorpion, spider, etc.

But only a few reports focused on the venom of centipedes, which was a neglected group of venomous animals

(Rates et al., 2007; Undheim and King, 2011). Although some active peptides from centipede venom were identified, most of the results were obtained using milked venom and gel electrophoresis analysis (Peng et al., 2010; Malta et al., 2008; Antoniazzi et al., 2009). Many proteins or peptides with molecular masses ranging from 1.3 to 22.6 kDa were found by 2D chromatographic analysis of *S. viridicornis* nigra and *Scolopendra angulata* venoms. N-terminal sequencing of 13 and 11 of these protein molecules from *S. viridicornis* nigra and *S. angulata*, respectively, yielded a total of 10 protein families (Rates et al., 2007). However, few full-length sequences of these venom proteins were clearly identified. The reason may be that the amount of the milked venom is not enough to sequence and analyze. So, it is an important and easy path that the venomous gland cDNA library was constructed and used to screen the venom protein/peptide genes for acquiring the complete sequences (Peng et al., 2010; Rates et al., 2007). Especially, it would be most relevant to survey comprehensively the active proteins/peptides in the venom through the cDNA cloning method. So *S. subspinipes* dehaani venoms were systematically investigated by transcriptomic and proteomic analysis coupled with biological function assays. The purified proteins/peptides showed different pharmacological properties, including platelet aggregating, anticoagulant, phospholipase A(2) and trypsin inhibiting activities (Liu et al., 2012).

Serine proteases are common constituents of venom proteomes and venom gland trancriptomes of viperid species (Francischetti et al., 2004; Kashima et al., 2004; Cidade et al., 2006; Jin et al., 2007; Vilca-Quispe et al., 2010). Abundant serine proteases have been isolated and characterized from snake venoms (Serrano and Maroun, 2005). But there is no report that serine proteases were isolated and cloned from the venom gland of *S. subspinipes* mutilans. In the present paper, we obtained the complete cDNA sequence encoding a new tryptase, Ssmase, which was cloned and characterized from the venom gland of *S. subspinipes* mutilans for the first time.

The homology of Ssmase was searched through the non-redundant protein sequences (nr) and Swiss-Prot databases. The results showed that 40% significant similarities were found between Ssmase and Scolonase, which was purified and characterized from the tissue of the Korean centipede, *S. subspinipes* mutilans (You et al., 2004). The ORF of Ssmase cDNA was found to be composed of 259 amino acid residues, including a signal peptide sequence of 19 amino acid residues and a mature protein of 240 amino acid residues, using the online SignalP 4.0 server. The data indicated that Ssmase should be a typical secretory protein. Residues 1 to 19 represent a signal peptide and residues 20 to 259 possess the typical domain of a tryptase. Based on the assignment proposed for batroxobin (Itoh et al., 1987), the 19 amino acid residues prepro-peptide of Ssmase putatively composed of 14 amino acids of pre-peptide and 5 amino acids of propeptide. Moreover, two putative cleavage sites were found in the prepro-peptide region of Ssmase, as shown in Figure 2. The five-residue propeptide between the signal peptide and the mature enzyme is a putative activation peptide. Among the serine proteases from viperid and crotalid snake venoms, they share a highly conserved activation peptide sequence: QR/KSSDR (Itoh et al., 1987; Serrano and Maroun, 2005). Three of these residues (15Q, 17S and 18S) in Ssmase are conserved, compared with other snake venom serine proteases (SVSPs), which these three residues may play an important role in executing the function of activation peptide.

No apparent match to any of the deposited nucleotide sequences was found in the current GenBank/EMBL databases, indicating that the cDNA clone encodes a new serine protease distinct from the known enzymes. Serine proteases are among the best-characterized components of living organisms. All tryptases share a substrate preference for a basic P1 residue, lysine (Lys) or arginine (Arg). This is mainly caused by the presence of a negatively charged Asp189 at the bottom of the S1 pocket (Serrano and Maroun, 2005). SVSPs possess an identical trypsin containing the conserved catalytic triad, His57, Asp102 and Ser195 (Vitorino-Cardoso et al., 2006). Multiple alignment analysis of Ssmase and serine proteases revealed that Ssmase had low amino acid sequence homology with typical tryptases. However, the amino acid residues in their functional domains were almost identical and remarkably conserved in the carboxy-terminal regions. As was shown in Figures 1 and 3, Ssmase also contains the conserved His61, Asp108 and Ser208, which are the principal residues of the catalytic center. Recently, a number of sequence motifs surrounding serine proteinase evolutionary markers and active site residues for the S1 family of clan SA were identified (Krem and Di Cera, 2001; Vitorino-Cardoso et al., 2006). These motifs were 54TAAHC58 surrounding the catalytic His57, 102DIAL105 at Asp102, and 193GDSGGP198 around Ser195 (Vitorino-Cardoso et al., 2006), which were also highly conserved in the Ssmase as shown in Figure 3. Together, Ssmase is a new member of trypsin-like serine protease from the venom of the centipede *S. subspinipes* mutilans.

Despite the sharing of similar structural features, venom serine proteases display a highly diverse pharmacological profile, which includes actions on proteins of the coagulation cascade, such as thrombin-like activity on fibrinogen, activation of factor V, activation of protein C, fibrinogenolysis, activation of plasminogen, and induction of platelet aggregation (Serrano and Maroun, 2005). Venom serine proteinases are commonly glycosylated, where the carbohydrate moiety is usually Asn-linked. The extent of N- or O-glycosylation appears to be significant but quite variable, and the functional

