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# DD&T

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Topic:
Glycoconjugates

Guest Editor:
Fengshan Wang

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# DD&T

# Drug Discoveries & Therapeutics

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**Editorial**

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# Glycoconjugates, promising subjects for medical science

**Munehiro Nakata***

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A long period of studies on carbohydrates such as glycans and carbohydrate moieties of glycoconjugates has brought us a lot of understandings on various biological phenomena in the molecular level. Many of biological phenomena, such as cell-cell recognition, clearance of glycoproteins in blood, infection of pathogens, and initiation of innate immunity, are now recognized as those triggered *via* carbohydrate recognition. These studies have developed not only understandings on biological rolls of carbohydrate moieties of glycoconjugates but also established glycotechnology including glycan analyses, modification of oligosaccharide structure in glycoconjugates, and creation of novel glycosylated substances. Nowadays, glycotechnology and knowledge on carbohydrate-related biological phenomena are applied in a wide range of fields, especially medical science.

This issue carries a series of articles including reviews on immunomodulation by polysaccharides and advanced technologies for structural analysis of glycans as well as some original studies on carbohydrate-related biological functions aiming medical use. These biochemical and/or physiological understandings on carbohydrates or glycoconjugates are expected to progress new approaches to overcome diseases. Finally, I express my gratitude to Dr. Fengshan Wang, Shandong University, Ji'nan, China, for his active management of these articles.

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Review

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# Carbohydrate drugs: current status and development prospect

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**Summary** **In recent years, there has been a great effort devoted to the investigation of the roles of carbohydrates in various essential biological processes and the development of carbohydrates to therapeutic drugs. This review summarizes the carbohydrate drugs which have been recorded in several pharmacopoeias, marketed, and under development. A prospect of the future development of carbohydrate drugs is discussed as well.**

***Keywords:*** Carbohydrate drugs, pharmacopoeia, carbohydrate modification, carbohydrate vaccine, oligosaccharide synthesis

## 1. Introduction

Carbohydrates are the essential constituents of organisms and play crucial roles in many important physiological and pathogenic processes, such as cell surface recognition, signal transduction, tumor metastasis, *etc.* However, carbohydrates, the third major class of biopolymers, have drawn much less attention from a drug development perspective compared to nucleic acids and proteins due to our very recent understanding of the fundamental glycobiology (*1,2*). Nevertheless, carbohydrate drugs still pave the way to a non-ignorable area of the world of therapeutics owing to their natural significance and the vast effort devoted by glycoscientists in recent decades.

This review mainly focuses on carbohydrate drugs, including polysaccharides, oligosaccharides, monosaccharides and their derivatives. The status of carbohydrate drugs included in several pharmacopoeias, marketed, or under development is summarized and the development prospect of carbohydrate drugs is discussed.

## 2. Carbohydrate drugs included in important pharmacopoeias

To compile the recorded carbohydrate drugs, four pharmacopoeias including United States Pharmacopoeia 37/National Formulary 32 (USP 37/NF 32), European Pharmacopoeia 7.0 (EP 7.0), Japanese Pharmacopoeia Sixteenth Edition (JP 16), Chinese Pharmacopoeia 2010 (CP 2010) and Chinese ministerial standards 1998 were meticulously searched and the acquired data was analyzed (*3-7*). A total of 131 carbohydrate drugs have been included in the four pharmacopoeias, demonstrating the fundamental role of carbohydrate pharmaceuticals both in crude drugs and pharmaceutical excipients (*8,9*). As indicated in Figure 1A, polysaccharides derived from natural animal, plant and microorganism sources still

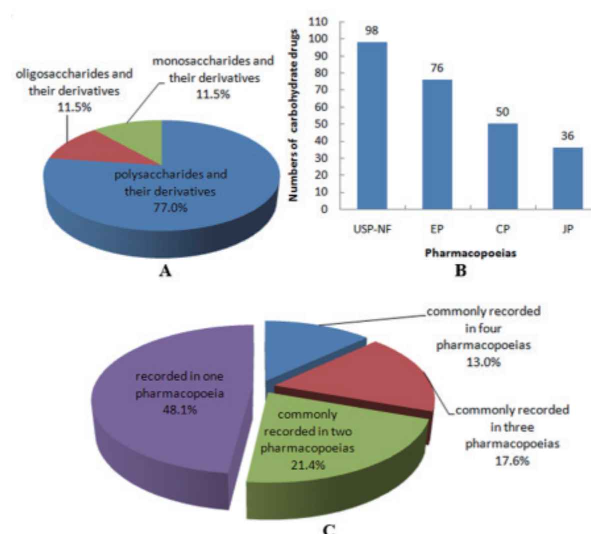


**Figure 1. Data analysis of carbohydrate drugs recorded in USP, EP, JP, and CP. (A)** Composition of carbohydrate drugs recorded in the pharmacopoeias. **(B)** Numbers of carbohydrate drugs recorded in each pharmacopoeia. **(C)** Carbohydrate drugs commonly recorded in the pharmacopoeias.

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**Table 1. Carbohydrate drugs commonly recorded in four or three of the pharmacopoeias**

| Drugs | Pharmacopoeias | Dosage forms | | | Structures |
|---|---|---|---|---|---|
| | | USP | CP | JP | |
| Acacia | USP EP CP JP | PE | PE | CD | P |
| Dextran 40 | USP EP CP JP | BD I | BD I | I | P |
| Dextran 70 | USP EP CP JP | BD I | BD I | I | P |
| Dextrin | USP EP CP JP | PE | PE | | P |
| Cellacefate | USP EP CP JP | PE | PE | | P |
| Corn Starch | USP EP CP JP | PE | PE | | P |
| Fructose | USP EP CP JP | PE BD I | PE | I | M |
| Heparin Sodium | USP EP CP JP | BD I S | BD I CR | | P |
| Hydroxypropyl Cellulose | USP EP CP JP | PE | PE | | P |
| Hypromellose | USP EP CP JP | PE BD OPS | PE | | P |
| Lactose Monohydrate | USP EP CP JP | PE | PE | | O |
| Lactulose | USP EP CP JP | BD S | OS | | O |
| Meglumine | USP EP CP JP | PE BD | BD | | M |
| Methylcellulose | USP EP CP JP | PE BD OPS OS T | PE | | P |
| Microcrystalline Cellulose | USP EP CP JP | PE | PE | | P |
| Sucralfate | USP EP CP JP | BD T | BD OSU DT CT C | | O |
| Sucrose | USP EP CP JP | PE | PE | | O |
| Alginic Acid | USP EP CP | PE | PE* | | P |
| Betadex | USP EP CP | PE | PE | | P |
| Carboxymethylcellulose Sodium | USP EP CP | PE BD T P | PE | | P |
| Cellulose Acetate | USP EP CP | PE | PE | | P |
| Croscarmellose Sodium | USP EP CP | PE | PE | | P |
| Ethylcellulose | USP EP CP | PE | PE | | P |
| Glucose Monohydrate | USP EP CP | PE BD I | BD I | | M |
| Heparin Calcium | USP EP CP | BD I | BD* I* | | P |
| Hydroxypropyl Betadex | USP EP CP | PE | PE | | P |
| Maltodextrin | USP EP CP | PE | PE | | P |
| Pregelatinized Starch | USP EP CP | PE | PE | | P |
| Sodium Alginate | USP EP CP | PE | PE | | P |
| Xanthan Gum | USP EP CP | PE | PE | | P |
| Agar | USPCP JP | PE | PE | CD | P |
| Simple Syrup | USPCP JP | PE | PE | | O |
| Anhydrous Glucose | EP CP JP | | BD I | I | M |
| Anhydrous Lactose | USP EP JP | PE | | | O |
| Hypromellose Phthalate | USP EP JP | PE | | | P |
| Potato Starch | USP EP JP | PE | | | P |
| Powderd Cellulose | USP EP JP | PE | | | P |
| Rice Starch | USP EP JP | PE | | | P |
| Tragacanth | USP EP JP | PE | | | P |
| Wheat Starch | USP EP JP | PE | | | P |

*, collected by Chinese ministerial standards 1998; USP, United States Pharmacopoeia; EP, European Pharmacopoeia; CP, Chinese Pharmacopoeia; JP, Japanese Pharmacopoeia; PE, pharmaceutical excipient; BD, bulk drug; T, tablet; C, capsule; DT, dispersible tablet; CT, chewable tablet; CR, cream; I, injection; S, solution; OS, oral solution; OSU, oral suspension; OPS, ophthalmic solution; CD, crude drug; M, monosaccharide or its derivative; O, oligosaccharide or its derivative; P, polysaccharide or its derivative. Dosage forms are not recorded in EP, and PE and BD are not distinguished in EP and JP.

represent the largest percentage of more than two thirds of all the recorded carbohydrate drugs (101, 77.0%), followed by oligosaccharides, monosaccharides and their derivatives, contributing an equal occupation of 11.5% (*15*).

The comparison of the numbers of carbohydrate drugs recorded by each pharmacopoeia reveals that USP-NF covers the largest number of 98, followed by 76 of EP, 50 of CP, and 36 of JP, respectively (Figure 1B). Among which, most carbohydrate drugs (63, 48.1%) are recorded in only one of the four pharmacopoeias and only 17 (13.0%) carbohydrate drugs are commonly recorded in all of the four pharmacopeias with 23 (17.6%) carbohydrate drugs commonly recorded in three of them as shown in Figure 1C and Table 1.

In the course of analyzing the differences among carbohydrate drugs recorded by different pharmacopoeias, we noted that instead of entirely different active ingredients, diverse modifications of carbohydrate drugs such as salinization, esterification, and derivatization contribute most to the diversity of each pharmacopoeia (*10,11*). For example, powdered cellulose is recorded in JP only while celluloseacetate is commonly recorded in all of the other three pharmacopoeias. Although hydroxypropyl cellulose is commonly recorded in all of the four pharmacopoeias, low-substituted hydroxypropyl cellulose is missed in EP compared with USP and JP. Even USP which records the most carbohydrate drugs contains glucose monohydrate only while anhydrous glucose is commonly collected by all the other three

**Table 2. Carbohydrate drugs marketed**

| Drug (Trade name) | Country | Indications |
|---|---|---|
| Astragalus polysaccharide | China | Cancer |
| Coriolus versicolor polysaccharide | Japan | Cancer |
| Ginseng polysaccharide | China | Cancer |
| Lentinan | Japan | Cancer, hepatitis B |
| Pachyman | China | Cancer |
| Tremella polysaccharide | China | Cancer, chronic bronchitis |
| Hydroxyethyl starch 130/0.4 (Voluven) | Germany | Blood volume expansion |
| Hydroxyethyl starch 200/0.5 (Haes) | Germany | Blood volume expansion |
| Fondaparinux sodium (Arixtra) | England | Thrombus |
| Swine duodenum mucopolysaccharide | China | Coronary atherosclerosis |
| Icodextrin (Extraneal) | Germany | Peritoneal dialysis |
| Hai Kun Shen Xi Capsule | China | Chronic renal failure |
| Pentosan polysulfate sodium (Elmiron) | America | Interstitial cystitis |
| Mucopolysaccharide polysulfate (Hirudoid) | Germany | Phlebitis |
| Zanamivir | England | Influenza |
| Miglitol | Germany | Diabetes |
| Testa triticum tricum purify (Fiberform) | Sweden | Constipation |
| Polysacharidum of G. lucidum Karst | China | Neurosis, polymyositis |

pharmacopoeias. In terms of molecular weight and the degree of polymerization, dextran 40 and dextran 70 are commonly recorded in all of the four pharmacopoeias while dextran 20 and dextranomer are recorded in CP and EP, respectively. Besides, broader sources and more sophisticated categories also make contribution to the diversity of the recorded carbohydrate drugs. Starches from corn, potato, rice, wheat, tapioca, and pea are all recorded in USP, while tapioca starch is not accepted by EP or JP, and only tapioca and corn starch are accepted by CP. Heparin sodium is also commonly recorded in all of the four pharmacopoeias, while in USP heparin sodium is obtained from the intestinal mucosa or other suitable tissues of domestic mammals used for food by human, and the EP heparin sodium is prepared either from the lungs of cattle or from the intestinal mucosa of pigs, cattle or sheep, and the CP heparin sodium comes from the intestinal mucosa of pigs or cattle, and the JP heparin is obtained from the livers, the lungs and the intestinal mucosa of healthy edible animals. For the different degrees of categories, sodium starch glycolate is categorized into type A, B and C in USP and EP according to their different ranges of pH and contents of sodium, while CP records just one type which has the same range of pH but different content of sodium from type A.

Additionally, in certain countries, some carbohydrate drugs commonly recorded in the foreign pharmacopoeias have already been used in clinic although have not been recorded in the native pharmacopoeia. On the other hand, certain commonly recorded carbohydrate drugs have not yet been used in clinic in other countries. Besides, some recorded pharmaceutical excipients with excellent properties have only been used as food additives in other countries.

Hence, in spite of the considerable number and diversity of carbohydrate drugs recorded in the pharmacopoeias, more comprehensive and normalized standards for carbohydrate drugs are urgently needed to accelerate their internationalized utilization. As far as we concerned, adoption and quotation of uncommonly recorded carbohydrate drugs especially for the diverse carbohydrate derivatives among all the pharmacopoeias are feasible ways to achieve this goal.

## 3. Carbohydrate drugs marketed

Except for the pharmacopoeia recorded carbohydrate drugs, numbers of carbohydrate drugs have already been marketed and used in clinic for years. We combed through the databases of Chinese Food and Drug Administration (CFDA) and United States Food and Drug Administration (FDA) for carbohydrate drugs marketed without included in the pharmacopoeias and eighteen of them were found (Table 2) (*12-14*). Their indications comprise a vast array of maladies ranging from cancer to constipation.

Thirty three percentage of the marketed carbohydrate drugs are exploited as clinical antitumor adjuvants benefited from their immunomodulatory effects and low cytotoxicity. To point out, approximately all of the adjuvants are first marketed in China as polysaccharides extracted from traditional Chinese medicinal plants or fungi except for *Coriolusversicolor* polysaccharide and lentinan, which are developed by Japan. Nevertheless, none of the above antitumor adjuvants have been approved by FDA so far.

Drugs for the treatment of blood and cardiovascular diseases account for 22% of the marketed carbohydrate drugs, which are in the second position as the indications concerned. Fondaparinux, approved by FDA in 2001, is a fully synthetic analog according to the pentasaccharide domain of heparin and widely used for the treatment of deep vein thrombosis in clinic. It is the first selective antithrombin III (AT III) mediating Xa inhibitor with AT III as its only binding target in plasma

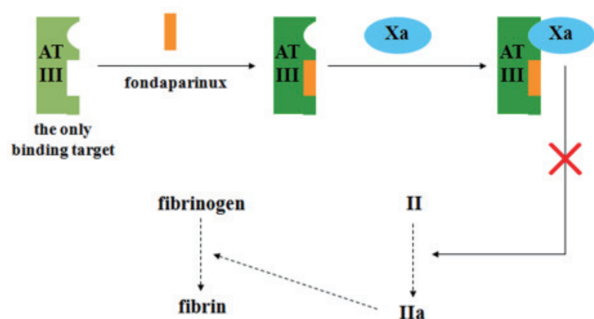


**Figure 2. The anticoagulant mechanism of fondaparinux.**

compared with unfractionated heparin (UFH) and other low molecular weight heparins (LMWHs) (Figure 2) (*15*). Consequently, both improved subcutaneous bioavailability and reduced risk of contamination were achieved (*16*). Voluven, registered by Fresenius Kabi (Bad Homburg, Germany), is a profitable novel hydroxyethyl starch plasma volume expander (*17*). Reduced coagulation impairment was gained by Voluven with a lower molecular weight (130 kDa) and a lower molar substitution (0.4) compared with Haes (average molecular weight 200 kDa, degree of substitution 0.5; Fresenius Kabi) (*18*). Besides, swine duodenum mucopolysaccharide extracted from the duodenum of healthy swines with a glucosamine content of more than 20.0%, is produced in China as an anti-coronary atherosclerosis pharmaceutical.

Urinary diseases are also a relatively hot field for currently marketed carbohydrate drugs, occupying a portion of 17%. Icodextrin, a cornstarch-derived glucan with a molecular weight of 16 kDa, is a biocompatible colloid osmotic agent for peritoneal dialysis for the treatment of chronic renal failure approved by FDA in 2002. Compared with the traditional glucose solution, more stable ultrafiltration rate and lower risk of peritoneal fibrosis were achieved owing to the difficulties in reabsorption and fewer glucose degradation products of icodextrin solution (*19-21*). Elmiron, developed by Ortho Mcneil Janssen, is the only oral drug approved by FDA for interstitical cystitis. Pentosan polysulfate sodium, the active ingredient of Elmiron, is a semi-synthetic heparin-like derivative (*22*). It is possibly considered that the glycosaminoglycan-like pentasaccharide of Elmiron can rebuild the highly hydrophilic and protective glycosaminoglycans (GAGs) layer on the bladder urothelium (Figure 3) (*23*). Hai Kun Shen Xi Capsule, with sulfated fucans as its active ingredient, is another Chinese medicine unapproved by FDA for the treatment of chronic renal failure together with hemodialysis.

