African Journal of Pharmacy and Pharmacology

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The Introduction should provide a clear statement of the problem, the relevant literature on the subject, and the proposed approach or solution. It should be understandable to colleagues from a broad range of scientific disciplines.

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The Discussion should interpret the findings in view of the results obtained in this and in past studies on this topic. State the conclusions in a few sentences at the end of the paper. The Results and Discussion sections can include subheadings, and when appropriate, both sections can be combined.

The Acknowledgments of people, grants, funds, etc should be brief.

Tables should be kept to a minimum and be designed to be as simple as possible. Tables are to be typed double-spaced throughout, including headings and footnotes. Each table should be on a separate page, numbered consecutively in Arabic numerals and supplied with a heading and a legend. Tables should be self-explanatory without reference to the text. The details of the methods used in the experiments should preferably be described in the legend instead of in the text. The same data should not be presented in both table and graph form or repeated in the text.

Figure legends should be typed in numerical order on a separate sheet. Graphics should be prepared using applications capable of generating high resolution GIF, TIFF, JPEG or Powerpoint before pasting in the Microsoft Word manuscript file. Tables should be prepared in Microsoft Word. Use Arabic numerals to designate figures and upper case letters for their parts (Figure 1). Begin each legend with a title and include sufficient description so that the figure is understandable without reading the text of the manuscript. Information given in legends should not be repeated in the text.

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Examples:

Cole (2000), Steddy et al. (2003), (Kelebeni, 1983), (Bane and Jake, 1992), (Chege, 1998; Cohen, 1987a,b;

Tristan, 1993,1995), (Kumasi et al., 2001)
References should be listed at the end of the paper in alphabetical order. Articles in preparation or articles submitted for **publication**, unpublished observations, personal communications, etc. should not be included in the reference list but should only be mentioned in the article text (e.g., A. Kingori, University of Nairobi, Kenya, personal communication). Journal names are abbreviated according to Chemical Abstracts. Authors are fully responsible for the accuracy of the references.

Examples:

Ansell J, Hirsh J, Poller L (2004). The pharmacology and management of the vitamin K antagonists: the Seventh ACCP Conference on Antithrombotic and Thrombolytic. Therapy. 126:204-233

Ansell JE, Buttaro ML, Thomas VO (1997). Consensus guidelines for coordinated outpatient oral anticoagulation therapy management. Ann Pharmacother 31 : 604-615

Charnley AK (1992). Mechanisms of fungal pathogenesis in insects with particular reference to locusts. In: Lomer CJ, Prior C (eds) Pharmaceutical Controls of Locusts and Grasshoppers: Proceedings of an international workshop held at Cotonou, Benin. Oxford: CAB International, pp 181-190.

Jake OO (2002).Pharmaceutical Interactions between Striga hermonthica (Del.) Benth. and fluorescent rhizosphere bacteria Of Zea mays, L. and Sorghum bicolor L. Moench for Striga suicidal germination In Vigna unguiculata . PhD dissertation, Tehran University, Iran.

Furmaga EM (1993). Pharmacist management of a hyperlipidemia clinic. Am. J. Hosp. Pharm. 50 : 91-95

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Review

Antioxidant compounds, assays of determination and mode of action

Emad A. Shalaby[1]* and Sanaa M. M. Shanab[2]

[1]Biochemistry Department, Faculty of Agriculture, Cairo University, Giza, 12613, Egypt.
[2]Department of Botany, Faculty of Science, Cairo University, Giza, 12613, Egypt.

The human body uses an antioxidant defense system to neutralize the excessive levels of reactive oxygen species. This system consists of enzymatic and non enzymatic antioxidants, catalase, peroxidase, superoxide dismutase and gluatathion s-transferease as major defense enzymes. However, ascorbic acid, tocopherol, and phenolic compounds are considered as examples for non-enzymatic antioxidants. Increasing research on natural antioxidants in foods and development of new assays has prompted critical reflection on the field. It has been common practice to identify health benefits from antioxidant activity on the cellular level with antioxidant capacity of food measured *in vitro.* The use of antioxidants and their positive effects on food quality has been demonstrated in a large variety of foods and beverages using various methods for detection of lipid and protein oxidation or various assays based on electron transfer or hydrogen-atom transfer. There is a need for screening studies in order to identify the mode of action of different antioxidant compounds (enzymatic and non-enzymatic in addition, comparing between synthetic and natural antioxidant compounds) by different assays, in addition to highlighting the advantage and disadvantage of it. Some of these assays depend on hydrogen atom transfer methods or electron transfer methods in addition, metal chelating compounds and free radical scavenging activity.

Key words: Synthetic and natural antioxidant compounds, assays, mechanism.

INTRODUCTION

Natural and synthetic antioxidants are widely used in modern medicine. Some of them proved to be efficient geroprotectors, that is they extend the life span of laboratory animals when added to food or drinking water on a regular basis. *In vitro*, antioxidants inhibit free radical chain oxidation reactions, resulting in oxidation of fatty acids, edible fats, etc. However, their efficiency as scavengers of oxygen free radicals in cells and tissues is negligible as compared with natural antioxidant enzymes (Koltover, 2010). An antioxidant is a chemical that prevents the oxidation of other chemicals. They protect the key cell components by neutralizing the damaging effects of free radicals (Figure 1), which are natural by-products of cell metabolism (Miller et al., 2000). The oxidative stress (OS) induced by reactive oxygen species (ROS) can be described as a dynamic imbalance between the amounts of free radicals generated in the body and levels of antioxidants to quench and/or scavenge them and protect the body against their deleterious effects (Shirwaikar et al., 2006).

Excessive amounts of ROS may be harmful because they can initiated bimolecular oxidations which lead to cell injury and death, and create oxidative stress which results to numerous diseases and disorders such

*Corresponding author. E-mail: dremad2009@yahoo.com. Tel: 002-01001203313, 00966581241911.
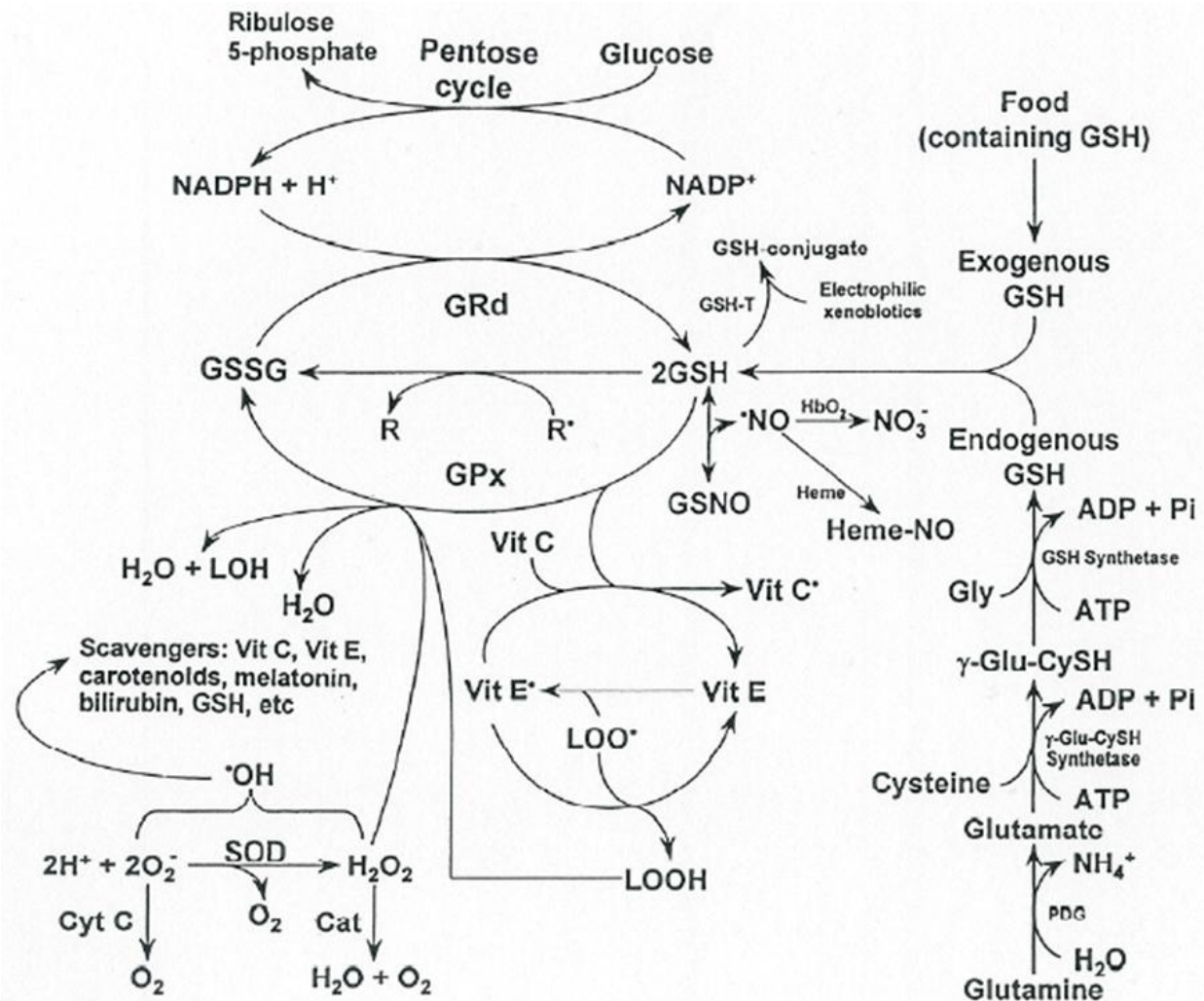
Figure 1. Free radicals and other reactants are enzymatically removed from cells by a series of antioxidative enzymes. Source: Reiter et al. (2003).

as aging, cancer therosclerosis, cirrhosis and cataracts (Halliwell and Gutteridge,2000). There has been a growing considerable interest to identify new sources of safe and inexpensive antioxidant and antimicrobial potential of natural origin (Anwar et al., 2009). Free radicals are formed when oxygen is metabolized or formed in the body and are chemical species which posses an unpaired electron in the outer (valance) shell of the molecule. This is the reason why the free radicals are highly reactive and can react with proteins, lipids, carbohydrates and DNA.

These free radicals attack the nearest stable molecules, stealing its electron. When the attacked molecule loses its electron, it becomes a free radical itself, beginning a chain reaction, finally resulting in the description of a living cell (Prior et al., 1998; Cao et al., 1995).

Free radicals may be either oxygen derived (ROS, reactive oxygen species) or nitrogen derived (RNS, reactive nitrogen species). The oxygen derived molecules are O^{-2} (superoxide), $HO^{.}$ (hydroxyl), HO_2 (hydroperoxyl), $ROO^{.}$ (peroxyl), $RO^{.}$ (alkoxyl) as free radicals and H_2O_2 oxygen as non-radical. Nitrogen derived oxidant species are mainly $NO^{.\,-}$ (nitric oxide), $ONOO^{.\,-}$ (peroxy nitrate), NO_2 (nitrogen dioxide) and N_2O_3 (dinitrogen trioxide) as shown in Figure 2.

In a normal cell, there is appropriate oxidant: antioxidant balance. However, this balance can be shifted, when production of oxygen species is increased or when levels of antioxidants are diminished. This stage is called oxidative stress (Vinson et al., 1998; Cuvelier et al., 1992). Oxidative stress results in the damage of biopolymers including nucleic acids, proteins, poly-

Figure 2. Oxygen and nitrogen-based free radicals and associated reactants that are generated in cells by various processes. Source: Reiter et al. (2003).

unsaturated fatty acids and carbohydrates.

Lipid peroxidation is an oxidative deterioration of polyunsaturated lipids and it involves ROS and transition metal ions. It is a molecular mechanism of cell injury leading to the yielding of a wide range of cytotoxic products, most of which are aldehydes, like malondialdehyde (MDA), 4- hydroxynonrnal (HNE). Oxidative stress causes serious cell damage leading to a variety of human diseases like Alzheimer's disease, Parkinson's disease, atherosclerosis, cancer, arthritis, immunological incompetence and neurodegenerative disorders, etc. Nutritional antioxidant deficiency also leads to oxidative stress which signifies the identification of natural anti-oxidative agents present in die consumed by human population (Hogg et al., 1961; Cao et al., 1966). Plant extracts rich in phenol acids exhibit strong antioxidant and antiradical activity *in vitro* (Mary et al., 2003) and *in vivo* (Rajlakshmi et al., 2003).

Among the compounds with strong antiradical features, one could mention tannins, flavonoids, and phenolic acids. Tannins are high-molecular compounds with complicated and variable structure, exhibiting usually strong antiradical and antioxidant activity. An interesting group of compounds are derivatives of catechins and gallic acid, the so-called gallocatechins, which are present in green tea (Polovka et al., 2003). Research has shown that these compounds have strong antioxidant activity as well as some anticancer features. The present work aims to make comparison between synthetic and natural antioxidant in addition to antioxidant determination assays and its mode of actions.

ANTIOXIDANT COMPOUNDS

The chemical compounds which can delay the start or slow the rate of lipid oxidation reaction in different biological systems are known as antioxidant compounds.

Types of antioxidant compounds

The human body uses an antioxidant defense system to neutralize the excessive levels of reactive oxygen

Figure 3. Classification of antioxidants.

species. This system consists of enzymatic and non enzymatic antioxidants. Some of the antioxidant enzymes that are found to provide a protection against ROS are superoxide dismutases, catalases, and glutathione peroxidases, in addition to numerous non-enzymatic small molecules distributed widely in the biological system and capable of scavenging free radicals (Figure 3). These non enzymatic molecules include glutathione, tocopherol (vitamin E), vitamin C, β-carotene, and selenium. The antioxidants may be natural or synthetic ones. Polyhydroxy flavones, flavanones, flavanols, isoflavones, chalcones and many members are of these groups of natural substances which proved to have a high degree of antioxidant activity and they are found to be widely spread in plant material (Rajani, 2004). The antioxidant characteristics of plant derived materials can be attributed to their content of polyphenols (Andrea et al., 2003).

Natural and synthetic antioxidants

Natural antioxidants: Natural antioxidants are the cell's defense mechanisms that scavenge reactive species, and they can be classified into different groups according to their properties: endogenous antioxidants, and natural antioxidants. Endogenous antioxidants include glutathione, alpha-lipoic acid, coenzyme Q, ferritin, uric acid, bilirubin, metallothionein, l-carnitine, melatonin, enzymatic superoxide dismutase (SOD), catalase (CAT), glutathione peroxidases (GPXs), thioredoxins (TRX) and peroxiredoxins (PRXs). PRXs are a ubiquitous family of antioxidant enzymes (PRX I-VI) that also control cytokine-induced peroxide levels and mediate signal transduction in mammalian cells (Yoshida et al., 2003). For example, PRX III scavenges up to 90% of H_2O_2, and PRX V behaves more effectively as a scavenger of peroxynitrite. Natural antioxidants coexist in a delicate

balance with oxidative inputs. Other antioxidants can be obtained from the diet, such as ascorbic acid (Vitamin C), tocopherol (Figure 4a), β-carotene (Vitamin A), lipoic acid, uric acid, glutathione and polyphenol metabolites.

Synthetic antioxidants: Butylated hydroxy anisole (BHA); butylated hydroxy toluene (BHT); Propyl Gallate (PG); and tertiary butyl hydroquinone (TBHQ) all fall into this class. In 1975, a research scientist who worked on a project commissioned by the Food and Drug Administration (FDA) to investigate the safety/side effects of BHT/BHA observed that the laboratory rats used in this study developed cancerous tumors at an alarming rate. BHA and BHT are laboratory synthesized phenolic compounds (Figure 4b) that are often added to foods to preserve fats. However, the same chemical properties which make BHA and BHT excellent preservatives may also be implicating in health effects. The oxidative characteristics and/or metabolites of BHA and BHT may contribute to carcinogenicity or tumorigenicity. Extensive research has shown high doses of this ingredient to cause significant damage to the lungs, liver and kidneys. Oral consumption of this ingredient has also been shown to have toxic effects on the body's blood coagulation system. Evidences are also available to prove that metabolizing activity can be difficult with BHA and BHT, resulting in health and behavior changes. The Feingold Association maintains that BHA/BHT promotes lung cancer. They also found that it may be the causing agent of developmental neurobehavioral toxicity in their experiments. Kate Murphy, in an article entitled "Do Food Additives Subtract From Health?" for Business Week, noted that:

"Repeated studies have shown that BHA and BHT increase the risk of cancer as well as its accumulation in body tissue, cause liver enlargement, and retard the rate of DNA synthesis and thus, cell development" (Koltover, 2010; U.S. National Library of Medicine, 2010; Baur et al., 2001).

These substances are petroleum derived and created as a by-product in the petroleum refining process. The International Agency for Research on Cancer, and part of the World Health Organization, considers BHA to be possibly carcinogenic to humans, and the State of California has listed it as a carcinogen. Studies showed the same cancer causing possibilities for BHT. The compound has been banned for use in food in Japan, Romania, Sweden, and Australia. The US has barred it only from infant foods.

Despite all these dangers, butylated hydroxyanisole (BHA; tert-butyl-4-hydroxyanisole) is perhaps the most extensively used antioxidant in the food industry. BHA is used in fats and oils, fat-containing foods, confectioneries, essential oils, food-coating materials, and waxes. Butylated hydroxytoluene (BHT; 2,6-di-*tert*-butyl-p-cresol) on the other hand, is one of the antioxidants used extensively in the food industry. It is used in low-fat foods, fish products, packaging materials, paraffin, and mineral oils. BHT is also widely used in combination with other antioxidants such as BHA, propyl gallate, and citric acid for the stabilization of oils and high-fat foods (Shalaby et al., 2010; Shanab et al., 2010).

Some non-enzymatic antioxidants like uric acid, vitamin E, glutathione and CoQ_{10} are synthesized in the human body and they can also be derived from dietary sources. Polyphenols are the major class of antioxidants which are derived from diet (Venkat Ratnam et al., 2006) (http://dx.doi.org/10.1016/j.jconrel.2006.04.015).

Identifying the antioxidant mechanism

The different types of methods published in the literature for the determinations of antioxidant activity of different biological systems involve more than one mechanism (Tables 1 and 2). So, when you determine the antioxidant activity you must use more than one method for comparing the mode of action of crude or pure compounds.

TLC autography technique: The antiradical screening by thin layer chromatography (TLC) autography technique provides an easy, effective and rapid way to study plant extract profiles. No sample purification is needed as this technique provides a simultaneous separation and radical scavenging activity measurement of antioxidative compounds in plant extract. Qualitative as well as semi quantitative analysis of antioxidants can be done by this technique for example, dot blot assay of the crude and various fractions of *Phellinus merrillii* on a silica sheet stained with a 1,1-diphenyl-2-picrylhydrazyl (DPPH) solution in methanol (Chang et al., 2007; Wang et al., 1996) as shown in Figure 5.

Electron spin resonance (ESR) method: These analytical methods measure the radical scavenging activity of antioxidants against free radicals like the 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical, the superoxide anion radical (O_2), the hydroxyl radical (OH), or the peroxyl radical (ROO). The various methods used to measure antioxidant activity of food products can give varying results depending on the specific free radical being used as a reactant. There are other methods which determine the resistance of lipid or lipid emulsions to oxidation in the presence of the antioxidant being tested.
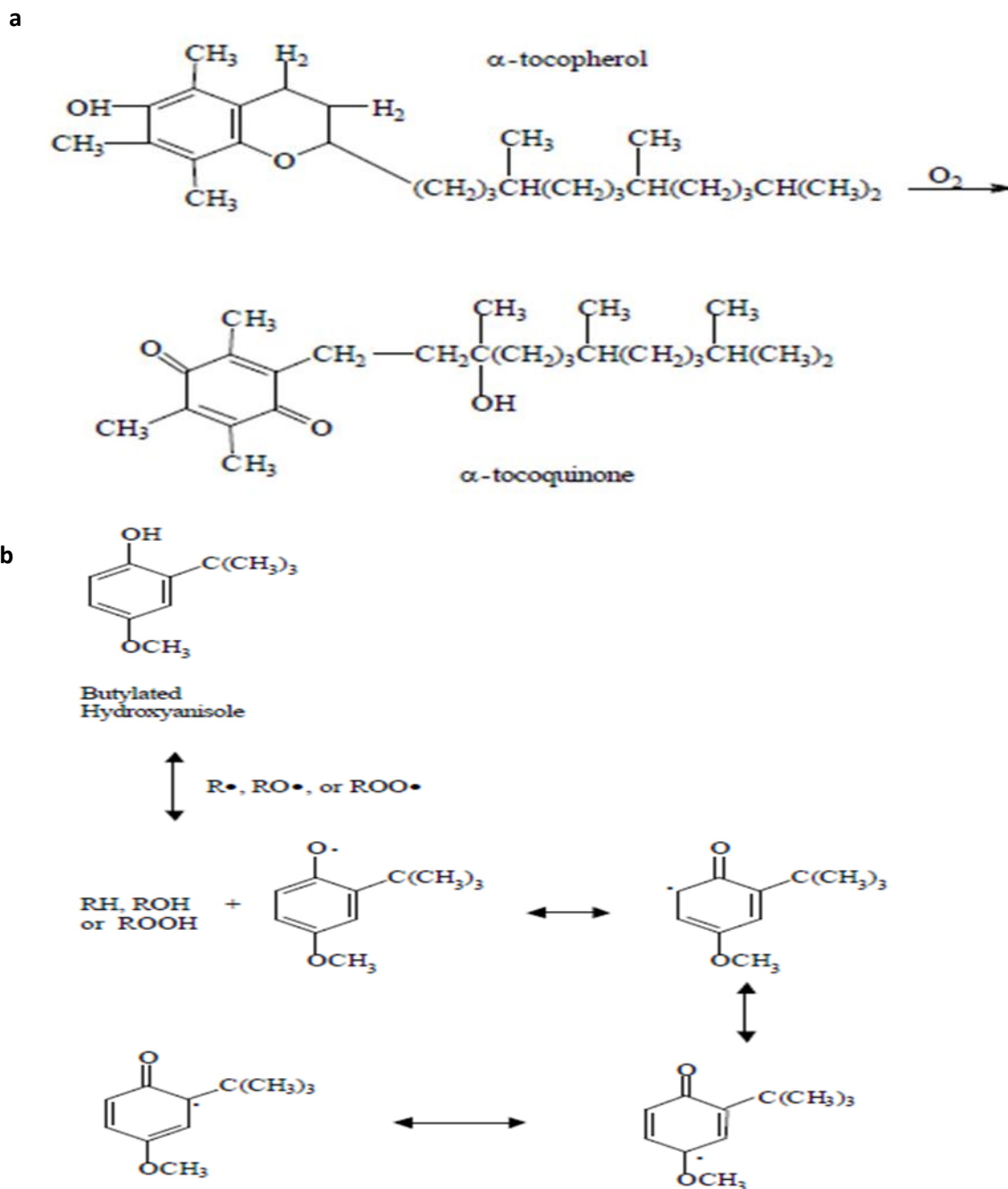
Figure 4. (a) Natural antioxidant (Tocopherol), (b) synthetic antioxidant (BHA).

Enhanced chemiluminescence (ECL): ECL has been used to measure antioxidant capacity in biological fluids. The assay involves the chemiluminescent substrate luminal. Light emission occurs when the luminal is oxidized by hydrogen peroxide that is generated in a reaction catalyzed by horseradish peroxidase (HRP). This method can quantify the antioxidant capacity of a substance which is sensitive to radical scavenging antioxidants that reduce the light output. A method of assay of the antioxidant activity of biological sample suspected of having such activity, is under patent and this method comprises the steps of: (a) initiating a chemiluminescent reaction and allowing reaction to progress, thereby to generate a level of luminescence,
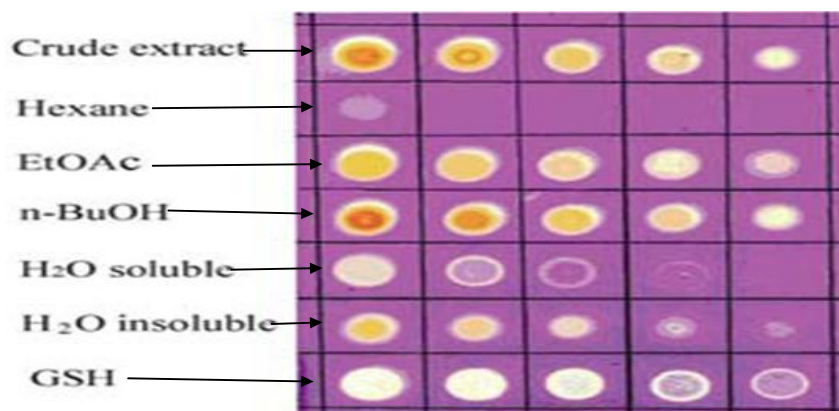
Figure 5. Dot blot assay of the crude and various fractions of *Phellinus merrillii* on a silica sheet stained with DPPH solution in methanol. The crude and fractions of *P. merrillii* (2, 1, 0.5, 0.25, and 0.125 mg/ml) were applied from left to right in sample row; GSH (2, 1, 0.5, 0.25, and 0.125 mg/ml) were applied from left to right in control row.

Table 1. List of *in vitro* antioxidant methods.