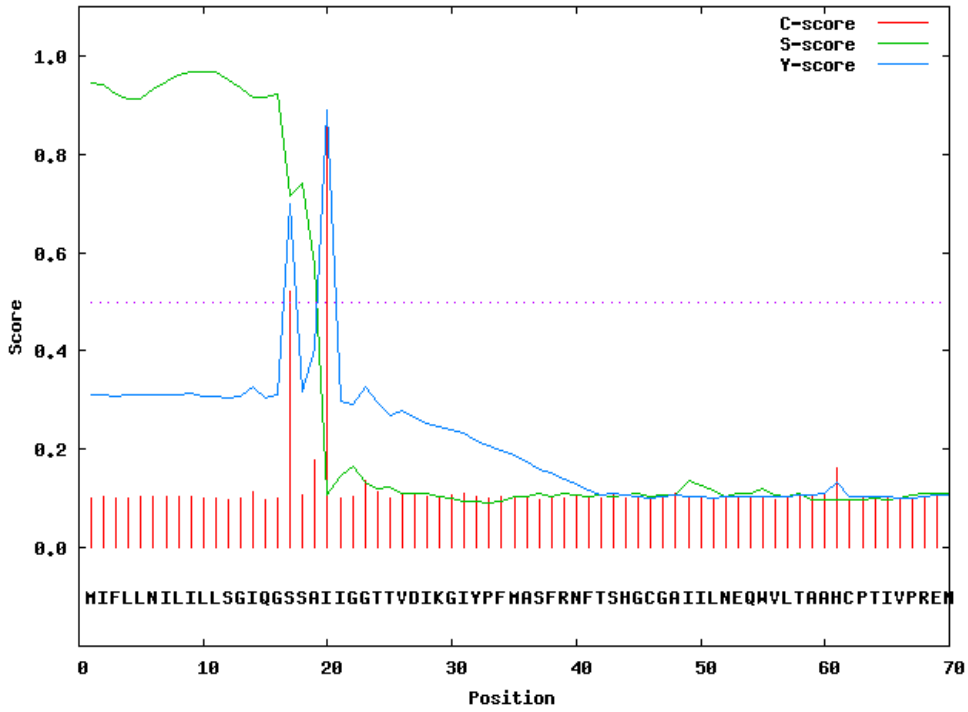

Figure 2. The predicted cleavage sites of the Ssmase pre-peptide peptide and pro-peptide signals using the online Signal P 4.0 server. The cleavage site of Ssmase between the signal peptide and the pro-peptide was predicted to be 14 to 15. The cleavage site of Ssmase between the pro-peptide and the mature protein was predicted to be 19 to 20.

```
          Ssmase  ----MIFLLNILILLSG----IQGS------SAIIGGTTVDIKGIYPFMASFRNFTSHG-    45
       Scolonase  MNSFTILIVTYFSLAFGSRCGIKNGPMLDEFNRIVGGEAAEP-GEFPWQISLQVVSWYGS    59
         Tryptase  ---MLNLLVLALPLLVSLVHTAPAPGQALERAGIVGGKEAPG-HKWPWQVSLRCLDQYWK    56
     Kallikrein1  ----MRFLILFLALSLGGIDAAPPVQ-----SRIVGGFNCEK-NSQPWQVAVYRFTKYQ-    49
      Batroxobin  ----MVLIRVIANLLILQVSYAQKSS-----ELVIGGDECDI-NEHPFLAFMYYSPRYF-    49
                  1.......10.........20.........30.........40.........50.........60

          Ssmase  ---CGAIILNEQWVLTAAHCPTIVPRENIMIIAGRLSLDETESFEQTRQVMEVYKHPEYN   102
       Scolonase  YHYCGGSILDESWVVTAAHCVEGMNPSDLRILAGEHNFKKEDGTEQWQDVIDIIMHKDYV   119
         Tryptase  -HFCGGSLIHPQWVLTAAHCFGPE-KADPLYIRVQLGEQHLYYQDRLLLVSRIIVHPNYY   114
     Kallikrein1  ---CGGILLNANWVLTAAHCHNDK----YQVWLGKNNFLEDEPSAQHRLVSKAIPHPDFN   102
      Batroxobin  ---CGMTLINQEWVLTAAHCNRRF----MRIHLGKHAGSVANYDEVVRYPKEKFICPNKK   102
                  ........70.........80.........90.........100.........110.........120

          Ssmase  ------------DTLENDIALLKLDQAFELN-NYVGKALLPTDKNQVFEGDCTVIGWGTV   149
       Scolonase  Y------------STLENDIALLKLAEPLDLTPTAVGSICLPSQNNQEFSGHCIVTGWGSV   168
         Tryptase  D------------EVNGADIALLELEDPVNLSSHVQPVTLPPASETFPKGTRCWVTGWGDV   163
     Kallikrein1  MSLLNEHTPQPEDDYSNDLMLLRLKKPADITDVVKPIDLPTEEPK--LGSTCLASGWGSI   160
      Batroxobin  KN----------VITDKDIMLIRLDRPVKNSEHIAPLSLPSNPPS--VGSVCRIMGWGAI   150
                  .......130.......140.......150.......160.......170.......180

          Ssmase  KFDAVSAKYMNSTYSSQLRKVNVPLWSNFDCDNIYPI--------MNITDSMICAGAQ--   199
       Scolonase  REGGNSP--------NILQKVSVPLMTDEECSEYY----------NIVDTMLCAGYAEG   209
         Tryptase  HSGWPLPP------PYPLKQVRVPIVENSECDMQYHLGLSTGDNIPIVRDDMLCAGSE--   215
     Kallikrein1  TPVKYEYP-------DELQCVNLKLLPNEDCAKAH---------IEKVTDDMLCAGDMDG   204
      Batroxobin  TTSEDTYP-------DVPHCANINLFNNTVCREAYN---------GLPAKTLCAGVLQG   193
                  ......190.......200.......210.......220.......230.......240

          Ssmase  GRDACQGDSGGPMVCKKNEDQDIISGISIWG-LNCG-DIHPGVYTRVSYFLDWINQTINT   257
       Scolonase  GKDACQGDSGGPLVCPNGDGTYSLAGIVSWG-IGCAQPRNPGVYTQVSKFLDWIRNTNID   268
         Tryptase  GHDSCQGDSGGPLVCRVN-GTWLQAGVVSWG-EGCALPNRPGIYTRVTHYLDWIHQCIPR   273
     Kallikrein1  GKDTCAGDSGGPLICDGV-----LQGITSWGPSPCGKPNVPGIYTRVLNFNTWIRETMAE   259
      Batroxobin  GIDTCGGDSGGPLICNGQ-----FQGILSWGSDPCAEPRKPAFYTKVFDYLPWIQSIIAG   248
                  ........250.......260.......270.......280.......290.......300

          Ssmase  HS-------    259
       Scolonase  GSNVIEFII    277
         Tryptase  ES-------    275
     Kallikrein1  ND-------    261
      Batroxobin  NKTATCP--    255
                  .........
```

Figure 3. Multiple sequence alignment of Ssmase and representative serine proteases. Sequence alignment of Ssmase and representative serine proteases was carried out by the ClustalX1.83 software. The deduced amino acid sequence of Ssmase was compared with those of other serine proteases, such as Scolonase (Centipede, GenBank: AAD00320.1), Tryptase (Pig, UniProtKB/Swiss-Prot: Q9N2D1.1), Kallikrein-1 (Mouse, UniProtKB/Swiss-Prot: P15947.3) and batroxobin (Snake, GenBank: CAA31240.1). Amino acid residues were denoted by one-letter symbols.