Additionally, the physical or biochemical properties of carbohydrates and glycomimetic drugs are also exploited for the treatment of gastro-intestinal diseases, virus infections, and diabetes. Fiberform, registered by Recip AB, is the top choice for constipation recommended by

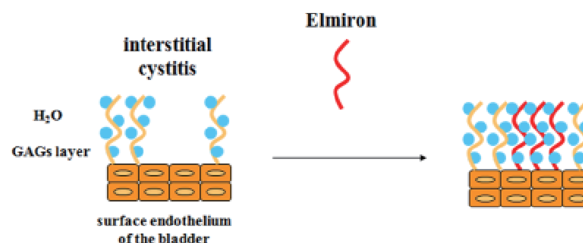


**Figure 3. The possible mechanism of Elmiron for the treatment of interstitial cystitis.**

the World Gastroenterology Organization. The natural wheat fiber in this pharmaceutical is able to soften the stool by absorbing large amount of water without diarrhea which is the common side effect of other laxatives. The sialic acid mimic, zanamivir (registered by GlaxoSmithKline), is widely used for clinical prevention and treatment of influenza by inhibiting the viral neuraminidase (*24,25*). Another monosaccharide mimic, miglitol, is designed mimicking the structure of glucose to inhibit the carbohydrate digestion activity of α-glucosidase (*26-28*). Miglitol has been used for the treatment of non-insulin-dependent-diabetes mellitus (NIDDM) since registered by Bayer in 1996.

Noticeably, most of the marketed carbohydrate drugs exhibit favorable pharmacological activities with low cytotoxicity in the treatment of various human diseases based on their physical and biochemical properties. However, the applications of most polysaccharide derived pharmaceuticals such as the polysaccharide antitumor adjuvants, Elmiron, and Hai Kun Shen Xi Capsule are still regional restricted due to their mixed compositions, ambiguous mechanisms, and the difficulties in quality control. Hence, more efforts are needed to put in the structure characterization, pharmacological mechanism investigation, and precise quality control of carbohydrate drugs for their more extensive utilization.

## 4. Carbohydrate drugs in development

Carbohydrate-based therapeutics are extremely potential and a vast number of carbohydrate drugs are in the stage of preclinical or clinical studies. Several carbohydrate drugs in Phase III clinical studies are listed in Table 3.

Cancer is also the hottest field of current carbohydrate drug research and development with several potential therapeutics in development. Synthetic tumor associated carbohydrate antigens are considered to be ideal candidates to elicit antitumor immune responses. Theratope, a conjugation of sialyl Tn oligosaccharide epitope and keyhole limpet hemocyanine (KLH), was developed to be a promising carbohydrate vaccine for metastatic colon cancer and breast cancer. However, the Phase III clinical trial of Theratope failed after ten years of hard work because of its low efficiency (*29-32*). On the other hand, PI-88 which is primarily composed of

**Table 3. Examples of carbohydrate drugs in development**

| Drugs | Indications | Institutes | Phase of development |
|---|---|---|---|
| Theratope (sialylTn Ag conjugate vaccine) | Metastatic colon cancer, breast cancer | Biomira, Memorial Sloane-Kettering | Phase III discontinued |
| PI-88 | Liver cancer | Progen, Medigen | Phase III |
| Vevesca (Miglustat) | Fabry's disease, Gaucher's disease, HIV | Oxford Glycoscience/Celltech | Phase III |
| Naturlose (D-tagatose) | NIDDM | Spherix | Phase III |
| Betafectin (beta-glucan) | Postoperative infectioin | Alpha-Beta Tech | Phase III |
| Therafectin (amiprilose) | Rheumatoid arthritis | Boston Life Science | Applying |

highly sulfated oligomannose generated from yeast turns to be the most potential novel antitumor pharmaceutical exhibiting excellent antiangiogenic and anti-metastatic activities (*33*). PI-88 was initially developed by Progen while the Phase III clinical trial of PI-88 in hepatocellular carcinoma patients has been conducted all around the world by Taiwan Medigen Biotechnology Corporation since 2011 (*34-36*). Medigen reported that the treatment of patients was nearly completed and desirable results were expected.

Moreover, monosaccharides and their mimics are intensively studied as inhibitors of enzymes associated with carbohydrate metabolism. Vevesca, an orally effective amino sugar, is able to inhibit the glucosyltransferase in the biosynthetic pathway of glycosphingolipid. It has already been approved by FDA for the treatment of mild or moderate type 1 Gaucher's disease in adult, while the anti-HIV trial of Vevesca unfortunately failed in preclinical stage (*37-39*). Besides, D-tagatose is also proposed to reduce the absorption of carbohydrates by inhibiting the disaccharidases in intestine and interfering with the transportation of glucose (*40*). It is a promising antidiabetic and obesity controlling pharmaceutical which has achieved statistical significance in a Phase III trial for NIDDM conducted by Spherix (*41,42*).

Additionally, betafectin, a glucan derived from yeast cell walls, is able to bind with the specific receptors on monocytes and neutrophils, promoting cell proliferation and increasing cell activities (*43*). Consequently, it is able to significantly prevent postoperative infection by enhancing the immunity of patients.

As described above, numbers of potential carbohydrate drugs in development display promising activities. Challengingly, carbohydrate-based therapeutics including carbohydrate vaccines towards cancers and complicated metabolic diseases are the main tasks of current carbohydrate research and development.

## 5. Development prospects of carbohydrate drugs

There is an increasing interest in the development of carbohydrate candidates for novel drug discovery since most of the currently used carbohydrate drugs display remarkable pharmacological activities and low toxicity. Different approaches can be followed for the discovery and design of carbohydrate drugs (*2,44*).

### 5.1. *Discovery of novel carbohydrate drugs*

Naturally occurring polysaccharides and their degradation fragments especially for those generated from traditional Chinese medical herbs and fungi possessing diverse structures and bioactivities are always the most potential library for carbohydrate drug discovery. Several successful carbohydrate pharmaceuticals derived from native glycan structures have already been used in clinic for years, such as LMWHs, alginic acid, hyaluronic acid *etc*. (*45-47*). Moreover, a host of natural polysaccharides and oligosaccharides are at the stage of basic research guiding the carbohydrate drug development. For example, polysaccharides from Chinese wolfberry, *Agaricusblazei* Murill and meretrix are all intensively studied on their anti-tumor, anti-oxidative, and immunomodulatory activities (*48-50*).

However, the development of most natural polysaccharides encounters difficulties in structure characterization, mechanism unraveling, and quality control, which hamper their universal approval. Besides, the construction of the huge glycan library is also rendered tough by the difficulties to obtain glycans either from natural extraction or from artificial synthesis.

### 5.2. *Modification of carbohydrates*

There is convincing evidence that appropriate molecular modification of natural glycans could generate novel bioactivities, enhance the original activities, or improve the solubility and pharmacokinetic properties. A lot of efficient methods have been developed for the modification of polysaccharides from various sources.

Sulfation is considered to be one of the most convenient and feasible methods of polysaccharide modification endowing glycans with anti-virus, anti-tumor, and anti-inflammation activities. A recent investigation demonstrated that curdlan sulfate could effectively inhibit the HBV infection of HepG2 and HepaRG cells and could be developed as a vaccine adjuvant for the treatment or prevention of HBV infection (*51,52*). SIP-SII, a sulfated *Sepiella maindroni* ink polysaccharide, demonstrated significant suppressing activity for the metastasis of melanoma by inhibiting tumor adhesion and angiogenesis compared to the unsulfated SIP (*53*). Besides, selenylation,

phosphorylation, acetylation, carboxymethylation, and alkylation are all efficient strategies for the modification of polysaccharides such as *Atractylodes macrocephala* polysaccharide, Chinese angelica polysaccharide, and achyranthes polysaccharide, which endue them varieties of bioactivities (*54,55*).

### 5.3. *Carbohydrates mediated drug modification and delivery*

Recently, the specific recognition reactions mediated by saccharide ligands and their specific receptors on the corresponding organs, tissues, and cells are extensively studied for pharmaceutical modification and targeting drug delivery (*2,56,57*). For example, lactosylated microspheres with high specificity for the asialoglycoprotein lectin expressed on mammalian hepatocytes have been created for liver-specific delivery of drugs and genes (*58,59*). Mannose modified liposomes selectively targeting their mannose receptors have also been developed as efficient alveolar macrophage selective drug carriers (*60,61*). Moreover, a novel lectin-directed enzyme-activated prodrug therapy (LEAPT) has been developed for regio-selective drug release by carbohydrate-lectin interaction mediated localization of a glycosidase enzyme to the target cells (*62*).

In addition, natural polysaccharides, as well as their derivatives, are also attractive pharmaceutical and biomedical materials for better solubility, stability and lower toxicity based on their biodegradability, biocompatibility, and non-immunogenic properties (*63,64*). It has been demonstrated that oligomannose modification of the asparagine residue of C34, a 34-mer peptide derived from the C-ectodomain of HIV-1 envelope glycoprotein, dramatically improved its solubility and stability as a promising candidate for anti-HIV agents (*65,66*). Based on the hydrophilic outside surface and the hydrophobic internal cavity, cyclodextrins have been successfully exploited to form inclusion complexes with various hydrophobic drugs to dramatically improve their water solubility (*67*). Besides, both chitosan and hyaluronic acidare intensively investigated on their unique biomedical properties as novel materials for drug delivery (*68*).

### 5.4. *Carbohydrate vaccines*

Both specific polysaccharides located on the surface of pathogenic microorganisms andtumor associated carbohydrate antigens can be specifically recognized by the host immune system and exploited as the basis for the design of carbohydrate vaccines (*69-71*). Conjugated carbohydrate vaccines have been proved to be effective in the prevention or treatment of a range of human diseases.

By far, several carbohydrate vaccines based on capsular polysaccharides have already been commercialized to preventthe corresponding bacterial or viral infections. For example, *Haemophilus influenzae* type B (HiB) conjugate vaccine, a conjugation of purified capsular polysaccharide from HiB and tetanus toxoid, has been recommended for the prevention of childhood meningitis and recorded in CP and EP. At present, many promising anti-tumor and anti-HIV vaccines are intensively studied with chemically synthesized clusters of oligosaccharide as antigen epitopes. Globo-H, a synthetic hexasaccharide from the cell surface of breast cancer, was covalently linked to KLH with QS-21 as an adjuvant and displayed favorable results in the phase I trial (*72,73*). Carbohydrate vaccines based on oligomannose clusters are also intensively studied for their anti-HIV activities (*74,75*).

### 5.5. *Oligosaccharide synthesis*

Chemical or enzymatic synthesis is considered to be the most potential strategy to acquire sufficient amount of homogeneous and structurally well-defined oligosaccharides for carbohydrate drug development due to the limitation of the natural sources. However, unlike polymerase chain reaction (PCR) or solid phase peptide synthesis (SPPS), the synthesis of oligosaccharides is rendered extremely challenging by the structural complexity of carbohydrates without any universal automatic strategy (*76,77*).

Currently, a great effort is invested in establishing efficient oligosaccharide synthetic approaches and a lot of oligosaccharides have been successfully synthesized such as $Le^x$, $Le^y$, and Globo-H (*78-80*). However, most of the developed chemical strategies are time consuming and labor intensive with poor region- or stereo-selectivity and low yield in spite of their flexibility (*81*). On the other hand, enzymatic approaches with high efficiency and selectivity are usually limited by the sources of the enzymes desired. Consequently, chemoenzymatic synthesis which combines the flexibility of the chemical method and the efficiency and selectivity of the enzymatic method is considered to be one of the most efficient approaches for complex oligosaccharide preparation (*82,83*). A disialyl tetrasaccharide epitope has been regioselectively synthesized based on a chemical manipulation strategy mediated by a bacterial sialyltransferase Pd2,6ST (*84*). The chemoenzymatic strategy based on bacterialβ1-3 galactosetransferase and α2-3sialyltransferase has also been developed for the efficient synthesis of fluorinated T-antigens and their sialylated derivertives (*85*).

In spite of all the difficulties currently encountered, the successful synthesis of highly pure pentasaccharide, fondaparinux, in multi-kilogram scale has convincingly demonstrated the feasibility and potential of artificial synthesis for industrial production. It is expected that efficient synthetic protocols will be developed, which will open access to various oligosaccharide structures

for the development of carbohydrate drugs (*76,86*).

## 6. Conclusions

Despite the structural complexity of carbohydrates, a vast array of carbohydrate drugs have already been included in important pharmacopoeias, marketed, and in development for the treatment of a variety range of human diseases such as cancer, AIDS, diabetes *etc*. However, more efforts should be put on the study of comprehensive and normalized standards, carbohydrates with defined structures, precise mechanisms and practical quality control for the application and development of carbohydrate drugs.

Additionally, drug discovery and design based on natural occurring glycans, carbohydrate modification, carbohydrate mediated drug modification and delivery, carbohydrate vaccines, and oligosaccharide synthesis are all highly potential approaches for the future development of carbohydrate-based therapeutics. We anticipate that carbohydrate drugs will be one of the most attractive fields of novel drug research and development and play more important roles in the treatment of human diseases in the future.

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**Review**

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# Polysaccharides: Candidates of promising vaccine adjuvants

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**Summary** **Aluminium-based adjuvants remain the only adjuvants approved for human use in the USA for over 80 years because of alum's simplicity, tolerability, safety and cost-efficiency. Recent development of vaccines, especially the increasing applications of recombinant subunit and synthetic vaccines, makes aluminium adjuvants cannot stimulate enough immunity to the antigens, since aluminium adjuvants can only induce Th2 type immune responses. So, novel adjuvants are urgent to make up the disadvantages of aluminium adjuvants. However, some major hurdles need to be overcome, not only the scientific knowledge of adjuvants but also unacceptable side-effects and toxicity. A number of carbohydrate-based polysaccharides from plant, bacterial, yeast and synthetic sources can act as pathogen-associated molecular patterns (PAMPs) and recognize pattern recognition receptors (PRRs) on immune cells, followed by triggering innate immunity and regulating adaptive immunity. What is more, polysaccharides are safe and biodegradable without tissue deposits as observed in aluminium adjuvants. Therefore, polysaccharide-based compounds and formulations are potential vaccine adjuvant candidates. Here, we mainly review polysaccharide-based adjuvants investigated in recent years.**

***Keywords:*** Polysaccharide, vaccine, adjuvant, immunity

## 1. Introduction

Vaccination is the most effective strategy to prevent and control the spread of infectious diseases through generation of sufficient protective immune response. Vaccines based on inactivated pathogens, live attenuated pathogens, surface molecules such as proteins, carbohydrates and lipids, or recombinant antigens can induce neutralizing antibodies against the particular pathogen by intradermal, oral, or intranasal administration route. In many cases, the vaccine consisting of antigen alone can only stimulate too weak immunogenicity to prevent infection, therefore, an adjuvant is needed to strengthen the immune response (*1*). At present, aluminum-based adjuvant (aluminum hydroxide) is the only one approved for human use by FDA. Although it has been used for more than 80 years, it only contributes to the induction of a good Th2 immune response but has little capacity to induce cellular (Th1) response, which limits its applications (*2*). Besides, high aluminum levels that may be accumulated in persons with reduced renal function, may affect the brain and bone tissues resulting in neurological syndrome and dialysis-associated dementia (*3*). MF59 is another adjuvant approved for human use in Europe, which is added to influenza vaccines to stimulate the immune response (*4*). MF59 can induce the body to generate both Th1 and Th2 type immune response (*5*), however, as an oil emulsion adjuvant, it also has the problem of local injection site reactogenicity. To enhance the immunogenicity and safety of vaccines, novel adjuvants and formulations are needed to improve the cell mediated immune response to antigens.

So far, the licensed vaccine adjuvants and formulations are rare and precious, which include aluminum with no special indication, MF59 used in influenza (Fluad), liposomes in HAV/influenza, AS03 in pandemic flu (Pandemrix), AS04 in HBV (Fendrix)

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and HPV (Cervarix) (*2,6*). These so-called generation 1 adjuvants share common mechanism of action based on their particulate structure and dimensions: as a depot of antigen to increase uptake and stability of the antigen at the site of injection and as a carrier to deliver antigens to the appropriate immune cells. These actions are effective in some cases, but in poorly immunogenic antigens, are not able to induce sufficient protective immune response, therefore, an immune potentiator may be needed to assist the antigens (*6*). A number of carbohydrate polymers from plants, microbes, and synthetic sources have been studied to possess immunomodulating activities, as well as the performance of high biocompatibility and low toxicity (*7*). These characteristics of carbohydrates, especially polysaccharides, have drawn the attention of many experts to explore the possibility to develop polysaccharides as successful vaccine adjuvants (*7,8*).