S/No	Name of the method
I	**Hydrogen atom transfer methods (HAT)**
1	Oxygen radical absorbance capacity (ORAC) method
2	Lipid peroxidation inhibition capacity (LPIC) assay
3	Total radical trapping antioxidant parameter (TRAP)
4	Inhibited oxygen uptake (IOC)
5	Crocin bleaching Nitric oxide radical inhibition activity
6	Hydroxyl radical scavenging activity by p-NDA (p-butrisidunethyl aniline)
7	Scavenging of H_2O_2 radical
8	ABTS radical scavenging method
9	Scavenging of super oxide radical formation by alkaline SASA
II	**Electron transfer methods (ET)**
1	Trolox equivalent antioxidant capacity (TEAC) decolourization
2	Ferric reducing antioxidant power (FRAP)
3	DPPH zree radical scavenging assay
4	Copper (II) reduction capacity
5	Total phenols by Folin-Ciocalteu
6	N,N-dimethyl-p-Phenylenediamine (DMPD) assay
III	**Other assays**
1	Total oxidant scavenging capacity (TOSC)
2	Inhibition of Briggs – Rauscher oscillation reaction
3	Chemiluminescence
4	Electrochemiluminescence
5	Fluorometric Analysis
6	Enhanced chemiluminescence (ECL)
7	TLC bioautography
8	Cellular antioxidant activity (CAA) assay
9	Dye-substrate oxidation method

Table 2. Comparison between the advantage and disadvantage of different antioxidant methods

| Methods	Advantage	Disadvantage
2,2-Diphenyl-1-picrylhydrazyl (DPPH)	This technique is easy, effective, and rapid way to study plant extract profiles. No sample separation is needed. Potency of sample can be known	Time consuming, costly
Cellular antioxidant activity (CAA) assay	More accurate guage of antioxidant power of whole foods and individual antioxidant nutrients and compounds. This approach is more biologically relevant as it accounts for uptake, metabolism, distribution	Time consuming, costly
Dye-substrate oxidation method	It provides fro-concurrent multisample analysis with automated data storage, regression analyses, and calculation of oxidation inhibition rates. For screening crude extracts and typical assay results are presented	Nil
Cupric Ion Reducing antioxidant capacity (CUPRAC)	It requires sophisticated instrumentation. As a more convenient and less costly alternative. The developed method is less lengthy, more specific and of a higher yield than the classical TBARS assay	Sophisticated instruments are required which are more expensive
Cellular antioxidant activity	Antioxidant capacity of number of non-refined seed oils is compared with that of refined oils by using this simple technique	Nil
Enhanced chemiluminescence (ECL)	ECL has been used to measure antioxidant capacity of biological fluids. This method can quantify the antioxidant capacity of a substance which is sensitive to radical scavenging antioxidants that reduce the light output	This method is cumbersome and time-consuming because fresh signaling reagent solution must be prepared. Also, expensive instrumentation is needed to measure the chemiluminescence (for example, Luminometer)
Ferric-reducing antioxidant power (FRAP) assay	It is simple, speedy, inexpensive, and robust does not required specialized equipment. It can be performed using automated, semiautomated, or manual methods	FRAP cannot detect species that act by radical quenching (H transfer), particularly SH group containing antioxidants like thiols, such as glutathione and proteins.
Total radical trapping antioxidant parameter (TRAP)	Used for measurements of *in-vivo* antioxidant capacity in serum or plasma because it measures nonenzymatic antioxidants such as glutathione, ascorbic acid.24	Many different end points have been used, so comparisons between laboratories are difficult. It is relatively complex and time consuming. It also requires a high degree of expertise and experience
Oxygen radical absorbing capacity (ORAC) assay	The advantage of the AUC approach is that it implies equally well for both antioxidants that exhibit distinct lag phase and those that have no lag phases. ORAC assay has been broadly applied in academy and in the food and dietary supplement industries as a method of choice to quantify AOC	ORAC is limited to measurement of hydrophilic chain but ignores lipophilic antioxidants. It requires fluorometers, which may not be routinely available in analytical laboratories.Temperature control decreases reproducibility
Trolox equivalent antioxidant capacity (TEAC), ABTS.+	The ABTS method has the extra flexibility in that it can be used at different pH levels (unlike DPPH, which is sensitive to acidic pH) and thus is useful when studying the effect of pH on antioxidant activity of various compounds. It is also useful for measuring antioxidant activity of samples extracted in acidic solvents. Additionally, ABTS is soluble in aqueous and organic solvents and is thus useful in assessing antioxidant activity of samples in different media and is currently most commonly used in simulated serum ionic potential solution (pH 7.4 phosphate buffer solution containing 150 mM NaCl) (PBS). Another advantage of ABTS+ method was that samples reacted rapidly with ABTS in the aqueous buffer solution (PBS) reaching a steady state within 30 min	The price of ABTS reagent is high

Table 3. Simplicity, instrument required, biological relevance, mechanism and time required for different antioxidant assays. Source: Reiter et al. (2003).

Antioxidant assays	Simplicity	Instrumentation required	Biological relevance	Mechanism	Time required
ORAC	++	+	+++	HAT	++
TRAP	+++	--	--	HAT	+++
FRAD	+++	+++	--	SET	--
TEAC	++	++	-	SET	-
F-C	+++	-	-	SET	+
TLC autography	+++	+	---	HAT, SET	---
CAA assay	-	-	+++	HAT	+++
Dye substrate oxidation method	+	++	++	HAT	+
CUPRIC	+++	+++	-	HAT	+
Flourometric analysis	++	++	+	HAT	+
ECL	---	+++	+++	HAT	+++
ABTS	+	+	+	HAT	+

+, ++, +++ = Desirable to highly desirable characteristic. -, --, --- = Less desirable to highly undesirable characteristic.
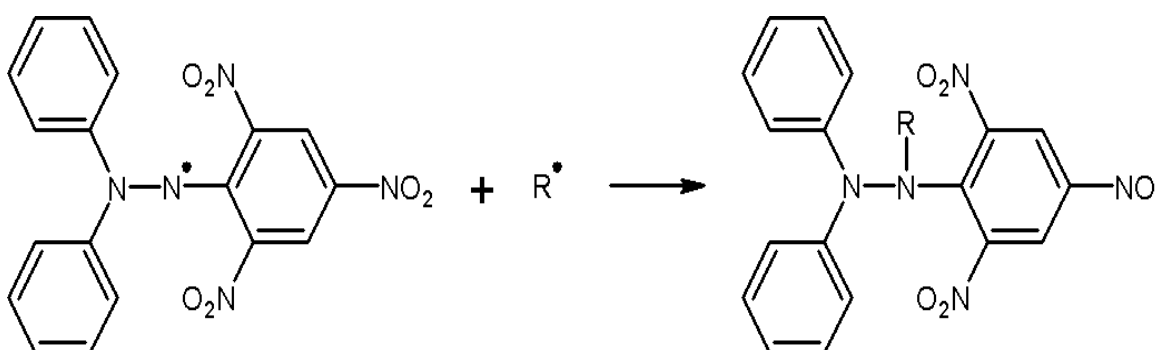
Figure 6. Mode of action of DPPH radical with antiradical compound (RH).

the level being selected from the group consisting of (i) A rising level between 90 to 100 % of maximum; (ii) the maximum; (iii) a postmaximum substantially constant plateau level, (b) adding sample to progressing chemiluminescent reaction, sample causing the level of luminescence generated by the reaction to change when the sample has antioxidant activity, (c) monitoring the change in the level of luminescence, and (d) determining the antioxidant activity of said sample assayed by reference to that of samples of known antioxidant activity subjected to steps (a) to (c). The principle behind the enhanced chemiluminescent assay for total antioxidant capacity (TAC) measurement is best described in the work by Whitehead et al. (1992). To perform the enhanced chemiluminescence assay, a signal reagent (luminal plus para-iodophenol), which is a source of chemiluminescence, is mixed with horseradish peroxi-dase (HRP)-linked immunoglobulin to produce ROS,

which in turn is mixed with a substrate, hydrogen peroxide (H_2O_2).

4-2,2-Diphenyl-1-picrylhydrazyl (DPPH): A rapid, simple and inexpensive method to measure antioxidant capacity of food involves the use of the free radical 2,2-Diphenyl-1-picrylhydrazyl (DPPH). DPPH is widely used to test the ability of compounds to act as free radical scavengers or hydrogen donors (Figure 6), and to evaluate antioxidant activity of foods. It has also been used to quantify antioxidants in complex biological systems in recent years. The DPPH method can be used for solid or liquid samples and is not specific to any particular antioxidant component, but applies to the overall antioxidant capacity of the sample. A measure of total antioxidant capacity helps understand the functional properties of foods.
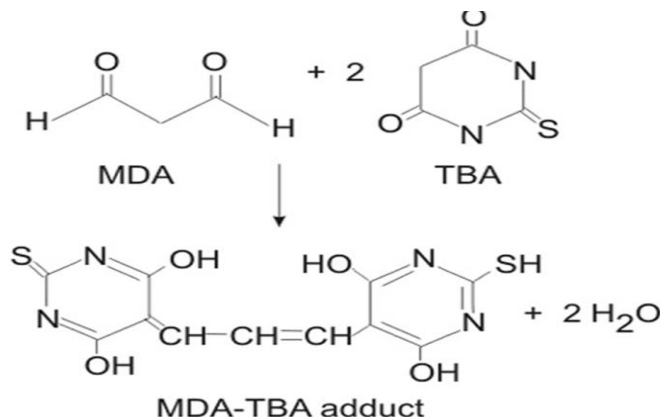
Figure 7. MDA-TBA reaction.
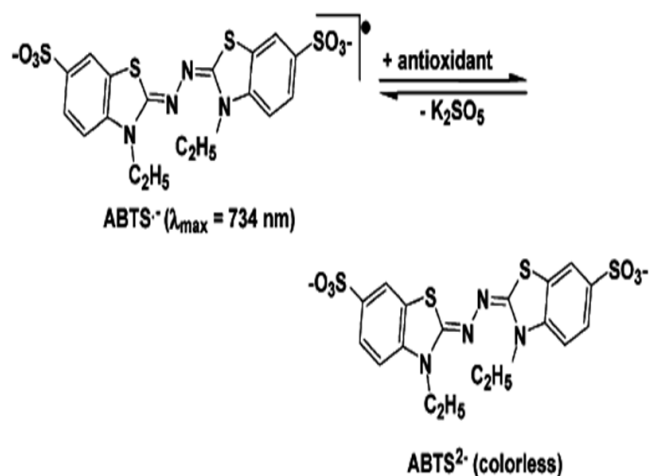
Figure 8. Reaction between ABTS radical and antioxidant compound.

$$Cu(II) + AOH \rightarrow Cu(I) + AO^\bullet + H^+$$

$$AO^\bullet + L-H \rightarrow AOH + L^\bullet$$

$$L^\bullet + O_2 \rightarrow LOO^\bullet$$

$$LOO^\bullet + L-H \rightarrow LOOH + L^\bullet$$

$$Cu(I) + LOOH \rightarrow Cu(II) + LO^\bullet + HO^-$$
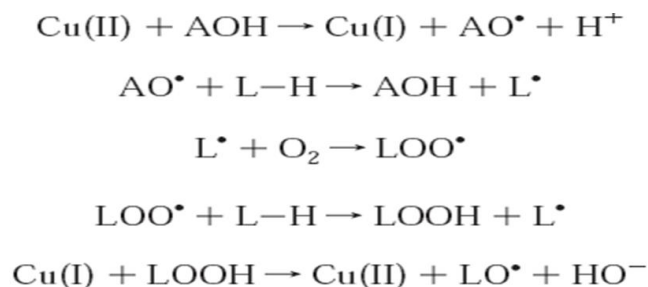
Figure 9. The reaction of cupric ion with antioxidant compound (AOH).

The malondialdehyde (MDA) or thiobarbituric acid-reactive-substances (TBARS): These assays have been used extensively since the 1950's to estimate the

peroxidation of lipids in membrane and biological systems. These methods can be time consuming because they depend on the oxidation of a substrate which is influenced by temperature, pressure, matrix etc. and may not be practical when large numbers of samples are involved. Antioxidant activity methods using free radical traps are relatively straightforward to perform (Figure 7).

The ABTS [2,2'- azinobis(3-ethylbenzothiazoline-6-sulfonic acid)] radical cation: This has been used to screen the relative radical-scavenging abilities of flavonoids and phenolics. ABTS is a better choice than DPPH and more sensitive than DPPH. The ABTS method has the extra flexibility in that it can be used at different pH levels (unlike DPPH, which is sensitive to acidic pH) and thus is useful when studying the effect of pH on antioxidant activity of various compounds. It is also useful for measuring antioxidant activity of samples extracted in acidic solvents. Additionally, ABTS is soluble in aqueous and organic solvents and is thus useful in assessing antioxidant activity of samples in different media and is currently most commonly used in simulated serum ionic potential solution (pH 7.4 phosphate buffer solution containing 150 mM NaCl) (PBS). Another advantage of ABTS+ method was that samples reacted rapidly with ABTS in the aqueous buffer solution (PBS), reaching a steady state within 30 min. The DPPH reacted very slowly with the samples, approaching but not reaching steady state after 8 h. This slow reaction was also observed when ABTS was reacted with samples in alcohol (Figure 8).
.

The Oxygen Radical Absorbance Capacity (ORAC): This procedure is used to determine antioxidant capacities of fruits and vegetables. In the ORAC method, a sample is added to the peroxyl radical generator, 2,2'-azobis(2-amidinopropane)dihyd rochloride (AAPH) and inhibition of the free radical action is measured (Cao et al., 1995) using the fluorescent compound, B-phycoery-thrin or R-phycoerythrin. Phenolic and polyphenolic compounds constitute the main class of natural antioxidants present in plants, foods, and beverages and are usually quantified employing Folin's reagent. Vinson et al. (1998) have measured phenolics in fruits and vege-tables colorimetrically using the Folin-Ciocalteu reagent and determined the fruit and vegetables antioxidant capacity by inhibition of low density lipoprotein oxidation mediated by cupric ions.

Cupric assay: Cu (II) may act as a catalyst in the presence of excessive antioxidants, and the antioxidants

$$[Fe(III)(TPTZ)_2]^{3+} \qquad [Fe(II)(TPTZ)_2]^{2+}, \lambda_{max} = 593 \text{ nm}$$
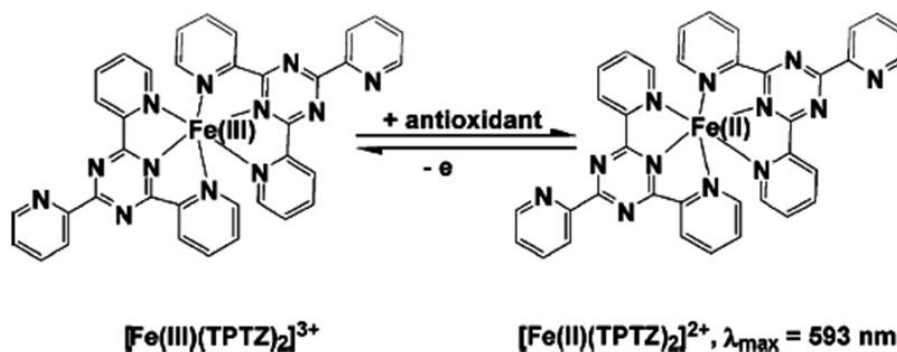
Figure 10. Antioxidant reaction with ferric salt, Fe(III)(TPTZ)2Cl3 (TPTZ) 2,4,6-tripyridyl*s*-triazine.

may act as pro-oxidants. Thus, Cu (II) is a questionable initiator for assaying the radical chain-breaking capacity of antioxidants (Figure 9).

Ferric reducing antioxidant power (FRAP): FRAP assay also takes advantage of electron-transfer reactions. Here, a ferric salt, Fe (III) (TPTZ)2Cl3 (TPTZ) 2,4,6-tripyridyl*s*-triazine), is used as an oxidant. The redox potential of Fe (III) salt (-0.70 V) is comparable to that of ABTS¥- (0.68 V). Therefore, essentially, there is no much difference between TEAC assay and the FRAP assay except that TEAC is carried out at neutral pH and FRAP assay under acidic (pH 3.6) conditions (Figure 10).

REFERENCES

Andrea L, Hóvári J, Katalin VS, Lajos B (2003). The role of antioxidant phytonutrients in the prevention of diseases, Acta biologica szegediensis 47:119-125.

Anwar F, Ali M, Hussain AI, Shahid M (2009). Antioxidant and antimicrobial activities of essential oil and extracts of fennel (*Foeniculum vulgare* Mill.) seeds from Pakistan. Flav. Frag. J. 24:170-176.

Baur AK, Dwyer-Nield LD, Hankin JA, Murphy RC, Malkinson AM (2001). "The lung tumor promoter, butylated hydroxytoluene (BHT), causes chronic inflammation in promotion-sensitive BALB/cByJ mice but not in promotion-resistant CXB4 mice," Toxicology 169, no. 1 (December 2001):1-15.

Cao G, Sofic E, Prior RL (1966). Antioxidant capacity of tea and common vegetables. J. Agric. Food Chem. 44:3426-3431.

Cao G, Verdon CP, Wu AHB, Wang H, Prior RL (1995). Automated assay of oxygen radical absorbance capacity with the COBAS FARA II. Clin. Chem. 41:1738-1744.

Chang H, Ho Y, Sheu M, Lin Y, Tseng M, WU S, Huang G, Chang Y (2007). Antioxidant and free radical scavenging activities of *Phellinus merrillii* extracts. Bot. Stud. 48:407-417.

Cuvelier ME, Richard H, Berset C (1992). Comparison of the Antioxidative Activity of Some Acid-Phenols: Structure-Activity Relationship. Biosci. Biotech. Biochem. 56:324-325.

Halliwell B, Gutteridge JMC (2000). Free Radicals in Biology and Medicine. Oxford University Press, Oxford.

Hogg JS, Lohmann DH, Russell KE (1961). The kinetics of reaction of

2,2-diphenyl-1-picrylhydrazyl with phenols. Can. J. Chem. 39:1588-1594.

Koltover VK (2010). Antioxidant biomedicine: from free radical chemistry to systems biology mechanisms. Russian Chemical Bulletin, Int. Ed. 59(1):37-42.

Mary NK, Achuthan CR, Babu BH, Padikkala J (2003). *In vitro* antioxidant and antithrombotic activity of *Hemidesmus indicus* (L) R. Br. J. Ethnopharmacol. 87:187–191.

Miller HE, Rigelhof F, Marquart L, Prakash A, Kanter M (2000). Antioxidant content of whole grain breakfast cereals, fruits and vegetables. J. Am. Coll. Nutr. 19(3):S312-S 319.

Polovka M, Brezova V, Stasko A (2003). Antioxidant properties of tea investigated by EPR spectroscopy. Biophys. Chem. 106:39–56.

Prior RL, Cao G, Martin A, Sofic E, McEwen J, O'Brien C, Lischner N, Ehlenfeldt M, Kalt W, Krewer G, Mainland CM, (1998). Antioxidant capacity as influenced by total phenolic and anthocyanin content, maturity, and variety of Vaccinium species. J. Agric. Food Chem. 46, 2686-2693.

Rajani M (2004)."brahmi (bacopa monnieri (l.) Pennell) - a medhya rasaayana drug of ayurveda" in ramawat, K. G. Biotechnology of medicinal plants: vitalizer and therapeuticenfield, new hampshire science publishers, inc.

Rajlakshmi D, Banerjee SK, Sood S, Maulik SK (2003). *In-vitro* and *in-vivo* antioxidant activity of different extracts of the leaves of *Clerodendron colebrookianum* Walp in the rat. J. Pharm. Pharmacol. 55:1681-1686.

Ratnam VD, Ankola DD, Bhardwaj V, Sahana DK, Kumar MNVR (2006). Role of antioxidants in prophylaxis and therapy: A pharmaceutical perspective. J. Controlled Release 113(3):189–207.

Reiter RJ. Tan D. Mayo JC. Sainz RM. Leon J, Czarnocki Z (2003). Melatonin as an antioxidant: biochemical mechanisms and pathophysiological implications in humans. Acta Biochemica polonica 50(4):1129-1146.

Shalaby EA, Shanab SMM, Singh V (2010). "Salt stress enhancement of antioxidant and antiviral efficiency of Spirulina platensis," J. Med. Plants Res. 4(24):2622–2632.

Shanab SMM, Shalaby EA, Lightfoot DA, El-Shemy HA (2010) Allelopathic Effects of Water Hyacinth [Eichhornia crassipes]. PLoS ONE 5(10): e13200. doi:10.1371/journal.pone.0013200.

Shirwaikar A, Shirwaikar A, Kuppusamy R, Isaac SR (2006). *In Vitro* Antioxidant Studies on the Benzyl Tetra Isoquinoline Alkaloid Berberine. Biol. Pharm. Bull. 29(9):1906-1910.

U.S. National Library of Medicine (2010). In Haz-Map: Occupational Exposure to Hazardous Agents, 2010, http://hazmap.nlm.nih.gov.

Vinson JA, Hao Y, Su X, Zubik L (1998). Comparison of the Antioxidative Activity of Some Acid-Phenols: Structure-Activity Relationship. J. Agri. Food Chem. 46:3630-3634.

Wang H, Cao G, Prior RL (1996). Total Antioxidant activities of fruits. J.

Agric. Food Chem. 44:701-705.

Whitehead TP, Thorpe GHG, Maxwell SRJ (1992). Enhanced Chemiluminescent assay for antioxidant capacity in biological fluids, Ana. Chim. Acta 266:265-277.

Yoshida T, Oka S, Masutani H, Nakamura H, Yodoi J (2003). The role of thioredoxin in the aging process: involvement of oxidative stress. Antioxidants and Redox Signaling 5:563–570.

Full Length Research Paper

Safety and efficiency of valsartan and combination of valsartan plus hydrochlorothiazide for high blood pressure

Bunc M[1,2]*, Mohorko Pleskovič P[1] and Pleskovič A[1]

[1]Clinical Department for Cardiology, University Medical Center Ljubljana, Zaloška c. 004, 1000 Ljubljana, Slovenia.
[2]Medical Center Cerklje, Trg Davorina Jenka 14, 4207 Cerklje na Gorenjskem, Slovenia.

The essential hypertension is one of the most common cardiovascular risk factors in adult population. The duration of hypertension influences the risk of stroke, heart failure, atherosclerosis, and kidney disease. Diuretics, beta blockers, calcium-antagonists, ACE-inhibitors, and sartans may be used to treat arterial hypertension. In this study, the efficacy and safety of a prompt pharmacologic treatment with valsartan (VAL) or combination of valsartan plus hydrochlorothiazide (VAL/HYCT) in middle aged population was demonstrate with the most frequent cardiovascular risk factors. The results of the 4 months study period showed that treatment with VAL or VAL/HYCT in middle aged 1059 patients significantly reduce the systolic and diastolic blood pressure. Average systolic and diastolic blood pressure were lowered from 161.2 (±16.5) and 92.9 (±9.5) mmHg to 136.1 (±12.5) and 81.6 (±7.8) mmHg, respectively. Side effects were observed only in few patients: elevated serum creatinine (4.6%), elevation of HbA1c (1%) and serum potassium values (0.3%). It may be concluded that VAL or VAL/HYCT is safe and very effective potent antihypertensive drug.

Key words: Valsartan, hydrochlorothiazide, high blood pressure.

INTRODUCTION

Hypertension is defined as a systolic blood pressure (SBP) of > 140 mmHg and/or diastolic blood pressure (DBP) of > 90 mmHg. It is one of the most common cardiovascular risk factors in adult population (Kearney et al., 2005; Ong et al., 2007; Fields et al., 2004). The most common is essential hypertension with no known cause. Secondary hypertension is usually due to a renal disorder. Often, no symptoms develop unless hypertension is severe or long-standing. The duration of hypertension and values of blood pressure influence the risk of stroke, heart failure, atherosclerosis and kidney disease (Kannel, 1996; Vasan et al., 2001; Lindeman et al., 1984; Wollom and Gifford, 1976). The Framingham Heart Study showed that people with blood pressure (BP) value of 130 to 139/85 to 89 mmHg have more than two times more risk for cardiovascular diseases as patients with BP≤ 120/80 mmHg.

In the United States (US), about 65 million people have hypertension, 59% are being treated, and only 34% have adequately controlled BP (Kearney et al., 2005). Lack of diagnosis, inadequate treatment and poor compliance to pharmacologic therapies are the main reasons for poor control of BP. Inadequate compliance of some patients may be due to unpleasant side effects of prescribed drugs (Bangalore et al., 2007; Beto and Bansal 1992).

The European Guidelines recommend prompt treatment of patients affected by hypertension to reduce cardiovascular risk (Summary of the 2007 European Society of Hypertension (ESH) and European Society of Cardiology (ESC) guidelines for the Management of

*Corresponding author. E-mail: mbuncek@yahoo.com.

Arterial Hypertension, 2007; Bonny et al., 2008). Diure-tics, beta blockers, calcium-antagonists, angiotensin-converting enzyme inhibitors (ACEI), and sartans may be used to treat arterial hypertension (Kearney et al., 2005; Plum et al., 1998; Cutler et al., 1989).

The aim of this study was to demonstrate the efficacy and safety of a prompt pharmacologic treatment with valsartan (VAL) or combination valsartan plus hydrochl-orothiazide (VAL/HYCT) in middle aged population with most frequent cardiovascular risk factors.

MATERIALS AND METHODS

In open-label, multicenter, prospective, observational, non-interventional and post-marketing surveillance study, we evaluated safety and effectiveness of the treatment with valsartan 320 mg alone and the fixed combination of valsartan and hydrochlo-rothiazide 320 mg/12.5 mg or 320 mg/25 mg in reaching blood pressure goals. Duration of treatment in the study was 4 months. Paired Students t-test was used to calculate the statistical significance of the differences between the systolic and diastolic blood pressure at the end of the study and the systolic and diastolic blood pressure at the beginning of the experiment between patients that were treated with VAL alone or/and with VAL/HYCT. All data was expressed as means±standard deviation (SD). Statistical significance was set at $P<0.05$.220 doctors recruited up to 5 patients for the study. The total number of patients analyzed was 1059; they were 527 men and 530 women. The mean age of the patients in the study was 63.5 years (SD=11 years), ranging from 28 to 94.