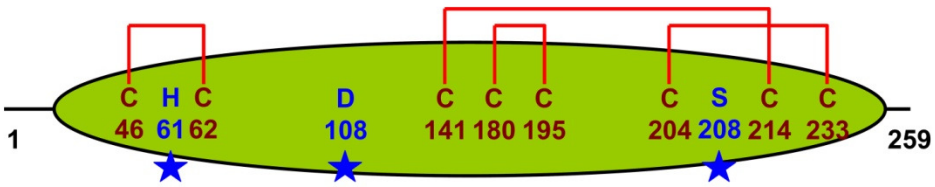

Figure 4. The trypsin domain analysis of the Ssmase using the online software ExPASy. Cysteine residue (C) was indicated with purple color, while active residues (H61, D108 and S208) were blue and highlighted with blue five-pointed star. Four pairs of disulfide bridges of Ssmase were linked by red lines.

significance of this variation is incompletely understood (Serrano and Maroun, 2005). In the case of Ssmase, five potential N-glycosylation sites, Asn-Xaa-Ser/Thr, were located at amino acid residues 40 to 42, 102 to 104, 160 to 161 and 188 to 190.

Snake venom thrombin-like enzymes are serine endopeptidases, which are structurally constrained by the presence of six highly conserved disulfide bridges, five of which are common to all S1 serine proteinases (Vilca-Quispe et al., 2010). However, eight half-cysteine residues were found for the mature Ssmase, suggesting the presence of four disulfide bonds in this protein (Figure 4). Recently, twenty-six neurotoxin-like peptides were identified from the centipede venoms, *S. subspinipes* mutilans L. Koch by peptidomics combined with transcriptome analysis. These neurotoxins each contain 2 to 4 intra-molecular disulfide bridges, and in most cases the disulfide framework is different to that found in neurotoxins from the venoms of spiders, scorpions, marine cone snails, sea anemones, and snakes (Yang et al., 2012).

The online ExPASy analysis of Ssmase conserved domain showed that the cysteine residues of Ssmase at 46 to 62, 141 to 214, 180 to 195 and 204 to 233 form four disulfide bonds, which may be very important and indispensable for Ssmase to maintain the dimensional structure and the activity as a tryptase. Thus, Ssmase represents a new family of trypsin-like serine proteinase with four disulfide bond motif from centipede venom, significantly distinct from the known serine proteinase with 5/6 disulfide bonds.

Majority of proteolytic activity in centipede venoms seems to originate from metalloproteases, gelatinolytic activity from non-metalloproteases, and most likely serine proteases, detected in the venoms of *O. pradoi* and *Conus iheringi* (Malta et al., 2008; Undheim and King, 2011). Ssmase has low amino acid sequence homology with tryptases, but it shares the amino acid residues of functional domains and the carboxy-terminal regions with serine proteases.

The finding led us to consider that novel serine proteinases remain to be unveiled in the venom of the centipede *S. subspinipes* mutilans. To gain insights into the structures and activities of the novel enzyme, studies to obtain enough amounts of the protein are in progress using both yeast and *E. coli* expression systems.

ACKNOWLEDGEMENT

This work was supported by a grant from the National Natural Sciences Foundation of China (No.30800571).

REFERENCES

Acosta MD, Cazorla (2004). [Centipede (*Scolopendra sp.*) envenomation in a rural village of semi-arid region from Falcon State, Venezuela]. Rev. Investi. Clin. 56:712-717.

Antoniazzi MM, PedrosoCM, Knysak I, Martins R, Guizze SP, Jared C, Barbaro KC (2009). Comparative morphological study of the venom glands of the centipede *Cryptops iheringi, Otostigmus pradoi* and *Scolopendra viridicornis*. Toxicon 53:367-374.

Bush SP, King BO, Norris RL, Stockwell SA (2001). Centipede envenomation. Wilderness Environ. Med. 12:93-99.

Cidade DA, Simao TA, Davila AM, Wagner G, Junqueira-de-Azevedo IL, Ho PL, Bon C, Zingali RB, Albano RM (2006). *Bothrops jararaca* venom gland transcriptome: analysis of the gene expression pattern. Toxicon. 48:437-461.

Dóra Dienes, Johan Börjesson, Per H¨agglund c, Folke Tjerneld b, Gunnar Lid´en d, ∗, Henrik St°albrand (2007). Identification of a trypsin-like serine protease from *Trichoderma reesei* QM9414. Enzyme, Microbial Technol.

Francischetti IM, My-PhamV, Harrison J, Garfield MK, Ribeiro JM (2004). *Bitis gabonica* (Gaboon viper) snake venom gland: toward a catalog for the full-length transcripts (cDNA) and proteins. Gene 337:55-69.

Gomes A, Datta A, Sarangi B, Kar PK, Lahiri SC (1982). Pharmacodynamics of venom of the centipede *Scolopendra subspinipes dehaani* Brandt. Ind.J. Exp. Biol. 20:615-618.

Gonzalez-Morales LE, Diego-Garcia L, Segovia C, Gutierrez Mdel LD, Possani (2009). Venom from the centipede *Scolopendra viridis Say*: purification, gene cloning and phylogenetic analysis of a phospholipase A2. Toxicon 54:8-15.

Itoh NN, Tanaka S, Mihashi I, Yamashina (1987). Molecular cloning and sequence analysis of cDNA for batroxobin, a thrombin-like snake venom enzyme. J. Biol. Chem. 262:3132-3135.

Jin Y, WH, Lee Y, Zhang (2007). Molecular cloning of serine proteases from elapid snake venoms. Toxicon, 49:1200-1207.