**2. Polysaccharide-based adjuvants**

2.1. *Inulin-based adjuvants*

Inulin is found in the roots of Compositae, consisting of a linear β-D-(2→1)polyfructofuranosyl α-D-glucoses, with up to 100 fructose moieties linked to a single terminal glucose (*9*). Inulin contains four forms distinguished by solubility: alpha, beta, gamma and delta, in which gamma and delta forms have been demonstrated to exhibit adjuvanticity with enhancement of both cellular and humoral immunity, whereas no toxicity. Petrovsky group (Flinders) has done a lot of work to discover and verify adjuvant activities of inulin against different infectious pathogens (10-16). Cooper *et al*. (10) found that delta inulin showed stronger immune enhancement effect when combined with recombinant hepatitis B surface antigen (HBsAg) than gamma inulin. Advax is the latest generation of adjuvants made from delta inulin developed through the National Institutes of Health's Adjuvant Development Program (*11*). Advax has been demonstrated to enhance immunogenicity as an adjuvant in many vaccines in animal models and human clinical trials. Based on preclinical studies (*10,12*), Gordon *et al*. (*13*) designed a Phase I trial to evaluate immunogenicity and safety of Advax when formulated with HBsAg. The results revealed that Advax was safe and well tolerated in healthy adult subjects, and also enhanced HBsAb titers and $CD4^+$ T-cell proliferation compared to HBsAg containing aluminium adjuvant. Lobigs *et al*. (*14*) reported the Advax-adjuvant Vero cell culture vaccine could elicit strong neutralizing immunity against challenge of live Japanese encephalitis virus (JEV) both in mice and horse models. The Advax-adjuvanted vaccine stimulated a balanced Th1/Th2 immune response and produced neutralizing antibody not only against JEV, but also Murray Valley encephalitis virus (MVEV), another virus belonging to JEV serocomplex. These results indicated that Advax as an adjuvant was able to improve the efficiency and application range of JEV vaccine compared to aluminium adjuvants.

Advax has also been shown to be effective in influenza vaccines when challenged by the particular influenza virus. Vaccination of ferrets with split-virion H5N1 vaccine combined with Advax improved protection effect against H5N1 virus significantly by enhanced immunogenicity, survival, as well as reduced morbidity, compared with vaccine alone (*15*). Similarly, Advax increased both humoral and cellular responses of mice immunized intramuscularly with trivalent human influenza vaccine (TIV), and provided significant antigen-sparing effect, in contrast to influenza antigen alone (*11*). A randomized clinical trial confirmed that Advax adjuvant used in H1N1/2009 vaccine could enhance recombinant hemagglutinin immunogenicity, and this vaccine system may be effective in acute influenza pandemic (*16*).

The possible mechanism of Advax working as an adjuvant in aforementioned vaccines is that Advax can bind to antigen-presenting cells (APCs), such as mononuclear cells and dendritic cells (DCs), resulting in antigen presentation upregulation and antigen-specific T- and B-cell activation (*11*). Advax administered with vaccines induces the generation of both Th1 (IgG2a) and Th2 (IgG1, IgA) antibody responses, and the production of Th1 (IL-2, IFN-γ) and Th2 (IL-5, IL-6) cytokines, which makes up the limitation of aluminum adjuvant. Advax itself does not stimulate innate immune response, therefore, there are no obvious inflammation reactions in tested subjects. The properties of safety and tolerability of Advax are superior to emulsion formulation adjuvants.

2.2. *Chitosan-based adjuvant*

Chitosan is a linear β-(1→4)-linked copolymer of D-glucosamine and *N*-acetyl-D-glucosamine (GlcNAc), prepared by alkaline partial deacetylation of chitin (*17*). Chitosan possesses good biological characteristics *in vivo*, such as favorable biocompatibility and biodegradability, no toxicity and no reactogenicity. Studies have found that chitosan is capable of inducing immunity, and chitosan-based powders and micro/nanoparticles used in parenteral and mucosal delivery of antigen vaccines can enhance antigen-presenting functions, which exhibits its vaccine immunoadjuvant property (*18*). An *in vitro* assay indicated that BSA- or OVA-encapsulated chitosan particles could stimulate RAW264.7 macrophages and BMDCs activation compared to soluble antigens. The expressions of surface activation markers MHCI, MHCII and co-stimulatory cytokines CD40, CD80, CD86, and CD54, were up-regulated significantly than those of soluble antigens in the two APCs. Meanwhile, chitosan

particles also enhanced the release of proinflammation cytokines, IL-1β, IL-6, TNF-α, MCP-1 and MIP-1α, as well as the proliferation of $CD4^+$ and $CD8^+$ T cells (*19*). Dry-powder chitosan nanospheres encapsulated with influenza virus, CpG oligodexynucleotide (CpG ODN) and *Quillaja saponins* (QS) induced humoral and cellular immune responses after nasal administration to rabbits (*20*). It is thought that dry powder particulate system provides long lasting protective immunity, and offers physical and chemical stability for antigens (*21,22*). Chitosan nanospheres delivery system elevated both local sIgA and systemic IgG titers against the virus, suggesting this nanoparticulate chitosan may be an efficient adjuvant delivery system for immunization against influenza virus (*20*). Similar to chitosan, its trimethylated derivative (TMC) nanoparticles loaded with HBsAg given to mice intranasally and intramuscularly induced higher nasal and serum antibody titers than HBsAg solution. Both chitosan and TMC nanoparticles induced higher level of IgG1 and IgG2a after intramuscular injection of mice than those of aluminum absorbed HBsAg (*23*).

Neimert-Andersson and his colleagues (*24,25*) have prepared a hydrogel named ViscoGel from one kind of soluble chitosan and investigated its immune adjuvant activity. ViscoGel together with vaccine against Haemophilus influenza type b (Act-HIB) could induce stronger humoral and cellular response to antigen than those induced by vaccine alone. The titers of IgG1 and IgG2a in serum were significantly enhanced, and the productions of Th1-, Th2-, and Th17-type cytokines tested were also elevated. ViscoGel recruited neutrophils at the injected site and generated a pro-inflammatory environment which is important for early immune response (*24*). ViscoGel may trigger immune response in the similar way as chitin, since chitin particles were reported to function as PAMPs and recognize TLR-2 receptor on macrophages to induce innate immune response (*26*). Furthermore, the safety and efficacy of ViscoGel as an adjuvant with Act-HIB vaccine has been evaluated in the Phase I/IIa clinical trial (*25*). No serious adverse events were observed in all of the subjects, and a larger percentage of subjects appeared local, transient reactions at the injection site in groups received ViscoGel than those vaccinated with Act-HIB alone. These side effects were mild and resolved within short time, therefore, it was thought ViscoGel was safe and well tolerated in combination with Act-HIB. The efficacy study of ViscoGel showed that ViscoGel stimulated cell-mediated response through enhancement of interferon-γ (IFN-γ) response to Act-HIB in peripheral blood monouclear cells (PBMCs). However, it did not significantly elevate anti-HIB antibody level and this may be somewhat related to inter-individual variation. This clinical trial revealed that ViscoGel was safe as a vehicle for vaccine delivery, but the adjuvant effect was not so ideal in human and may need further study afterwards.

The immunomodulating activity of chitosan endows its application not only in the pathogen antigen vaccine adjuvant, but also anti-tumor adjuvant. Chitosan substrate was able to induce mouse bone marrow-derived monocytes differentiation into DCs, and vaccination of mice with tumor lysate-pulsed DCs cultured on chitosan system increased cytotoxic T lymphocyte (CTL) activity against inoculated tumor, which indicated chitosan may be useful for DC vaccines in the treatment of tumor (*27*). Highton *et al*. (*28*) applied vaccine consisted of hydrogel chitosan with OVA antigen and Quil-A adjuvant to against melanoma challenge in mice. Chitosan exhibited protection effect by stimulating stronger OVA-specific $CD8^+$ T cell memory response than those receiving DC vaccination instead of chitosan hydrogel. Cytotoxic $CD8^+$ T cells are critical for cancer therapy and prevention. Ma *et al*. (*29*) investigated the adjuvant effect of water soluble chitosan (WSC) on the immunity of radiotherapy patients suffered from lung cancer. The study found that oral administration of WSC to radiotherapy patients increased CD3, CD4, NK cells, and CD4/CD8 ratio, as well as IL-6, TNF-α levels remarkably compared with control group. These results indicated the possibility of chitosan and its formulation action as an immune adjuvant in anti-tumor therapy in future.

### 2.3. *Glucan-based adjuvant*

Glucans are polysaccharides from plants and microorganisms made up of repeating D-glucose units linked by glycosidic bonds, and can be divided into α- and β-glucans according to conformations (*7*). β-Glucans are one of the most abundant polysaccharides in bacteria, fungi, and plants, such as zymosan, lentinan, and algal glucan. They have been reported to possess immunological, anti-tumor, anti-infection activities, and also enhance the immune response of vaccines. β-Glucans can be recognized as PAMPs by the innate immune system through binding of immune cell receptors on neutrophils, macrophages, and DCs, such as toll-like receptors (TLRs), dectin-1, CR3 and CD5. The interactions between β-glucans and receptors trigger intracellular signalings activation followed by expression of immune-related molecules and regulate innate and adaptive immune responses (*30*).

The adjuvant and immunomodulatory effect of β-glucan in fish has been studied when β-glucan was injected intramuscularly with recombinant glyceraldehyde-3- phosphate dehydrogenase (rGAPDH) vaccine (*31*). The results showed that fish immunized with rGAPDH combined with β-glucan produced high level antibody, and up-regulated transcription levels of immunomodulatory molecules involved in innate and adaptive immune responses significantly, compared to rGAPDH immunization group. When challenged with *E. tarda* after immunization, fish in the group of rGAPDH containing β-glucan showed the

highest relative percentage survival compared to the other groups. These results indicated the adjuvant and protective effects of β-glucan when vaccinated fish with rGAPDH. Sulfated glucan from *Saccharomyces cerevisiae* could induce chicken splenic lymphocyte proliferation *in vitro*, and when injected to chickens as the adjuvant with Newcastle disease (ND) vaccine, sulfated glucan enhanced serum antibody titers, as well as improved serum IL-2 and IFN-γ concentrations (*32*). Another kind of β-glucan, curdlan, the extracellular polysaccharide from the bacteria of *Alcaligenes faecalis* var. *myxogenes* 103K with the structure of β-(1→3)-D-glucan, has been demonstrated to have good immunomodulating activity by recognization of dectin-1 receptor on immune cell surface (*33*). Our study found that its sulfation product, curdlan sulfate, could stimulate splenic lymphocyte proliferation, RAW264.7 cell activation, DCs maturation, and increase TNF-α, IL-6, and IL-1β cytokines secretion (*34*). Compared to aluminum adjuvant, curdlan sulfate enhanced recombinant HBsAg vaccine immunogenicity, and increased both cellular and humoral responses in mice. A higher IgG2a/IgG1 ratio within anti-HBs antibodies was produced in mice received HBsAg plus curdlan sulfate than those in mice immunized with HBsAg and an aluminum adjuvant, which indicated that curdlan sulfate induced a shift toward a Th1-biased immune response (*35*). β-Glucan particles (GP) from *Sacharomyces cerevisiae* has been shown to regulate innate immunity and can be phagocytized by DCs *via* dectin-1 receptor. The hollow structure of GP allows encapsulation of antigen, and OVA can be electrostatically complexed inside the hollow GP shells formed GP-OVA antigen. Mice immunized with GP-OVA produced substantially higher antigen-specific $CD4^+$ T cell proliferation than those with alum/OVA. Moreover, GP-OVA induced strong secretions of IgG1 and IgG2c anti-OVA antibodies, and Th1 and Th17 biased $CD4^+$ T cell immune responses (*36*). Further study found that oral administration of GP-OVA by mice stimulated IL-17 expression significantly in the spleen, and increased OVA-specific IgA, secretory-IgA and secretory component production in intestinal fluids, suggesting GPs were potent delivery vehicles to deliver OVA *via* an oral route and resulted in Th17-biased cellular and humoral responses (*37*).

In addition to β-glucans, some α-glucans also exhibit immune adjuvant functions. Lu *et al*. (*38*) found that a dendrimer-like α-D-glucan nanoparticle made from chemical modification of phytoglycogen, Nano-11, could adsorb negatively charged protein antigens by electrostatic interaction. The Nano-11 as the antigen delivery vehicle could enhance antigen-uptake by DCs and stimulate activation of DCs *in vitro*. Intramuscular injection of Nano-11-antigen formulations increased the immune responses to antigens obviously, since Nano-11 not only enhanced the antibody titers to antigen, but also recruited large number of monocytes at the injection sites. Compared to aluminum adjuvant, Nano-11 induced fewer neutrophils at the injection sites, which indicated Nano-11 as a vaccine delivery vehicle was safe to be an effective immune adjuvant. Acetalated-dextran (Ac-DEX) microparticles were used to encapsulate the TLR7 ligand, imiquimod, to stimulate immune cells. Compared to the free form, encapsulated imiquimod significantly increased IL-1β, IL-6, TNF-α, inducible nitric oxide synthase (iNOS) and PD1-L1 expression and nitric oxide (NO) level of macrophages, as well as IL-1β, IL-6, IL-12p70, and MIP-1α of BMDCs. These results suggested that Ac-DEX microparticles encapsulation could increase the potency of TLR ligands, and may be an effective delivery vehicle in vaccine formulations for future *in vivo* study (*39*).

### 2.4. *Other polysaccharide adjuvants*

The actions of polysaccharides based on mannose possessing adjuvanticity may be dependent on the binding of polysaccharides by mannose-binding lectin (MBL) and other C-type lectins of the mannose receptor family on macrophages and DCs, since the binding interactions can induce complement activation, opsonization and phagocytosis, which play important roles in innate immunity (*40*). Vaccination with mannan or mannan-BSA conjugate could protect mice against systemic aspergillosis (*41*). In mice, oxidized mannan-MUC1 stimulated Th1 type responses mediated by $CD8^+$ T cells with IFN-γ secretion and mainly IgG2a antibody response, whereas reduced mannan-MUC1 stimulated Th2 type responses with IL-4 production and a high IgG1 antibody response (*42*). A phase III clinical trial revealed that oxidized mannan-MUC1 as the immunotherapy adjuvant in the treatment for breast cancer patient could decrease the cancer recurrence rate and prolong recurrence time compared to the placebo group during the 12-15-year follow-up, without any evidence toxic or autoimmune adverse effects (*43*). Moreover, oligomannose-coated liposomes were able to deliver antigens to peripheral phagocytic cells and induce antigen-specific Th1 immune responses and CTLs (*44*). Besides mannan, fructooligosaccharide (FOS) was also reported to recognize MBL receptor. The chickens with high serum MBL concentrations (L10H) immunized with infectious bronchitis vaccine (IBV) plus FOS enhanced higher IBV-specific IgG antibody than that of IBV alone, indicating adjuvant effect of FOS in IBV vaccine (*45*). Lipopolysaccharide (LPS) derivative, monophosphoryl lipid A (MPL), has been demonstrated to be a non-toxic and immunoactive adjuvant *via* binding to TLR4 complex (*46*). AS04 adjuvant, consisting of MPL and aluminum phosphate, produced by GlaxoSmithKline (GSK), has been licensed in the vaccines of HBV and HPV as an adjuvant (*6*). The adjuvant system composed

of MPL and QS-21 applied in HBV vaccine has also been studied in clinical trials in recent years (*47*).

### 3. Conclusions

The potent immune adjuvants derived from polysaccharides have been the hot topic in the pathway to a successful vaccine adjuvant, due to the advantages of non-toxic, biodegradation, good biocompatibility, strong immune enhancement and low reactogenicity. Polysaccharide-based compounds and formulations are promising candidates for vaccine adjuvants in prevention and treatment of infectious pathogen diseases or cancers. Although a large number of studies on novel adjuvants have been carried out, further efforts are needed to select one adjuvant to challenge the monopoly of aluminum adjuvants in human vaccine adjuvant usage.