Besides high blood pressure (92%), the most frequent cardiovas-cular risk factors were dyslipidemia (62%), diabetes mellitus (34%), obesity (40%), and smoking (20%). The number of existing risk factors per patient is given in Figure 1. Almost 60% of the patients have more than 4 (4/9) risk factors for atherosclerosis.

For the patients in primary prevention (73%), total absolute risk for development of coronary disease in the next years was more than 20%. In the group of patients for secondary prevention (27%), coronary artery disease, periferial artery disease or cerebro-vascular disease were found in 57, 16 and 24%, respectively. The patients were included in the study mostly due to ACEI intolerance or inadequate blood pressure control on their medical treatment. Many different antihypertensive combinations were used. Most frequently ACEI, angiotensin receptor II blockers (ARB), calcium channel blockers, diuretics and beta blockers were used. 17.8% patients were treated with ACEI as mono-therapy. Most of the patients have some co-morbidity and therefore concomitant medications, most frequently, aspirin and statins were administrated. There were some changes of medical treatment in patients at inclusion to the study. VAL/HYCT was introduced most frequently in 160/12.5 mg (40%) and VAL 320 mg (30%) doses. After the final treatment the most frequent used doses of VAL/HYCT were 160/12.5 mg (24.7%) and 320/12.5 mg (34.5%). The changing of the dose is presented in Figure 2.Patients' compliance to the treatment with VAL/HYCT was good, more than 93% of the patients take tablets nearly always (>90%). The patients' compliance with the treatment is presented in Figure 3.

RESULTS

There were statistical significant difference between the systolic and diastolic blood pressure at the end of the

study and the systolic and diastolic blood pressure at the beginning of the experiment between patients that were treated with VAL alone or/and with VAL/HYCT.

Average systolic blood pressure at the beginning of the study was 161.2 (±16.5) mmHg and at the end of the study was 136.1 (±12.5) mmHg (P<0.05), average diastolic blood pressure was 92.9 (±9.5) mmHg and at the end of DBP was 81.6 (±7.8) mmHg (P<0.05).

These patients (65%) had SBP over 160 mmHg or DBP over 100 mmHg. Only 3.5% of the patients had BP below 140/90 mmHg. At the end of the study, only 5% of the patients have SBP over 160 mmHg or DBP over 100 mmHg. 27% of the patients have BP below 120/80 mmHg and additional 35% below 140/90 mmHg. The comparison of the blood pressure distribution between the first and the last visit is as shown in Figure 4.

The most frequent non-serious adverse event reported was elevated serum creatinine (4.6%), elevation of HbA1c (1%) and serum potassium values (0.3%). All together non-serious adverse effects were reported in 79 cases (7.5%[).

DISCUSSION

The results of this study showed that treatment with VAL or VAL/HYCT in middle aged population with most frequent cardiovascular risk factors is safe, very effective and with good compliance (Bangalore et al., 2007; Ruvolo et al., 2010; Mallion et al., 2003; Pool et al., 2007; Wagstaff, 2006). There were no important changes in HbA1c, serum creatinine and serum potassium values. Side effects were observed only in 7.5% patients. The reduction of SBP and DBP was excellent. The average reduction of SBP was 25 mmHg and DBP was 11 mmHg.

Hypertension is a multifactor disease. Combining therapies with different mechanisms of action can additively reduce BP (Schmidt et al., 2001). VAL blocks the activation of angiotensin II receptors. This causes vasodilatation, reduces secretion of vasopressin, secre-tion and production of aldosterone, what reduces blood pressure. VAL and HYCT in combination has additional BP lowering effects as compared to the monotherapy (Pool et al., 2007; Ruvolo et al., 2010; Mallion et al., 2003; Weir et al., 2007). The benefit of adding HYCT to VAL may be explained by the fact, that diuretics decrease intravascular volume and activate renin-angiotensin-aldosterone system resulting in a diminished antihypertensive response.

Fixed-dose combination of VAL/HYCT was used due to low side effects; the treatment compliance was good in more than 92% of the patients. Fixed-dose combination improves medication compliance for 24 to 26% (Bangalore et al., 2007).

It may be concluded that VAL or VAL/HYCT is a potent antihypertensive medication. It is indicated both in non diabetic and diabetic patient due to its potency and
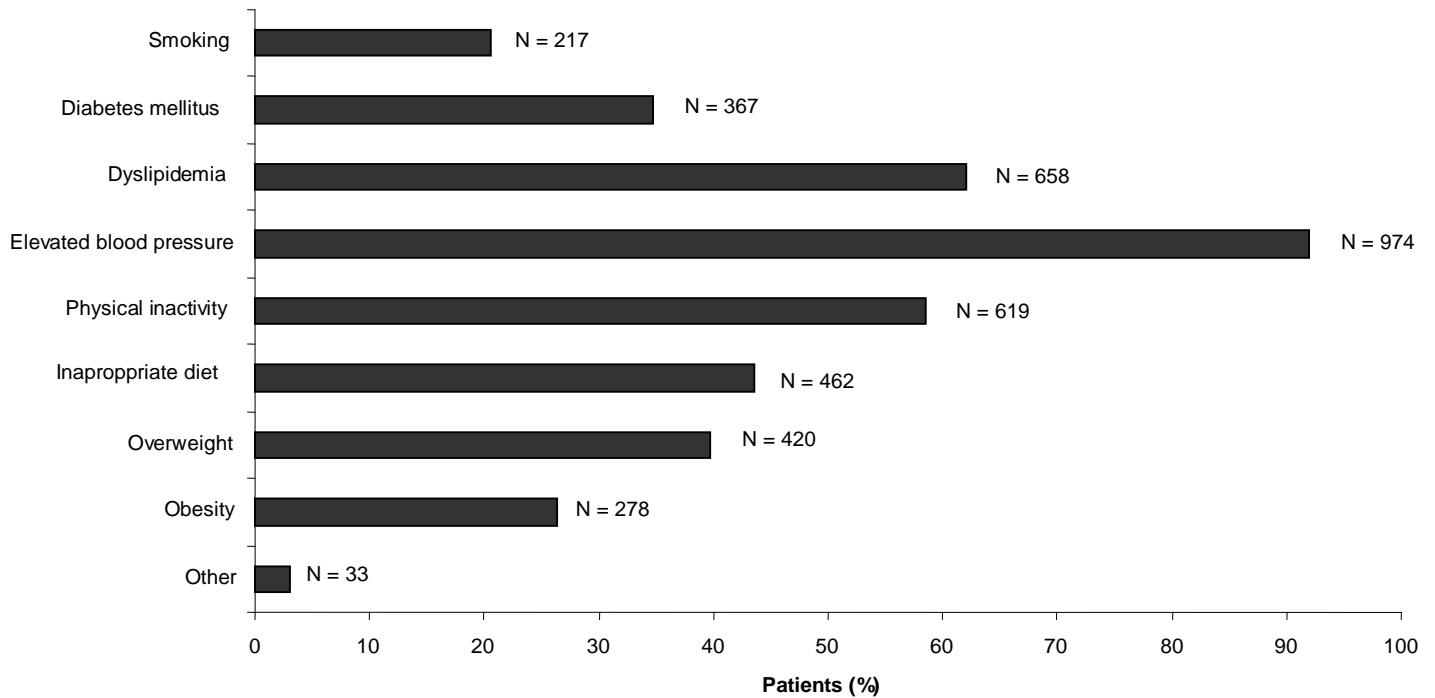
Figure 1. Existing risk factors.
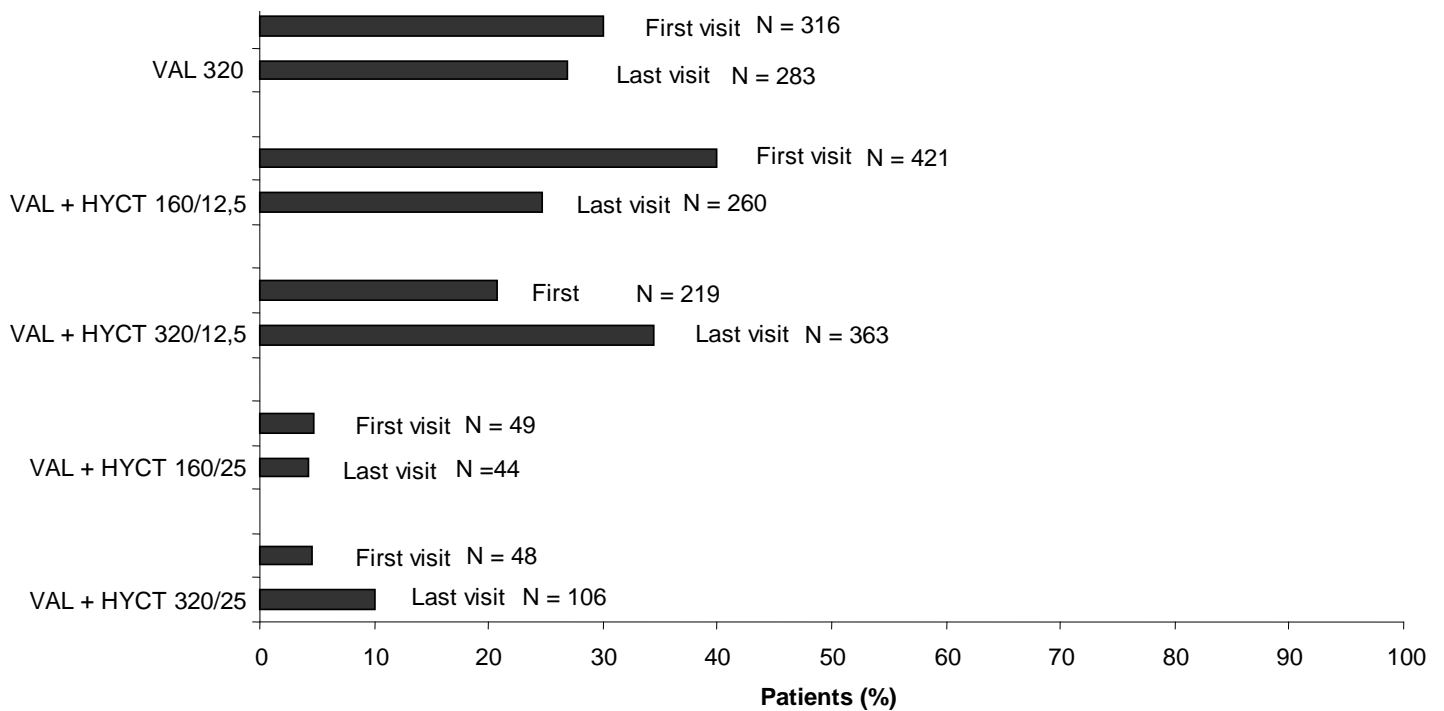
Figure 2. VAL/VAL+HYCT dose changing.

metabolic neutral action. Long term influence of the treatment on mortality, target organs ischemia, heart failure and 24 h antihypertensive profile could not be analyzed from our data because the duration of the study
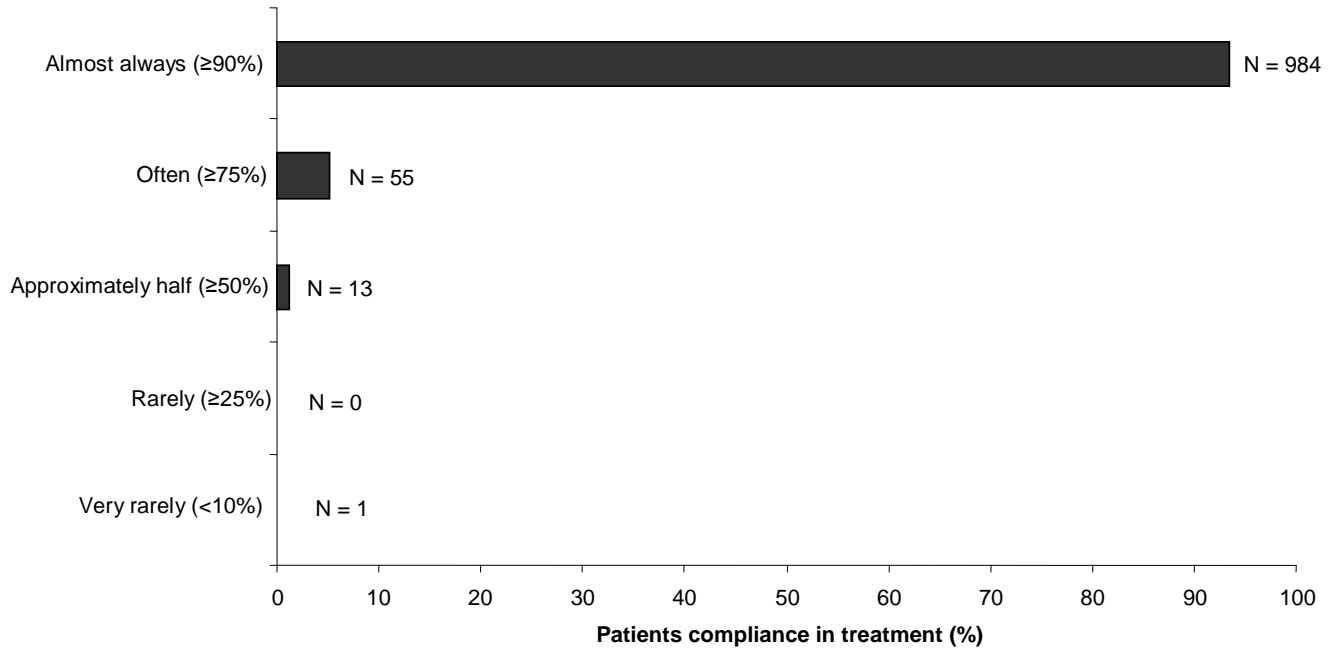
Figure 3. Patient compliance with treatment.
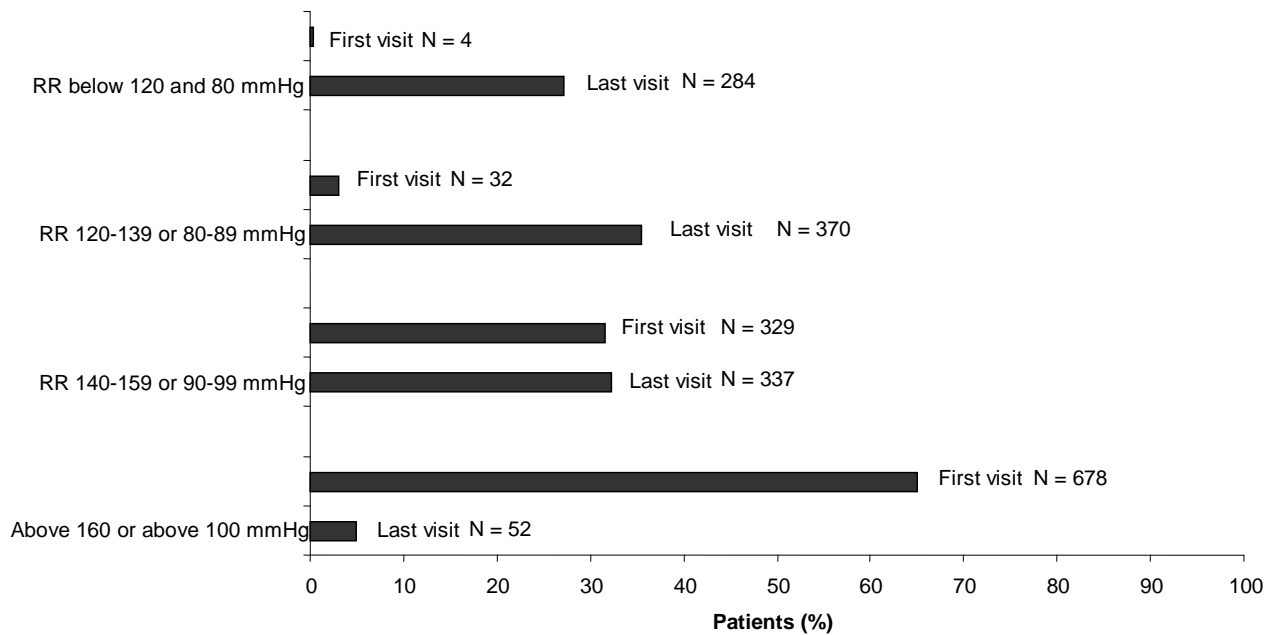
Figure 4. Comparison of blood pressure values.

was only four months.

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REFERENCES

Bangalore S, Kamalakkannan G, Parkar S, Messerli FH (2007). Fixed dose combinations improve medication compliance: a meta-analysis. Am. J. Med. 120:713-719.

Beto JA, Bansal VK (1992). Quality of life in treatment of hypertension. A metaanalysis of clinical trials. Am J Hypert. 5:125-133.

Bonny A, Lacombe F, Yitemben M, Discazeaux B, Donetti J, Fahri P, Megbemado R, Estampes B (2008). The 2007 ESH/ESC guidelines

for the management of arterial hypertension. J. Hypertension , 26: 825-826.

Cutler JA, MacMahon SW, Furberg CD (1989). Controlled clinical trials of drug treatment for hypertension. A review. Hypertension , 13:136-144.

Fields LE, Burt VL, Cutler JA, Hughes J, Roccella EJ, Sorlie P (2004). The burden of adult hypertension in the United States 1999 to 2000: a rising tide. Hypertension. 44:398-404.

Kannel WB (1996). Blood pressure as a cardiovascular risk factor: prevention and treatment. JAMA ., 275:1571-1576.

Kearney PM, Whelton M, Reynolds K, Muntner P, Whelton PK, He J (2005). Global burden of hypertension: analysis of worldwide data. Lancet.. 365:217-223.

Lindeman RD, Tobin J, Shock NW (1984). Association between blood pressure and the rate of decline in renal function with age. Kidney Int. 26:861-868.

Mallion JM, Carretta R, Trenkwalder P, Martinez JF, Tykarski A, Teitelbaum I, Oddou P, Fagan T (2003). Valsartan/ Hydrochlorothiazide is Effective in Hypertensive Patients Inadequately Controlled by Valsartan Monotherapy. Blood Pressure*, 2:36*-43.

Ong KL, Cheung BM, Man YB, Lau CP, Lam KS (2007). Prevalence, awareness, treatment, and control of hypertension among United States adults 1999–2004. Hypertension. 49:69-75.

Plum J, Bunten B, Nemeth R, Grabensee B (1998). Effects of the angiotensin II antagonist valsartan on blood pressure, proteinuria, and renal hemodynamics in patients with chronic renal failure and hypertension J. Am. Soc. Nephrol. 9:2223-2234.

Pool JL, Glazer R, Weinberger M, Alvarado R, Huang J, Graff A (2007). Comparison of valsartan/hydrochlorothiazide combination therapy at doses up to 320/25 mg versus monotherapy: a double-blind, placebo-controlled study followed by long-term combination therapy in hypertensive adults. Clin. Ther. 29:61-73.

Ruvolo A, Mercurio V, Fazio V, Carlomagno G, Russo T, Affuso F, Fazio S (2010). Efficacy and safety of valsartan plus hydroclo-rothiazide for high blood pressure. World J. Cardiol. 26: 125-130.

Summary of the 2007 European Society of Hypertension (ESH) and European Society of Cardiology (ESC) guidelines for the management of arterial hypertension (2007) Vasc . Health Risk Manag. 3:783-795.

Vasan RS, Larson MG, Leip EP, Evans JC, O'Donnell CJ, Kannel WB, Levy D (2001). Impact of high-normal blood pressure on the risk of cardiovascular disease. N. Engl. J. Med .45:1291-1297.

Wagstaff Antona J. (2006). Valsartan/Hydrochlorothiazide: A Review of its Use in the Management of Hypertension. Drugs. 6:1881-1901.

Weir MR, Crikelair N, Levy D, Rocha R, Kuturu V, Glazer R (2007). Evaluation of the Dose Response with Valsartan and Valsartan/Hydrochlorothiazide in Patients With Essential Hypertension. J Clin Hypert. 9:103–112.

Wollom GL, Gifford RW (1976). The kidney as a target organ in hypertension. Geriatrics. 31:71–79.

Full Length Research Paper

Determination of levo-tetrahydropalmatine in rat plasma by HPLC and its application to pharmacokinetics studies

Xiao-bo WU[1], Yong-huang LUO[1], Jia-hong XU[2], Shu-ying GU[2], Xian-qin [LUO2], Hao-ling WU[1] and Gui-jun ZHAO[3*]

[1]College of Pharmaceutical Sciences, Southwest University, Chongqing 400715, China
[2]Chongqing Academy of Chinese Materia Medica, Chongqing 400065, China
[3]Chongqing institute of pharmaceutical plant, Chongqing 408435, China

The objective of this study was to establish a high performance liquid chromatography (HPLC) method for the determination of levo-tetrahydropalmatine (*l*-THP) in rat plasma, and to investigate the pharmacokinetics after oral administration of *l*-THP in rats. The plasma samples were extracted by ethyl acetate. The mobile phase consisted of a mixture of phosphoric acid (0.1%) and methanol (60: 40 v/v). The flow rate was 1.0 ml/min and the ultraviolet detection wavelength was at 280 nm. Plasma concentrations at different time were determined after oral administration at the dose of 20, 40 and 80 mg/kg. The data of concentration-time were fitted and the pharmacokinetics parameters were calculated with 3p97 program (Chinese Pharmacology Society). The limit of quantitation was 0.02 µg/ml, the linear range was 0.02-20.0 µg/ml (R^2= 0.9989). The mean absolute recoveries of *l*-THP at three different concentrations (0.04, 5.00 and 20.00 µg/ml) were 97.5 ± 4.9, 98.2 ± 3.6 and 99.2 ± 3.2%, respectively. The relative standard deviations (RSD) of intra-day and inter-day were both less than 10%. The parameters of low, middle and high doses were as follows: $t_{1/2}\alpha$ were (0.79 ± 0.04), (0.66 ± 0.02), (4.42 ± 0.07) h, $t_{1/2}\beta$ were (20.26 ± 1.21), (19.28 ± 1.04), (31.96 ± 0.85) h, while AUC were (6.95 ± 0.98), (9.91 ± 1.11), (19.19 ± 3.35) mg·h/L, respectively. The proposed method was found to be convenient, accurate and reliable, and it can be used for determination of *l*-THP in rat plasma. The pharmacokinetics studies also provided the theoretical foundation and reference for the safe and reasonable clinic exploitation of *l*-THP.

Key words: High performance liquid chromatography (HPLC), levo-tetrahydropalmatine, pharmacokinetics, rat.

INTRODUCTION

Rhizoma Corydalis (yanhusuo), the dried tuber of Corydalis yanhusuo W.T. Wang, has been traditionally used in China for the treatments of chest pain, epigastric pain, dysmenorrheal, and traumatic swelling and pain for thousands of years (Pharmacopoeia commission of RPC, Chinese Pharmacopoeia, 2000). Tetrahydropalmatine (THP) is one of the active ingredients isolated from *R. Corydalis*, which possesses the functions of anodyne and hypnosis without drug addiction.

It has also been reported that THP has other functions, such as hypotensive effect, anti-arrhythmia, inhibiting the aggregation of thrombocytes and the secretion of gastric acid (Hu et al., 1999a, b; Hong et al., 2005; Huang et al., 1999). THP, including both *d*- and *l*-conformations, belongs to the isoquinoline alkaloid family. The two enantiomers act on different targets in the CNS; *d*-THP acts as a dopamine (DA) depletory, while *l*-THP acts as a brain DA antagonist (Hu et al., 1999a, b). The pharma-

*Corresponding author. E-mail: zhaoguijun307@163.com Tel/Fax:+86-23-71480136
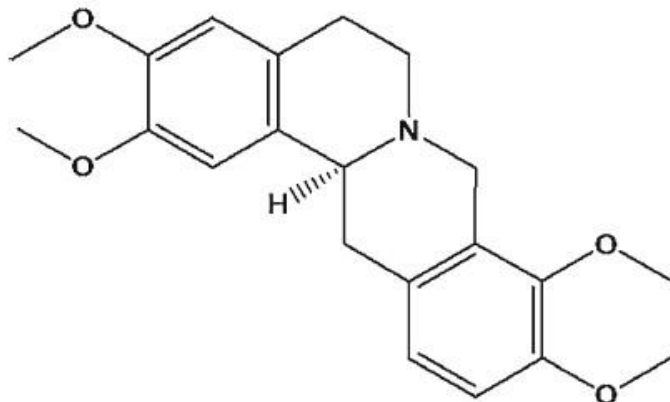
Figure 1. Chemical structure of levo-tetrahydropalmatine (*l*-THP).

ceutical industry has synthetically produced plenty of potent enantiomers: levo-tetrahydropalmatine (*l*-THP), which has been marketed worldwide under different brand names as an alternative to anxiolytic and sedative drugs of the benzodiazepine group and analgesics such as opiates (Li et al., 2011). The chemical structure of *l*-THP is shown in Figure 1. Methods for the determination of THP in rat plasma by e high-performance liquid chromatography electrospray ionization (-) tandem mass spectrometry (NP-(HPLC–ESI–MS) or HPLC–MS/MS have been described (Lu et al., 2006; Deng et al., 2008), because THP exists as the racemic mixture in Chinese herb. Hong et al. (2005) established a chiral HPLC method using an achiral column to separate and quantify THP enantiomers in dog plasma. As far as we know, there has been no report published for the determination of *l*-THP in rat plasma or its pharmacokinetics.

In this study, a robust, sensitive, simple and accurate HPLC method for the determination of *l*-THP in rat plasma was developed and the pharmacokinetics of *l*-THP in rats was also investigated at three different doses of 20, 40 and 80 mg/kg after oral administration, which provides the theoretical foundation and reference for the safe and reasonable clinic exploitation of *l*-THP.