Jin Y, QM, Lu WY, Wang YL, Xiong (2002). Actions of two serine proteases from *Trimeresurus jerdonii* venom on chromogenic substrates and fibrinogen. Comp Biochem. Physiol. B Biochem. Mol. Biol. 132:529-534.

Kashima S, PG, Roberto AM, Soares S, Astolfi-Filho JO, Pereira S, Giuliati M, Faria Jr, MA, Xavier MR, Fontes JR, Giglio SC, Franca (2004). Analysis of *Bothrops jararacussu* venomous gland transcriptome focusing on structural and functional aspects: I--gene

expression profile of highly expressed phospholipases A2. Biochim. 86:211-219.
Krem MM, E, Di Cera (2001). Molecular markers of serine protease evolution. EMBO J. 20:3036-3045.
Liu ZCR, Zhang F, Zhao ZM, Chen HW, Liu YJ, Wang P, Jiang Y, Zhang Y, Wu JP, Ding WH, Lee (2012). Venomic and transcriptomic analysis of centipede *Scolopendra subspinipes* dehaani. J. Proteome Res. 11:6197-6212.
Malta MB, Lira MS, Soares SL, Rocha GC, Knysak I, Martins R, Guizze SP, Santoro ML, Barbaro KC (2008). Toxic activities of Brazilian centipede venoms. Toxicon 52:255-263.
Medeiros CR, Susaki TT, Knysak I, Cardoso JL, Malaque CM, Fan HW, Santoro ML, Franca FO, Barbaro KC (2008). Epidemiologic and clinical survey of victims of centipede stings admitted to Hospital Vital Brazil (Sao Paulo, Brazil). Toxicon 52:606-610.
Mohamed AH, G. Abu-Sinna HA, El-Shabaka AA, El-Aal (1983). Proteins, lipids, lipoproteins and some enzyme characterizations of the venom extract from the centipede *Scolopendra morsitans*. Toxicon 21:371-377.
Peng K, Kong Y, Zhai L, Wu X, Jia P, Liu J, Yu H (2010).Two novel antimicrobial peptides from centipede venoms. Toxicon 55:274-279.
Qiu Y (2012). Molecular cloning and fibrin(ogen)olytic activity of a bumblebee. J. Asia-Pacific Entomol. 15:21-25.
Rates B, Bemquerer MP, Richardson M, Borges MH, Morales RA, De Lima ME, Pimenta AM (2007). Venomic analyses of *Scolopendra viridicornis nigra* and *Scolopendra angulata* (Centipede, Scolopendromorpha): shedding light on venoms from a neglected group. Toxicon 49:810-826.
RojnuckarinP, Muanpasitporn C, Chanhome L, Arpijuntarangkoon J, Intragumtornchai T (2006). Molecular cloning of novel serine proteases and phospholipases A2 from green pit viper (*Trimeresurus albolabris*) venom gland cDNA library. Toxicon 47:279-287.
Serrano SM, Maroun RC (2005). Snake venom serine proteinases: sequence homology vs. substrate specificity, a paradox to be solved. Toxicon 45:1115-1132.
Stankiewicz M, Hamon A, Benkhalifa R, Kadziela W, Hue B, Lucas S, Mebs D, Pelhate M (1999). Effects of a centipede venom fraction on insect nervous system, a native Xenopus oocyte receptor and on an expressed Drosophila muscarinic receptor. Toxicon, 37:1431-1445.
Undheim EA, King GF (2011). On the venom system of centipedes (Chilopoda), a neglected group of venomous animals. Toxicon 57:512-524.
Vilca-Quispe A, Ponce-Soto LA, Winck FV, Marangoni S (2010). Isolation and characterization of a new serine protease with thrombin-like activity (TLBm) from the venom of the snake *Bothrops marajoensis*. Toxicon, 55:745-753.
Vitorino-Cardoso AF, Pereira OH, Ramos MI, Homsi-Brandeburgo, Selistre-de-Araujo HS (2006). Insights into the substrate specificity of a novel snake venom serine peptidase by molecular modeling. Comp. Biochem. Physiol. B Biochem. Mol. Biol. 144:334-342.
Yang S, Liu Z, Xiao Y, Li Y, Rong M, Liang S, Zhang Z, Yu H, King GF, Lai R (2012). Chemical punch packed in venoms makes centipedes excellent predators. Mol. Cell Proteom. 11:640-650.
You WK, Sohn YD, Kim KY, Park DH, Jang Y, Chung KH (2004). Purification and molecular cloning of a novel serine protease from the centipede, *Scolopendra subspinipes* mutilans. Insect. Biochem. Mol. Biol. 34:239-250.
Yuan Z, Dahms HU, Han LL, Li QY, Zhang QZ, Wu RJ, Tan J, Zou XY, Hou L (2012). Cloning and characterization of a trypsin-like serine protease gene, a novel regeneration-related gene from *Apostichopus japonicus*. Gene 502:46-52.

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Vol. 7(17), pp. 1061-1066, 8 May, 2013
DOI 10.5897/AJPP12.1449
ISSN 1996-0816 © 2013 Academic Journals
http://www.academicjournals.org/AJPP

African Journal of Pharmacy and Pharmacology

Full Length Research Paper

Simultaneous determination of organic acids and iridoid glycosides in traditional Chinese medicine (TCM) preparation reduning injection by rapid resolution liquid chromatography (RRLC) with ultraviolet (UV) detection under segmental monitoring mode

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Accepted 15 April, 2013

A sensitive method of the simultaneous determination of organic acids and iridoid glycoside in reduning injection was described by rapid resolution liquid chromatography (RRLC) with ultraviolet (UV) detection under segmental monitoring mode. RRLC separation was achieved by use of a C_{18} analytical column packed with sorbent of particle size 1.8 µm while the injected sample volume was set to 2 µl. A binary gradient elution program of aqueous phosphoric acid (0.1%, v/v) versus acetonitrile was selected for the quantitative analysis of nine major components. The RRLC-UV method under segmental monitoring mode showed satisfactory linearity, the limit of detection and the limit of quantitation. The method had been successfully applied to the determination of nine major components content in reduning injection samples. The analytical time of the proposed protocol is less than that of conventional HPLC-UV analytical methods, thus RRLC-UV method under segmental monitoring mode could function as an attractive alternative method to be used to evaluate the quality of reduning injection in the process of production.