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**Review**

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# Quality control of sweet medicines based on gas chromatography -mass spectrometry

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**Summary** **Sweet medicines are a relatively untapped source of new drugs. Their biological activities are closely correlated to their chemical characteristics. However, accurately defining the chemical characteristics of glycans is a challenge due to their chemical heterogeneity and diversity. Gas chromatography-mass spectrometry (GC-MS) is an excellent technique for the analysis of glycans even though the preparation of adequate derivatives is necessary. We reviewed and discussed the most important methodologies currently used for glycan analysis in sweet medicines based on GC-MS, including the derivatization for monosaccharide analysis, hydrolysis methods for polysaccharide analysis, glycosidic linkage analysis based on methylation, and pyrolysis gas chromatography in carbohydrate analysis. Finally a strategy for quality control of sweet medicines based on quantification analysis is proposed.**

***Keywords:*** Carbohydrate, gas chromatography, derivatization, methylation, hydrolysis, pyrolysis gas chromatography

## 1. Introduction

Sugars occur in a variety of forms and locations in nature. Besides their roles in metabolism and as structural building blocks, sugars are fundamental constituents of every cell surface, which play critical roles in many cellular functions and disease. Sugar-based drugs are a relatively untapped source of new drugs and therefore offer an exciting new generation of drug therapies (*1,2*). Depending on their degree of polymerization (DP), simple sugars are often referred to as monosaccharides such as glucose and disaccharides (DP 1-2). Oligosaccharide typically refers to a bit longer chains (DP 3-9), whereas much larger molecules are defined as polysaccharides (DP > 9). Those attached with proteins or lipids are known as glycoconjugates or, more specifically, glycoproteins and glycolipids (*3,4*). Although studies of those activities lag behind research into genes and proteins, several carbohydrate-based molecules are known for their wide range of pharmacological activities and have been clinically used to treat different ailments (*5,6*).

Naturally occurring sugars are abundant, and can be derived from plants, fungi, bacteria, algae and animals (*1,7*). Low-molecular-weight heparin, derived from animal tissue, is the prominent example that successfully developed as clinical medicine for anticoagulants (*8*). Carbohydrates have also established themselves as the most clinically relevant antigens of those tested and subsequently developed for vaccines against infectious diseases, which initially isolated from bacteria (*9*). So far carbohydrate vaccines are widely derived from bacteria, protozoa, helminths, viruses, fungi and especially from cancer cells for immunotherapy on cancer (*10*). Lentinan, isolated from the fruit body of *Lentinula edodes*, is one of the host-mediated anti-cancer drugs and has been shown to affect host defense immune systems (*11*). Structure-activity relationship studies showed that (1→3)-*β*-D-glucan with (1→6)-glucosyl side groups and triple-

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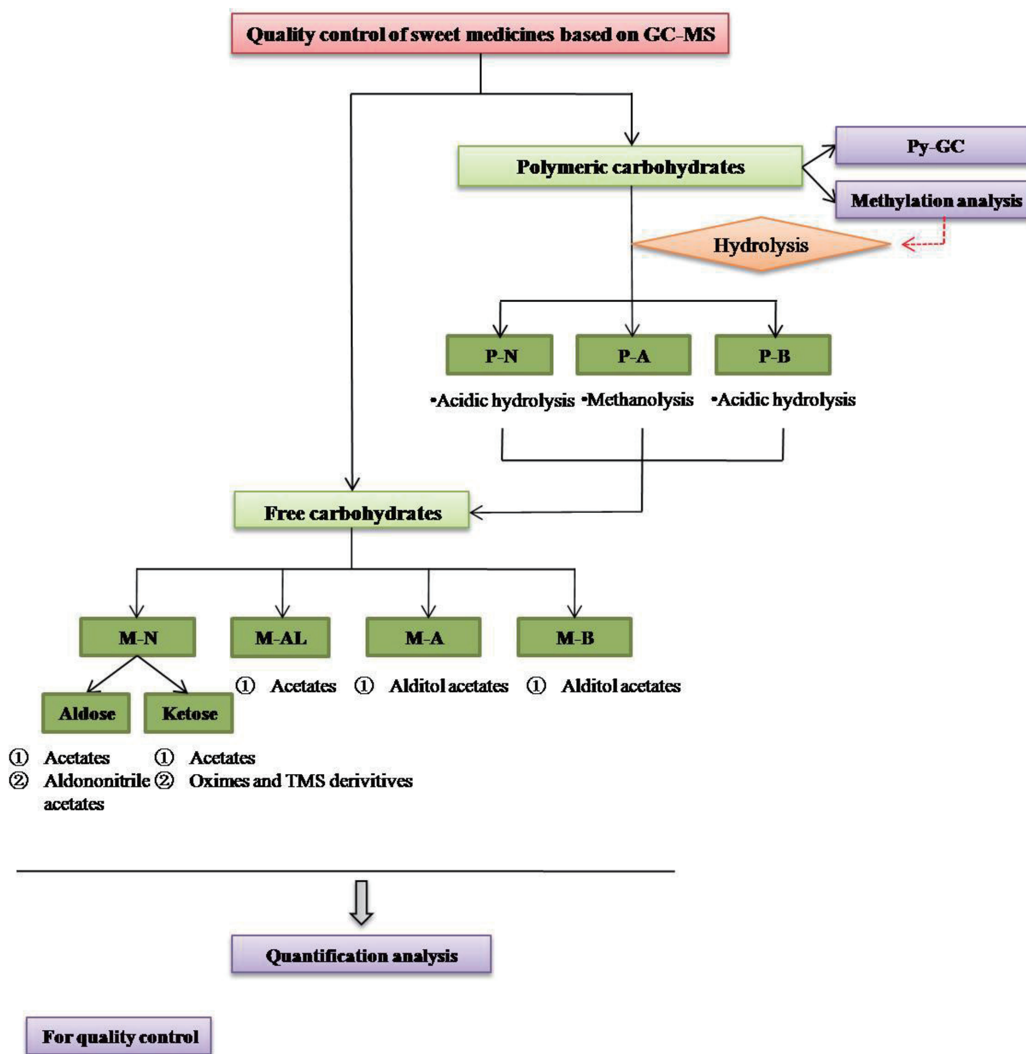


**Figure 1. Quality control of sweet medicines based on GC-MS. P-N:** polysaccharides composed of neutral monosaccharides, **P-A**: polysaccharides contained sugar acids, **P-B**: polysaccharides contained amino sugars or iminosugars, **M-N**: neutral monosaccharides, **M-AL**: alditols, **M-A**: sugar acids, **M-B**: amino sugars or iminosugars.

helical types play the decisive roles in its anti-cancer activity (*12*).

The structure of naturally occurring carbohydrates is often complex. The biological activities of them are closely correlated to their physico-chemical properties, such as molecular size, types and ratios of constituent monosaccharides, and features of glycosidic linkages (*e.g.*, configuration and position of glycosidic linkages, and sequence of monosaccharides) (*13*). Characterization of carbohydrates is therefore necessary to ensure their efficacy and safety (*14*). Gas chromatography – mass spectrometry (GC-MS) is an excellent technique for analysis of carbohydrates for its high resolution and high sensitivity. It is irreplaceable for both qualitative and quantitative analysis of structurally similar monosaccharides (*14*). However, the preparation of volatile derivatives is required for different functional groups in carbohydrates. This review aims to collect the most important methodologies currently used for the carbohydrates analysis of sweet medicines based on GC-MS. The aspects include the derivatization for monosaccharide analysis, hydrolysis methods during polysaccharide analysis, glycosidic linkages analysis based on methylation, and pyrolysis gas chromatography in carbohydrate analysis. Finally a strategy for quality control (QC) of sweet medicines based on quantification analysis is proposed.

## 2. Qualitative analysis of monosaccharides

Monosaccharides are the simplest carbohydrates, which are the basic unit to compose disaccharides, oligosaccharides and polysaccharides. Monosaccharides can be found naturally as free carbohydrates or are produced by the hydrolysis of polymeric carbohydrates including oligosaccharides, polysaccharides and glycoconjugates (*15*). Generally analysis of polymeric carbohydrates in sweet medicines based on GC-MS would convert to monosaccharides analysis after various derivatization and hydrolysis (Figure 1). The strategy for carbohydrates analysis of sweet medicines based on GC-MS is shown in Figure 1. The targets include

**Table 1. Derivatization methods available for neutral carbohydrates and alditols in sweet medicines (data from 243 journal articles collected in Web of Science mainly dated 2010-2014)**

| Derivatiztion Methods | Reaction steps | Neutral carbohydrates | | Alditols | Applications |
|---|---|---|---|---|---|
| | | Aldose | Ketose | | |
| Acetates | One | M[a]/S[b] | M/S | S | 9 |
| TMS derivatives | One | M | M | S | 58 |
| Alditol acetates | Two | S | D[c] | S | 120 |
| Oximes and derived compounds | Two | D | D | S | 8 |
| Aldononitrile acetates | Two | S | -[d] | S | 42 |
| Others | | | | | 6 |

[a]M: multiple peaks; [b]S: single peak; [c]D: double peaks; [d]-: not applicable.

neutral carbohydrates (aldoses and ketoses), alditols, sugar acids, amino sugars and iminosugars in both of free and polymeric carbohydrates in sweet medicines. Derivatization of carbohydrates for gas chromatography (GC) and GC-MS analyses was reviewed recently (*14*). Herein we discuss the aspects related to QC of sweet medicines and updated applications based on GC-MS.

2.1. *Derivatization*

The common sugars existed in nature in free and polymeric forms mainly include neutral carbohydrates (aldoses and ketoses), alditols, acid sugars, amino and iminosugars. Derivatization is crucial for non-volatile carbohydrates converted to volatile derivatives amenable to GC analysis. Due to the relatively low volatility of carbohydrates, GC analysis is limited to derivatized sugars of low molecular weight, mainly mono-, di- and trisaccharides (*14*). Generally, the diversity of naturally occuring carbohydrates makes the derivatization difficult to cover all kinds of sugars. Therefore adopting a suitable choice based on the individual samples is very important. The most used derivatization method available for different kind of sugars in sweet medicines especially the chromatographic behaviors and thier applications were summarized in Table 1 and disscussed as follows.

2.1.1. *Neutral carbohydrates (aldoses, ketoses) and alditols*

Neutral carbohydrates are the most common sugars existed in sweet medicines, such as aldoses including arabinose, xylose, ribose, fucose, rhamnose, mannose, glucose, galactose and ketoses such as fructose. Alditols such as erythritol, rhamnitol, mannitol, sorbitol, xylitol, *etc*. However the different chemical properties of aldoses, ketoses and alditols, which are induced by a high number of functional groups in the molecule and tautomeric forms in solution, lead to different derivatives and chromatographic behavior.

A variety of derivatives, including acetates, trimethylsilyl (TMS) derivatives, alditol acetates, aldononitrile acetates and oxime derived compounds, have been widely used for the analysis of carbohydrates in sweet medicines. One-step reaction, including acetylation and silylation, focuses on the increase of volatility by substituting the polar groups in carbohydrates. It is preferred because of its simple and time-saving derivatization procedure.

Acetates are prepared directly by reaction of the sugar with acetic anhydride or together with a basic solvent such as pyridine. It is rapid and applicable for aldoses, ketoses and alditols (*14,16*). Especially when catalyst such as 1-methylimidazole is used, the reaction will be sped up (*17,18*). Multiple peaks formed corresponding to one sugar limit the application of this method. Researchers focused on this problem recently developed a methyl sulfoxide (Me2SO)/1-methylimidazole system to esterification reactions, and 23 free saccharides (80% MeOH extracts) including aldoses, ketoses, alditols, amino sugars as well as trehalose and sucrose were acetylated. Only one peak was formed of each analyte for quantification analysis Figure 2a (*17*). Besides microscale sampling and derivatization is environmentally friendly and speed up the total sample preparation procedure for GC-MS analysis, therefore promising for the future carbohydrates analysis in sweet medicines (*18*).

TMS ethers have better volatility and stability than acetates and are more popularly applied for GC analysis of carbohydrates in sweet medicines Table 1. TMS ethers also prepared directly with derivatization regents or together with aprotic solvents. Generally, pyridine is the most commonly used solvent among several aprotic solvents for good solubility of the carbohydrates. Pyridine and silylation reagents are volatile and can be easily evaporated before the sample is analyzed. Sometimes complete reaction mixture can be injected directly into the gas chromatograph, thus avoiding any cleanup stages. There are many silylation reagents that have been applied for the analysis of carbohydrates in sweet medicines at different temperatures for different reaction times (*19-22*). Hexamethyldisilazane (HMDS), *N*,*O*-bis(trimethylsilyl)trifluoroacetamide (BSTFA), trimethylchlorosilane (TMCS) and *N*-methyl-*N*-trimethylsilyltrifluoroacetamide (MSTFA) are the most commonly used reagents. Alkylsilyl derivatives for gas chromatography are summarized in a previous review (*23*). Sometimes, a mixture of different silylation regents is also used. HMDS and chlorotrimethylsilane (TMSCI)

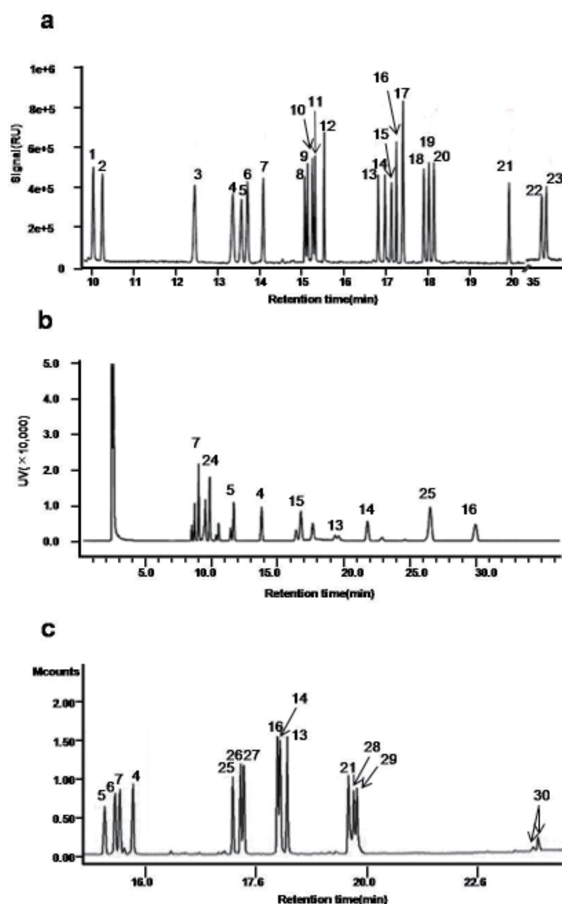


**Figure 2. Typical GC chromatograms of acetates of aldoses, ketoses, alditols, amino sugars and disaccharides (a), TMS derivatives of aldoses, ketoses and sugar acids (b), alditol acetates of aldoses, amino sugars and sugar acids (c). (a)** (*17*), **(b)** (*22*), **(c)** (*26*), respectively, with permission. 1. erythritol, 2. 2-deoxy-β-D-ribose, 3. 2-Deoxy-D-ribitol, 4. xylose, 5. rhamnose, 6. fucose, 7. arabinose, 8. rhamnitol, 9. ribitol, 10. fucitol, 11. arabinitol, 12. xylitol, 13. galactose, 14. glucose, 15. fructose, 16. mannose, 17. inositol, 18. mannitol, 19. sorbitol, 20. galactitol 21. *N*-acetyl-D-glucosamine, 22. trehalose, 23. sucrose, 24. ribose, 25. galacturonic acid, 26. mannuronic acid, 27. glucuronic acid, 28. *N*-acetyl-D-galactosamine, 29. *N*-acetyl-D-mannosamine, 30. *N*-acetyl-neuraminic acid.

were together used for the derivatization of aldoses, ketoses, and uronic acid simultaneously to characterize the polysaccharides from Kadsura marmorata fruits, which is a commonly used traditional Chinese medicines (TCMs) Figure 2b (*22*). Different silylation regents including HMDS, MSTFA and BSTFA were compared for determination of carbohydrates in medicinal plants. Mono-, di- and tri-saccharides (glucose, sucrose, and raffinose) were all taken into the consideration. The results showed that BSTFA delivered both satisfactory chromatographic behavior (two signals of glucose and one signal for sucrose and raffinose) and signal intensity. MSTFA delivered comparable results with BSTFA, however with lower signal intensity. HMDS has drawn negative attention because of multiple peaks gained and very low signal intensity (*24*). Furthermore, trimethylsilyl cyanide (TMSCN) was developed for evaluating the carbohydrates simultaneously with amino acids, small organic acids, phenolic acids, flavonoids and triterpenoids in plant extracts. The results indicated that TMSCN achieved 8.8 times higher intensities than MSTFA (*25*). However, TMSCN should be employed with special care since it hydrolyzes to give hypertoxic hydrogen cyanide, which limits its applications. For TMS derivatizations, it should be noted that the silylation reagents are moisture sensitive, the entire derivatization process needs to avoid the introduction of water.