MATERIALS AND METHODS

Drugs and Chemicals

l-THP (purity 99.72%) was provided by Shanghai Winherb Medical S&T Development Co. Ltd. (Shanghai, China). HPLC-grade methanol was obtained from Merck Company (Darmstadt, Germany). All other reagents were of analytical graded. Phosphoric acid was purchased from Shanghai Reagents Company (Shanghai, China). Double-distilled water was used for the preparation of all solutions and 0.45 μM pore size filters (Millipore, MA) were used to filter the solutions. Blank rat blood was collected from healthy, drug-free rats. Plasma was obtained by centrifugation of blood treated with anticoagulant. Plasma was prepared and stored at approximately -20°C.

Instruments and chromatographic conditions

The HPLC analysis was carried out according to a Waters 2695 HPLC system (Waters Associates, Milford, MA), which consisted of a photodiode array detector and an autosampler. The apparatus was interfaced to a DELL PC compatible computer with Empower Pro software. A Diamonsil C_{18} column (250 × 4.6 mm i.d; pore size 5 μM) was used. The mobile phase consisted of a mixture of phosphoric acid (0.1%) and methanol (60: 40 v/v). The flow rate was set at 1 ml·min^{-1} and a 20 μL aliquot was injected into the HPLC column. Column temperature was maintained at 30°C by a column heater controller and the peaks were monitored at 280 nm.

Standard solutions

The *l*-THP was weighed and dissolved in HPLC-grade methanol at room temperature to obtain a stock solution of 1.0 mg/ml. Serial dilutions of the stock solutions were made for spiking the calibration standards. The calibration curve for *l*-THP was prepared in rat plasma at eight concentrations: 0.02, 0.04, 0.08, 1.25, 2.50, 5.00, 10.00 and 20.00 μg/ml. The simulated samples were prepared by adding appropriate volumes of the diluted solutions to drug-free rat plasma. Stock and working standard solutions were protected from light and stored at -20°C until being used.

Sample preparation

To determine *l*-THP in rat plasma, blood samples were taken from the tail vein and placed in the tubes containing sodium heparin. The tubes were centrifuged (6000 rpm for 5 min) at 27°C in order to separate the plasma elements and then stored at -20C for the posterior analysis. The plasma samples (0.2 ml) were alkalinized with 50 μL of 1 M NaOH and shaken for 20 s. The mixture was extracted with 2.0 ml ethyl acetate by a vortex mixer for 3 min and then centrifugated at 4000 rpm for 10 min. An accurately measured 1.2 ml of the supernatant organic layer was evaporated to dryness in a stream of nitrogen on a 45°C water bath. The residue was reconstituted in 100 μL mobile phase, then centrifugated at 15,000 rpm for 5 min. A 20 μL aliquot of the supernatant was directly injected into the HPLC system.

Assay validation

To validate the assay in rat plasma, the following parameters were investigated: selectivity, sensitivity, recovery, precision and accuracy, linearity and Stability.

Selectivity and sensitivity

Selectivity was defined as the lack of interfering peaks at the retention times of the assayed drug. The specificity of the method was determined by comparing the chromatograms obtained from the samples containing *l*-THP with those obtained from blank plasma samples. The limit of quantification (LOQ) for *l*-THP was defined as the lowest concentration of spiked plasma that can be determined with sufficient precision and accuracy (RSD < 20% and -20% < RE < 20%) for both intra-day and inter-day runs.

Recovery and linearity

In the analysis of *l*-THP in rat plasma, the analytical recovery of *l*-THP was determined at concentrations of 0.04, 5.00 and 20.00 μg/ml (n=6). The samples of the plasma without drug were spiked

with known amounts of the drug to achieve the specified concentration. These samples were processed with the analytical method described above and peak areas were compared to that obtained by direct injection of the drug in the mobile phase. To calculate linearity, calibrations curves were constructed by linear regression within the range of 0.02-20.00µg/ml of *l*-THP, with eight standard solutions.

Precision and accuracy

Precision was determined as the coefficient of variation (CV) and accuracy as the percent relative error (RE). Intraday precision and accuracy data were obtained by analyzing aliquots of plasma samples at low (0.04 µg/ml), medium (5.00 µg/ml) and high (20.00 µg/ml) levels of the *l*-THP concentration (n=6). Inter-day reproducibility was determined over three days.

Stability

The ambient stability and freeze-thaw stability of *l*-THP in rat plasma were assessed with the samples of three different concentrations (0.04, 5.00 and 20.00µg/ml). The ambient stability was assessed by leaving the samples at room temperature for 24 h. The freeze-thaw stability was assessed over three cycles, thawed at room temperature and refrozen at -20°C.

Pharmacokinetics study

The analytical method was applied to evaluate the pharmaco-kinetics parameters of *l*-THP after oral administration in rats. The plasma pharmacokinetics of *l*-THP was studied in 30 Wistar rats with body weight 180 – 220 g (fifteen males and fifteen females, respectively). Wistar rats were from the Laboratory Animal Center of Third Military Medical University, Chongqing, China. All the rats were treated in accordance with the guidelines approved by Chongqing Science and Technology Commission (SYXK/2009-002).

The animals were randomly divided into three groups of ten, which were orally administered *l*-THP at the doses of 20, 40, and 80 mg/kg, respectively. The rats were fasted for 12 h prior to and during the experiments, and were allowed free access to water. *l*-THP solution was then orally administered to rats at different doses of *l*-THP (20, 40 and 80 mg/kg). Heparinized blood samples (0.5 ml) were collected before drug administration and 15 min, 30 min, 1, 1.5, 2, 3, 5, 7, 9, 12, 24, 36 and 48 h after oral administration. Thirty rats were used for each time point. After each sampling, the removed volume of blood was supplemented with an equal volume of sodium chloride. Plasma samples were obtained after centrifugation (6000rpm for 5 min) and were stored at -20°C until being analyzed.

Pharmacokinetics and statistical analysis

The pharmacokinetics parameters, including the area under the plasma concentration-time curve (AUC), elimination rate constant (Ke), elimination half-life ($t_{1/2}\beta$), apparent volume of distribution (Vd), and clearance (CL) were calculated with 3p97 program (Chinese Pharmacology Society). An appropriate pharmacokinetics model was chosen based on the lowest Akaike's information criterion (AIC) value, lowest weighed squared residuals, lowest standard errors of the fitting parameters, and dispersion of the residual under equal weight scheme. All data were expressed as mean ± standard deviation (S.D).

RESULTS

Method validation

Figure 2 shows the chromatograms of the blank rat plasma, blank rat plasma spiked with *l*-THP and rat plasma obtained 9 h after oral *l*-THP administration. The retention time for *l*-THP was 8 min, at a flow rate of 1 ml/min. The method described was shown to be selective for the analyte. The limit of quantification (LOQ) was defined as the lowest concentration of spiked plasma samples that can be determined with sufficient precision and accuracy, relative standard deviation (RSD) less than 20% and relative error (RE) of -20 to 20%. The LOQ was estimated at 0.02 µg/ml. The calibration curve for *l*-THP was constructed by plotting the area under peak versus drug concentration.

It was found to be linear over a concentration range from 0.02 to 20.00 µg/ml ($y=23422x$ - 5061.1, R^2= 0.9989, y is the peak area of *l*-THP, and x is the plasma concentration of *l*-THP). The mean absolute recovery of *l*-THP at three concentrations was 97.5 ± 4.9, 98.2 ± 3.6 and 99.2 ± 3.2%, respectively. The results are shown in Table 1, which indicate a lack of interference from the sample preparation procedure. The analytical precision and accuracy for intraday (n=6) and inter-day (n=6) assays of three quality controls (0.04, 5.00 and 20.00 µg/ml) are presented in Table 2.

Stability investigation (Table 3) demonstrates that the concentrations of *l*-THP in processed samples had no significant difference to nominal values within 24 h at 25°C and between the three freeze-thaw cycles (room temperature to -20°C). These results indicates that the analyte in rat plasma was stable for up to 24 h at 25°C with the maximal loss of 2.5% and was stable over at least three freeze-thaw cycles with no significant loss(≤10.3%).

Pharmacokinetics study

The developed HPLC analytical method has been successfully used for the pharmacokinetics study after oral administration of *l*-THP in rats. The mean plasma concentration-time curves of *l*-THP after oral administration at doses of 20, 40 and 80 mg/kg in rats are shown in Figure 3, and the mean pharmacokinetics parameters (mean ± S.D) are summarized in Table 4. Pharmaco-kinetics analysis of *l*-THP concentrations in plasma was performed with two-compartment model methods via the 3p97 software package (Chinese Pharmacology Society).

DISCUSSION

There is an asymmetric carbon on the chemical structure of *l*-THP, as shown Figure 1. It reveals that the two enantiomers acted in different pharmacological activities.
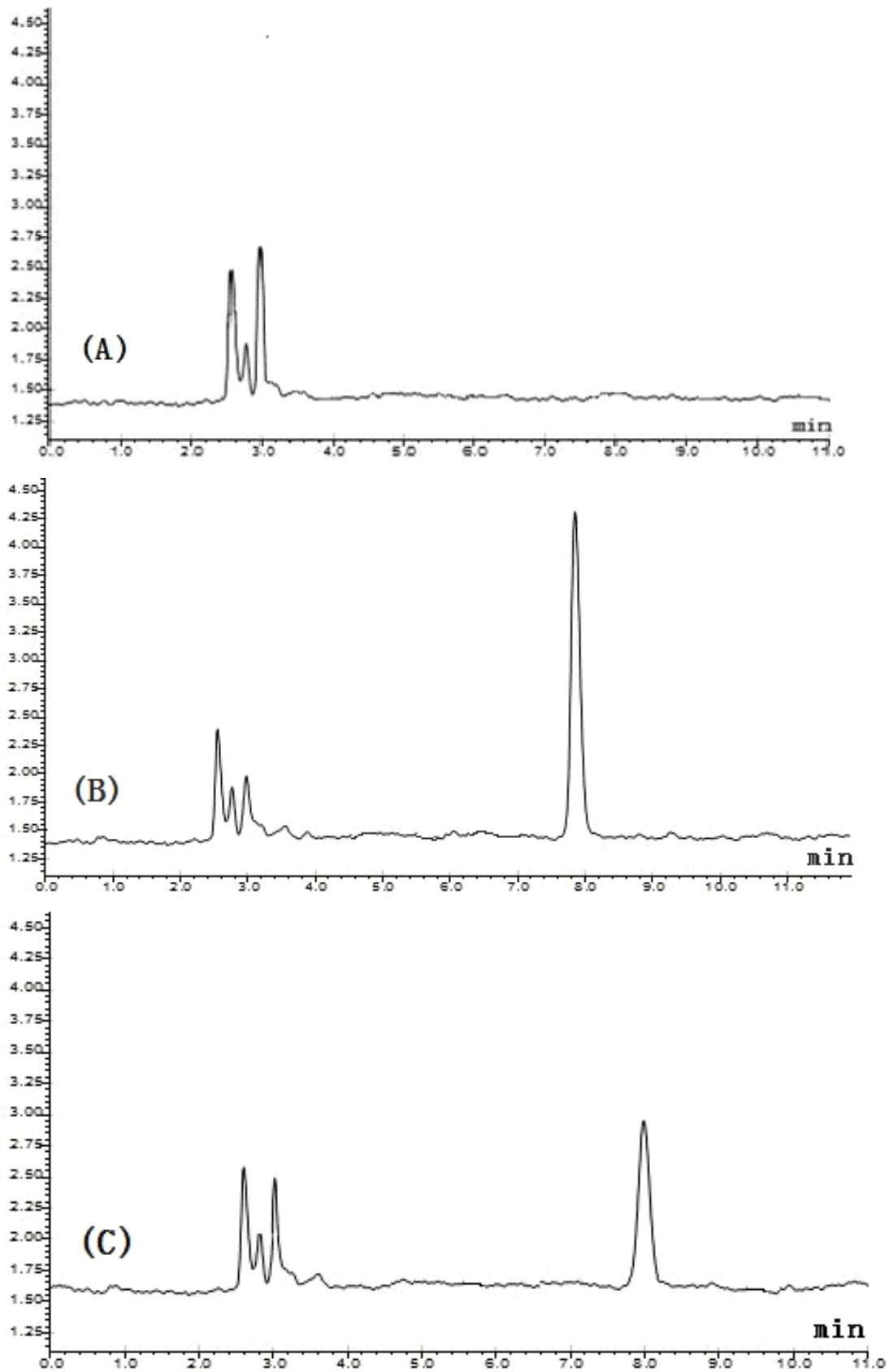
Figure 2. Chromatograms demonstrating selectivity: (A) blank rat plasma; (B) blank rat plasma spiked with *l*-THP; (C) plasma from rat 9 h after oral administration of *l*-THP.

Table 1. Recovery of l-THP from rat plasma (n=6).

Nominal concentration (µg·mL^{-1})	Measured concentration (µg·mL^{-1})	Recovery (%)	RSD
0.04	0.039 ± 0.002	97.5 ± 4.9	3.25
5.00	4.91 ± 0.008	98.2 ± 3.6	2.19
20.00	19.84 ± 0.348	99.2 ± 3.2	3.46

Table 2. Intra- and inter-day precision and accuracy of l-THP in rat plasma (n=6).

Parameter	Nominal concentration (µg·mL^{-1})	Measured concentration (µg·mL^{-1})	Accuracy (RE%)	Precision (RSD%)
	0.04	0.038 ± 0.003	95	4.67
Inter-day	5.00	4.96 ± 0.012	99.2	3.56
	20.00	20.12 ± 0.416	100.6	3.39
	0.04	0.039 ± 0.006	97.5	2.73
Intra-day	5.00	4.93 ± 0.006	98.6	3.29
	20.00	20.20 ± 0.371	101	2.42

Table 3. Assessment of stability in rat plasma.

Condition	Concentration (µg·mL^{-1})		
	0.04	5.00	20.00
Freeze-thaw stability(-20°C)*			
Cycle 1	97.6	96.9	98.2
Cycle 2	93.5	90.8	94.7
Cycle 3	89.9	89.7	92.6
Short-term stability(25°C)*			
Time=2.0h	98.2	99.4	99.9
Time=4.0h	99.4	99.0	100
Time=8.0h	97.8	102	99.7
Time=24.0h	97.5	101	99.6

*Expressed as the mean percentage change from time zero (nominal concentration).

Though THP exists as a racemic mixture in Chinese herbs, it is necessary to evaluate the pharmacokinetic behavior of each enantiomer rather than that of the racemate to use the racemic drug effectively and safely (Li et al., 2011). Early publications have described methods for the determination of racemic THP concentration in rat plasma by HPLC-ESI-MS (Ma et al., 2009) or HPLC-MS/MS (Deng et al., 2008; Lin et al., 2008). As it is well known, HPLC-MS or HPLC-MS/MS method is more expensive than HPLC-UV and the matrix effect is also insuperable. A sequential achiral-chiral HPLC method has been established to determine the enantiomer pharmacokinetics of THP in dogs. Due to the interference by biological matrix components, the reten-tion times of the enantiomers were increased, drastically

diminishing the sensitivity of the method and the life of the column (Hong et al., 2005). In this paper, a simple and effective HPLC method was established and used for l-THP pharmacokinetic studies.

The chromatograms showed a good baseline separation and the mobile phase used resulted in optimal separation. The method was selective for l-THP since it showed that no interfering peaks appeared near the retention time (8 min) of the compound of interest. The LOQ values were low (0.02 µg/ml), indicating the good sensitivity of this HPLC method. Moreover, the precision met the expected range. Accuracy and recovery were also in good agreement with acceptable values for the validation of an analytical procedure (100 ± 20%). The sample preparation used in this study involved only a
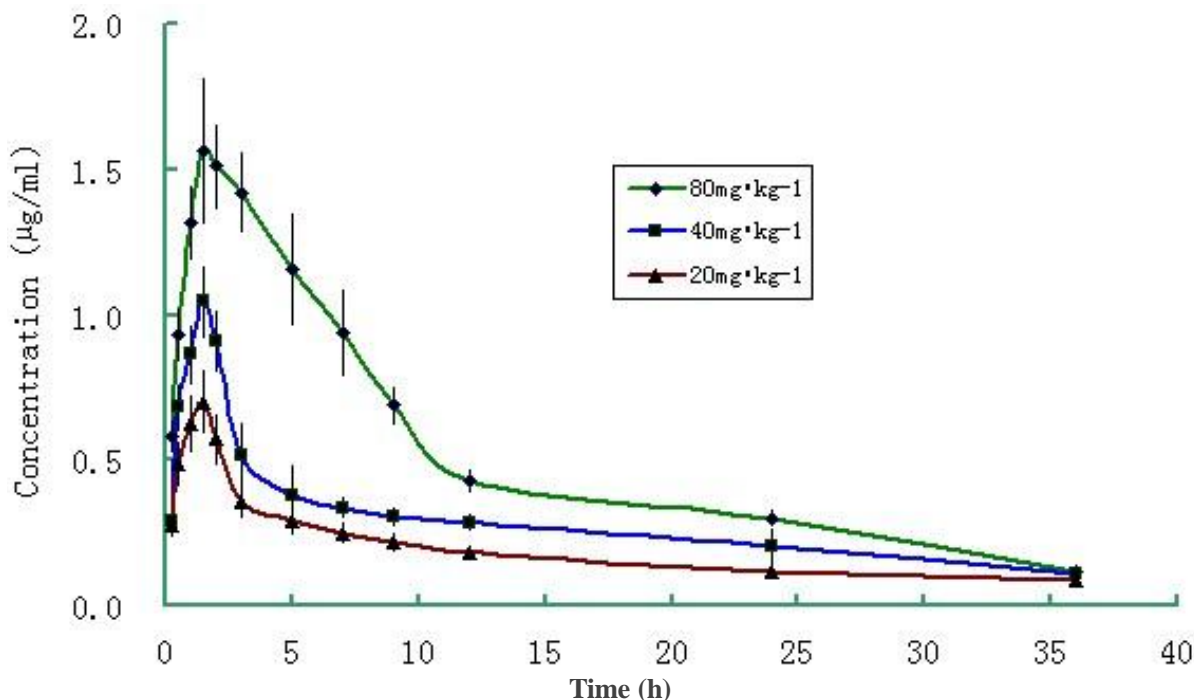
Figure 3. Mean Plasma concentration-time profile of *l*-THP after oral administration of three dosages.

Table 4. The pharmacokinetics parameters of *l*-THP after single oral administration of three dosages in rats (n=10).

Parameter	Dosage (mg·kg⁻¹)		
	20	40	80
$t_{1/2}\alpha$/h	0.79 ± 0.04	0.66 ± 0.02	4.42 ± 0.07
$t_{1/2}\beta$/h	20.26 ± 1.21	19.28 ± 1.04	31.96 ± 0.85
$t_{1/2}k\alpha$/h	0.44 ± 0.05	0.47 ± 0.08	0.60 ± 0.06
K_{12}/ h⁻¹	0.55 ± 0.02	0.69 ± 0.03	0.04 ± 0.03
K_{21}/ h⁻¹	0.22 ± 0.01	0.23 ± 0.01	0.04 ± 0.02
K_{10}/h⁻¹	0.13 ± 0.01	0.17 ± 0.02	0.10 ± 0.01
$K\alpha$/ h⁻¹	1.57 ± 0.21	1.48 ± 0.32	1.16 ± 0.16
V_{d}/ L·kg⁻¹	15.82 ± 1.22	18.22 ± 1.33	39.32 ± 2.09
CL/ L·kg⁻¹·h⁻¹	2.12 ± 0.32	3.10 ± 0.45	3.74 ± 0.28
AUC/ mg·h·L⁻¹	6.95 ± 0.98	9.91 ± 1.11	19.19 ± 3.35
Tmax/h	1.5 ± 0.08	1.5 ± 0.07	1.5 ± 0.06
Cmax/mg·L⁻¹	0.70 ± 0.03	1.04 ± 0.05	1.56 ± 0.09

single step- that is extraction with ethyl acetate. This condition was optimal for sample preparation as it resulted in clean chromatograms. The validated method was employed in pharmacokinetic analysis of *l*-THP after oral administration in rats. The result (Table 4) shows that there was a significant difference in the elimination half-life $t_{1/2}\beta$ (20.26, 19.28, 31.96 h, respectively) when the oral dose of *l*-THP was increased from 20 to 80 mg/kg. These results suggested that the pharmacokinetics of *l*-THP is a nonlinear process. The nonlinear increasing of

$t_{1/2}\beta$ with the increased dosage suggested the saturated elimination of *l*-THP and the nonlinear pharmacokinetics in rats from 20 to 80 mg/kg. The AUC were calculated to be 6.95, 9.91 and 19.19 mg·h/L at doses of 20, 40 and 80 mg/kg, respectively. The $t_{1/2}a$ were approximately 0.79, 0.66, 4.42 h after three dosages, indicating that *l*-THP was distributed quickly in rats. The value of V_{d} after three dosages were 15.82, 18.22 and 39.32 L/kg, respectively. Li et al. (2011) reported that the Tmax was 1.25 h and the $t_{1/2}\beta$ for *l*-THP was about 11.42 h in healthy Chinese after

oral administration. A similar Tmax of 1.5 h and a longer $t_{1/2}\beta$ was obtained in this study, which may be explained by species variation. In conclusion, a sensitive, specific, accurate and reproducible HPLC method for the determination of *l*-THP in rat plasma was developed, which had been successfully applied in the study of pharmacokinetics of *l*-THP in rat. This method can be applied efficiently to large number of biological samples. Pharmacokinetics studies provide the theoretical foundation and reference for the safe and reasonable clinic exploitation of *l*-THP.

ACKNOWLEDGEMENTS

REFERENCES

Deng YT, Liao QF, Li SH, et al (2008). Simultaneous determination of berberine, palmatine and jatrorrhizine by liquid chromatography–tandem mass spectrometry in rat plasma and its application in a pharmacokinetic study after oral administration of coptis–evodia herb couple. J. Chromatography B. 863:195-205.

Hong ZY, Fan GR, Chai YF, et al (2005). Chiral liquid chromatography resolution and stereoselective pharmacokinetic study of tetrahydropalmatine enantiomers in dogs. J.Chromatography B. 826:108-113.

Hong ZY, Fan GR, Chai YF, et al (2005). Chiral liquid chromatography resolution and stereoselective pharmacokinetic study of tetrahydropalmatine enantiomers in dogs. J. Chromatography B. 826:108-113.

Hu JY, Jin GZ (1999a). Effect of tetrahydropalmatine analogs on Fos expression induced by formalin-pain. Acta Pharmacolog. Sin. 20:193-200.

Hu JY, Jin GZ (1999b). Supraspinal D-receptor involved in antinoci-ception induced by L-tetrahydropalmatine. Acta Pharmacolog. Sin. 20:715-719.

Huang K, Dai GZ, Li XH, et al. (1999). Blocking L-calcium current by L-tetrahydropalmatine in single ventricular myocyte of guinea pigs. Acta Pharmacolog. Sin. 20:907-911.

Li Chao-Wu, Zhang Shuo, Gao Hai-Qing, et al (2011). Determination of L-tetrahydropalmatine in human plasma by HPLC and pharmacokinetics of its disintegrating tablets in healthy Chinese. Eur. J. Drug Metab Pharmacokinet. 36:257-262.

Lin L, Liu JX, Zhang Y, et al (2008). Pharmacokinetic studies of tetrahydropalmatine and dehydrocorydaline in rat after oral administration of Yanhusuo extraction by LC-MS/MS method. Acta Pharmacologica Sinica. 43:1123-1127.

Lu T, Liang Y, Song J, et al (2006). Simultaneous determination of berberine and palmatine in rat plasma by HPLC-ESI-MS after oral administration of traditional Chinese medicinal preparation Huang-Lian-Jie-Du decoction and the pharmacokinetic application of the method. J. Pharmaceut. Biomed. Anal. 40:1218-1224.

Ma HD, Wang YJ, Guo T, et al. (2009). Simultaneous determination of tetrahydropalmatine, protopine, and palmatine in rat plasma by LC-ESI-MS and its application to a pharmacokinetic study. J. Pharmaceut. Biomed. Analy. 49:440-446.

Pharmacopoeia commission of RPC, Chinese Pharmacopoeia (Part I, 2000 edition), Chemical Industry Publishing House, Beijing, 2000, p.216.

Full Length Research Paper

Penta-O-galloyl-beta-D-glucose enhances antitumor activity of imatinib and suppresses the growth of K562 cells in mice

Tae-Rin Kwon, Min ho Lee, Soo Jin Jeong, Eun Jung Sohn, Duckgu Lee, Eun Ah Shin, Hyo-Jeong Lee, Eun-Ok Lee, Ji Hoon Jung, Ji-Hyun Kim, Deok-Beom Jung and Sung-Hoon Kim*

College of Oriental Medicine, Kyung Hee University, Seoul 130-701, Republic of Korea.

This study involves the antitumor potential of 1,2,3,4,6-penta-O-galloyl-beta-D-glucose (PGG) to induce apoptosis and enhance antitumor activity of imatinib in K562 cells. Though PGG was reported to have antitumor activities in breast, prostate, kidney, liver cancers and HL-60 leukemia cells, there is no report on synergistic antitumor effect of PGG with imatinib until now. In the present study, PGG significantly enhanced the cytotoxicty and cleavage of poly (ADP-ribose) polymerase (PARP) in imatinib induced chronic myeloid leukemia K562 cells. Furthermore, oral administration of PGG or imatinib significantly inhibited the growth of K562 cells inoculated in Balb/c athymic nude mice and also immunohistochemistry revealed decreased expression of Ki67 (proliferation), CD34 (blood density) and death domain-associated protein (DAXX) and increased terminal deoxynucleotide transferasemediated dUTP nick end labeling (TUNEL) positive cells as one of the apoptotic feature in tumor sections of K562 mouse xenograft model comparable to imatinib treated group. Overall, our findings suggest the potency of PGG to induce apoptosis and enhance antitumor activity of imatinib in K562 cells.

Key words: Chronic myeloid leukemia, apoptosis, 1,2,3,4,6-penta-O-galloyl-beta-D-glucose (PGG), imatinib.