Key words: Reduning injection, organic acids, iridoid glycosides, rapid resolution liquid chromatography-ultraviolet (RRLC–UV).

INTRODUCTION

Reduning injection made from the aqueous extracts of *Flos Lonicerae*, *Herba Artemisiae Annuae* and *Gardenia jasminoides* was one of most widely used traditional Chinese medicines preparations to treat upper respiratory tract infection in china. It also has good clinical efficacy on herpangina, hand-foot-mouth disease, bronchiolitis and aphthous stomatitis (Guo, 2010; Heng et al., 2010; Shao, 2010; Luo et al., 2009). The chemical constituents of reduning injection mainly included three chemical types: essential oil, organic acids and iridoid glycoside.

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The organic acids of reduning injection which are mainly derived from *Flos Lonicerae* and *Herba A. Annuae*, include chlorogenic, 4-caffeoylquinic, 5-dicaffeoylquinic, caffeic and isochlorogenic acids A, B, C. It was reported that these organic acids have been associated with several biological effects, including antioxidant, antibacterial, antiviral and antipyretic properties (Chen et al., 2008; Hung et al., 2006; Mishima et al., 2005). Iridoid glycosides in reduning injection included secoxyloganin and geniposide. The pharmacological studies showed that geniposide had certain analgesic effect and anti-inflammatory effect (Koo et al., 2006). But in the quality standard of reduning injection, only chlorogenic acids and geniposide were quantified and used as chemical markers for the quality control of the preparation, owing to their antipyretic and antibiotic activities as well as their high content in this injection. Therefore, this existed quality evaluation method could not reflect the real and comprehensive active constituents of reduning injection, and is inadequate to control the quality of reduning injection. Consequently, determinations of all types of components such as chlorogenic acid and its analogues and iridoid glycosides in reduning injection could be a better strategy for the comprehensive quality control of reduning injection.

Recently, the rapid-resolution and ultra-performance liquid chromatography (RRLC/UPLC) methods have been established in many laboratories for qualitative and quantitative determinations of active components in TCM materials and preparations (Liu et al., 2010). In contrast to the conventional high performance liquid chromatography (HPLC), the proposed method is an attractive method for analysis, because the improved resolution, shorter retention times, low consumption of harmful organic solvent, higher sensitivity and better performance could be achieved for certain herbal medicines by using RRLC or UPLC (Liang et al., 2010; Qi et al., 2008) .

To the best of our knowledge, there were no methods reporting the RRLC determination of organic acids and iridoid glycosides in reduning coupled to UV detection in literatures. In order to comprehensively control the quality of reduning injection, a RRLC-UV method as reported under segmental monitoring mode for simultaneous determination of two category's compounds in the traditional Chinese medicine preparation reduning injection.

MATERIALS AND METHODS

Reference substances, reagents and chemicals

Three reference compounds including chlorogenic acid, caffeic acid and geniposide were purchased from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, P.R. China). Six reference standards including secoxyloganin, isochlorogenic acids A, B, C, 4-caffeoylquinic acid and 5-dicaffeoylquinic acid were offered by Jiangsu Kanion Pharmaceutical Co., Ltd (Lianyungang, China). Their structures were elucidated by their spectra data (MS, 1H NMR and 13C NMR). The purities of nine reference standards were analyzed on Eclipse plus C_{18} column (4.6 × 50 mm, 1.8 µm, Agilent Technologies, USA). The purity of each compound was determined to be higher than 98% by normalization of the peak areas as detected by HPLC-UV. All of the standard substances were stored in the refrigerator at 4°C. Acetonitrile was chromatographic pure and was purchased from Dima Technology Inc. (USA). De-ionized water was purified with a Milli-Q Academic ultra-pure water system (Billerica, MA, USA) prior to usage as RRLC mobile phase. Phosphoric acid was purchased from the First Chemical Company of Tianjin (Tianjin, China); chromatographic grade methanol was purchased from Hanbang Science & Technology Company (Nanjing, P.R. China). All samples were kindly offered by Jiangsu Kanion Pharmaceutical Co., Ltd, produced in the year 2009 to 2010.

Preparation of standard solutions

Nine standard stock solutions were prepared by accurately weighed amounts of the each reference compounds and dissolving them in 50% methanol (*v/v*). Working standard solutions were prepared by diluting the mixed standard solution to give six different concentrations for the calibration curves. The standard stock and working solutions were all stored in dark brown calibrated flasks at 4°C.

Preparation of sample solutions

Reduning injection was diluted with 50% methanol at the ratio of 1:100 and filtrated through a membrane filter (0.45 µm), and an aliquot of 2 µl of the filtrate was injected for RRLC–UV analysis.

Apparatus and chromatographic conditions

RRLC analyses were performed on an Agilent 1200 SL HPLC (Agilent Technologies, USA) equipped with a binary pump and a micro-vacuum degasser, a multi-wavelength (MW) detector, an autosampler, a column temperature controller. The column configuration composed of an Agilent Zorbax Extend reversed-phase C_{18} column (100 × 3.0 mm I.D., 1.8 µm particle size) and an Agilent Zorbax C_{18} guard column. The UV detection wavelength at 0 to 5 min was set at 225 nm for 4-caffeoylquinic acid, 5-dicaffeoylquinic acid, chlorogenic acid, caffeic acid and geniposide; at 237 nm from 5 to 8 min for secoxyloganin; from 8 to 10 at 324 nm for isochlorogenic acid A, isochlorogenic acid B and isochlorogenic acid C. A constant flow rate of 0.7 ml/min was employed throughout the analysis. All analyses were performed at 30°C and volume of solution injected into the column was 2 µl. The mobile phase comprised of (A) aqueous phosphoric acid (0.1%, *v/v*) and (B) acetonitrile using a gradient elution of 9.5% B at 0 to 4 min, 9.5 to 19% B at 4 to 6min, 19% B at 6 to 10 min and the re-equilibration time of gradient elution was 5 min.