Both acetates and TMS ethers are achieved by one-step derivatization and could be applied for the analysis of aldoses, ketoses and alditols. However, the anomeric centre leads to isomers peaks make the chromatography complicated and embarrass the accurate identification. For more authentic identification and accurate quantification, two-step derivatization is appreciated: one step to modify the anomeric centre, another to improve the volatility. The commonly used modification methods about the anomeric centre of carbohydrates are 1) reduction or 2) oximation.

Reduction of the carbonyl group in aldoses and subsequent acetylation to form alditol acetates could simplify chromatograms by producing a single peak for each aldose. The reduction commonly achieved by $NaBH_4/NaBD_4$ or $NaBH_4/NaBD_4$ in $NH_4OH$ (usually cost several hours) and acetylation with pyridine-acetic anhydride or 1-methylimidazole-acetic anhydride. The aldoses, sugar acids and amino sugars were successfully derivatized as single peaks corresponding to every sugar by this method (except *N*-acetyl-neuraminic acid (NAcNeu)) (Figure 2c) (*26*). One of the main drawbacks of these derivatives, which make it lose the original information of complex samples, is that ketoses produce two alditol acetates. What's more, aldoses and ketoses could yield the same alditol acetates. Take fructose as an example, it produces mannitol and glucitol after reduction, while glucose also produces glucitol after the reduction. In fact, fructose could be reduced into glucitol and mannitol in a fixed proportion and samples containing glucose and fructose simultaneously could be quantified with acceptable reproducibility (*27*). Another difficulty in preparation of alditol acetates is that the step of reduction is time-consuming; furthermore, removing the excess $NaBH_4$ makes the procedure tedious to perform. Nevertheless, alditol acetate is still the most commonly used approach for carbohydrate analysis in sweet medicines (Table 1), because of good chromatographic behavior for identification and quantification. Additionally, the achieved derivatives are stable.

Oxime derivatives are obtained by oximation, which commonly uses hydroxylamine hydrochloride in pyridine (other regents including methoxiamine hydrochloride, *O*-ethylhydroxylamine hydrochloride and *O*-benzylhydroxylamine hydrochloride also could be used (*28*)) and subsequent silylation to form trimethylsilyl oximes (TMSO). In some cases, trifluoroacetylation (*28*) and acetylation (*29*) also used for

subsequent derivatization. All of these oximes derivatives produce two peaks corresponding to the *syn (E)* and *anti (Z)* forms per reducing sugar and applicable to aldoses, ketoses and alditols (except aldononitrile acetates). As relative simple chromatograms are achieved, these derivatives have also been adopted for carbohydrate analysis in complex mixtures (*30-33*). The comparison of single-step derivatization trimethylsilylation and two-step approaches including ethoximation-trimethylsilylation (EO-TMS), ethoximation-trifluoroacetylation (EO-TFA), benzoximation-trimethylsilylation (BO-TMS) and benzoximation-trifluoroacetylation (BO-TFA) have been comprehensively studied for derivatization of aldoses and ketoses with regard to chromatographic characteristics. Results showed that two-step EO-TMS was superior to other approaches due to the low number of peaks obtained per carbohydrate, abundant structural information of mass spectra, low limits of detection and quantitation (*28*). Derivatization of aldose and ketose to their respective *O*-methyloxime acetates (oximation by methoxiamine hydrochloride and acetylation by acetic anhydride) for GC-MS analysis is also a facile method for the determination when aldoses and ketoses simultaneously existed, and more stable and sensitive than TMSO. Moreover, *O*-methyloxime acetates derivatives of glucose and fructose showed characteristic fragments both in chemical ionization (CI) and electronic ionizaton (EI) mode of mass spectrometry (*31*).

Especially, when aldose oximes are subsequently acetylated with acetic anhydride and dehydrated to aldononitrile acetates, a unique peak is achieved for every aldose. The derivatization procedure is relatively rapid (oximation with hydroxylamine hydrochloride-pyridine at 90°C for 30 min and acetylation by acetic anhydride at 90°C for 30 min). The produced aldononitrile acetates are more stable than TMS derivatives and have better sensitivity, accuracy and reproducibility in the qualitative and quantitative analysis of carbohydrates in complex matrix (*34-37*). The derivatization of aldose and alditols to aldononitrile acetates was successfully archived single peaks and quantification analysis of carbohydrates in *Ganoderma* (*38*). However, the validity of quantitative analysis using these derivatives also has the drawback that they cannot be applied for analysis of ketoses (*14,31*). Actually, when aldoses and ketoses exist simultaneously in the matrix, samples can go through oximation first and subsequent derivatization by acetylation and silylation, respectively, and finally converted to aldononitrile acetates and TMSO respectively. The developed method showed good chromatographic behavior and quantitative results (*39*).

2.1.2. *Sugar acids*

Sugar acids are monosaccharides with one or more carboxyl group and also known as polyhydroxy carboxylic acids. Generally, sugar acids include following classes: *i*) aldonic acids, in which the aldehyde functional group of an aldose is oxidized; *ii*) uronic acids, in which the terminal hydroxyl group of an aldose or ketose is oxidized; *iii*) aldaric acids, in which both ends of an aldose are oxidized (*40*). Among them uronic acids such as glucuronic acid (GlcA) and galacturonic acid (GalA) are most commonly found in nature and present as parts of structural and/or extracellular polysaccharides or glycoconjugates. Analysis of these compounds requires hydrolysis or methanolysis before derivatization, which will discuss in section 2.2. Conventionally, colorimetric methods using different chromogens including carbazole, 3-phenylphenol and 3,5-dimethylphenol (DMP) are most commonly used methods explored for uronic acid estimation in polysaccharides but these methods counter numerous difficulties when neutral sugars are present in substantial amount (*41*). GC analysis despite time consuming for derivatization procedure remains the finest method for precise estimation of uronic acids (*41,42*). The derivatives including acetates (*43*), TMS derivatives (*22,44*), oximes derivatives (*45*), and alditol acetates (*26*) have been used for the analysis of sugar acids. However, because hydroxyl and carboxyl groups simultaneously exist in one molecule, different lactones will be formed, and furthermore, coupled with anomeric centre, complex chromatograms will be generated when uronic acids presented in the samples. The methyl ester alditol acetate is the alternative solutions for decreasing the multiple peaks. Guilherme L. Sassaki proposed methyl ester alditol acetate for simultaneously determined neutral, uronic acids and amino sugars. The mixture was firstly de-lactonizated with $NH_4OH$ at room temperature, subsequently reduced by $NaBH_4$ to form alditols, and then methyl esters were formed by 0.5 M HCl in MeOH. Finally acetylation of the Me-alditols was performed in pyridine-acetic anhydride ($Ac_2O$) (1:1, v/v) and uronic acids gave characteristic ions at m/z 143, 156 and 173 (*26*).

2.1.3. *Amino sugars and iminosugars*

Amino sugars are the hydroxyl group of monosaccharides replaced by the amino group, and sometimes by the *N*-acetyl-amino group. As with the deoxy sugars, theoretically any hydroxyl group can be replaced. The most commonly occurring amino sugars are D-glucosamine (2-amino-2-deoxy-D-glucose, GlcN), D-galactosamine (2-amino-2-deoxy-D-galactose, GalN), *N*-acetylglucosamine (*N*-acetyl-D-glucosamine, GlcNAc) and *N*-acetylgalactosamine (*N*-acetyl-D-galactosamine, GalNAc) (*40*). Iminosugars are found both free or as part of glycoproteins, glycolipids or polysaccharides, therefore, a previous hydrolysis step before their analysis is commonly necessary. Iminosugars are monosaccharides where the O atom in the cycle has been replaced by N atom such as fagomine and deoxynojirimycin (DNJ). The derivatives

including acetates (*29*), TMS derivatives (*29,46,47*), oximes derivatives (*29*) and alditol acetates (*47,48*) have been used for the analysis of amino sugars and iminosugars in sweet medicines. Derivatization methods of aminoglycosides have been reviewed before (*49*). Alditol acetates have been widely used for determination of neutral and basic monosaccharides simultaneously (*50-52*) however failed in uronic acids detection without forming methyl esters (*52*). The derivatization procedure including silylation, acetylation, oximation + acetylation and oximation + silylation have been compared for the analysis of iminosugars (DNJ and fagomine) and other low molecular weight carbohydrates. Results indicated that two-step derivatization including oximation + acetylation and oximation + silylation allowed the separation of target compounds, whereas TMS and acetylated derivatives showed several co-elutions. Oximation + acetylation were discarded for giving inaccurate results for ketoses. TMSO formed by oximation + silylation was successfully applied for simultaneous determination of iminosugars and other carbohydrates including mono-, di-saccharides and alditols (*29*).

Currently, there is considerable interest in developing the simple and quick method for derivatization and separation of carbohydrates in complex matrices. Microwave-assisted derivatization has been successfully applied for carbohydrates analysis in complex matrix (*53-56*). Taking the advantage of high efficiency of microwave, the derivatization procedure could be significantly shortened. Silylation was finished within 4 min with HMDS, BSTFA or MSTFA as derivative reagents (*24*). Microwave-assisted derivatization combined with comprehensive two-dimensional gas chromatography-time-of-flight mass spectrometry (2D GC-TOF-MS) has been successfully applied for carbohydrate analysis in complex extracts (*57*).

Although some of the existing procedures for preparing GC derivatives are quite satisfactory, and some of them have even been improved, one of the goals of these methods to achieve only one chromatographic peak for each individual sugar seem to need further work. Generally, when the analytes composed of neutral carbohydrates (ketose and aldose), alditols and amino sugars, novel developed methyl sulfoxide (Me2SO)/1-methylimidazole system to acetylation is recommended (Figure 2a). Aldononitrile acetate is also a good choice for quantitative analysis of aldose (Figure 1, Table 1). Oximes and TMS derivitives are the alternative methods when ketose and aldose simultaneously existed (Figure 2b, Figure 1). When aldose, alditols, sugar acids, amino sugar as well as iminosugars taken into the considerations alditol acetates should be an ideal choice (Figure 2c and Figure 1).

2.2. *Hydrolysis*

Hydrolysis is a necessary and crucial step both in the compositional monosaccharide analysis and linkage analysis of polysaccharides. The hydrolysis conditions are varying depending on the nature of samples and their compositional sugars. The compositional sugars in sweet medicines are diverse, and additionally their chemical properties are varying. Therefore, different hydrolysis methods are developed for the accurate identification and quantification of sugars in sweet medicines.

2.2.1. *Acidic hydrolysis*

Acidic hydrolysis is the most commonly used methods for releasing monosacchardies. Two of the most common reagents for acidic hydrolysis are trifluoroacetic acid (TFA) and sulfuric acid. TFA is most commonly used for soluble polysaccharides such as isolated polysaccharides and secreted polysaccharides. It is volatile therefore easily be removed. It accounts 73% of hydrolysis in characterization of polymeric carbohydrates in sweet medicines shown in Figure 3, which is based on the data from 224 journal articles in web of science. While sulfuric acid commonly used for insoluble samples such as plant cell walls or samples difficult to complete hydrolysis (*14*). What's more the hydrolysis based on sulfuric acid need further cleanup to remove excessive and involatile sulfuric acid, which make the hydrolysis procedure more complex and time-consuming. However this problem partly solved when microscale analysis applied, samples hydrolyzed by sulfuric acid were neutralized with *N,N*-dioctylamine (DOM) in chloroform, followed by successive washes with the same solution. This procedure effectively removed the sulfuric acid and allowed derivatization of monosaccharides in one tube. However the chromatography achieved by this procedure is not as clean as the TFA hydrolysis, and unknown peaks will appear in the chromatogram (*58*). It should be noted that some acid-sensitive sugars would decompose during the acidic hydrolysis. Therefore, identification and quantification of these sugars by hydrolysis should be performed carefully. Fructose is easily decomposed under acidic conditions in both acid hydrolysis and methanolysis conditions (*59,60*). Some alternative methods have been developed to solve the problem such as enzymatic hydrolysis (*61,62*) or

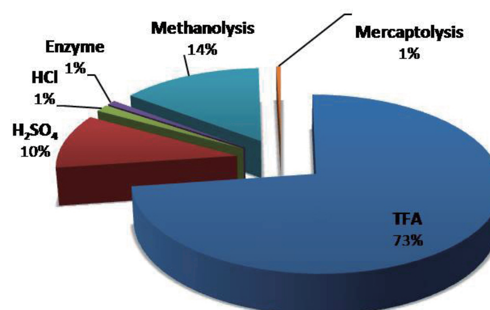


**Figure 3. Hydrolysis methods used for releasing monosacharides (data from 224 journal articles collected in Web of Science mainly dated 2010-2014).**

determined by phenol-acetone-boric acid reagent (PABR) which introduced by Boratynski (*60,63*). Besides, anhydrosugars that are common in red/brown algal cell walls such as 3,6-anhydrogalactose need to be analyzed by reductive hydrolysis (*64*). Mild methanolysis is required for the detection of 3-deoxy-D-manno-oct-2-ulosonic acid (KDO), which is used by bacteria in the synthesis of lipopolysaccharides (*65-67*). GlcNAc also easily destroyed during hydrolysis therefore mild acid condition or methanolysis is needed (*68-71*). On the contrary, liberation of all monosaccharides from polysaccharides is also not easily achieved, especially uronic acid-containing polysaccharides because glycosidic linkages between uronic acids and other monosaccharides are acid resistant during acid treatments. Therefore the reduction of carboxylic groups of uronic acids into their corresponding hexoses or methanolysis is recommended to carry out to allow the complete liberation of monosaccharides (*14,58*). The strategy for releasing neutral, amino sugars and acidic sugars was proposed. Firstly for releasing the neutral and amino sugars from lipopolysaccharides, samples were hydrolyzed with 2 M TFA (120°C, 2 h). While to release acidic sugar components, lipopolysaccharides were subjected to methanolysis (1 M HCl in methanol, 85°C, 16 h), finally the hydrolysis products were converted to alditol acetates for GC-MS analysis (*66*).

Microwave-assisted hydrolysis of polymeric carbohydrates showed its power in complete glycosidic cleavage and conversion of polysaccharides into monosaccharides (*72*). The optimization of microwave-assisted hydrolysis and derivatization of hydroxyethylstarch showed that hydrolysis could be finished in 2 min at 1,200 W, 100°C and derivatization could be achieved within 5 min at 1,020 W, 100°C. The sample preparation time is greatly shortened by this procedure, compared with traditional hydrolysis and derivatization (*73*). It should be noted that optimization procedure should be carefully carried out to avoid the degradation of monosaccharides during the hydrolysis.

2.2.2. *Enzymatic hydrolysis*

Although it is not commonly used as acidic hydrolysis (only accounting for 1% of hydrolysis in Figure 3), enzymatic hydrolysis plays an irreplaceable role for its mild hydrolysis condition and avoiding sugar degradation. Enzymatic hydrolysis is commonly used for the analysis of fructose-rich carbohydrates (*61,74*) such as inulin and fructans. The amount of inulin in the samples was quantified for the QC as the amounts of hydrolyzed sugars (fructose, glucose and sucrose) after inulinase treatment minus the amounts of free sugars (the existing sugars in the original sample) (*61*). Enzymatic hydrolysis has also been used for releasing carbohydrates from plant-derived arabinoxylans and uronic acid-containing polysaccharides (*62,75-77*). The applications of enzymatic hydrolysis in the utilization and analysis of carbohydrates have been summarized in previous reviews (*78-81*).

2.2.3. *Others*

Methanolysis is another commonly used approach for releasing monosaccharides (accounting for 14% of hydrolysis in Figure 3) and is usually performed with HCl in anhydrous methanol. Monosaccharides are liberated as methyl glycosides and the carboxyl groups are esterified. Polysaccharides with the inclusion of uronic acid residues can be determined by methanolysis (*14*). Prebiotic oligosaccharides from *Corylus avellana* L., composed mainly of GalA and GalNAc, have been successfully quantified by methanolysis (1 M MeOH-HCl at 80°C for 24 h) combined with TMS derivatives (*82*).