INTRODUCTION

Chronic myelogenous leukemia (CML) is a myeloproliferative cancer with *BCR-ABL* fusion genes in hematopoietic progenitor cells. Bcr-Abl selective tyrosine kinase inhibitor imatinib mesylate (Glivec or Gleevec) has substantially improved the treatment landscape for CML (Druker et al., 1996). Nonetheless, some patients are refractory to imatinib and eventually develop imatinib resistance (Hochhaus et al., 2007). The resistance to imatinib is related with *BCR-ABL* dependent mechanism via point mutations on *Bcr-ABL* fusion protein or independent mechanism via activation of Src family kinase (Valent, 2007; Ramirez and DiPersio, 2008).

1,2,3,4,6-penta-O-galloyl-beta-D-glucose (PGG), a naturally occurring gallotannin polyphenolic compound from *Rhus chinensis* Mill, showed anti-proliferative, anti-angiogenic, anti-diabetic and apoptotic activities (Zhang et al., 2009).

Also, it is of note that PGG exerts antitumor activity in various cancers such as prostate (Hu et al., 2008), lung (Huh et al., 2005), breast (Chen et al., 2003), melanoma (Ho et al., 2002), liver cancers (Oh et al., 2001) and sarcomas (Miyamoto et al., 1987). Nevertheless, there are no evidences regarding the synergistic antitumor effect of PGG with imatinib so far. Thus, in the current study, the antitumor potential of PGG to induce apoptosis and enhance antitumor activity of imatinib in K562 cells and animal study using immunohistochemistry was elucidated *in vitro* and *in vivo*.

*Corresponding author. E-mail: sungkim7@khu.ac.kr. Tel: 82-2-961-9233. Fax: 82-2-964-1064.

MATERIALS AND METHODS

Isolation of PGG

PGG was isolated from the gallnut of *Rhus chinensis* Mill as previously described (Chai et al., 2010). The yellowish active compound was identified as PGG by nuclear magnetic resonance (NMR) and fast atom bombardment mass spectrometry (FAB-MS) analyses. The purity of PGG was estimated to be >96% by high performance liquid chromatography (HPLC).

Cell culture

K562 (CML) cells purchased from American Type Culture Collection (ATCC) (Rockville, MD) were maintained in Roswell Park Memorial Institute (RPMI) 1640 medium containing 10% fetal bovine serum (FBS).

Cell viability assay

Cytotoxicity of PGG was determined using sodium 2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide inner salt (XTT) assay. Cells (2×10^4 cells/100 µl/well) were seeded on 96-well microplates, treated with various concentrations of PGG (0, 10, 20, 40 µM) for 24 h and incubated with XTT labeling mixture [125 µM XTT/25 µM phenazine methosulphate (PMS)] at 37°C for 2 h. Optical density (OD) was measured using a microplate reader (Molecular Devices Co., Sunnyvale, CA) at 450 nm. Cell viability was calculated as a percentage of viable cells in PGG-treated group compared with untreated control by following equation.

Cell viability (%) = [OD (PGG) − OD (Blank)] / [OD (Control) − OD (Blank)] × 100

Western blotting

Western blotting was performed as previously described (Seo et al., 2011). Whole cell extracts were prepared using cell lysis buffer (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1 mM Ethylenediaminetetraacetic acid (EDTA), 1% NP-40, 0.25% deoxycholate acid) containing protease inhibitor cocktail (Roche Applied Science, Inndianapolis, IN). Protein samples were quantified by using Bio-Rad DC protein assay kit II (Bio-Rad, Hercules, CA), separated onto 8 to 15% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) gels and electro-transferred to a nitrocellulose membranes. After blocking with 5% nonfat skim milk, the blots were probed with antibodies against Poly (ADP-ribose) polymerase (PARP) (Santa Cruz Biotechnology, Santa Cruz, CA), and β-actin (Sigma, St. Louis, MO), and exposed to horseradish peroxidase (HRP)-conjugated anti-mouse or rabbit secondary antibodies. Protein expression was detected by using enhanced chemiluminescence (ECL) system (Amersham Pharmacia, Piscataway, NJ).

Mouse xenograft model

The animal studies were conducted under guidelines approved by Institutional Animal Care and use Committee, Kyung Hee University [KHUASP(SE)-11-005]. Five-week-old female athymic nude mice were purchased from Jung Ang lab animal (Seoul, Korea) and maintained under conventional conditions. K562 cells (5×10^6 cells) were mixed with matrigel (Becton Dickinson, 50%, in 100 µl) and

injected subcutaneously on the right flank of the mice. After 5 days of inoculation, the mice were given intraperitonial (i.p) injection of PGG at 4 or 10 mg/kg body weight or imatinib at 25 or 50 mg/kg body weight in 50% PEG 400/50% saline (v/v) every 2 days for 18 days. Control mice were administered the solvent vehicle. Tumor volume was measured every other day with caliper and calculated according to the formula; $V = 0.52a^2b$, where *a* is the smallest superficial diameter and *b* is the largest superficial diameter.

Immunohistochemistry

The animals were sacrificed 18 days after inoculation of K562 cells. Tumors were immediately removed, fixed in 4% PFA, paraffin-embedded, and sectioned at 4 µm. Antigen retrieval was performed after dewaxing and dehydration of the tissue sections by microwaving them for 10 min in 10 mM citrate buffer. Sections were cooled to room temperature, treated with 3% hydrogen peroxide in methanol for 10 min, and blocked with 6% horse serum for 40 min at room temperature. Sections were then incubated with the primary antibodies for Ki-67 (Lab Vision Corporation, Fremont, CA), CD34 (Abcam, Boston, MA), TUNEL (Calbiochem, Darmstadt, Germany), and DAXX (Santa Cruz Biotechnology, Santa Cruz, CA) at 4°C overnight. Sections were washed in PBS and incubated with secon-dary antibody (biotinylated goat anti-rabbit (Vector laboratories, Burlingame, CA) or biotinylated rabbit anti-rat IgG (Abcam, Boston, MA) for 30 min. The antibodies were detected with the vector avidin biotin complex (ABC)/HRP kit (Vector Laboratories, Burlingame, CA) and color-developed with 3,3'-diaminobenzidine tetrahydrochloride (DAB·4HCl).

Statistical analysis

Data were presented as means ± standard deviation (SD) of a minimum of 3 or more replicates. The statistically significant differences between control and PGG-treated groups were calculated by Student's *t*-test using Sigmaplot software (Systat Software Inc., San Jose, CA).

RESULTS

PGG synergistically enhances the antitumor activity of imatinib in K562 cells

Imatinib is an anti-cancer drug currently used to treat chronic myeloid leukaemia (CML) patients by targeting Bcr-Abl activity (Druker and Lydon, 2000). We tested the possibility that PGG could stimulate apoptosis in CML cells induced by Imatinib. Cells were treated with various concentrations of PGG (0, 10, 20 or 40 µM) in the absence or presence of imatinib (0.25 µM) for 24 h. The cell viability was significantly decreased in combination of PGG with imatinib in a dose-dependent manner com-pared with the cells treated with either drug alone (Figure 1A). PGG obviously revealed the synergistic effect on imatinib-induced cell death with combination interval (CI) value = 0.180, 0.005 and 0.001 at 10, 20 and 40 µM, res-pectively (Figure 1B). In addition, PGG treatment further increased PARP cleavage by combination with imatinib (Figure 1C). These results suggest the potential of   PGG
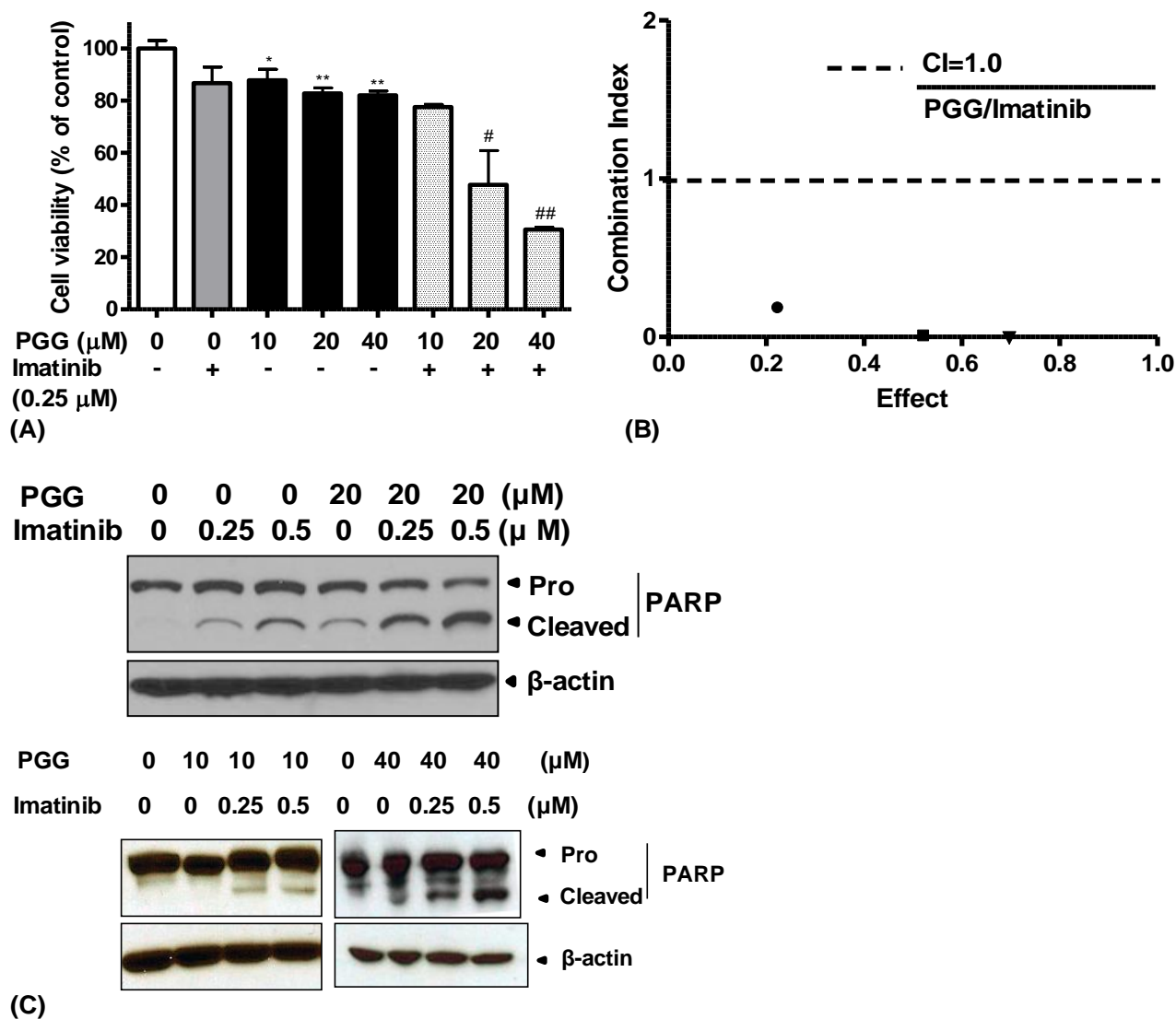
Figure 1. Combination of PGG and imatinib synergistically induces apoptosis in K562 cells. (A) Cells were treated with various concentrations of PGG (0, 10, 20 or 40 μM) and/or imatinib (0.25 μM) for 24 h. XTT assay was performed to measure the cytotoxicity of PGG and/or imatinib. Data are presented as means ± SD. *p < 0.05 and **p < 0.01 versus untreated control, and [#]p < 0.05 and [##]p < 0.01 versus imatinib control. (B) The combination index (CI) between PGG and imatinib was determined by Chou-Talalay method and CalcuSyn software (Biosoft, Ferhuson, MO). (C) Cells were treated with PGG (10, 20 or 40 μM) and/or imatinib (0, 0.25 or 0.5 μM) for 24 h. Cell lysates were prepared and subjected to Western blotting for PARP.

for combinational therapy suggest the potential of PGG for combinational therapy with imatinib for CML chemotherapy. Consistent with PARP cleavage, treatment of PGG (20 μM) with imatinib (0.25 μM) increased the accumulation of the sub-G1 apoptotic portion by 5.15 ± 0.34 compared to control (0.8 ± 0.08) or PGG alone (2.8 ± 0.08) (Figure 2).

PGG influences the expression of cancer biomarkers and DAXX in K562 mouse xenograft model

To verify the *in vivo* anti-tumor efficacy of PGG, K562

cells were subcutaneously injected into Balb/c athymic nude mice in the flank area, and starting after 5 days inoculation, the mice were intraperitoneally administered PGG (4 or 10 mg/kg) or imatinib (25 or 50 mg/kg) (a positive control) every 2 days. Tumor growth was monitored for 18 days. As shown in Figure 3, tumor size and weight in PGG- or imatinib-treated groups were decreased compared with untreated control without significant body weight loss. For analyzing biomarkers of anti-tumor efficiency, we conducted immunohistochemistry analysis with tumor sections from the mice (Figure 4).

A

Control

Imatinib 0.25 µM

PGG 20 µM
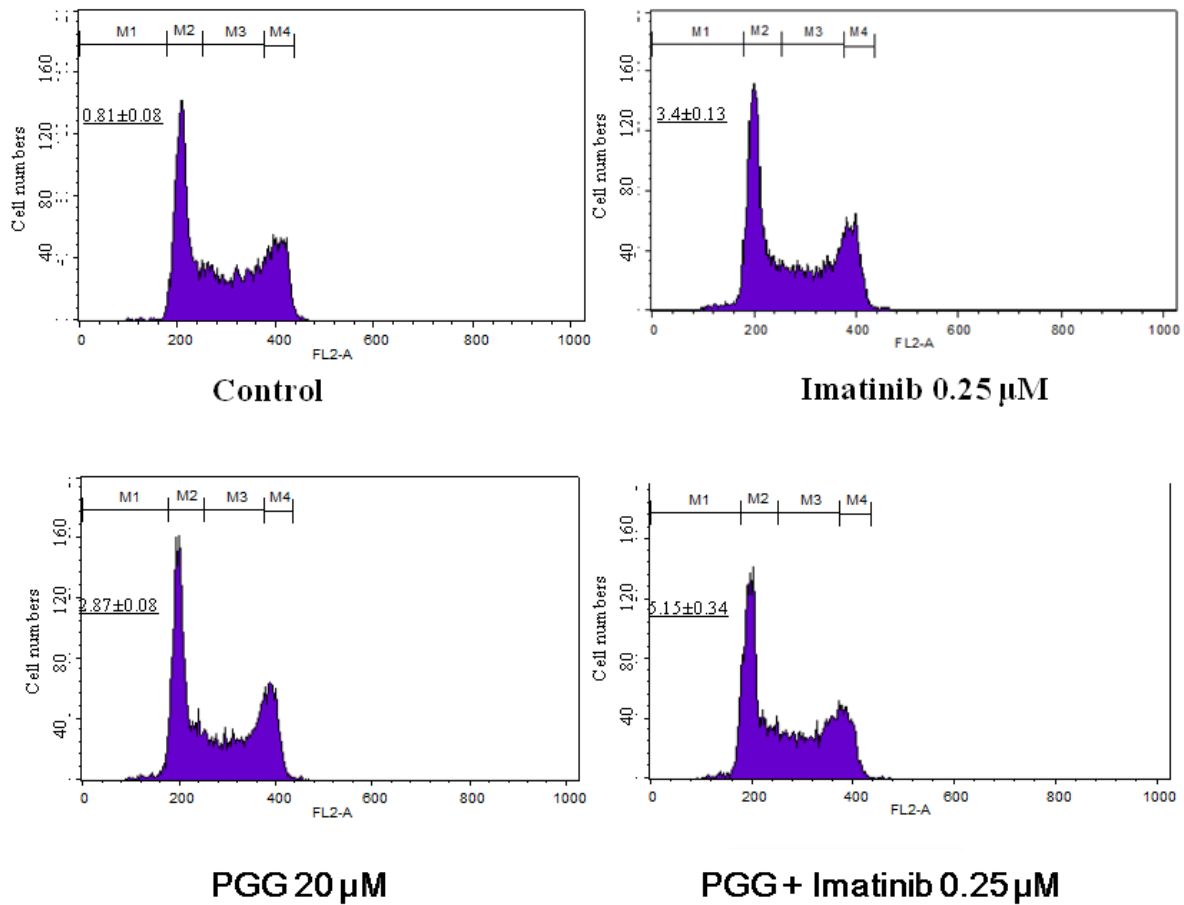
PGG + Imatinib 0.25 µM

B
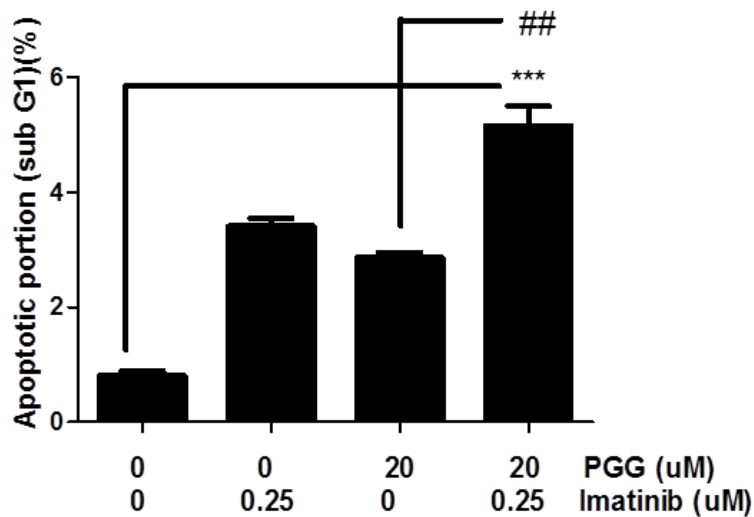
Figure 2. Combination of PGG and imatinib synergistically accumulates the sub-G1 population in K562 cells. (A) FACS analysis with propidium iodide (PI) staining. Cells were treated with PGG (20 µM) and/or imatinib (0.25 µM) for 24 h and stained with PI. (B) Bar graphs represent the percentages of sub-G1 DNA contents. Data represent means ± S.D. ***p < 0.001 versus untreated control, ##p < 0.01 versus PGG alone.

(A)

(B)

(C)
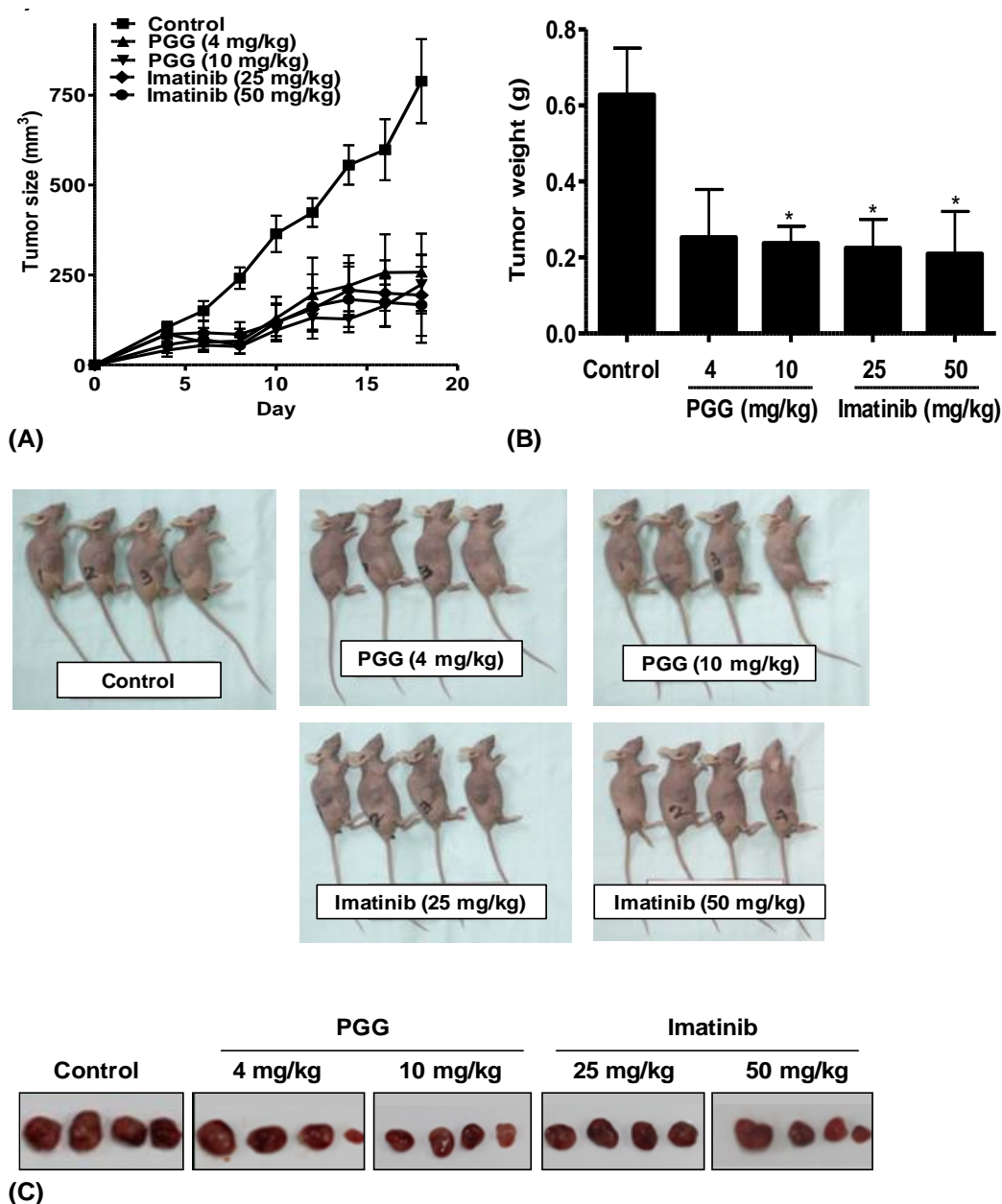
Figure 3. PGG suppresses K562 xenograft growth in female athymic nude mice. Five-week-old Balb/c nude mice were subcutaneously injected in the flank area with 5×10^6 cells in 100 μl of matrigel mixed with PBS. Five days after inoculation, mice (n = 4/group) were treated with vehicle (50% PEG400/50% saline (v/v)), PGG (4 or 10 mg/kg body weight) or imatinib (25 or 50 mg/kg body weight) by i.p. injection every 2 days for 18 days. (A) Tumor volume was calculated according to the formula $V = 0.25a^2b$, where a is the smallest superficial diameter and b is the largest superficial diameter. (B) Tumors were immediately removed and weighed at termination of experiment. Data are presented as means ± SD. *p < 0.05 versus untreated control. Photographs display selected tumor bearing mice and their dissected tumors.

The indicators of proliferation marker Ki-67 (first column) and vascular endothelial marker CD34 (second column) were significantly decreased in both PGG and imatinib-treated groups. Also, TUNEL positive stained cells were significantly increased in both PGG- and imatinib-treated mice (third column). Furthermore, positive cells for DAXX which are known as a proapoptotic protein were significantly reduced in PGG- and imatinib-treated

(A)

(B)
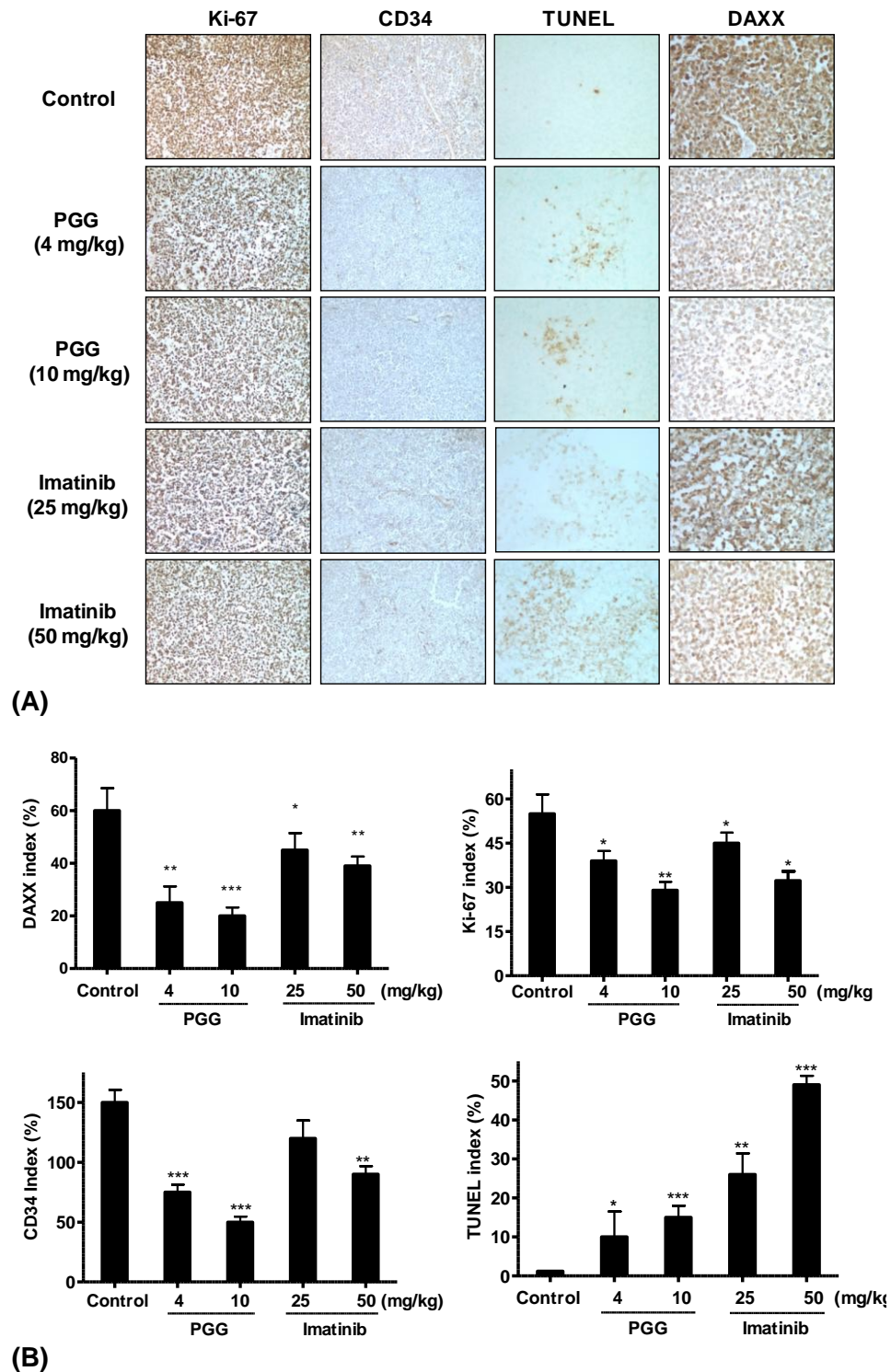
Figure 4. PGG exhibits anti-tumor activity in K562 mouse xenograft model. Mice were sacrificed on day 18 after cell inoculation, and tumors were immediately removed, fixed, embedded and sectioned at 4 μm for immunostaining of biomarkers Ki-67, CD34, TUNEL and DAXX at ×400 (Ki-67 and CD34) or ×200 (TUNEL and DAXX) of original magnification. The sections were detected as DAB substrate staining. Graphs show the Ki-67 index (proliferation), CD34 index (angiogenesis), TUNEL index (apoptosis) and DAXX index in tumor sections. Data are presented as means ± SD. $*p < 0.05$, $**p < 0.01$ and $***p < 0.001$ versus untreated control.

mice (fourth column).