Validation procedure

Calibration curves, limits of detection (LOD) and limits of quantification (LOQ)

The linear dynamic range, LOD, recovery and precision were evaluated for the method developed. 50% methanol stock solution containing the nine reference components were prepared and diluted to an appropriate concentration for the construction of calibration curves. Six concentrations of the mixed standard solution were injected in triplicate, and their regression equations were calculated in the form of $Y = A \times X + B$. The dilute solution was further diluted to a series of concentrations with 50% methanol

to obtain the LOD and LOQ. The LOD and LOQ under the present chromatographic conditions were determined at a signal-to-noise (*S/N*) ratio of 3 and 10, respectively.

Precision, repeatability, stability and recoveries

The precision was determined by analyzing the six replicates on the same day and inter-day variation was determined in three consecutive days. The relative standard deviation (RSD) of retention time and peak areas was taken as a measure of precision.

Stability was tested with an extract solution of the same sample at 4°C and analyzed at 0, 4, 8, 12, 24, 48, and 72 h within 3 days. To confirm the repeatability, five different working solutions prepared from the same batch of reduning injection were analyzed. The RSD was also taken as the measures of stability and repeatability.

Recovery test was used to evaluate the accuracy of this method. Each sample was analyzed in triplicate. The total amount of each component was calculated from the corresponding calibration curve. The average recoveries were counted by the formula:

Recovery (%) = (amount found − original amount)/amount spiked ×100%, and RSD (%) = (SD/mean) ×100%.

The percentage recovery of the samples was also calculated.

RESULTS AND DISCUSSION

Optimization of chromatographic separation of RRLC

Method for rapid, highly resolving and efficient determination of reduning injections was of great interest. RRLC methods have been accepted to offer greater resolution, good sensitivity and high speed of analysis for complex system, thus RRLC-UV method was investigated to control the quality of reduning injections.

In order to obtain good chromatographic separation and ideal peak distribution of the sample, the chromatographic conditions including mobile phase, flow rates, column temperature and detection wavelength, were investigated, respectively. Firstly, the analytical performance of the compositions of mobile phases (methanol-water, methanol-0.1% formic acid, 0.1% (*v/v*) phosphoric acid and acetonitrile, 0.1% formic acid and acetonitrile) were compared in terms of separation and ability to suppress tailing peaks for each compound. Thus, 0.1% (*v/v*) phosphoric acid and acetonitrile were finally preferred as the optimized composition of mobile phases. It was well known that the flow rate was a crucial factor for shortening analytical time in RRLC (Nakajima et al., 2007), therefore the effect of flow rates was studied in the range of 0.3 to 1.0 ml/min in 0.1 units, and finally 0.7 ml/min was selected. The effects of column temperature on the separation were investigated in the range 20 to 50°C. The result showed that the running time decreased when the column temperature increased, but the resolution of isochlorogenic acid A and isochlorogenic acid B become worse. To obtain the best separation, it was fixed at 30°C throughout the analysis. The detection wavelength of these two category's compounds was then investigated. It was reported that the segmental monitoring strategy based on variable wavelength detection (VWD) was very useful for comprehensive quality control of herbal medicines (Ma et al., 2012). Based on the retention time and the maximal UV absorption of each target compound, segmental monitoring was designed as in apparatus and chromatographic conditions. The choice of detection with segmental monitoring in a run could provide an optimum *S/N* for simultaneous analysis of nine target components. Based on chromatographic conditions optimized, nine main active components could be eluted with baseline separation in 10 min. The typical chromatographic profiles of the standard solution and the real sample solution are shown in Figure 1. Almost no interference was presented in the chromatographic separation, and each target peak in reduning injection had a good resolution. The target components in the chromatographic profile of the sample solution were identified by comparing the retention times and the characteristic of the UV spectra of these peaks with those presented in the chromatogram of the mixed standard solution.

Comparison of LC-UV and RRLC-UV method

The analytical performance of newly established RRLC-UV and conventional LC-UV methods were compared in terms of analytical time and solvent saving. The result demonstrated that the chromatographic analysis time for RRLC on columns packed with 1.8-μm particles was less than 10 min without a loss in resolution. It was found that a great improvement in analytical time was obtained comparing with up to 60 min when LC-UV method was used in our previous research. The reduced solvent consumption was also friendly to environment and financial expense. Thus RRLC method had its advantages over LC- UV in terms of time saving and solvent saving, and subsequently was a powerful tool for the analysis of complex system such as Chinese herbal prescription reduning injection.

Validation of the method

All detailed descriptions of the regression curves are listed in Table 1. The good linearities (Coefficient of determination r^2>0.999) were achieved for nine active components. Meanwhile, as clearly shown in Table 1, the LOD and LOQ for seven phenolic acids and two iridoid glycosides range from 0.0003 to 0.005 μg/ml and from 0.001 to 0.016 μg/ml, respectively. The RSD values of peak areas were lower than 3.0% for both precision and stability tests. Moreover, all the RSD values of the analysis' repeatability for the solution of real sample were

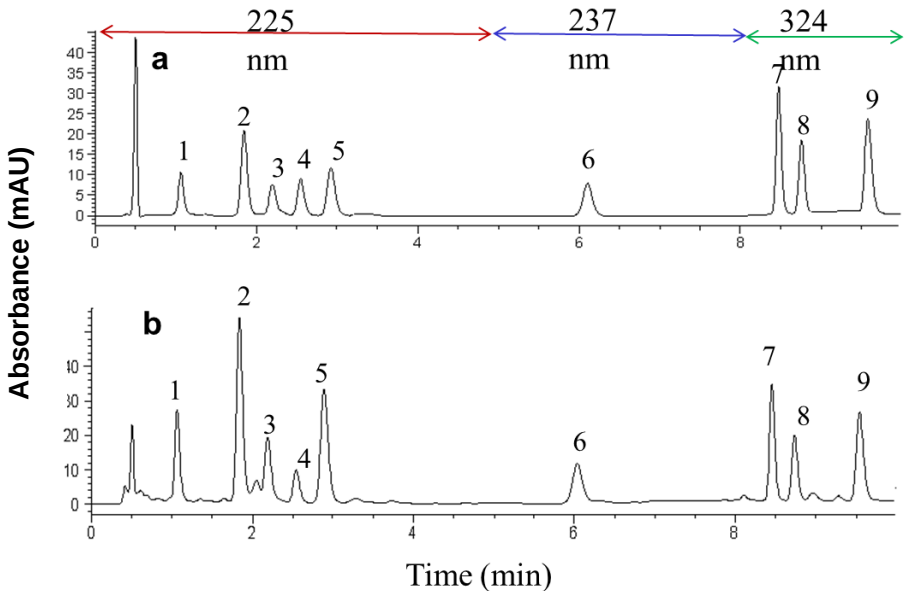

Figure 1. Typical RRLC-UV chromatograms of nine authentic standards mixed and reduning injection, (a). nine authentic standards mixed, (b). the real sample solution. 1 = 4-caffeoylquinic acid, 2 = 5-dicaffeoylquinic acid, 3 = chlorogenic acid, 4 = caffeic acid, 5 = geniposide, 6 = secoxyloganin, 7 = isochlorogenic acid B, 8 = isochlorogenic acid A, 9 = isochlorogenic acid C.