Generally, methanolysis and prereduction are highly recommended for uronic acids containing polysaccharides. Acidic hydrolysis is effective in most cases, when neutral and amino sugars are the compositional monosaccharides (Figure 1). Actually, the combination of different hydrolysis and derivatization methods certainly makes the results more reliable (*47,83*). The methanolysis (1 M MeOH-HCl at 80°C for 16 h) coupled with TMS derivatives and acid hydrolysis (4 N HCl at 100°C for 6 h) couple with alditol acetate derivatives were successfully applied for the identification of rare monosaccharides in *O*-antigen capsular polysaccharide from *Francisella tularensis*. GC-MS analyses of TMS derivative, confirmed the presence of 2-acetamido-2,6-dideoxy-*O*-D-glucose (QuiNAc) in the sample. While GC-MS analyses of alditol acetates showed the presence of QuiNAc and 4,6-dideoxy-4-formamido-D-glucose (Qui4NFm). Besides, two ionization modes were used in the identification, which CI could get fragments related to molecular weight whereas EI could get more fragment ion information (*47*). A combination of reductive acid hydrolysis and anhydrous mercaptolysis (0.5 M HCl in EtSH:MeOH (2/1, v/v) at 60°C, 6 h) was applied for selective hydrolysis of the 3,6-anhydrogalacotosidic linkage in red algal galactan (*83*). Acid hydrolysis, methanolysis, and enzymatic hydrolysis were compared for depolymerization of different plant materials containing uronic acids. Besides GC (using both HP-1 and HP-5 capillary columns and FID and MS detectors), HPAEC-PAD and HPAEC-Borate techniques also were compared for subsequent analysis of the released monosaccharides. It was shown that methanolysis combined with GC analysis is a convenient method for obtaining the sugar unit composition from uronic acids containing polysaccharides (*76*).

2.3. *Methylation analysis*

Since permethylation reaction was developed for the

linkage analysis between sugar residues in the 1960s, it is a crucial analytical approach for the structural analysis of carbohydrates, called "methylation analysis" (*84-86*). Methylation analysis traditionally including permethylation-hydrolysis- reduction-acetylation procedures (Figure 4a) and complete permethylation is critical for the correct analysis (*16*). There are two most commonly used permethylation methods for carbohydrates analysis. One is the method introduced by Hakomori in 1964 (*86*), and the other is Ciucanu and Kerek introduced in 1984 (*87*). In Hakomori's method carbohydrates in dimethylsulfoxide (DMSO) is reacted with methyl iodide catalysed by the methylsulfinyl carbanion, which is prepared from sodium hydride (*86*). Sometimes with modification for the use of methylsulfinyl carbanion made with bases such as potassium hydride or butyl-lithium (*16,21,88*). Several years later, Ciucanu and Kerek developed a simple, rapid and quantitative procedure used finely powdered sodium hydroxide as base catalyst and DMSO as solvent (*87*). These two methods have been compared for the analysis of β-cyclodextrin (β-CD) and Hakomori method showed superior base catalyst than NaOH-DMSO suspension. Under the latter condition, premethylation of β-CD occurs selectively at 3-hydroxy groups, which may because the 3-hydroxy groups are buried within the relatively hydrophobic torus of β-CD where they are excluded from deprotonation by the NaOH base (*89*). In the same study, however, consistent with this, maltoheptoase, which is a linear form of β-CD, is permethylated equally well using either two methods. Even several mannose oligosaccharides are more completely permethylated using NaOH-DMSO (*89*). The conclusion is that permethylation conditions are not universally applicable to all carbohydrate types, and it is therefore recommended that the completeness of permethylation of carbohydrate samples should be checked before the acid hydrolysis step such as using infrared spectroscopy to monitor the hydroxyl residues. Besides many researches cited the methods described by Needs and Selvendran in 1993 (*90*). It is a modified sodium hydroxide-catalysed procedure, in which methylation with sodium hydroxide and methyl iodide is sequentially rather than simultaneously added into samples. The results showed that it was not prone to the oxidative deficiencies of the original and that, given its reduced tendency towards polysaccharide undermethylation. The preparation of permethylated carbohydrates for GC and LC analysis has been the subject of several reviews (*91-93*).

For the subsequent derivatization, hydrolysis is also necessary in methylation analysis. TFA is still the most frequently used hydrolysis reagents. And what should be noted is that acid-sensitive sugars such as fructose also need hydrolysis in mild conditions after permethylation (*94*). Then the hydrolyzed free methylated monosaccharide residues are commonly reduced by $NaBH_4/NaBD_4$ or $NaBH_4/NaBD_4$ in $NH_4OH$ and converted into alditols. Then the remaining hydroxyl groups acetylated with $Ac_2O$/pyridine or $Ac_2O$/1-methylimidazole. The final product partially methylated alditol acetates, known as PMAAs are subject to the GC-MS analysis (Figure 4b). The glycosidic linkage is concluded based on the retention time and mass spectrometry data (Figure 4c). Some databases have been built up to assist the analysis of these data. Such as Spectral Database for PMAA's which is initiated by Complex Carbohydrate Research Center is available online (*95*). Recently, researchers attempt to synthesize partially *O*-methylated alditol acetate standards of galactofuranose. These PMAAs could be used as GC-MS standards for simultaneous identification of galactofuranose units with diverse linkages in complex carbohydrates (*96*).

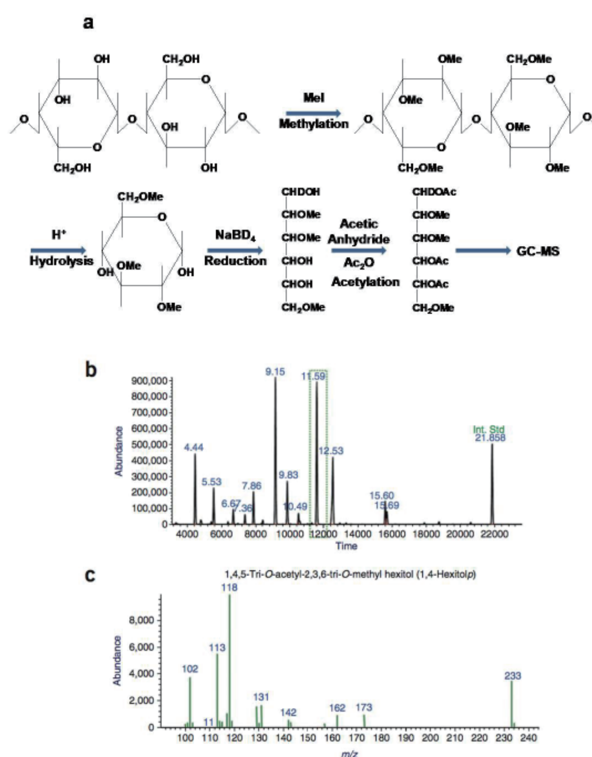


**Figure 4. Methylation analysis of sweet medicines. a,** procedures of methylation analysis; **b**, GC-MS chromatogram of PMAAs; **c**, mass spectrum of PMAAs. From *Ref.* (*58*), with permission.

Traditional methylation analysis commonly costs several days, of which the permethylation process takes most (*97*). Microwave-promoted methylation significantly shortens and simplifies this procedure. It was demonstrated that permethylation of plant seed gum with NaOH-dimethyl sulfate was completed in 4 min after exposure to microwave power. And subsequently hydrolysis with 70% aqueous formic acid and 0.5 N $H_2SO_4$ was finished in 3.32 min (*97*). Therefore microwave assistant methylation analysis is probably a wise choice to improve the methylation

analysis efficiency. However, still, there are time-consuming steps. By-products and excessive methylation agents should be separated with partially methylated carbohydrates before hydrolysis. And dialysis or extraction with organic solvents such as dichloromethane and chloroform are performed. Extraction partially methylated carbohydrates by organic solvents is simple and time-saving. After three or more times extraction with organic solvents and wash with water, the organic layer could be separated and obtained the purified partially methylated carbohydrates. However, solvent extraction is not suitable for the high molecular weight polysaccharides because of poor solubility (*58*). Dialysis is commonly adopted for these polysaccharides. However, dialysis is usually performed "over night". So far, it has still been the rate-limited step in methylation analysis, which needs further improvements.

Even though methylation analysis could provide abundant information about the characterization of carbohydrates, it still could not give the definite linkage of polymeric carbohydrates for the mass spectrum of some PMAAs are highly similar. Accurate identification of structures must combine with other approaches such as MALDI-TOF-MS (*16*), characteristic enzymatic digestion (*13*) and NMR (*65*).

### 2.4. *Pyrolysis-gas chromatography (Py-GC)*

Pyrolysis-gas chromatography (Py-GC) has been well established as a simple, quick and reliable analytical technique for a range of applications including the analysis of polymeric materials (*98,99*). The most important application of Py-GC in carbohydrates analysis is characterization of cellulose, hemicellulose and plant gum (*100-103*). Derivatizations including methylation and silylation are also necessary of Py-GC for improving the behavior of analytes during separation in the column, modifying the thermal degradation pathway or enhancing detectability (*98*). The derivative reagent including TMCS (*103*), BSTFA (*103,104*) and HMDS (*105,106*) are most commonly used. Recently, on-line analysis of thermally assisted hydrolysis and methylation (THM) gas chromatography commonly used tetramethylammonium hydroxide (TMAH) (*107-109*) as base reagents, made the analysis simpler and faster (*99*). Pyrolysis GC-MS as a novel analysis technique to determine the biochemical composition including carbohydrate has been applied for microalgae. The results showed that a linear trend was observed and the method could give a quick estimation of carbohydrate contents (*110*). The medicinal plant Ginkgo biloba was also successfully identified by THM-GC (*111*). The greatest advantage of pyrolysis is that, in most cases, only minimal sample preparation is required. Therefore, Py-GC is a promising method for quick identification of sweet medicines and is useful in the QC of sweet medicines (Figure 1).

## 3. Quantification analysis

Quantitation is crucial for QC of sweet medicines. Compared with free carbohydrates, polymeric carbohydrates such as oligosaccharides and polysaccharides are more difficult to quantify due to their large molecular weights, complex structures and rare of chemical standards (*4*). However, separation and quantification are possible for free carbohydrates (including mono-, di- and trisaccharides) by GC-MS (*24,112*). Therefore, suitable hydrolysis of polymeric carbohydrates (discussed in Section 2.2) combined with efficient derivatization methods (discussed in Section 2.1) is an alternative method to quantify the carbohydrates in sweet medicines for QC.

Monosaccharide profile has been successfully applied for QC of *Dendrobii Officinalis* Caulis, which is a rare medicinal plant (*113*). Chinese Pharmacopoeia (2010 Edition) documented that the ratio of mannose and glucose in *Dendrobii Officinalis* Caulis should be 2.4-8.0 (*114*). The monosaccharide profiles released from polysaccharides have also been used to discriminate different sweet medicines and identify their origins (*115-118*). The results of those studies indicate that free sugars or sugar profiles obtained after acidic or enzymatic hydrolysis (*i.e.*, amounts and composition of monosaccharides) are crucial for QC of polysaccharides. On the other hand, the characteristic chromatography of PMAAs achieved by GC-MS which reflects the glycosidic linkages is also could be applied for the discrimination of original for sweet medicines, however many works should be carry on to make the procedures involved in methylation analysis more efficient, automated and high-throughput.

## 4. Conclusion

Biological activities of sweet medicines are highly correlated with their chemical characteristics. The qualitative and quantitative analyses of both free and polymeric active carbohydrates are necessary for QC of sweet medicines. GC coupled with MS, which provides abundant structure and quantitative information, is very helpful in improving QC of sweet medicines.

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## Original Article

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# A novel polysaccharide isolated from *Litchi chinensis* by using a simulated gastric medium and its immunomodulatory activity

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**Summary** **A novel polysaccharide (LCPA50-S1) with immunomodulatory activity was extracted with simulated gastric medium from *Litchi chinensis*, and purified by DEAE-52 cellulose column, Sephadex G-50 column and Sephacryl S-300 HR chromatography. The structural characteristics of LCPA50-S1 were expounded through complete acid hydrolysis, partial acid hydrolysis, methylation and instrumental analysis. The results demonstrated that LCPA50-S1 is a heteropolysaccharide with a molecular weight of 1.58 × $10^5$ Da. The backbone was composed of (1→4)-linked β-D-glucopyranosyl residues, (1→6)-linked β-D-galactopyranosyl, (1→3,6)-linked β-D-galactopyranosyl residues, (1→4,6)-linked α-D-glucopyranosyl residues and branched at O-6. The branches were consisted of (1→2)-linked α-L-rhamnopyranosyl residues, (1→4)-linked β-D-glucopyranosyl residues, and (1→6)-linked β-D-galactopyranosyl, terminated with (1→)-linked α-L-arabinopyranosyl residues and (1→)-linked β-D-galactopyranosyl residues, respectively. The immunoregulatory activity of LCPA50-S1 was evaluated through determination the effect of LCPA50-S1 on nitric oxide (NO) production of RAW264.7 macrophages and spleen lymphocyte proliferation as well as its cytokines secretion level. The results demonstrated that LCPA50-S1 increased NO and TNF-α production in RAW264.7 macrophages significantly, enhanced the proliferation as well as the interleukin-2 (IL-2) production of splenocytes. The data indicated that LCPA50-S1 had the potential to be explored as a novel natural immunomodulator for application in functional foods and medicine.**

***Keywords:*** *Litchi chinensis*, polysaccharide, structure characterization, immunomodulatory activity

## 1. Introduction

Litchi (*Litchi chinensis* Sonn.), originating from China, is a subtropical fruit belonging to Sapindaceae. As a kind of health-care fruit, litchi contains plenty of nourishments and is considered to be of excellent medicinal value. Recent investigations indicated that the polysaccharides from litchi pulp had antioxidant and immunomolulatory effects *in vitro* (1,2). In addition, litchi has been a kind of favorite fruit from ancient times due to its unique flavor and cultural background.

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Polysaccharides are a class of biomacromolecules composed of more than ten monosaccharide units, which generally exist in plants, animals, algae and microorganisms such as fungi and bacteria. Because polysaccharides provide nutritional support for developmental process, they play an important role in the growth of living organisms. Moreover, the interest in natural resources and the regulation of antioxidant, immune ability, anti-tumor effects as well as low toxicity leads to the wide applications of polysaccharides in the medicine and food fields (*3*).

The development of modern science, cytobiology and molecular biology makes people realize that the disorder of the immune system can result in a variety of diseases. Some crude polysaccharides also had effect on the lymphocyte proliferation (*4*). LCPA50W, isolated from *litchi chinensis*, was confirmed to promote the

proliferation of mouse splenocytes and enhance the cytotoxicity of NK cells (*2*). The production of cytokines is a key event in the initiation and regulation of an immune response. Therefore, we tried to explore the effect of LCPA50-S1 on the secretion of cytokines from macrophages and spleen lymphocytes.

The polysaccharides reported in the research paper mentioned above were extracted by hot water method. Although the traditional extraction is simple, the high temperature may have an impact on the bioactivity of polysaccharides (*5*). Furthermore, different specific structures and biological activity of polysaccharides may be obtained from different extractions (*6*). Therefore, in our study, we chose the stimulated gastric medium to isolate the polysaccharides of litchi, and purify through DEAE-52 cellulose, Sephadex G-50, and Sephacryl S-300 HR column chromatography. Then detailed structure characterization and immunomodulatory activity were also investigated.

## 2. Materials and Methods

### 2.1. *Plant materials and reagents*

*Litchi chinensis* was obtained from Guangzhou Yide Food Market, China. The material was identified to be Feizixiao by Professor R. M. Yu, Biotechnological Institute of Chinese Materia Medica, Jinan University, Guangzhou, China. The seed and peel were removed manually to get the pulp tissues. DEAE-52 cellulose and Sephadex G-50 were purchased from Whatman Ltd. (England). Sephacryl S-300 HR was purchased from Amersham Biosciences (Swenden). Standard monosaccharides, T-series dextrans were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Pepsin was purchased from Guangzhou Qiyun Biotechnology Company. All chemicals and reagents were of analytical grade.

Lipopolysaccharides (LPS) were obtained from Sigma Chemical Co. (USA). RPMI 1640 medium, RPMI DMEM medium, Trizol reagent and fetal bovine serum (FBS) were purchased from Gibco Invitrogen Corp. (USA). ELISA test kits for interleukin-2 (IL-2) and tumor necrosis factor (TNF-α) were obtained from RayBiotech Systems (USA). The NO assay kit was obtained from Beyotime Biotech Inc. (China).

### 2.2. *Animals and cells*

Kunming mice (grade II, SCXK 2011-2015, 20-25 g, 6-8 weeks old) were obtained from the Experimental Animal Center, Sun Yat-sen University, China. Animals were acclimatized for at least 7 days prior to use and maintained in a temperature-controlled environment (22 ± 2°C) with a 12 h light-dark cycle and provided with free access to water and standard rodent chow. Raw264.7 cell line was obtained from The Cell Bank of Type Culture Collection of Chinese Academy of Sciences (Shanghai, China). It was maintained in RPMI DMEM medium supplemented with100 IU/mL penicillin, 100 μg/mL streptomycin, and 10% FBS at 37°C under humidified air with 5% $CO_2$.