DISCUSSION

Several groups reported anti-cancer effects of natural products against CML through the reactive oxygen species (ROS) signaling. For instance, Mao et al. (2008) reported that shikonin induced apoptosis through the ROS/ c-Jun N-terminal kinases (JNK)-mediated process in CML cell lines K562 and LAMA84. Rakshit et al. (2010) reported the role of ROS in chlorogenic acid (Chi)-induced cell death in CML cell lines as well as primary leukemia cells from CML patients. In addition, Zhang et al. (2008) reported that beta-phenylethyl isothiocyanate (PEITC) exerted the cytotoxic effect in Glivec-resistant CML cells by regulating redox signaling. These evidences suggest the potential of natural products for CML treatment.

Combination cancer therapy has been thought as an effective process to increase the therapeutic efficiency of anti-cancer agents and reduce their adverse effects. Interestingly, PGG potentiated imatinib induced apoptosis as a combination therapy for CML with the value of CI > 1, determined by Chou-Talalay method and CalcuSyn software, implying significant synergism between PGG and imatinib. We recently found the protective effect of PGG on anti-cancer drug cisplatin-induced apoptosis in normal renal epithelial cells (data not shown), supporting the potential of PGG as a supplement of imatinib for a combination therapy for CML.

We finally found that PGG administration suppressed the growth of K562 xenograft without significant body weight loss. Immunohistochemistry showed decreased expression of Ki67 (proliferation), CD34 (angiogenesis), DAXX and increased TUNEL (apoptosis) positive cells in tumor sections of PGG treated mice, indicating the antitumor effects PGG were associated with anti-proliferation, anti-angiogenesis and apoptosis induction.

PGG suppressed the growth of K562 cells in xenograft nude mice and also immunohistochemistry confirmed antitumor effects PGG were associated with anti-proliferation, anti-angiogenesis and apoptosis induction. However, it still requires further study to confirm *in vitro* synergistic antitumor activity of PGG with imatinib in a large number of athymic nude mice and also elucidate molecular mechanism in another resistant CML cell lines in the near future.

Conclusion

PGG revealed the synergistic effect on imatinib-induced cell death in K562 cells *in vitro* and oral administration of PGG or imatinib significantly inhibited tumor size and weight of K562 cells inoculated in Balb/c athymic nude

mice. Furthermore, immunohistochemistry assay revealed that the expressions of proliferation marker Ki-67 and vascular endothelial marker CD34 and (DAXX) were inhibited and also apoptosis marker TUNEL stained cells were significantly increased from the tumor tissues from PGG or imanitib treated groups. Overall, our findings suggest that PGG showed anticancer potential to induce apoptosis and enhance antitumor activity of imatinib in K562 CML cells.

Abbreviations: CML, Chronic myelogenous leukemia; **DAXX,** death domain associated protein; **PGG,** 1,2,3,4,6-penta-O-galloyl-beta-D-glucose.

REFERENCES

Chai Y, Lee HJ, Shaik AA, Nkhata K, Xing C, Zhang J, Jeong SJ, Kim SH, Lu J (2010). Penta-O-galloyl-beta-D-glucose induces G1 arrest and DNA replicative S-phase arrest independently of P21 cyclin-dependent kinase inhibitor 1A, P27 cyclin-dependent kinase inhibitor 1B and P53 in human breast cancer cells and is orally active against triple-negative xenograft growth. Breast Cancer Res. 12(5):R67.

Chen WJ, Chang CY, Lin JK (2003). "Induction of G1 phase arrest in MCF human breast cancer cells by pentagalloylglucose through the down-regulation of CDK4 and CDK2 activities and up-regulation of the CDK inhibitors p27(Kip) and p21(Cip)". Biochem. Pharmacol. 65(11):1777-1785.

Druker BJ, Lydon NB (2000). "Lessons learned from the development of an abl tyrosine kinase inhibitor for chronic myelogenous leukemia". J. Clin. Investig. 105(1):3-7.

Druker BJ, Tamura S, Buchdunger E, Ohno S, Segal GM, Fanning S, Zimmermann J, Lydon NB (1996). "Effects of a selective inhibitor of the Abl tyrosine kinase on the growth of Bcr-Abl positive cells." Nat. Med. 2(5):561-566.

Ho LL, Chen WJ, Lin-Shiau SY, Lin JK (2002). "Penta-O-galloyl-beta-D-glucose inhibits the invasion of mouse melanoma by suppressing metalloproteinase-9 through down-regulation of activator protein-1." Eur. J. Pharmacol. 453(2-3):149-158.

Hochhaus A, Erben P, Ernst T, Mueller MC (2007). "Resistance to targeted therapy in chronic myelogenous leukemia." Semin Hematol 44(1 Suppl 1):S15-S24.

Hu H, Lee H J, Jiang C, Zhang J, Wang L, Zhao Y, Xiang Q, Lee EO, Kim SH, Lu J (2008). "Penta-1,2,3,4,6-O-galloyl-beta-D-glucose induces p53 and inhibits STAT3 in prostate cancer cells HJ in vitro and suppresses prostate xenograft tumor growth in vivo". Mol. Cancer Ther. 7(9):2681-2691.

Huh JE, Lee EO, Kim MS, Kang KS, Kim CH, Cha BC, Surh YJ, Kim SH (2005). "Penta-O-galloyl-beta-D-glucose suppresses tumor growth via inhibition of angiogenesis and stimulation of apoptosis: roles of cyclooxygenase-2 and mitogen-activated protein kinase pathways." Carcinogenesis 26(8):1436-1445.

Mao X, Yu CR, Li WH, Li WX (2008). "Induction of apoptosis by

shikonin through a ROS/JNK-mediated process in Bcr/Abl-positive chronic myelogenous leukemia (CML) cells." Cell Res. 18(8):879-88.

Miyamoto K, Kishi N, Koshiura R, Yoshida T, Hatano T, Okuda T (1987). "Relationship between the structures and the antitumor activities of tannins." Chem. Pharm. Bull. 35(2):814-822.

Oh GS, Pae HO, Oh H, Hong SG, Kim IK, Chai KY, Yun YG, Kwon TO, Chung HT (2001). "*In vitro* anti-proliferative effect of 1,2,3,4,6-penta-O-galloyl-beta-D-glucose on human hepatocellular carcinoma cell line, SK-HEP-1 cells". Cancer Lett. 174(1):17-24.

Rakshit S, Mandal L, Pal BC, Bagchi J, Biswas N, Chaudhuri J, Chowdhury AA, Manna A, Chaudhuri U, Konar A, Mukherjee T, Jaisankar P, Bandyopadhyay S (2010). "Involvement of ROS in chlorogenic acid-induced apoptosis of Bcr-Abl+ CML cells". Biochem. Pharmacol. 80(11):1662-1675.

Ramirez P, DiPersio JF (2008). "Therapy options in imatinib failures." Oncologist 13(4):424-434.

Seo HJ, Huh JE, Han JH, Jeong SJ, Jang J, Lee EO, Lee HJ, Ahn KS, Kim SH (2011). "Polygoni Rhizoma Inhibits Inflammatory Response through Inactivation of Nuclear Factor-kappaB and Mitogen Activated Protein Kinase Signaling Pathways in RAW264.7 Mouse Macrophage Cells". Phytother. Res. 26(2):239-245

Valent P (2007). "Imatinib-resistant chronic myeloid leukemia (CML): Current concepts on pathogenesis and new emerging pharmacologic approaches". Biologics 1(4):433-448.

Zhang H, Trachootham D, Lu W, Carew J, Giles FJ, Keating MJ, Arlinghaus RB, Huang P (2008). "Effective killing of Gleevec-resistant CML cells with T315I mutation by a natural compound PEITC through redox-mediated mechanism." Leukemia 22(6):1191-1199.

Zhang J, Li L, Kim SH, Hagerman AE, Lu J (2009). "Anti-cancer, anti-diabetic and other pharmacologic and biological activities of penta-galloyl-glucose." Pharm. Res 26(9): 2066-2080.

Full Length Research Paper

Pharmacokinetics of piperine following single dose administration of benjakul formulation in healthy Thai subjects

Piracha Jumpa-ngern[1], Somboon Kietinun[1,2], Intouch Sakpakdeejaroen[1], Anurak Cheomung[2] and Kesara Na-Bangchang[2]*

[1]Department of Applied Thai Traditional Medicine, Faculty of Medicine, Thammasat University (Rangsit campus), Pathumthani, Thailand.
[2]International College of Medicine, Thammasat University (Rangsit campus), Pathumthani, Thailand.

Benjakul formulation is a Thai traditional medicine preparation, has also been used as an adaptogenic drug for cancer patients. No toxicity, either acutely or chronically has been reported in experimental animals and humans. The study was to preliminarily investigate the pharmacokinetics of piperine, the major active component of Benjakul formulation, following the administration of single oral doses of 100 (group 1) and 200 (group 2) mg Benjakul tablets in 20 healthy Thai subjects. Venous blood samples were collected before and after dosing. Serum concentrations of piperine were measured using HPLC-UV method. Pharmacokinetic analysis was performed by using model-independent analysis approach. Benjakul formulation was well tolerated in all subjects, with no apparent adverse events. Piperine was rapidly absorbed following the administration of both dosage levels. The pharmacokinetics of piperine was dose–independent. The observed median first maximum concentration (Cmax-1st) of piperine of 1,078 ng/mL following the dose of 200 mg Benjakul was significantly higher than (p< 0.001) that of 100 mg dose (467 ng/mL). Median time to first maximum concentration (tmax-1st) was about 1 hours in both groups. The area under serum concentration-time curve (AUC 0-48hr) of 10,216 ng.hr/mL following 200 mg dose was also significantly greater than (p< 0.001) that of 100 mg dose (4,288 ng.hr/mL). It was noted however for the second maximum concentration (Cmax-2nd) of piperine at about 9 hours post-dosing observed in 9 (median: 203 ng/mL) and 7 (median: 499 ng/mL) subjects who received 100 and 200 mg Benjakul formulation, respectively.

Key words: Benjakul formulation, piperine, Piper chaba Hunter., Piper sarmentosum Roxb., Piper interruptum Opiz., Plumbago indica Linn., and Zingiber officinale Roscoe., pharmacokinetics.

INTRODUCTION

Cancer is one of the leading causes of death in Thailand, and the trend is continuously increasing (National Cancer Institute, 2012). Several approaches are currently being applied for treatment of cancer including chemotherapy, radiation, and surgery. The major problem of cancer chemotherapy is drug-associated toxicities due to non-selectivity of most chemotherapeutics to normal cells

(Rebucci and Michiels, 2013). The use of herbs as complementary and alternative medicine has increased dramatically in the past decades. Plant-derived com-pounds or dietary phytochemicals have emerged as an accessible and promising approach to cancer control and management (Surh, 2003). A growing trend among cancer patients, especially those living in the rural areas

*Corresponding author. E-mail: karbwangj@nagasaki-u.ac.jp. Tel: 6629869203 ext. 7172. Fax: 662 9869217.

is to combine conventional therapy with some forms of complementary therapy (Vapiwala et al., 2006). These products may act synergistically with other chemothera-peutics or other treatment approaches to kill tumor cells by reducing angiogenesis, inflammation, and/or metastasis.

Benjakul formulation is one Thai traditional medicine used as complementary medicine in conjunction with other herbal medicines for treatment of cancer. This therapeutic application is believed to be through balancing "Dhatu" (regulating body chemical and physical function) before cancer chemotherapy (Itharat et al., 1999; Sriyakul et al., 2010). Benjakul formulation is composed of parts from five plants, that is *Piper chaba* Hunter. (fruit), *Piper sarmentosum* Roxb. (root), *Piper interruptum* Opiz. (stem), *Plumbago indica* Linn. (root), and *Zingiber officinale* Roscoe. (rhizome). Bioassay guided fractionation of the ethanolic extract of this herbal formulation revealed active constituents, of which piperine from *P. chaba* Hunter., *P. sarmentosum* Roxb., and *P. interruptum* Opiz. were the major components (78.69%), followed by plumbagin from *P. indica* Linn. (17.05%), and 6-gingerol from *Z. officinale* Roscoe. (4.26%) (Itharat et al., 2010).

The formulation including their active constituents, particularly piperine, have been demonstrated to exhibit a wide range of pharmacological and biological activities. Apart from cytotoxic and anticancer activities against lung, breast and prostate cancers, Benjakul formulation and piperine also possess antioxidant, anti-inflammatory, analgesic and anti-pyretic, central nervous system de-pressant, antiplatelet, antihypertensive, hepatoprotective, antithyroid, immuno-stimulating (promoting natural killer cell activity) activities, as well as inhibitory activity on nitric oxide production (Bhitre et al., 2008; Vaghasiya et al., 2007; Veeru et al., 2009; Wu et al., 2004). Acute and chronic toxicity tests in animals and humans have demonstrated the preparation to be practically non-toxic and well-tolerated (Amorndoljai et al., 2011; Itharat et al., 2010). Nevertheless, there has been no information regarding its pharmacokinetics in humans.

The aim of the present study was therefore to preliminarily investigate the pharmacokinetics of Benjakul formulation in healthy Thai subjects, using piperine as a pharmacological marker.

MATERIALS AND METHODS

Study subjects

Twenty healthy Thai subjects (10 males and 10 females), aged between 20 and 38 years, weighing 42 to 84 kg, with body mass index (BMI) between 17.9 and 25.9 kg/m^2 participated in the study. Inclusion criteria included: non-lactating and non-pregnant verified by urine β-hCG urine pregnancy test (females), normal laboratory tests (haematology, serum biochemistry, and urinalysis), no significant abnormal findings on physical and clinical examination particularly liver, kidney, cardiovascular diseases, or peripheral neuropathy, no previous consumption of foods containing the five composition of

Benjakul formulation within two weeks, and no previous administration of other drugs within three months. None was a smoker, alcohol drinker, or was on regular medication. Written informed consent for participation was obtained from each volunteer before initiation of the study. The study was approved by the Human Ethics Committee of the Faculty of Medicine, Thammasat University, Thailand.

At enrollment, a medical history was taken, including a full physical examination; each volunteer had a thorough physical examination and routine laboratory investigations (haematology, serum biochemsitry, and urinalysis).

Preparation of Benjakul formulation

Plant materials, that is fruits of *P. chaba* Hunter. (from Thongphaphoom District, Kanchanaburi Province, voucher number SKP 146160301), roots of *P. sarmentosum* Roxb. (from Hadyai District, Sonkhla Province, voucher number SKP 146161901), stems of *P. interruptum* Opiz. (Mae-rim District, Chaingmai Province, voucher number SKP 146160901), roots of *P. indica* Linn. (Bankoknoi District, Bangkok, voucher number SKP148160901), and rhizomes of *Z. officinale* Roscoe. (Khaokho District, Petchaboon Province, voucher number SKP206261501) were collected from all parts of Thailand during January to March, 2006. Authentication of plant materials was carried out at the herbarium of the Department of Forestry, Bangkok, Thailand, where the herbarium vouchers have been kept. The plant materials were rinsed thoroughly with tap water to remove extraneous contami-nants and cut into small pieces, oven-dried at 50°C until stability of dry weight was observed, and then ground into powder with an electric-grinder.

Extraction was carried out by macerating the powdered plant materials (100 g) in a flask containing 500 ml of 95% ethanol at room temperature (25 to 30°C) for 7 days. The extracted solvent was separated and filtered through Whatman no. 1 filter paper. After filtration, the extracts were evaporated under reduced pressure by rotary evaporation. The crude extracts were weighed and stored at -20°C until use. Standardization of the extract was performed using activity-guided fractionation. Piperine, the major component of Benjakul formulation was used as a marker for standardization of the formulation using high-performance liquid chromatography. Acceptable content of piperine was 27 ± 1 mg/g of dried weight plant materials with IC$_{50}$ (concentration that inhibits cell growth by 50%) value in lung cancer cell of 30 ± 1 μg/ml. Chroma-tographic separation condition used was as follow: Phenonemex™ Luna C18 column (5 μm particle size); mobile phase: a mixture of water and acetonitrile with gradient elution 0 min (60:40), 30 min (50:50), 50 min (5:95), and 60 min (0:100) at a flow rate of 1 ml/min.

Bajakul tablet was prepared and tested according to the United States Pharmacopeia USP Standard (weight variation, content uniformity, disintegration, and dissolution), as well as those not given in the USP Standard (hardness, thickness, and friability). The dried extract was formulated in tablets; each tablet contained 60 mg/g of total content (100 mg Benjakul formulation contained 6 mg piperine) (Itharat and Sakpakdeejaroen, 2010; Sakpakdeejaroen, 2009).

Drug administration

The subjects were randomly divided into two groups (5 males and 5 females in each group) to receive a single oral dose of 100 (group 1) or 200 (group 2) mg Benjakul formulation (100 and 200 mg tablets, respectively). Compliance with all drug intake was under investigators' supervision. No food was allowed until 2 h after drug intake. Volunteers were hospitalized at Thammasat Chalermprakiet Hospital, Pathumtanee Province one day prior to, and on the day of

pharmacokinetic study. No other concurrent drugs or alcohol were allowed during the study period. Meals with no composition of the five plant materials were provided to all subjects during the investigation period.

Adverse reaction monitoring

Following the administration of Benjakul formulation, all subjects were physically examined and adverse reactions during the study were recorded with the date and time at which they occurred and disappeared. These included gastrointestinal, central nervous, cardiovascular, dermatological effects, as well as other changes possibly attributable to the study drugs. Adverse effects were assessed on the basis of non-suggestive questioning by the study investigators. Routine blood investigations (haematology and biochemistry) and urinalysis were performed at baseline and 14 days after last drug administration (Amorndoljai et al., 2011).

Sample collection

An indwelling catheter was inserted into the antecubital vein of one arm of the patient, and patency maintained with heparin saline. Whole blood (5 ml) was collected from each patient and placed into sterile heparin tubes at 0.5, 1, 2, 4, 6, 9, 12, 18, 24, and 48 h after drug administration. Serum samples were prepared through centrifugation at 1,000 ×g for 15 min and stored at -80°C until analysis.

Drug analysis

Serum piperine concentrations were measured according to the methods of Bajad et al. (2002) and Sethi et al. (2009), with modifications. Piperine and the internal standard β-17-estradiol acetate (99% pure) were purchased from Merck Co. Ltd. (Darmstadt, Germany) and Sigma-Aldrich (CA, USA). Acetonitrile, methanol, ethyacetate, and propan-1-ol were of HPLC grade, which were purchased from Labscan Co. Ltd., Bangkok, Thailand. The following chemicals and solvents were obtained in the highest purity available: phosphoric acid and di-potassium hydrogen phosphate-3 hydrate obtained from Analytical Sciences Co. Ltd., Bangkok, Thailand. Deionized double distilled water was used for the preparation of working nicotine standard solutions. Serum from healthy volunteers used for standard curves was provided from the blood bank of Thammasat Chalermprakiet Hospital.

Standard stock solutions of both piperine and internal standard were prepared in methanol at a concentration of 1 mg/ml. Working solutions of 1 mg/ml for piperine and 3,000 ng/µl for internal standard were prepared and stored at -80°C. Calibration curves were constructed by analysis of 500 µl serum samples spiked with various concentrations of piperine (0, 25, 50, 100, 250, 500, and 1,000 ng/ml). The internal standard was used at a concentration of 15,000 ng/ml. Serum samples (500 µl) were spiked with internal standard, and the resultant mixture extracted with distilled water (500 µl), 12 mM phosphate buffer pH 3.4 (100 µl), and a mixture of ethyl acetate and propanol (9:1 v:v) (6 ml).

After centrifugation at 2000 ×g for 10 min, the clear organic layer was evaporated to dryness with nitrogen gas. The residue was then dissolved in 100 µl methanol and an aliquot of 40 µl was injected onto the HPLC system. Piperine and β-17-estradiol acetate were analyzed by HPLC using SpectraSystem™ P4000 Quaternary Solvent Delivery/Controller equipped with SpectraSystem SCM1000 Solvent Degasser, a SpectraSystem AS3500 Autosampler, and a SpectraSystem UV/Vis 3000 Detector (Thermo Fisher Scientific, CA, USA). The wavelength of UV-Vis detector was operated at 340 and 280 nm for piperine and β -17-estradiol acetate, respectively. Piperine and the internal standard were separated on a ZORBAX

Eclipse XDB-C18 (4.6 × 250 mm, 5 µm particle size) (Agilent Technologies™, CA, USA).

The HPLC system was operated under an isocratic mode at a flow-rate of 1 ml/min. The mobile phase was a mixture of 25 mM dipotassiumphosphate (pH 4.5, adjusted with orthophosphoric acid) and acetonitrile at the ratio of 35:65 (v:v). The retention times of piperine and β-17-estradiol acetate were 4.6 and 10.0 min, respectively. Piperine was quantified by using the ratio of the peak area of analyte to that of internal standard. Serum analysis was calibrated using concentration range of 25 to 4,000 ng/ml. All calibration ranges yielded linear relationships with correlation coefficients ($r^2 \geq 0.999$) or better. The limit of quantification (LOQ) for piperine was 25 ng/ml. The intra- and inter-day coefficients of variation (CV) of piperine were 3.0 versus 19.11, 13.59 versus 4.79, and 10.78 versus 10.79% at concentrations of 25, 250, and 500 ng/ml, respectively.

The intra- and inter-day accuracy values were 19.68 versus 0.48, 0.74 versus 11.98, and -4.43 versus -2.18% for the concentrations of 25, 250, and 1,000 ng/ml, respectively. The mean recoveries for piperine at 50, 500 and 1,000 ng/ml were 90.13, 90.06 and 92.18%, respectively. The recovery of β-17-estradiol acetate at the concentration of 3,000 ng/ml was 99.47 ± 0.15%. Quality control (QC) samples for piperine were made up in serum using a stock solution separated from that used to prepare the calibration curve, at the concentrations of with 50 (low), 500 (medium), and 1,000 ng/ml (high) piperine and 3,000 ng/ml internal standard. Samples were aliquoted into cryovials and stored frozen at -20°C for use with each analytical run. The results of the QC samples provided the basis of accepting or rejecting the run. At least four of the six QC samples had to be within ±20% of their respective nominal values. Two of the six QC samples could be outside the ±20% of their respective nominal value, but not at the same concentration.

Pharmacokinetic and statistical analysis

Serum concentration-time profile of piperine for each subject was plotted and the pharmacokinetic analysis was performed using model-independent analysis approach. Maximum concentration (the first and second: $C_{max-1st}$ and $C_{max-2nd}$) and time to maximum concentration (the first and second: $t_{max-1st}$ and $t_{max-2nd}$) were obtained directly from the concentration-time profile of each individual. The area under the curve from zero time to forty-eight hours of dosing ($AUC_{0-48\ h}$) was deduced using the linear trapezoidal rule for ascending data points and the log trapezoidal rule for descending data points. Data are presented as median (range) values. Comparison of the pharmacokinetic parameters obtained from subjects following administration of the two dosage regimens of Benjakul formulation was performed using Mann-Whitney U test for data not conforming to normal distribution (SPSS for windows Release 13, NY, USA.) at statistical significance level of α = 0.05.

RESULTS

Tolerability

All volunteers were healthy, verified by laboratory results, physical examination, and vital sign monitoring with no significant difference between the two dosing groups. The demographic characteristics (age, body weight, height, and BMI), vital signs (systolic and diastolic blood pressure, heart rate, respiratory rate) and laboratory investigations (haematology, biochemistry, and urinalysis) were comparable in both groups of subjects (p > 0.05) (Table 1).

Table 1. Baseline characteristics of healthy subjects in group 1 (100 mg Benjakul formulation) and group 2 (200 mg Benjakul formulation). Data are presented as median (range) values.