also lower than 3.0% for peak area. The recoveries of all investigated components ranged from 96.1 to 103%, and their RSD values were all less than 3.0%. It was concluded that the developed method possessed the reliability and accuracy for the measurement of these nine major components in reduning injection.

Application

Traditionally Chinese Medicines (TCM) could be selected for search and development of new antiviral TCM preparation on the basis of their medicinal use, e.g. against infections. Reduning injection was made from the aqueous extracts of *Flos Lonicerae*, *Herba A. annuae*, and *G. jasminoides* which were widely used as drug against infections. To our knowledge, the investigated analytes in reduning injection covered two chemical types, namely, seven caffeoylquinic acid derivatives (1, 2, 3, 4, 7, 8 and 9) and two iridoid glycosides (5 and 6). Ingredients 1, 2, 3, 4, 7, 8 and 9 were identified to be 5-dicaffeoylquinic acid, chlorogenic acid, 4-caffeoylquinic acid, caffeic acid, isochlorogenic acid B, isochlorogenic acid A, and isochlorogenic acid C. Caffeoylquinic acid derivatives are natural functional ingredients isolated from many herbal medicines and possess a broad range of pharmacological properties, including antioxidant, antibacterial, antihistaminic, anticancer, and other biological effects (Nakajima et al., 2007; Kwon et al., 2003). Recently, it has been demonstrated that isochlorogenic acid A and isochlorogenic acid C showed significant analgesic activity in the acetic acid-induced mouse writhing test (Dos Santos et al., 2005). Moreover, chlorogenic acid, isochlorogenic acid B and isochlorogenic acid A possessed potent anti-respiratory syncytial virus (RSV) activity against RSV (Li et al., 2005). Ingredients 5 and 6 were identified to be geniposide and secoxyloganin. It was also reported that geniposide had certain analgesic effect and anti-inflammatory effect (Koo et al., 2006). Thus, it would be useful for quality control of reduning injections to develop a method for comprehensive and accurate identification and determination organic acids and iridoid glycosides in this TCM preparation.

Thirteen batches of commercially available reduning injections were analyzed to assess the feasibility of the proposed method for the quality control of reduning injection. The typical RRLC chromatograms of mixed standard substances and reduning injection samples are shown in Figure 1. The contents of the nine active constituents in reduning injection samples from different batches are listed in Table 2. These results showed that the total content of nine major compounds varied from 26.51 to 41.29 mg/ml. Meanwhile, there are four more abundant components, namely 4-caffeoylquinic acid, 5-dicaffeoylquinic acid, chlorogenic acid and geniposide in reduning injection samples, whose contents were higher than 1.0 mg/ml and accounted for almost 85 to 95% of total contents acquired. Their contributions were more than 10% of total content. But another five components including caffeic acid, secoxyloganin, isochlorogenic acid

Table 1. The calibration curves, LODs and LOQs, Precision, repeatability , stability and recovery of the developed method for the nine active components of the assay

Component	Linear regression[a]	Linear range (μg/ml)	r^2	LOD[b] (μg/ml)	LOQ[c] (μg/ml)	Precision (RSD%)[d]	Stability (RSD%)	Repeatability (RSD%)	Recovery[e]	
									Mean (%)	RSD (%)
1	Y = 5.658X+5.7979	0.037-74.52	0.9997	0.003	0.010	1.26	0.80	0.20	100	1.89
2	Y = 4.6757X+9.1186	0.052-209.10	09997	0.001	0.003	1.49	1.27	0.19	100	2.27
3	Y = 4.2217X+3.7556	0.051-219.9	0.9998	0.002	0.005	1.96	0.90	2.28	103	1.95
4	Y = 9.0125X+2.2496	0.013-26.95	0.9994	0.002	0.008	1.58	0.50	0.78	103	0.67
5	Y =2.7618X+7.3283	0.063-253.8	0.9991	0.002	0.006	1.10	1.50	0.24	104	0.69
6	Y = 5.1673X+1.7106	0.059-59.85	0.9999	0.005	0.016	0.61	1.51	0.43	97.4	1.68
7	Y = 9.4589X+4.8758	0.037-74.00	0.9998	0.002	0.007	0.25	1.45	0.48	96.1	0.59
8	Y = 26.796X+5.5663	0.083-16.76	0.9996	0.003	0.008	1.26	1.72	1.39	96.6	0.81
9	Y = 8.5638X+6.3869	0.076-76.00	0.9996	0.002	0.007	0.68	1.89	0.64	102.3	0.69

[a]In the regression equation, the X value is the concentration of Compounds (μg/ml), the Y value is the peak area. [b]Limit of detection, [c]Limit of quantification, [d]The relative standard deviation (%), [e]Recovery (%) = (Amount determined – Amount original)/ Amount spiked ×100%

Table 2. Contents of nine components at samples from different batchs (mg/ml).