### 2.3. *Separation of crude polysaccharide LCPA50*

The pulp tissues of L. *chinensis* (500 g) were defatted with 80% ethanol for 6 h at 85°C, and dried in the open air, then immersed in a solution of simulated gastric juice (10 L, pH 2.0). The artificial gastric juice was used to mimic the conditions of the stomach. Simulated gastric juice was formulated from HCl (1.34 g/L), NaCl (2.16 g/L), $KH_2PO_4$ (0.63 g/L), $CaCl_2$ (0.12 g/L), KCl (0.39 g/L), and pepsin (0.53 g/L). Extraction was performed at 37°C for 4 h and repeated twice (*6*). All the extracts were put together, adjusted to a pH of 7 with NaOH and filtered to remove the insoluble material. Then 95% ethanol was added to a final concentration of 50%, and the solution was stored at 4°C overnight. The precipitate was dissolved in distilled water. To clear off the protein, we used Sevage method ($CHCl_3$/BuOH = 4:1, v/v) for 20 min, and repeated the procedure seven times. The supernatant was concentrated at 40°C by using a vacuum rotary evaporator and then passed through an ultrafiltration cell with membrane pores of 3000 Da. Finally, the crude polysaccharide named LCPA50 was collected after lyophilizing.

### 2.4. *Preparation and purification of LCPA50-S1*

To purify the crude polysaccharide, anion-exchange DEAE-52 cellulose chromatography was used. LCPA50 was dissolved in distilled water, then centrifuged and filtered through 0.45 μm filters. The filtrate was injected to DEAE-52 cellulose column (5.0 × 70.0 cm). The column was eluted by distilled water, a linear gradient from 0 to 0.5 M NaCl at a flow rate of 0.8 mL/min. Fractions (10 mL) were collected. Phenol-sulfuric acid method was used to monitor the carbohydrate content. Consequently, the two sharp peaks were combined respectively, dialyzed, concentrated and lyophilized (Figure 1). Afterwards, we chose size exclusion Sephadex G-50 column (1.6 × 70 cm) and Sephactyl

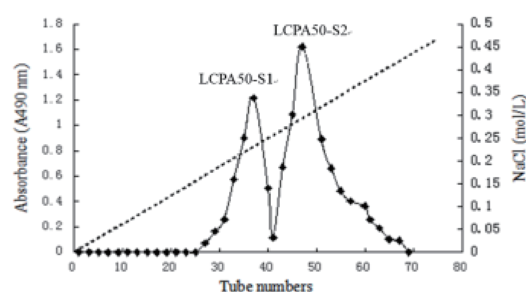


**Figure 1. Profile of LCPA50 in DEAE-52 cellulose chromatography.**

S-300 HR (1.2 × 90 cm) to purify the polysaccharides. By eluted with distilled water, two polysaccharides of high purity, namely LCPA50-S1, LCPA50-S2 were obtained. LCPA50-S1 was used in the structural analysis and bioactive evaluation.

2.5. *General quantification analysis of LCPA50-S1*

Phenol-sulfuric method was used for the detection of the content of polysaccharide, and glucose was used as the standard (*7*). Uronic acid content was measured by the carbazole-sulfuric acid method using gulcuronic acid as a standard. Protein content was determined by using Bradford reagent and bovine serum albumin was taken as standard.

IR spectra were recorded with a Tensor 27 Bruker instrument. 1 mg LCPA50-S1 was ground with KBr power and pressed into pellets for FT-IR measurement in the frequency range of 4,000-500 $cm^{-1}$ (*8*).

2.6. *Characterization of LCPA50-S1*

2.6.1. *Measurement of molecular weight*

The molecular weight of the LCPA50-S1 was determined by gel permeation chromatography (GPC). A Sephacryl S-300 HR (1.2 × 90 cm) was used, and the mobile phase was distilled water. The LCPA50-S1 molecular weight was measured by comparison to a calibration curve made with T-series Dextran (T-200, T-100, T-70 and T-7), glucose and dextran blue. Then by calculating we get the molecule of LCPA50-S1 (*9*).

2.6.2. *Analysis of monosaccharide composition and partial acid hydrolysis*

LCPA50-S1 (5 mg) was hydrolyzed with 2 mL 2 M trifluoroacetic acid (TFA) at 110°C in a sealed tube for 6 h. After the hydrolysis was completed, azeotropic distillation method was applied to remove the excess TFA by evaporation with methanol. The monosaccharide content was measured by high-performance anion exchange chromatography with pulsed amperometric detection (HPAEC-PAD). The hydrolysate (20 μL) was dissolved in distilled water (1 mL) and then passed through a 0.45 μm filter. Finally, the solution was injected into the HPAEC-PAD system (Dionex-5500, Dionex Corp., Canada) (*10*).

LCPA50-S1 (25 mg) was hydrolyzed with 0.05 M TFA for 6 h at 100°C in a sealed tube and then dialyzed with distilled water for 48 h. The distilled water outside the dialysis bag was collected, and concentrated up to dryness. To remove the extra TFA in fraction 1, methanol was added to evaporate with. The fraction in the dialysis bag (3,000 Da) was dried and then hydrolyzed with 0.5 M TFA. After dialyzing with distilled water for 48 h, we got the distilled water containing fraction 2 and the hydrolysate, fraction 3, respectively. Afterwards, fractions 1-3 were hydrolyzed with 2 M TFA and analyzed by HPAEC-PAD.

2.6.3. *Periodate oxidation and Smith degradation analysis*

LCPA50-S1 (15 mg) was dissolved in 25 mL distilled water, and then 80 mg $NaIO_4$ was added into the solution. The solution was kept in the dark at 4°C, 50 μL aliquots were withdrawn at 6 h intervals, diluted to 10 mL with distilled water and read in a spectrophometer at 223 nm. Consumption of $HIO_4$ was measured by a spectrophotometric method, and formic acid production was measured by titration with 0.041 M NaOH. The reaction mixture was dialyzed with distilled water. The nondialysate was concentrated and reduced with $NaBH_4$ (30 mg) for 24 h at room temperature. Then add 0.1 M HOAc to neutralize to pH 6.0. After dialysis and concentration to dryness, the mixture product was hydrolyzed with 2 M TFA (2 mL) at 110°C for 6 h and tested by HPAEC-PAD.

2.6.4. *Methylation analysis*

Methylation of LCPA50-S1 was carried out according to the method of Needs and Sevendran with minor modifications (*11*). Eight-hundred mg NaOH and 8 mg dry LCPA50-S1 were precisely weighed, and then dissolved in 8 mL DMSO, dispersed using a blender. The mixture was treated with an ultrasonic wave attached to an ultrasonic cleaner for 30 min. 4.5 mL methyl iodide was added for methylation after incubation for 1h at 25°C. The reaction was kept in darkness at room temperature for 6 h. Distilled water (4 mL) was added to terminate the reaction. The reaction product was extracted with 3 × 2 mL of chloroform and dried on a rotary evaporator at low pressure. The methylated polysaccharide was acid hydrolyzed using TFA and then reduced with $NaBH_4$. Finally, acetic anhydride was used to obtain the acetylated polysaccharide. Alditol acetates were analyzed by GC-MS.

2.6.5. *NMR spectroscopy*

LCPA50-S1 (20 mg) was dissolved in 0.6 mL of $D_2O$. $^{13}C$ NMR spectra was recorded on a Bruker AMX 500 FT spectrometer (Bruker, Germany) at 24°C. Chemical shift was expressed in ppm.

2.7. *Splenocyte proliferation assay in vitro (5)*

A spleen was separated from Kunming mice under aseptic condition and kept in phosphate-buffered saline (PBS) solution. The organ was crushed and passed through a steel mesh (200 mesh) to obtain a homogeneous cell suspension. The splenocytes were

collected and washed twice with PBS after removing the red blood cells through adding 0.83% $NH_4Cl$ in 0.01 M Tris-HCl (pH 7.2). Next, the pelleted cells were resuspended and diluted to 5 × $10^6$ cells/mL with RPMI-1640 containing 10% of FBS, and further inoculated into a 96-well microtiter plate (100 μL/well). Different concentrations of LCPA50-S1 was added and the final concentration was 0, 31.3, 62.5, 125, 250, and 500 μg/mL, respectively. ConA (5 μg/mL) was selected as the positive control. The plates were then incubated at 37°C in humidified incubator with 5% $CO_2$ for 48 h. All the tests were carried out in triplicate and the cell proliferation was evaluated by 3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay. Briefly, 20 μL of MTT solution (5 mg/mL) was added to each well and incubated for another 4 h in dark. The plates were centrifugated to remove the supernate and then 200 μL of DMSO was added into each well to dissolve the MTT-formazan crystals. The plates were shaken for 10 min to dissolve the crystals. Absorbance of each well was detected spectrophotometrically at 570 nm.

2.8. *Determination of IL-2 secretion from splenocytes*

Splenocytes were collected according to the reference (*2*) and was treated with different concentrations of LCPA50-S1 (0, 31.3, 62.5, 125, 250, and 500 μg/mL) for 48 h. The cell supernatant was collected by centrifugation at 1,500 rpm for 10 min at 4°C. IL-2 level in culture supernatant was measured with an ELISA kit according to manufacturer's instruction.

2.9. *Cytotoxicity assay*

MTT assay was selected to evaluate the effect of LCPA50-S1 on RAW264.7 cells. Briefly, RAW264.7 cells were seeded in 96-well microplates at 2 × $10^4$ cells/mL and treated by different concentrations of LCPA50-S1 (0, 31.3, 62.5, 125, 250, and 500 μg/mL) for 48 h. 20 μL of MTT (5 mg/mL) was added into each well and co-incubated for an additional 4 h in dark. Then the supernatant was discarded and 200 μL of DMSO was added to solubilize the MTT-formazan crystals. The absorbance of each well was detected by a microplate reader at 570 nm.

2.10. *Measurement of NO production*

RAW264.7 cells were seeded in a 96-well plate at 2 × $10^4$ cells/well in DMEM medium and incubated at 37°C in a humidified atmosphere with 5% $CO_2$. After incubation for 24 h, the cells were treated with series of concentrations of LCPA50-S1 (0, 15.6, 31.3, 62.5, 125, and 250 μg/mL) for 24 h. LPS (1 μg/mL) was selected as the positive control. The culture supernatant of RAW264.7 cells was collected after treatment of LCPA50-S1 for 24 h and the NO levels of supernatant were determined by Griess reaction. Briefly, the supernatant was distributed in a 96-well plate at 50 μL/well, and equal volumes of the Griess reaction solutions and Griess reaction solutions II were added. The absorbance of each well in the 96-well plate was detected spectrophotometrically at 540 nm and the contents of NO were calculated through a least squares linear regression analysis of a sodium nitrite standard curve.

2.11. *Determination of TNF-α secretion*

The RAW264.7 cells were treated with LCPA50-S1 (0, 31.3, 62.5, 125, 250, or 500 μg/mL) and LPS (1 μg/mL) for 24 h and then the supernatant was collected for the detection TNF-α secretion. TNF-α levels were detected *via* a commercial ELISA kit. The detailed procedure for determination TNF-α levels was carried out according to the manufacturer's instruction.

## 3. Results

3.1. *Purity and chemical compositions of polysaccharide*

Crude polysaccharide was purified by a 3,000 Da membrane, and the fraction with a molecular weight over 3,000 Da was collected and lyophilized. The crude polysaccharide (2.23 g) was obtained with a 0.44% yield from the raw plant material. After purification *via* DEAE-52 cellulose, Sephadex G-50 and Sephacryl S-300 HR column chromatography, the profile of LCPA50-S1 appeared as a single and symmetrical sharp peak (Figure 2), which was detected by the phenol-sulfuric acid method. The yield of LCPA50-S1 from crude polysaccharide was 37.7%.

Total carbohydrate content was measured to be 98.2% (w/w), and LCPA50-S1 did not contain any protein. The uronic acid content of LCPA50-S1 was below the detection limit. The optical rotation of LCPA50-S1 was

$$[\alpha]_D^{20} = +131° \text{ (c 1.0, } H_2O)$$

Complete acid hydrolysis of LCPA50-S1 performed by treatment with 2 M TFA allowed for the detection of

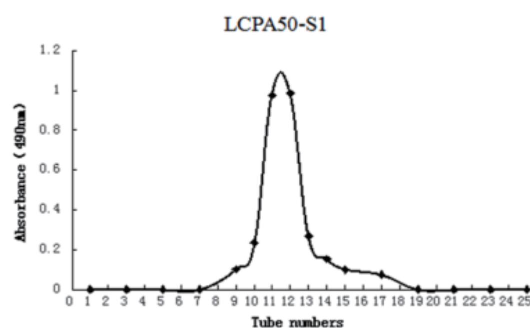


**Figure 2. Profile of LCPA50-S1 in Sephacryl S-300 HR column chromatography.**

the following monosaccharides in the hydrolysate, which were identified by HPAEC-PAD: rhamnose, arabinose, galactose and glucose with ratio of 1.00:1.37:9.96:8.44. The monosaccharide composition of LCPA50-S1 is presented in Table 1. Fractions 1-3 were obtained through partial acid hydrolysis.

The component of fraction 3 in Table 1 showed that galactose and glucose approximated to be the backbone of the structure of LCPA50-S1. The branched structure of LCPA50-S1 was composed of galactose, glucose and rhamnose. The LCPA50-S1 was terminated with arabinose and galactose from the analysis results of fractions 1 and 2.

### 3.2. *Molecular weight of LCPA50-S1*

The purified polysaccharide LCPA50-S1 was a white loose powder, odorless, and soluble in water and showed a single and relatively symmetrical peak on GPC, indicating its homogeneity (Figure 2).

From GPC analysis on a Sephacryl S-300 HR column, a calibration curve was obtained by using various Dextran T-series standards of known molecular weights. According to the calibration curve, logMw = 0.9209 − 0.4 Kav ($R^2$ = 0.991), Kav was the retention volume and the average molecular weight of the polysaccharide was calculated to be $1.58 \times 10^5$ Da.

### 3.3. *Structural Characterization of LCPA50-S1*

The UV absorption spectra of LCPA50-S1 showed no absorption at 280 or 260 nm, implying that there was no protein and nucleic acid in this polysaccharide. The infrared spectrum of LCPA50-S1 showed special absorption bands at 3,428, 2,925, 1,636, 1,422, and 1,076 $cm^{-1}$ (Figure 3). In the FT-IR spectrum, the characteristic bands in the regions of 3,428, 2,925, and 1,636 $cm^{-1}$ belonged to O-H bending, C-H bending, and associated water, respectively. The band at 1,076 $cm^{-1}$ indicated pyranose. The absorption bands at 890 and 810 $cm^{-1}$ showed that LCPA50-S1 contained both α- and β-type glycosidic linkages in its structure (*12*). There was no absorption at 1,740 $cm^{-1}$, indicating the absence of uronic acid in the polysaccharide structure, which was consistent with the result of the carbazole-sulfuric acid method.

The periodate-oxidized products were fully hydrolyzed and analyzed by HPAEC-PAD after periodate oxidation. The results shown in Table 1 indicated that there was only galactose in the oxidation products. The presence of galactose revealed that residues of galactose had (1→3)-linked, (1→2,3)-linked, (1→2,4)-linked, (1→3,6)-linked, or (1→2,3,4)-linked, which could not be oxidized, respectively. Moreover, the other monosaccharides could be inferred that linkages of were (1→), (1→2), (1→6), (1→4) and (1→4,6), which might be oxidized to produce glycerol (*7,12*).

**Table 1. Monosaccharide composition and molar ratio of LCPA50-S1**

| Items | Molar ratios | | | |
|---|---|---|---|---|
| | Rhamnose | Arabinose | Galactose | Glucose |
| LCPA50-S1 | 1.00 | 8.44 | 9.96 | 1.37 |
| fraction 1 | n.d. | 3.02 | 1.00 | n.d. |
| fraction 2 | 1.00 | n.d. | 1.21 | 2.55 |
| fraction3 | n.d. | n.d. | 5.62 | 1.00 |
| Smith degradation | n.d. | n.d. | 1.00 | n.d. |

n.d., not detected.