Parameter	Group 1	Group 2
Age (years)	23.5 (20-25)	24 (20-38)
Weight (kg)	52.5 (42-83)	54 (44-84)
Height (cm)	165.50 (148-178)	161.00 (155-180)
BMI (kg/m^2)	19.25 (17.95-26.20)	19.74 (17.95-25.92)
Haematology		
WBC (µl^{-1})	5.74 (4.27-7.31)	6.01 (4.45-8.04)
Neutrophil (%)	56 (38-64)	59 (43-73)
Lymphocyte (%)	35 (29-54)	31 (21-45)
Monocyte (%)	3.8 (2.9-7.2)	4.2 (2.3-7.6)
Eosinophil (%)	3.7 (1.3-13.3)	2.7 (0.6-8.1)
Basophil (%)	0.4 (0.2-0.6)	0.35 (0.2-0.7)
RBC (×10^6/µl)	4.86 (3.89-6.00)	4.91 (4.14-5.57)
Haemoglobin (g/dl)	13.0 (11.4-16.1)	13.9 (11.3-15.3)
Haematocrit (%)	38.1 (34.1-46.9)	40.8 (34.5-44.6)
Platelets (µl^{-1})	234 (179-338)	226 (163-326)
Biochemistry		
Glucose (mg/dl)	83 (73-89)	80 (76-95)
Blood urea nitrogen (mg/dl)	11.2 (7.0-15.4)	11.6 (7.7-13.5)
Creatinine (mg/dl)	1.00 (0.70-1.20)	0.95 (0.80-1.20)
Total cholesterol (mg/dl)	195 (150-208)	196 (157-208)
Triglyceride (mg/dl)	69 (44-109)	74 (30-125)
HDL-cholesterol (mg/dl)	55 (34-75)	51 (42-72)
LDL-cholesterol (mg/dl)	101 (92-106)	101 (91-108)
Globulin (mg/dl)	3.15 (2.90-3.80)	3.35 (2.70-3.70)
Albumin (mg/dl)	4.40 (4.10-4.70)	4.3 (4.00-4.60)
Total protein (mg/dl)	7.55 (7.20-8.30)	7.7 (7.00-8.90)
Total bilirubin (mg/dl)	0.95 (0.90-1.60)	0.95 (0.50-1.60)
Conjugated bilirubin (mg/dl)	0.20 (0.10-0.40)	0.2 (0.10-0.40)
Aspartate aminotranferase (mg/dl)	18 (14-23)	17.5 (12-30)
Alanine aminotranferase (mg/dl)	11 (6-24)	11.5 (9-18)
Alkaline Phosphatase (mg/dl)	63 (40-78)	59.5 (16-85)

Median plots of serum concentration-time profiles of piperine following the administration of the two dosage regimen of Benjakul formualtion in both groups of subjects are shown in Figure 1. Piperine was rapidly absorbed from gastrointestinal tract after oral dose administration with marked inter-individual variation. In most cases, the drug was detectable in plasma within 0.5 h of dosing, with median $t_{max-1st}$ of 1 h. It disappeared thereafter from systemic circulation within 48 h. The pharmacokinetic parameters of piperine calculated based on model-independent approach are summarized in Table 2. Large inter-individual variation among the pharmacokinetic parameters was noted, particularly with $C_{max-1st}$ and $AUC_{0-48\ h}$ as reflected by the values of coefficients of variation (CVs) for both parameters ($_{Cmax-1st}$

44 to 52%, and $AUC_{0-48\ h}$ 63 to 91%). Median $C_{max-1st}$ and $AUC_{0-48\ h}$ values were found to be about twice in the group receiving 200 mg dose compared with that of 100 mg dose (median $C_{max-1st}$ 1,078 versus 467 ng/ml; $AUC_{0-48\ h}$ 10,216 versus 4,288 ng h/ml; $p < 0.05$). It was noted however for the second maximum serum concentration ($C_{max-2nd}$) of piperine at about 9 h post-dosing observed in 9 (median: 203 ng/ml) and 7 (median: 499 ng/ml) subjects who received 100 and 200 mg Benjakul formulation, respectively.

DISCUSSION

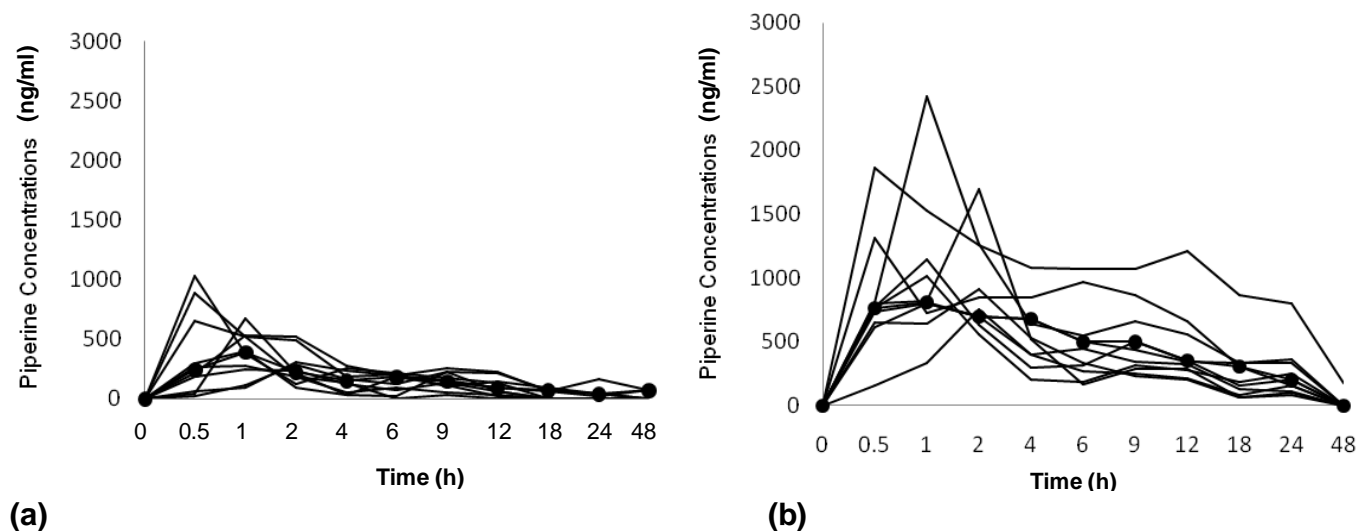This study is the first investigation of the pharmaco-

Figure 1. Median (filled circle) and individual serum concentration-time profiles (lines) of piperine following an oral administration of a single dose of (a) 100 mg (Group 1, n = 10), and (b) 200 mg (Group 2, n = 10) Benjakul formulation in healthy subjects.

kinetics of piperine following the administration of Benjakul formulation. The formulation was well tolerated with no apparent adverse drug reaction. The two dosage regimens, that is 100 and 200 mg Benjakul formulation were selected based on the doses used in phase I clinical study in 23 healthy Thai subjects. No significant adverse reaction except reversible gastrointestinal irritation was observed following the administration of 100 or 200 mg dose given three times daily (after meal) for 14 days (Amorndoljai et al., 2011).

Piperine was well absorbed from the gastrointestinal tract after oral dose of Benjakul formulation with median $t_{max-1st}$ of about 1 h. Pharmacokinetics of piperine following a single oral dose of 200 (12 mg piperine) or 100 (6 mg piperine) mg exhibited dose linearity as ascribed by the proportional increase in both dose-dependent pharmacokinetic parameters; $C_{max-1st}$ and $AUC_{0-48\,h}$ were increased by about 2-fold when the dose was doubled from 100 to 200 mg. Unfortunately, other pharmacokinetic parameters including terminal phase elimination half-life ($t_{1/2z}$), total clearance (CL/f) and apparent volume of distribution (V_z/F) could not be determined with accuracy due to limitation of blood sampling schedule during distribution and elimination phases.

The pharmacokinetic study in rats following a single oral dose of 100 mg/kg body weight alkaloids from *P. longum L.* (equivalent to 54.1 mg/kg bodyweight or approximately 12 mg piperine per 200 mg body weight rat) showed that the compound was rapidly absorbed and slowly eliminated (Liu et al., 2011). Mean C_{max}, t_{max}, $AUC_{0-\alpha}$, and $t_{1/2z}$ were 4,292 ng/ml, 2.45 h, 23.10 μg h/ml, and 4.10 h, respectively. The observed C_{max} ($C_{max-1st}$) in humans was thus only one-fourth of that reported in rats following an equivalent dose in rat (12 mg piperine). The

absorption appeared however to be more rapid in humans (median of 1 versus mean of 2.45 h).

A previous *in vitro* study has shown that piperine, as a weak base and highly lipophilic molecule, is absorbed very fast across the intestinal barrier through passive diffusion with short absorption clearance and high apparent permeability co-efficient (Khajuria et al., 1998). Investigation of tissue distribution and elimination of piperine in rats showed its absorption to be approximately 96% of the administered dose (170 mg/kg body weight) (Suresh and Srinivasan, 2010). Furthermore, pharmacokinetics of piperine in lipid nanospheres in mice showed a biexponential decline with a significantly high AUC, a lower rate of clearance and a smaller volume of distribution than piperine (Veerareddy and Vobalaboina, 2008).

Following absorption, piperine distributed throughout various tissues including liver, kidney, spleen, stomach, small intestine (Suresh and Srinivasan, 2010; Bhat and Chandrasekhara, 1986). It was noted for the observation of the second peak of piperine in almost all subjects (9 subjects in group 1 and 7 subjects in group 2) at about 6 h ($t_{max-2nd}$). This was not observed in any of the previous pharmacokinetic investigations in rats (Suresh and Srinivasan, 2010). The phenomenon is a common characteristics of drugs or compounds that undergo hepatic metabolism through phase II glucuronidation reaction, which is likely to exhibit entero-hepatic recycling. Urinary excretion and biliary excretion happen to be the main routes of piperine excretion (Bhat and Chandrasekhara, 1986). Glucuronidation and sulfation appeared to be the major pathways in the disposition of piperine, as glucuronides and conjugated sulfates were found in the urinary excretions of rats after oral administration of piperine (Bhat and Chandrasekhara,

Table 2. Pharmacokinetic parameters of piperine in individual subjects following an oral administration of a single dose of 100 mg (group 1, n = 10), and 200 mg (group 2, n = 10) Benjakul formulation in healthy subjects.

Subjects	Group 1					Subjects	Group 2				
	$c_{max-1st}$ (ng/ml)	$t_{max-1st}$ (h)	AUC_{0-48hr} (ng × h/ml)	$c_{max-2nd}$ (ng/ml)	$t_{max-2nd}$ (h)		$c_{max-1st}$ (ng/ml)	$t_{max-1st}$ (h)	AUC_{0-48hr} (ng × h/ml)	$c_{max-2nd}$ (ng/ml)	$t_{max-2nd}$ (h)
1 (male)	531	1	7902	225	9	1 (male)	1015	1	9881	312	9
2 (male)	654	0.5	4524	203	6	2 (male)	909	2	7263	-	-
3 (male)	891	0.5	1498	37	9	3 (male)	749	2	8230	440	6
4 (male)	251	1	1750	139	9	4 (male)	2423	1	18262	662	9
5 (male)	404	1	9811	184	6	5 (male)	1690	2	9021	289	9
6 (female)	283	1	1309	99	6	6 (female)	797	1	14936	-	-
7 (female)	289	2	3846	224	9	7 (female)	1141	1	10552	499	9
8 (female)	1030	0.5	8704	256	4	8 (female)	803	1	7907	-	-
9 (female)	674	1	5190	226	9	9 (female)	1862	1	57644	1212	12
10 (female)	310	2	4051	-	-	10 (female)	1314	0.5	29165	963	6
Median	467	1	4,288	203	9	Median	1,078*	1	10216*	499*	9
(Range)	(251-1,030)	(0.5-2)	(1,309-9,811)	(37-256)	(4-9)	(Range)	(749-2,423)	(0.5-2)	(7,263-57,644)	(289-1,212)	(6-12)

*Statistically significantly higher in group 2 compared with group 1 (p-value < 0.01, Mann-Whitney U Test).

1986). After oral administration of piperine at the dose of 170mg/kg body weight to rats, four metabolites of piperine, that is piperonylic acid, piperonyl alcohol, piperonal, and vanillic acid were identified in the free form in 0 to 96 h urine, whereas only piperic acid was detected in 0 to 6 h bile (Bhat and Chandrasekhara, 1987).

The most common concern in clinical application of piperine or herbal preparation containing piperine has been its inhibitory influence on hepatic and intestinal metabolic enzymes including cytochrome P450 (CYP)-mediated pathways (phase I) and phase II metabolism which results in potentially toxic concentrations of a number of concurrently administered drugs. Inhibitory effect of piperine on human CYP3A4 activity has been shown with phenytoin, propranolol, theophylline, and rifampin (Bano et al., 1987, 1991; Hu et al., 2005; Velpandian et al.,

2001). In addition, previous studies revealed that piperine could modulate the functional activity or gene expression of the efflux protein P-glycoprotein (P-gp: multidrug resistance protein 1 or ATP-binding cassette sub-family B member 1) (Bhardwaj et al., 2002; Han et al., 2008; Han, 2011; Lin and Han, 2010). The inhibition of this P-gp-mediated transportation was reported with digoxin, cyclosporine, fexofenadine (Bhardwaj et al., 2002).

Conclusion

The current pharmacokinetic study provides preliminary information on the absorption and disposition characteristics of piperine in humans, which would form the basis for further well-design pharmacokinetic-pharmacodynamic studies for

dose optimization of Benjakul formulation in healthy subjects and cancer patients. In addition, the impact of piperine on disposition of co-administered drugs which are substrates of CYP3A4, P-gp, and possibly other drug-metabolizing enzymes should be clarified to avoid any clinically relevant pharmacokinetic drug interactions in cancer patients who are likely to receive multiple drug therapy for cancer and concurrent complications.

for sample collection and laboratory work.

REFERENCES

Amorndoljai P, Kietinunand S, Somparn N (2011). Study on Safety of Benjakul Recipies Extract Tablets in Normal Volunteers. Tham. Med. J. 11(2):195-202.

Bajad S, Singla AK, Bedi KL (2002). Liquid Chromatographic Method for Determination of Piperine in Rat Plasma: Application to Pharmaco-kinetics. J. Chromatogr. B 776(2):245-249.

Bano G, Amla V, Raina RK, Zutshi U, Chopra CL (1987). The Effect of Piperine on Pharmacokinetics of Phenytoin in Healthy Volunteers. Planta Med. 53:568–569.

Bano G, Raina RK, Zutshi U, Bedi KL, Johri RK, Sharma SC (1991). Effect of Piperine on Bioavailability and Pharmacokinetics of Propanolol and Theophylline in Healthy Volunteers. Eur. J. Clin. Pharmacol. 41:615–617.

Bhat BG, Chandrasekhara N (1986). Studies on the Metabolism of Piperine: Absorption, Tissue Distribution and Excretion of Urinary Conjugates in Rats. Toxicol. 40(1):83-92.

Bhat BG, Chandrasekhara N (1987). Metabolic Disposition of Piperine in the Rat. Toxicol. 44(1):99-106.

Bhardwaj RK, Glaeser H, Becquemont L, Klotz U, Gupta SK, Fromm MF (2002). Piperine, a major constituent of black pepper, inhibits human P-glycoprotein and CYP3A4. J. Pharmacol. Exp. Ther. 302(2):645–650.

Bhitre MJ, Fulmali S, Kataria M, Anwikar S, Kadri H (2008). Anti-inflammatory activity of the fruits of *Piper longum* Linn. Asian J. Chem. 20(6):4357-4360.

Han Y, Chin Tan TM, Lim LY (2008). *In Vitro* and *In Vivo* Evaluation of the Effects of Piperine on P-gp Function and Expression. Toxicol. Appl. Pharmacol. 230:283–289.

Han HK (2011). The Effects of Black Pepper on the Intestinal Absorption and Hepatic Metabolism of Drugs. Expert Opin. Drug Metab. Toxicol. 7:721–729.

Hu Z, Yang X, Ho PC, Chan SY, Heng PW, Chan E, Duan W, Koh HL, Zhou S (2005). Herb-drug Interactions: a Literature Review. Drugs 65(9):1239-1282.

Itharat A, Singchangchai P, Rattanasuwan P (1999). Folk Wisdom of Traditional Doctors in South of Thailand. Songklanakarin J. Sci. Tech. 24:126-127

Itharat A, Kietinun S, Sireeratawong S, Tappayuthpijarn P, Sriyakul K, Ratanavalachai T, et al. (2010). Development of Benjakul extract as health products for cancer patients. Applied Thai Traditional Medicine, Faculty of Medicine, Thammasat University, Thailand.

Itharat A, Sakpakdeejaroen I (2010). Determination of Cytotoxic Compounds of Thai Traditional Medicine Called Benjakul Using HPLC. J. Med. Assoc. Thai. 93(7):S198-S203.

Khajuria A, Zutshi U, Bedi KL (1998). Permeability Characteristics of Piperine on Oral Absorption--An Active Alkaloid From Peppers and a Bioavailability Enhancer. Indian J. Exp. Biol. 36(1):46-50.

Liu J, Bi Y, Luo R, Wu X (2011). Simultaneous UFLC-ESI-MS/MS Determination of Piperine and Piperlonguminine in Rat Plasma After Oral Administration of Alkaloids From *Piper longum* L.: Application to Pharmacokinetic Studies in Rats. J. Chromatogr. B Analyt. Tech. Biomed. Life Sci. 879(27):2885-2890.

National Cancer Institute (2012). World Cancer Day 2012 Cancer Can Be Prevented. Thai Cancer J. 31(1):1-2.

Rebucci M, Michiels C (2013). Molecular aspects of cancer cell resistance to chemotherapy. Biochem. Pharmacol. 2952(13):121-124.

Sakpakdeejaroen I (2009). Study on Cytotoxicity and Chemical Fingerprint of Ethanolic Extract of Benjakul Preparation. Faculty of Medicine, Thammasat University, Thailand.

Sethi P, Dua KV, Mohanty S, Mishra KS, Jain R, Edwards G (2009). Development and Validation of a Reversed Phase HPLC Method for Simultaneous Determination of Curcumin and Piperine in Human Plasma for Application in Clinical Pharmacological Studies. J. Liq. Chrom. Rel. Tech. 32:2961–2974.

Sriyakul K, Kietinun S, Itharat A, Pattaraarchachai J, Kittipawong P, Sakpakdeejaroen I, Kamalashiran C, Sunopuk R, Chunthorng-orn J, Issarata T, Tonthong B, Chamnanaukson W, Chinsoi P (2010). Preliminary Comparative Study on the Efficacy and Side Effects of Benjakul in Normal and Imbalanced Dhatu Volunteers. Applied Thai Traditional Medicine, Faculty of Medicine, Thammasat University, Thailand.

Suresh D, Srinivasan K (2010). Tissue Distribution and Elimination of Capsaicin, Piperine and Curcumin Following Oral Intake in Rats. Indian J. Med. Res. 131:682-691.

Surh YJ (2003). Cancer Chemoprevention with Dietary Phytochemicals. Nat. Rev. Cancer. 3(10):768-780.

Vapiwala N, Mick R, Hampshire M, Metz J (2006). Patient Initiation of Complementary and Alternative Medical Therapies Following Cancer Diagnosis. Cancer J. 12(6):467-474.

Vaghasiya Y, Nair R, Chanda S (2007). Investigation of Some Piper Species for Anti-bacterial and Anti-inflammatory Property. Int. J. Pharmacol. 3:400-405.

Veerareddy PR, Vobalaboina V (2008). Pharmacokinetics and Tissue Distribution of Piperine Lipid Nanospheres. Pharmazie 63(5):352-355.

Veeru P, Kishor MP, Meenakshi M (2009). Screening of Medicinal Plant Extracts for Antioxidant Activity. J. Med. Plant Res. 3:608-612.

Velpandian T, Jasuja R, Bhardwaj RK, Jaiswal J, Gupta SK (2001). Piperine in Food: Interference in the Pharmacokinetics of Phenytoin. Eur. J. Drug Metab. Pharmacokinet. 26:241–247.

Wu S, Sun C, Pei S, Lu Y, Pan Y (2004). Preparative Isolation and Purification of Amides From the Fruits of *Piper longum* L. by Upright Counter-current Chromatography and Reversed-phase Liquid Chromatography. J. Chromatogr. A 1040:193-204.

Full Length Research Paper

PAMAM dendrimers affect the *in vitro* release of clotrimazole from hydrogels irrespective of its molecular state

Katarzyna Sosnowska* and Katarzyna Winnicka

Department of Pharmaceutical Technology, Faculty of Pharmacy, Medical University of Bialystok, Kilinskiego 1, 15-089 Bialystok, Poland.

Insufficient aqueous solubility of clotrimazole (CLO) is the main problem in designing pharmaceutical formulations and limits its therapeutic efficiency. Polyamidoamine (PAMAM) dendrimers give the opportunity to decrease this limitation because of their solubilising and permeation enhancement properties. The aim of this study was to examine the influence of PAMAM dendrimers on the release of CLO from hydrogels and water suspensions. PAMAM dendrimers improved permeation of CLO from all analyzed formulations in the following order: PAMAM-NH$_2$ G3 > PAMAM-NH$_2$ G2 > PAMAM-OH G3 > PAMAM-OH G2. The effect evoked by PAMAM dendrimers was the most potent from hydrogels containing dissolved drug.

Key words: Polyamidoamine (PAMAM) dendrimers, clotrimazole, hydrogels.

INTRODUCTION

Clotrimazole (CLO) is a local imidazole-derivative antifungal agent that is used for the treatment of skin and vaginal infections caused by various species of pathogenic dermatophytes and yeasts (Holt, 1974; Henry et al., 2000). An optimal therapeutic effect of dermal drug depends on the appropriate dosage system, physicochemical properties of the substance, type of vehicle (lipophilic, hydrophilic) and the presence of skin absorption enhancers, which influence diffusion of a medical agent from vehicle to skin.

One of the main problems in transdermal delivery is restricted permeation of the drug through *stratum corneum*. In order to improve the cutaneous penetration in dermal preparations, chemical enhancers are used (Pathan and Setty, 2009), which increase active substance's solubility in the vehicle, improve the partition coefficient or modify barrier properties of *stratum corneum*. Recently, dendrimers, a new class of polymers

were found to improve solubility of water-insoluble drugs and to expedite the transportation of drugs across biomembranes. Dendrimers are monodispersed, nanometer size range and hyperbranched macromolecules, which make them suitable as carriers binding guest molecules in the interior of dendrimers or with the surface groups (Nanjwade et al., 2009). One of the most examined families of dendrimers is polyamidoamine (PAMAM) dendrimers, which are being investigated as permeation enhancer and carriers for several routes of delivery: transdermal, oral, ocular or even intravenous (Cheng et al., 2007a; Borowska et al., 2012).

The aim of this work was to examine the influence of concentration and type of surface groups of PAMAM dendrimers generation 2 and 3 (G2, G3) on *in vitro* release of CLO. Additionally, the mechanism of permeation increase, the diffusion of CLO from hydrogels containing the drug

*Corresponding author. E-mail: katarzyna.sosnowska@umb.edu.pl. Tel/Fax: 0048 85 748 56 16.

in two molecular states and from water suspensions were also studied.

MATERIALS AND METHODS

Clotrimazole was provided by Aflofarm (Pabianice, Poland). PAMAM dendrimers G2 and G3 with -NH$_2$ or -OH surface groups and Tween 80 were provided by Sigma Aldrich (St. Louis, MO, USA), together with other chemicals and buffers used. Carbopol® 980 was donated by S&D Polska (Warsaw, Poland). Clotrimazolum GSK® cream is a product of GlaxoSmithKline Pharmaceuticals SA (Poznan, Poland). Cuprophan®, natural cellulose membrane (MWCO 10,000 Da) was purchased from Medicell (London, UK).

Preparation of hydrogels with CLO in different molecular states (dissolved or dispersed)

All hydrogels were prepared using mechanical stirrer model DT 200 (Witko, Lodz, Poland). Carbopol 980 was gradually added to the water and stirred for 45 min until homogenous mixture appeared, then mixture was neutralized by dropwise addition of 20% solution of sodium hydroxide to allow gel formation. Methyl p-hydroxybenzoate (0.1%) and propyl p-hydroxybenzoate (0.1%) dissolved in ethanol, Tween 80, and propylene glycol were added to the hydrogel bases. To obtain hydrogels with dissolved drug, CLO in aqueous solutions of PAMAM dendrimers G2 or G3 with -NH$_2$ or -OH surface groups was mixed up with hydrogels bases. Hydrogels with dispersed drug were prepared by mixing CLO with hydrogel bases, and then aqueous solutions of PAMAM-NH$_2$ G3 dendrimers were added. Final concentration of PAMAM dendrimers in hydrogels was 0.3, 3.0 and 30.0 mg/g. As CLO is stable in alkaline medium and in acidic environment hydrolyzes to (o-chlorophenyl)diphenyl methanol and imidazole (Hoogerheide and Wyka, 1982); pH of all preparations was adjusted to 6.9. Composition of prepared hydrogels is shown in Table 1.

Preparation of water suspensions of CLO

CLO was suspended in water or in mixture of water with aqueous solutions of PAMAM G2 or G3 with -NH$_2$ or -OH surface groups at concentration of 0.3, 3.0 and 30.0 mg/g. Composition of prepared suspensions is shown in Table 1.

The *in vitro* release of CLO from hydrogels

The *in vitro* release of CLO from prepared hydrogels and comer-cially available product was performed in enhancer cell (Agilent Technologies, Cary, NC, USA), using natural cellulose membrane (Cuprophan®, Medicell, London, UK), previously moistened with water. The diameter of the cell was 2.2 cm, providing 3.80 cm^2 effective constant area. The enhancer cell consisted of Teflon chamber with adjustable capacity (0.5 to 5.0 g) and a screw cap to hold the membrane. About 3 g of each formulation ("infinite dose") was placed in the drug reservoir on the top of the membrane (10 µm thick) without entrapped air at the interface of the gel and membrane. A United States Pharmacopeia (USP) Apparatus 2, Dissolution Tester (Agilent 708-DS, Agilent Technologies, Cary, NC, USA) with mini vessels (250 ml) and mini paddles with a rotating speed of 75 rpm were used to measure the release of CLO from the enhancer cell assembly. The receptor compartment was filled with 150 ml of acetate buffer pH 5.5 with sodium dodecyl sulfate

(SDS; 1%) to provide the sink conditions and maintained at 32 ± 0.5°C. Aliquots (1 ml) of the acceptor phase were collected at the predetermined time intervals (0.5, 1, 2, 3 and 4 h) and replaced with an equal volume of fresh buffer solution (Thakker et al., 2003). The drug content in the examined samples was determined by the high performance liquid chromatography (HPLC) method. The results are expressed as means (%) ± standard deviation (SD) for 6 independent experiments.