Sample batch	P1	P2	P3	P4	P5	P6	P7	P8	P9	Total content
S1080707	3.31	8.05	3.61	0.15	11.95	0.97	0.41	0.08	0.50	29.02
S2080309	3.27	8.32	4.88	0.11	12.79	0.62	0.39	0.07	0.39	30.85
S3091012	2.89	7.99	3.56	0.18	11.41	0.95	0.30	0.05	0.35	27.68
S4090907	3.60	10.3	4.37	0.24	13.77	0.34	0.41	0.18	0.38	33.56
S5091013	2.86	8.07	3.42	0.13	10.55	0.63	0.34	0.07	0.44	26.51
S6091105	3.80	10.5	3.69	0.22	13.89	1.91	0.70	0.15	0.78	35.61
S7091115	2.97	8.09	3.63	0.16	10.64	0.96	0.54	0.12	0.67	27.76
S8091206	3.78	8.66	4.45	0.19	11.15	1.40	0.81	0.18	0.91	31.52
S9091218	4.50	11.0	5.44	0.21	13.91	2.16	1.21	0.28	1.45	40.15
S10100201	3.07	8.00	3.68	0.13	10.48	0.27	0.77	0.17	0.32	26.88
S11100223	4.35	11.0	5.04	0.29	14.56	2.90	1.13	0.29	1.39	40.98
S12100206	3.89	10.9	4.7	0.29	14.81	2.72	0.90	0.24	1.09	39.53
S13100216	4.02	11.5	4.94	0.27	15.37	2.72	1.00	0.26	1.23	41.29

A, B and C which were less than 1 mg/ml could be the relatively minor components. Most of their contribution ranged from 1.0 to 10.0% of total content except for caffeic acid and isochlorogenic acid A. Considering the aforementioned pharmacological properties, it was indicated that 4-caffeoylquinic acid, 5- dicaffeoylquinic acid, chlorogenic acid, geniposide caffeic acid, secoxyloganin, isochlorogenic acid A, isochlorogenic acid B and isochlorogenic acid C should be selected as marker components for quality control of reduning injection. It was also

demonstrated that the established RRLC-UV method could be applied to evaluate the quality of reduning injection.

Conclusion

In this paper, a RRLC-UV method under segmental monitoring mode for rapid separation and quantitative analysis of nine compounds including seven organic acids and two iridoid glycosides, has been developed and successfully applied to determine nine major active components in reduning injection with good precision, accuracy and repeatability. Compared to the reported methods, the established RRLC-UV under segmental monitoring mode was not only suitable for its simplicity and reproducibility, but also specific enough for quality control of reduning injection. It could also be used to guarantee clinical efficacy of reduning injection.

ACKNOWLEDGEMENTS

This research was supported by the Key Project of Chinese Ministry of Education (No210013), Doctoral Fund of Ministry of Education of China (20101210120007), National Natural Science Foundation of China (No81001632), Tianjin Research Program of Application Foundation and Advanced Technology (12JCQNJC08800), China Postdoctoral Science Foundation (20100480657) and the Program for Changjiang Scholars and Innovative Research Team of the University (PCSIRT).

REFERENCES

Chen Y, Li SG, Lin XH, Luo HB, Li GG, Yao H (2008). On-Line Screening and Identification of Radical Scavenging Compounds Extracted from *Flos Lonicerae* by LC-DAD–TOF-MS. Chromatography 68:327-333.

Dos Santos MD, Gobbo-Neto L, Albarella L, de Souza GE, Lopes NP (2005). Analgesic activity of di-caffeoylquinic acids from roots of *Lychnophora ericoides* (Arnica daserra). J. Ethnopharmacol. l96:545–549.

Guo QJ (2010). Sixty curative effects of Reduning injection on hand-foot-mouth disease. Modern J. Integr. Tradit. Chin. Western Med. 19:933-934.

Heng YB, Wu LB, Chen GM, Zhu M (2010). Fifty curative effects of Reduning injection on herpangina. Modern. J. Integr. Tradit. Chin. Western. Med. 19:949-950

Hung TM, Na M, Thuong PT, Su ND, Sok D, Song KS, Seong YH, Bae K (2006). Antioxidant activity of caffeoyl quinic acid derivatives from the roots of *Dipsacus asper* Wall. J. Ethnopharmacol. 108:188-192.

Koo HJ, Lim KH, Jung HJ, Park EH (2006). Anti-inflammatory evaluation of gardenia extract, geniposide and genipin. J. Ethnopharmacol. 103:496–500

Kwon YS, Kim KO, Lee JH (2003). Chemical constituents of *Dipsacus asper* (II). Kor. J. Pharmacogn. 34:128–131.

Li YL, But PPH, Ooi VEC (2005). Antiviral activity and mode of action of caffeoylquinic acids from *Schefflera heptaphylla (L.) Frodin.* Antiviral. Res. 8:1-9.

Liang X, Zhang L, Zhang X, Dai W, Li HY, Hu LW, Liu H, Su J, Zhang WD (2010). Qualitative and quantitative analysis of traditional Chinese medicine Niu Huang Jie Du Pill using ultra performance liquid chromatography coupled with tunable UV detector and rapid resolution liquid chromatography coupled with time-of-flight tandem mass spectrometry. J. Pharm. Biomed. Anal. 51:565-571.

Liu J, Chen XY, Hu YH, Cheng G, Zhong DF (2010). Quantification of the major metabolites of bromhexine in human plasma using RRLC-MS/MS and its application to pharmacokinetics. J. Pharm. Biomed. Anal. 51:1134-1141.

Luo YM, Ma ZL, Qiu X (2009).Clinical observation of curative effects of Reduning injection on bronchiolitis. Chin. Modern Med.16:144-146.

Shao X (2010). Clinical observation of curative effects of Reduning injection on apthous stomatitis. Chin. Pract. Med. 5:144-145.

Ma J, Qi LW, Li HJ, Li P (2012). A segmental monitoring strategy based on variable wavelength detection for quality control of three Polygonaceae herbs. J. Pharm. Biomed. Anal. 62:155–161.

Mishima S, Yoshida C, Akino S, Sakamoto T (2005). Antihypertensive effects of Brazilian propolis: identification of caffeoylquinic acids as constituents involved in the hypotension in spontaneously hypertensive rats. Biol. Pharm. Bull. 28:1909–1914

Nakajima Y, Shimazawa M, Mishima S, Hara H (2007). Water extract of propolis and its main constituents, caffeoylquinic acid derivatives, exert neuroprotective effects via antioxidant actions. Life Sci. 80:370–377

Qi LW, Cao J, Li P, Yu QT, Wen XD, Wang YX. Li CY, Bao KD, Ge XX, Cheng XL (2008). Qualitative and quantitative analysis of *Radix astragali* products by fast high-performance liquid chromatography-diode array detection coupled with time-of-flight mass spectrometry through dynamic adjustment of fragmentor voltage. J. Chromatogr. A 1203:27–35.

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