The *in vitro* release of CLO from water suspensions

The experiments were performed using cellulose membrane (Cuprophan®, Medicell, London, UK) and acetate buffer pH 5.5 with SDS (1%). The dialysis bags containing 3 ml of investigated suspensions were placed in beakers with 150 ml of acceptor fluid preheated to 32 ± 0.5°C. The total area for diffusion was approximately 12 cm^2. The dialysis bags in beakers were shaken (75 rpm) in a water bath. Aliquots (1 ml) of the acceptor phase were collected at predetermined time intervals (0.5, 1, 2, 3 and 4 h) and replaced with an equal volume of fresh buffer solution. The drug content in examined samples was determined by the HPLC method. The results are expressed as means (%) ± SD for 6 independent experiments.

HPLC analysis of CLO

The amount of released CLO was determined by Agilent Technologies 1200 HPLC system equipped with a G1312A binary pump, a G1316A thermostat, a G1379B degasser and a G1315B diode array detector (Agilent, Waldbronn, Germany). Data collection and analysis were performed using ChemStation 6.0 software. Isocratic separation was obtained on a Zorbax Eclipse XDB–C18, 4.6 × 150 mm, 5 µm column (Agilent, Waldbronn, Germany). Mobile phase was methanol and phosphate buffer pH 7.4 (80:20 v/v); the flow rate was 1.0 ml/min and ultraviolet (UV) detection was performed at a wavelength of 210 nm (Hájková et al., 2007). The retention time for CLO was 5.5 min. The column's temperature was maintained at 25°C. For injection into the HPLC system, 20 µl of sample was used. Standard calibration curve was linear over the range of 1 to 100 µg/ml ($y = 154.4x + 6.0672$, $R^2 = 0.9995$). All reagents used for analysis were HPLC grade. The amount of CLO released (µg/cm^2) was plotted against square root of time ($\sqrt{h}$) and then linear regression analysis of the plot was accomplished (Higuchi's equation). The validity of applying Higuchi's equation was indicated by correlation coefficient ($R^2 \geq 0.998$).

Data analysis

The results were analysed by means of analysis of variance (ANOVA) and multiple comparisons were made to check statistical significance. The statistical significance between means was verified by Sheffe's comparison test accepting $p < 0.05$ as significant.

RESULTS AND DISCUSSION

The membrane permeability of drug depends on its solu-bility and *stratum corneum*/vehicle partition coefficient (Michaels et al., 1975). CLO belongs to lipophilic sub-stances, therefore is able to penetrate the skin relatively

Table 1. Composition of prepared formulations.

Ingredient (g)	Formulation code for hydrogels												
	H-1	H-2	H-3	H-4	H-5	H-6	H-7	H-8	H-9	H-10	H-11	H-12	H-13
CLO	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0
Carbopol 980	0.35	0.35	0.35	0.35	0.35	0.35	0.35	0.35	0.35	0.35	0.35	0.35	0.35
Sodium hydroxide (20%)	q.s.	q.s.	q.s.	q.s.	q.s.	q.s.	q.s.	q.s.	q.s.	q.s.	q.s.	q.s.	q.s.
Ethanol (760 g/l)	10.0	10.0	10.0	10.0	10.0	10.0	10.0	10.0	10.0	10.0	10.0	10.0	10.0
Propylene glycol	10.0	10.0	10.0	10.0	10.0	10.0	10.0	10.0	10.0	10.0	10.0	10.0	10.0
Methy p-hydroxybenzoate	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1
Propyl p-hydroxybenzoate	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1
Tween 80	2.0	2.0	2.0	2.0	2.0	2.0	2.0	2.0	2.0	2.0	2.0	2.0	2.0
PAMAM-NH$_2$ G2		0.03	0.3	3.0									
PAMAM-NH$_2$ G3					0.03	0.3	3.0						
PAMAM-OH G2								0.03	0.3	3.0			
PAMAM-OH G3											0.03	0.3	3.0
Purified water (up to)	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0

	Formulation code for suspensions												
	S-1	S-2	S-3	S-4	S-5	S-6	S-7	S-8	S-9	S-10	S-11	S-12	S-13
CLO	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0
PAMAM-NH$_2$ G2		0.03	0.3	3.0									
PAMAM-NH$_2$ G3					0.03	0.3	3.0						
PAMAM-OH G2								0.03	0.3	3.0			
PAMAM-OH G3											0.03	0.3	3.0
Purified water (up to)	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0

easier than hydrophilic ones and it should diffuse faster from hydrophilic vehicle than from lipophilic bases. However, poor aqueous solubility of CLO (0.49 µg/ml) (Pedersen et al., 1998) is the main problem in technology of effective dosage forms, because only dissolved fraction of drug is able to cross the barrier. Various methods, like forming microcapsules, liposomes (Ning et al., 2005), cyclodextrin complexes (Bilensoy et al., 2006) or solid dispersions (Balata et al., 2011) were used to improve the solubility of CLO and its permeation through the membranes.

PAMAM dendrimers are an interesting and relatively new class of compounds, which act not only as solubility enhancers (Filipowicz et al., 2011), but also possess the ability to improve anti-bacterial or antifungal activity of drugs (Strydom et al., 2013; Cheng et al., 2007b; Winnicka et al., 2011). Since properties of dendrimers depend on their size and surface charge (Malik et al. 2000), lower generation of PAMAM are more preferred for examination in dermal formulations, because of their stronger ability to enhance the permeation and lower toxicity (Heiden et al., 2007; Winnicka et al., 2009). PAMAM-NH$_2$ in contrast to neutral and anionic dendrimers can be accumulated in the skin upper layers (Yang et al., 2012), which makes them proper carriers for topical drug delivery. The effect of PAMAM dendrimers G2 and G3 with various surface groups on the release of
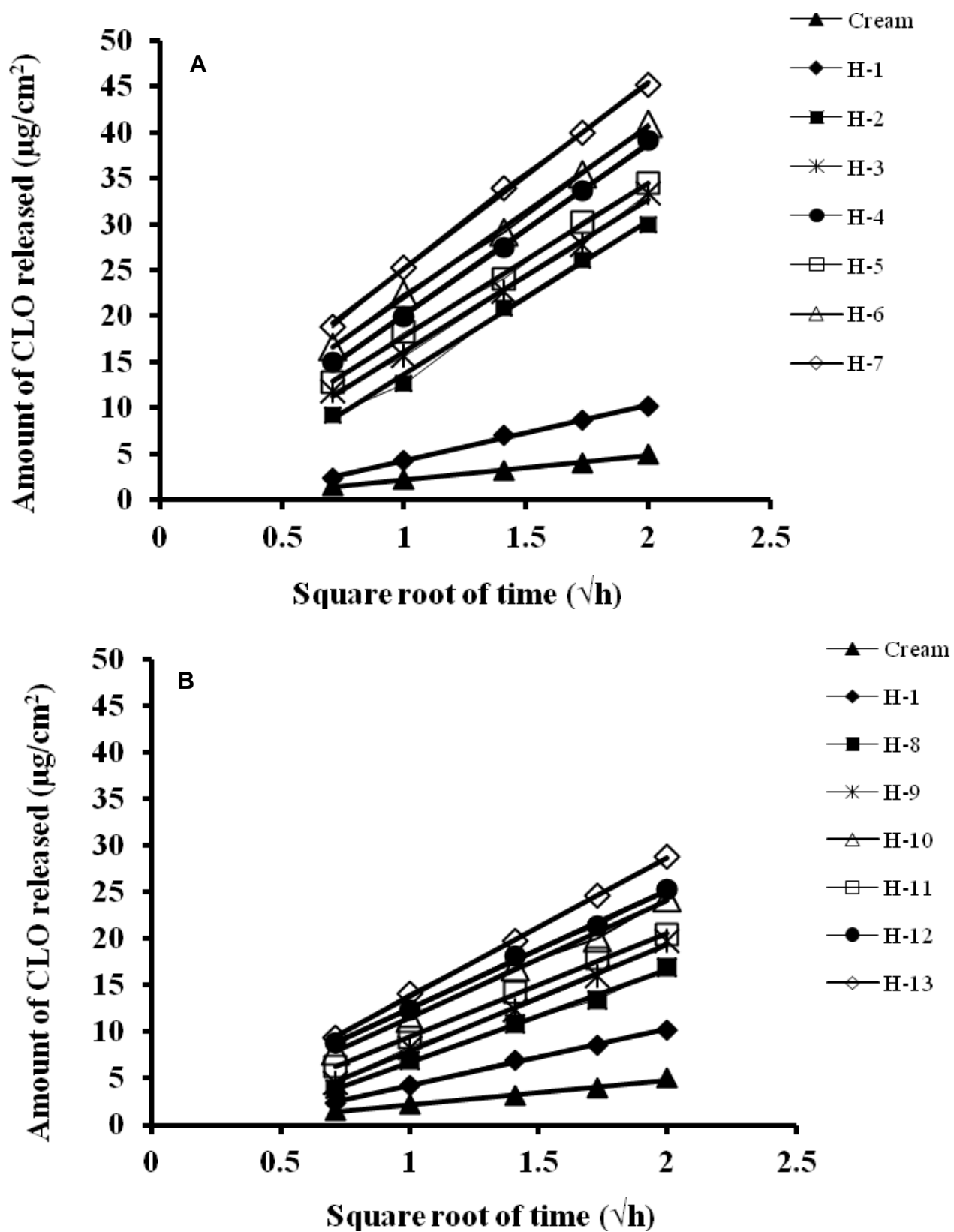
Figure 1. Cumulative amount of CLO released from hydrogels with PAMAM-NH$_2$ (A) or PAMAM-OH (B) containing dissolved CLO as a function of square root of time.
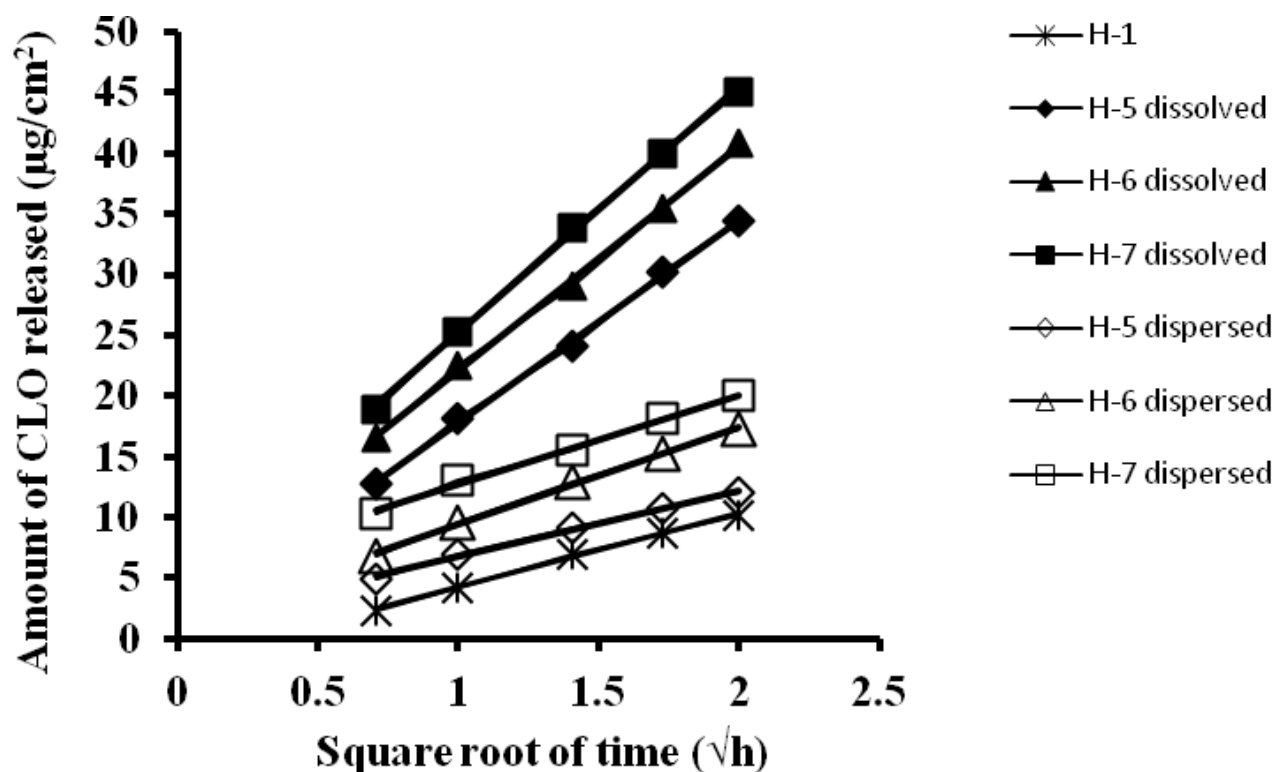
Figure 2. Comparison of cumulative amount of CLO released from hydrogels with PAMAM-NH$_2$ G3 containing dissolved or dispersed CLO as a function of square root of time.

CLO from hydrogels with CLO in two different molecular states and from water suspensions was studied, because the size and surface charge of dendrimers can influence the permeation of the drug. As shown in Figure 1, PAMAM-NH$_2$ dendrimers were more potent enhancers of CLO release than PAMAM-OH. After 4 h, the cumulative amount of CLO released from hydrogel with PAMAM-NH$_2$ G2 (H-4), hydrogel with PAMAM-OH G2 (H-10), hydrogel without PAMAM (H-1) and from the commercially available product was 39.11 ± 0.25, 24.34 ± 0.24, 10.16 ± 0.08 and 5.0 ± 0.05 µg/cm^2, respectively. Moreover, enhancing effect of PAMAM dendrimers on the release of CLO from hydrogels containing dissolved drug was generation and concentration dependent (Figure 1). For example, PAMAM-OH G3 induced 3-fold and PAMAM-from hydrogel with dispersed form of drug) (Figure 2). As it is shown in Figures 1 and 2, the straight plots with correlation coefficient ($R^2 \geq 0.998$) indicate that the data fit Higuchi's equation and the mechanism of release process is simply diffusion of the drug through membrane. Diffusion is a common mechanism controlling release and according to Higuchi's theory, the increase in thermodynamic activity of drug improves diffusion of the drug from topical formulation (Kobayashi et al., 1999).

To examine if dendrimers also affect the diffusion of CLO in dispersed form, the *in vitr o* release from water

OH G2 2- fold increase in diffusion of CLO in comparison with hydrogel without PAMAM. The increase in generation number affects growth in size and number of primary and tertiary amines groups available for interaction with the drug and can be responsible for its dissolution enhancement in a vehicle, which in consequence can improve the *in vitro* release of the drug (Devarakonda et al., 2004). Additionally, to examine if diffusion of CLO from hydrogels depends on its molecular state, CLO was dissolved or dispersed in hydrogels with the most potent PAMAM-NH$_2$ G3 dendrimers. The increase in release rate of CLO was higher from hydrogels with dissolved CLO (PAMAM-NH$_2$ G3 dendrimers 3.4 to 4.4-fold improved the *in vitro* release of CLO from hydrogel with dissolved and 1.19 to 2-fold suspensions of CLO with PAMAM, without additional excipients was studied. The results revealed that the enhancement in amount of CLO release evoked by PAMAM was size and concentration dependent (Figure 3). However, compared with hydrogels containing a dissolved form of the drug, the improvement in CLO release from water suspensions was definitely lower (PAMAM-NH$_2$ G3 caused 3.4 to 4.4-fold and 1.5 to 1.8-fold increase in diffusion of CLO from hydrogels with dissolved CLO and from suspensions, respectively).

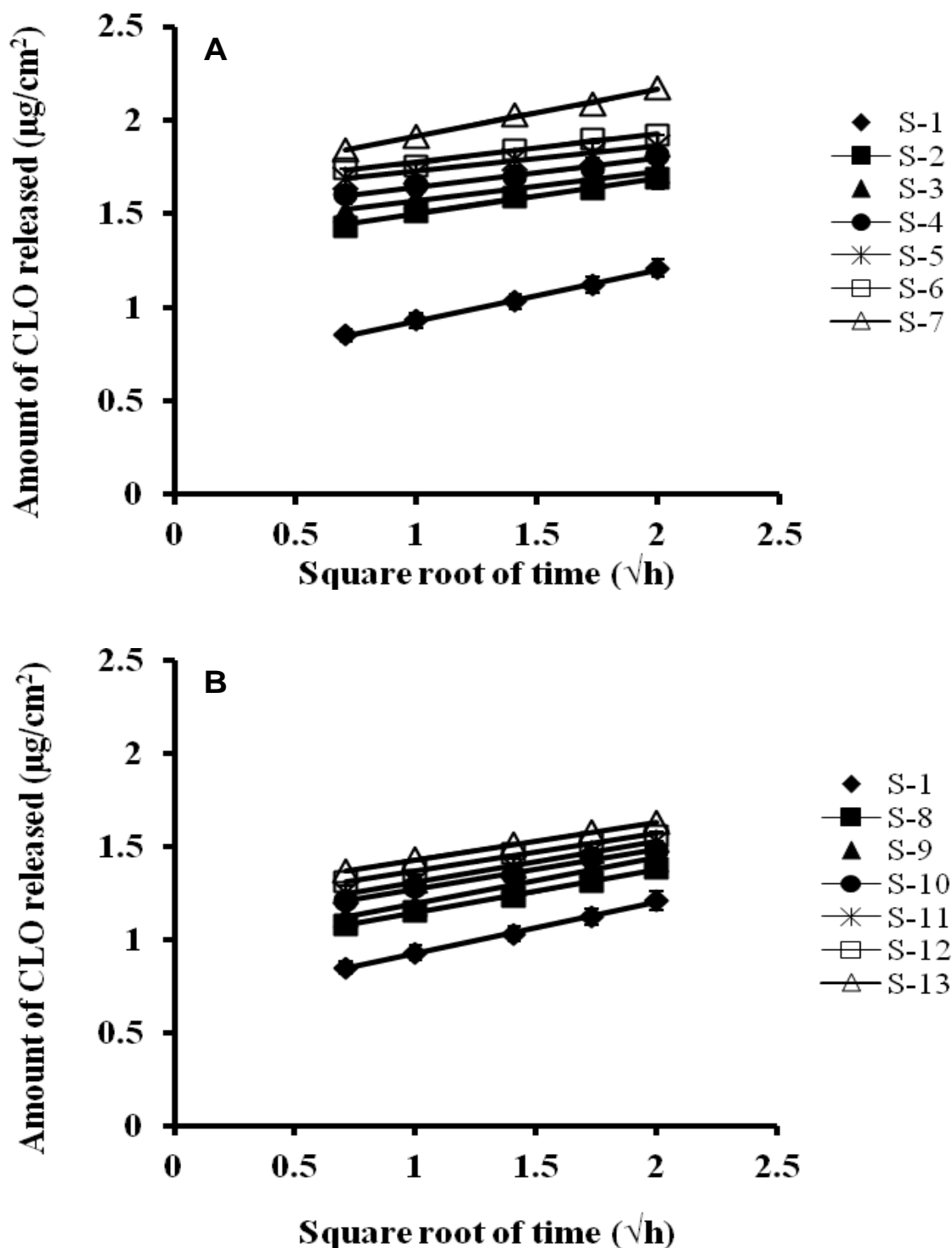The most potent enhancers of the *in vitro* release of

Figure 3. Cumulative amount of CLO released from water suspensions with PAMAM-NH$_2$ (A) or PAMAM-OH (B) as a function of square root of time.

CLO from hydrogels containing drug in two molecular states and suspensions were PAMAM-NH$_2$ G3. PAMAM dendrimers as co-solvent can generate saturated solution of the drug in the vehicle, maximize the thermodynamic activity of the drug, and as a result, improve the *in vitro* release of CLO from prepared formulations.

Conclusion

This study shows that PAMAM dendrimers with -NH$_2$ and -OH surface groups increased the release of CLO from both hydrogels, irrespective of its molecular state and suspensions.

REFERENCES

Balata G, Mahdi M, Bakera RA (2011). Improvement of solubility and dissolution properties of clotrimazole by solid dispersions and inclusion complexes. Indian J. Pharm. Sci. 73:517-526.

Bilensoy E, Abdur Rouf M, Vural I, Sen M, Atila Hincal A (2006). Mucoadhesive, thermosensitive, prolonged-release vaginal gel for clotrimazole: β-cyclodextrin complex. AAPS PharmaSciTech. 7(2):E54-E60.

Borowska K, Wołowiec S, Rubaj A, Głowniak K, Sieniawska E, Radej S (2012). Effect of polyamidoamine dendrimer G3 an12d G4 on skin permeation of 8-methoxypsoralene – In vivo study. Int. J. Pharm. 426:280-283.

Cheng Y, Man N, Xu T, Fu R, Wang X, Wang X, Wen L (2007a). Transdermal delivery of nonsteroidal anti-inflammatory drugs mediated by polyamidoamine (PAMAM) dendrimers. J. Pharm. Sci. 96:595-602.

Cheng Y, Qu H, Ma M, Xu Z, Xu P, Fang Y, Xu T (2007b). Polyamidoamine (PAMAM) dendrimers as biocompatible carriers of quinolone antimicrobials: an in vitro study. Eur. J. Med. Chem. 42:1032-1038.

Devarakonda B, Hill RA, de Villiers MM (2004). The effect of PAMAM dendrimer generation size and surface functional group on the aqueous solubility of nifedipine. Int. J. Pharm. 284:133-140.

Filipowicz A, Wołowiec S (2011). Solubility and in vitro transdermal diffusion of riboflavin assisted by PAMAM dendrimers. Int. J. Pharm. 408:152-156.

Hájková R, Sklenářová H, Matysová L, Šrecová P, Solich P (2007). Development and validation of HPLC method for determination of clotrimazole and its two degradation products in spray formulation. Talanta 73:483-489.

Heiden TCK, Dengler E, Kao WJ, Heideman W, Peterson RE (2007). Developmental toxicity of low generation PAMAM dendrimers in zebrafish. Toxicol. Appl. Pharm. 225:70-79.

Henry KW, Nickels JT, Edlind TD (2000). Upregulation of ERG genes in Candida species by azoles and other sterol biosynthesis inhibitors. Antimicrob. Agents Chemother. 44:2693-2700.

Holt RJ (1974). Laboratory and clinical studies with clotrimazole. Postgrad. Med. J. 50(Suppl. 1):24-27.

Hoogerheide JG, Wyka BE (1982). Clotrimazole. In: Florey K (ed.), Analytical Profiles of Drug Substances, Vol. 11. Academic Press, Salt Lake City. pp. 225-255.

Kobayashi N, Saitoh I (1999). Factors influencing the in vitro drug release from a liquid droplet dispersion system ointment. Chem. Pharm. Bull. 47:647-651.

Malik N, Wiwattanapatapee R, Klopsch R, Lorenz K, Frey H, Weener JW, Meijer EW, Paulus W, Duncan R (2000). Dendrimers: relationship between structure and biocompatibility in vitro and preliminary studies on the biodistribution of 125I-labelled polyamidoamine dendrimers in vivo. J. Control. Release 65:133-148.

Michaels AS, Chandrasekaran SK, Shaw JE (1975). Drug permeation through human skin: Theory and in vitro experimental measurement. J. AIChE 21:985-996.

Nanjwade BK, Bechra HM, Derkar GK, Manvi FV, Nanjwade VK (2009). Dendrimers: Emerging polymers for drug-delivery systems. Eur. J. Pharm. Sci. 38:185-196.

Ning M, Gu Z, Pan H, Yu H, Xiao K (2005). Preparation and in vitro evaluation of liposomal/niosomal delivery systems for antifungal drug clotrimazole. Indian J. Exp. Biol. 43:150-157.

Pathan IB, Setty CM (2009). Chemical penetration enhancers for transdermal delivery systems. Trop. J. Pharm. Res. 8:173-179.

Pedersen M, Bjerregaard M, Jacobson J, Sorensen AM (1998). A genuine clotrimazole γ-cyclodextrin inclusion complex-isolation, antimycotic activity, toxicity and an unusual dissolution rate. Int. J. Pharm. 176:121-131.

Strydom SJ, Rose WE, Otto DP, Liebenberg W, de Villiers MM (2013). Poly(amidoamine) dendrimer-mediated synthesis and stabilization of silver sulfonamide nanoparticles with increased antibacterial activity. Nanomedicine 9:85-93.

Thakker KD, Chern WH (2003). Development and validation of in vitro release tests for semisolid dosage forms–Case study. Dissolut. Technol. 10:10-15.

Winnicka K, Bielawski K, Rusak M, Bielawska A (2009). The effect on generation 2 and 3 poly(amidoamide) dendrimer on viability of human breast cancer cells. J. Health Sci. 55:169-177.

Winnicka K, Sosnowska K, Wieczorek P, Sacha P, Tryniszewska E (2011). Poly(amidoamine) dendrimers increase antifungal activity of clotrimazole. Biol. Pharm. Bull. 34:1129-1133.

Yang Y, Sunoqrot S, Stowell Ch, Ji J, Lee CW, Kim JW, Khan SA, Hong S (2012). Effect of size, surface charge, and hydrophobicity of poly(amidoamine) dendrimers on the skin penetration. Biomacromolecules 13:2154-2162.

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