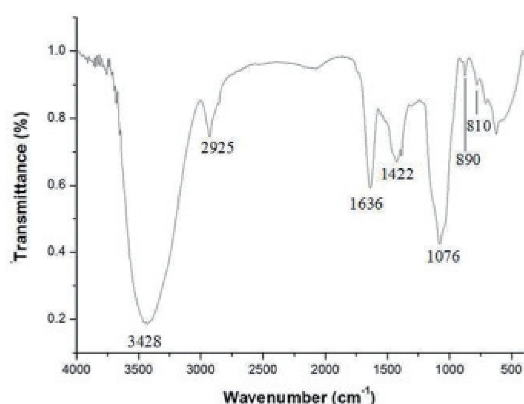


**Figure 3. FI-IR spectrum of LCPA50-S1.**

### 3.4. *Methylation and GC-MS*

Methylation analysis offers the information on the positions at which monosaccharide residues are substituted and their relative abundance in the polysaccharide. The polysaccharide was methylated completely, which was confirmed by the IR spectrum.

The profile of methylated LCPA50-S1 on GC-MS appeared seven peaks. Methylation analysis of fractions LCPA50-S1 showed the presence of seven components (Table 2), namely 2,3,5-$Me_3$-Ara, 2,3,4-$Me_3$-Gal, 2,3,6-$Me_3$-Glc, 2,3-$Me_2$-Glc, 3,4-$Me_2$-Rha, 2,3,4,6-$Me_4$-Gal, 2,4-$Me_2$-Gal. On the basis of the standard data in the CCRC Spectral Database for PMAA's, and according to the mass spectrum patterns from the literatures (*2,12-14*), the linkages of L-rhamnose, L-arabinose, and D-galactose were deduced as (1→), (1→2), (1→6), and (1→3,6), whereas the linkages of D-glucose were deduced as (1→4) and (1→4,6). This result showed correlation between terminal and branched residues. In addition, the

**Table 2. Methylation Analysis of LCPA50-S1**

| Methylated sugar | Major mass fragments (m/z) | Linkage pattern |
|---|---|---|
| 2,3,5-$Me_3$-Ara | 43, 45, 71, 87, 101, 117, 129, 161 | T→ linked Ara |
| 2,3,4,6-$Me_4$-Gal | 45, 87, 101, 117, 145, 161, 205 | T→ linked Gal |
| 2,3,6-$Me_3$-Glc | 43, 87, 99, 101, 113, 117, 161, 233 | 1→4 linked Glc |
| 3,4-$Me_2$-Rha | 87, 99, 129, 131, 189 | 1→2 linked Rha |
| 2,3,4-$Me_3$-Gal | 43, 71, 87, 101, 161, 189, 203, 233 | 1→6 linked Gal |
| 2,3-$Me_2$-Glc | 43, 58, 101, 117, 203, 261 | 1→4,6 linked Glc |
| 2,4-$Me_2$-Gal | 43, 87, 101, 189 | 1→3,6 linked Gal |

monosaccharide composition and linkages also agreed with the analysis of LCPA50-S1 described above.

### 3.5. *NMR analysis of LCPA50-S1*

The spectra of $^{13}C$ NMR of LCPA50-S1 is shown in Figure 4. Signals of LCPA50-S1 in spectra were analyzed on the basis of the data available in the literatures (*2,13,14,32*). The resonances in the region of 95.0-110.0 ppm in $^{13}C$ NMR were attributed to the anomeric carbon atoms of D-galactose (Galp), D-glucose (Glcp), L-rhamnose (Rhap), and L-arabinose (Arap). The peaks at 98.6 ppm corresponded to C-1 of (1→2)-linked α-L-Rha unit, 102.7 ppm corresponded to C-1 of (1→3,6)-linked β-D-Gal unit, 103.4 ppm corresponded to C-1 of T→linked β-D-Gal unit, 104.5 ppm corresponded to C-1 of (1→6)-linked β-D-Gal unit, 99.7 ppm corresponded to C-1 of (1→4,6)-linked α-D-Glc unit, 107.9 ppm corresponded to C-1 of (1→4)-linked β-D-Glc unit, and 109.2 ppm corresponded to C-1 of (1→)-linked α-L-Ara unit, respectively. The assignment of the carbon atom signals is shown in Table 3.

On the basis of the results of HPAEC-PAD, GC-MS and NMR, the backbone is composed of (1→4)-linked β-D-glucopyranosyl residues, (1→6)-linked β-D-galactopyranosyl, (1→3,6)-linked β-D-galactopyranosyl residues, and (1→4,6)-linked α-D-glucopyranosyl residues, which both branched at O-6. The branches are consisted of (1→2)-linked α-L-rhamnopyranosyl residues, (1→4)-linked β-D-glucopyranosyl residues, and (1→6)-linked β-D-galactopyranosyl, terminated with (1→)-linked α-L-arabinopyranosyl residues and (1→)-linked β-D-galactopyranosyl residues, respectively. From all above results, the predicated structure of the repeating unit of LCPA50-S1 could be proposed as Figure 5.

### 3.6. *Effect of LCPA50-S1 on splenocyte proliferation*

Splenocyte proliferation firmly reflects the immunity of biological organism. Therefore, we evaluated the effect of LCPA50-S1 on splenocyte proliferation *via* MTT assay. As shown in Figure 6, LCPA50-S1 significantly promoted splenocyte proliferation in a concentration-dependent manner, indicating that LCPA50-S1 had the potential of immunoregulation.

### 3.7. *Effect of IL-2 production of spleen lymphocyte cells*

We further detected the effect of LCPA50-S1 on production of IL-2, an important cytokine in the immune responses, *via* an ELISA kit. The results demonstrated that LCPA50-S1 significantly enhanced the IL-2 production of spleen lymphocyte cells in a concentration-dependent manner (Figure 7).

### 3.8. *Effect of LCPA50-S1 on viability of RAW264.7 cells*

MTT assay was selected to investigate the effect of LCPA50-S1 on the growth of RAW264.7 cells. As shown in Figure 8, LCPA50-S1 (31.5-500 μg/mL) had little cytotoxicity on RAW264.7 cells after treatment for 48 h.

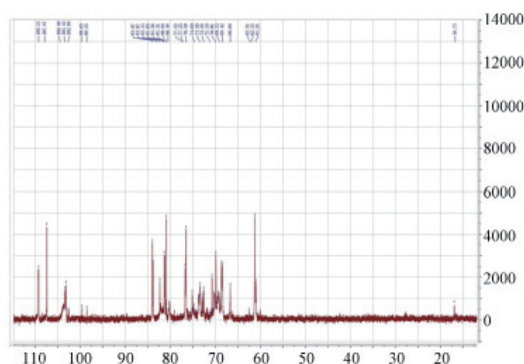


**Figure 4. $^{13}C$ NMR spectrum of LCPA50-S1.**

**Table 3. Chemical shifts of resonances in the $^{13}C$ spectra of LCPA50-S1**

| Sugar residues | Chemical shift (ppm) | | | | | | |
|---|---|---|---|---|---|---|---|
| | C1 | C2 | C3 | C4 | C5 | C6 | $CH_3$ |
| α-L-Ara(1→ | 109.2 | 81.6 | 74.7 | 83.9 | 66.7 | | |
| β-D-Gal(1→ | 103.4 | 69.1 | 81.3 | 71.2 | 74.7 | 61.2 | |
| →4)-β-D-Glc(1→ | 107.9 | 77.5 | 77.5 | 82.3 | 73.5 | 62.2 | |
| →2)-α-L-Rha(1→ | 98.6 | 81.7 | 68.4 | 70.0 | 69.1 | | 16.7 |
| →6)-β-D-Gal(1→ | 104.5 | 77.3 | 73.5 | 76.6 | 77.3 | 62.5 | |
| →4,6)-α-D-Glc(1→ | 99.7 | 71.2 | 72.6 | 76.5 | 71.2 | 74.7 | |
| →3,6)-β-D-Gal(1→ | 102.7 | 72.6 | 80.5 | 70.0 | 69.1 | 70.0 | |

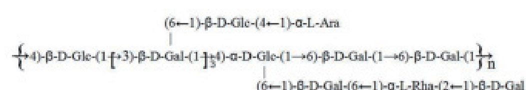


**Figure 5. Predicated structure of the repeating unit of LCPA50-S1.**

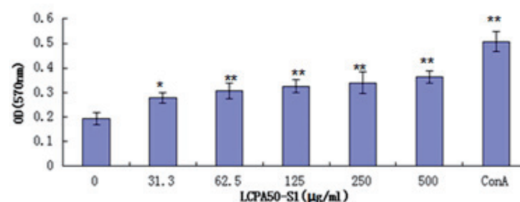


**Figure 6. Effect of LCPA50-S1 on splenocyte proliferation.**

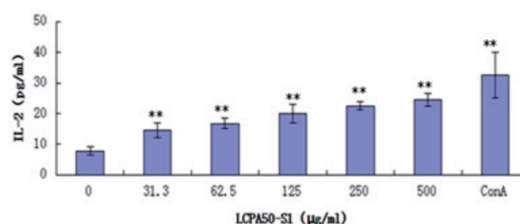


**Figure 7. Effect of LCPA50-S1 on the production of IL-2 of spleen lymphocytes.**

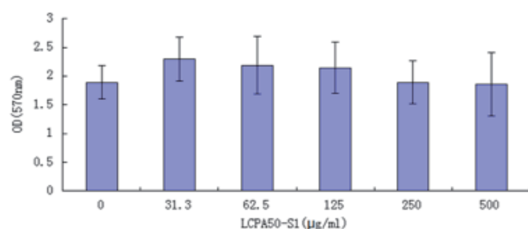


**Figure 8. Effect of LCPA50-S1 on cell viability of RAW264.7.**

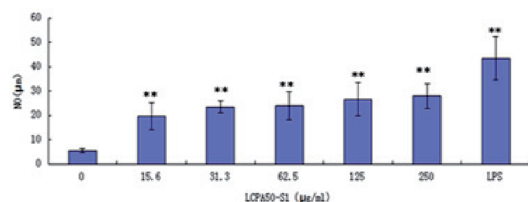


**Figure 9. Effect of LCPA50-S1 on the NO secretion from RAW264.7 cells.**

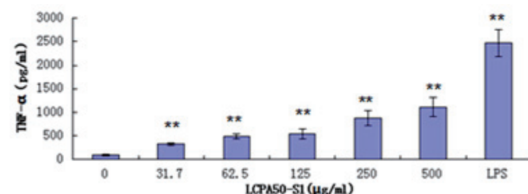


**Figure 10. Effect of LCPA50-S1 on the TNF-α secretion from RAW264.7 cells.**

3.9. *Effect of LCPA50-S1 on NO production of RAW264.7 cells*

NO is considered to be a major effector molecule produced by macrophages and reflects the activation capacity of macrophage. We subsequently detected the effect of LCPA50-S1 on NO production of RAW264.7 cells. As shown in Figure 9, comparing with the untreated group, the level of NO in the LCPA50-S1 treated group remarkably increased in a concentration-dependent manner.

3.10. *Effect of LCPA50-S1 on TNF-α secretion from RAW264.7 cells*

The effect of LCPA50-S1 on TNF-α secretion of RAW264.7 cells was also evaluated *via* an ELISA kit. As shown in Figure 10, LCPA50-S1 significantly increased the level of TNF-α in the supernatant of RAW264.7 cells after treatment for 24 h, indicating that LCPA50-S1 could remarkably enhance the TNF-α secretion from RAW264.7 cells.

**4. Discussion**

Hot water is commonly used to extract the crude polysaccharide of *Litchi chinensis*, and the water-soluble polysaccharide has the backbone substituted with glucose and rhamnose residues (*2,13*). However, to a large extent, the monosaccharide composition and structure of polysaccharide depend on extraction procedure. In the present study, we revealed the composition and structural features of LCPA50-S1, which was extracted from *Litchi chinensis* under conditions closely mimicking the normal fasting human stomach. The pulp tissues were incubated with simulated gastric juice (pH 1.5) at 37°C for 4 h. The pepsin is a protease, whose pH is 1.5-2.5 (*15*). Moreover, suitable dissolution media for simulating the fasted state gastric conditions will have pH values between pH 1.5 and pH 2.0. Therefore, pH 2.0 was chosen in this experiment. The extraction time of 4 h was selected to cover residence time for gastric exposure as most solids are emptied within 4 h (*16*).

Contrast with previous reports, the monosaccharide galactose was identified as the main constituents in the sugar chains of LCPA50-S1. It is generally known that the immunomodulating actions of polysaccharides are related to their molecular weight, chemical composition, glycosidic linkage, conformation, degree of branching and so on. This structural variability could affect the biological activities of these molecules profoundly. Many studies on pharmacology and phytochemistry have demonstrated that the polysaccharides from *Lycium barbarum* (LBP) had the backbones mainly composed of (1→3)-β-Galp, (1→4)-β-Galp, (1→6)-β-Galp, (1→6)-α-glucans, and (1→4)-α-polygalacturonans (*3*). Though the polysaccharides are terminated with different branches or sugar residues, the antioxidant activity and immunomodulatory activity were proved by many experimental results. The LCPA50-S1 monosaccharide ratio showed higher content of galactose than that isolated by hot water. Therefore, the LCPA50-S1 was supposed to have good immunomodulatory activity.

The immune system is the human's ultimate defense against infectious diseases, tumor and cancer growth. Immunostimulation is regarded as an important strategy for improving the body's defense mechanism in elderly people and cancer patients. The immunologic action of polysaccharides may begin with activating major subsets of immune cells such as lymphocytes and macrophages (*17*). Therefore, we tried to explore the effects of LCPA50-S1 on the activation of macrophages and lymphocyte proliferation.

The immune response including cellular and humoral immunity, characterized by T cells and B cells respectively, plays an important role in the host defense system such as anti-tumor and infectious defenses (*18*). The capacity to elicit an effective T and B cells immunity could be shown by the stimulation of lymphocyte proliferation response (*19*). The splenocyte proliferation is an indicator of immunoenhancement and related to immunity improvement of T-lymphocytes or B-lymphocytes (*19,20*). The splenic lymphocytes proliferation was considered as an important indicator

for studying immune response and evaluating cellular immune function. After stimulated by antigenic properties, the T and B lymphocytes, which are considered as the most important immune effector cells, can proliferate and differentiate, generate specific immune response, and produce lymphokine and antibody (*23*). To investigate the immunoregulatory function, we detected the effect of LCPA50-S1 on splenic lymphocyte proliferation. As shown in Figure 6, the A570 values of LCPA50-S1-treated cells were remarkably higher than those of the LCPA50-S1-untreated cells. It demonstrated that both T and B cells may be activated by LCPA50-S1.

The overall immune response elicited within each individual tumor results from the balance of the type of cells and cytokines secreted. Some cytokines may help to reject tumor cells whereas others promote tumor growth. Previous studies showed that polysaccharides from *Coriolus versicolor* can induce T cell proliferation and production of interfero-γ (INF-γ) as well as IL-2 in experimental animals (*24*). It is reported that Th1 cytokine IL-2 supports CTL reaction (*25*). IL-2 and TNF-α are two important cytokines in immune response in human body (*26*). In our present investigation, LCPA50-S1 was verified to increase the IL-2 level of spleen lymphocyte cells in a concentration-dependent manner remarkably, demonstrating that LCPA50-S1 had the potential of enhancing immune function.

NO is a gaseous molecule synthesized from L arginine by nitric oxide synthase (NOS) and acts as an inducer of immunoregulation (*27*). It is reported that NO is a major effector molecule produced by macrophages (*28,29*) and recognized as a quantitative index of macrophage activation (*30*). Several polysaccharides were reported to have the function of macrophage activation (*31*). An increased level of NO secretion reflects phagocytic stimulation. In our present study, LCPA50-S1 could increase the NO production of RAW264.7 cells after 24 h stimulation quickly, suggesting that LCPA50-S1 could act as an immunostimulant of innate immunity. Indeed, there are various surface receptors on macrophages, which are known as pattern recognition molecules such as polysaccharides and can recognize foreign ligands during initial phases of the immune response.

TNF-α is a cytokine mainly produced by the activated macrophages, which are exceedingly important for killing and degrading tumor cells. To investigate whether LCPA50-S1 could enhance TNF-α secretion of macrophages, RAW264.7 cells were incubated with LCPA50-S1 for 24 h. The result showed that LCPA50-S1 highly enhanced TNF-α secretion of RAW264.7 cells, demonstrating that LCPA50-S1 can enhance phagocytosis function of RAW264.7 cells. The results indicated that LCPA50-S1-treated macrophages could enhance LCPA50-S1 uptake capacity and TNF-α secretion, suggesting that this might be the mechanism by which litchi polysaccharide acts to stimulate the killing and degradation of invading pathogens and tumor cells.

In conclusion, the present experimental results indicated that LCPA50-S1 significantly enhanced NO and TNF-α production of RAW264.7 macrophages, promoted splenocyte proliferation as well as IL-2 production of splenocytes. These findings indicated that LCPA50-S1 had the potential to be an immunomodulator and applying in treating series of diseases associated with immunity disorder. Further research on the stereochemical structure, the bioactive effects on human subjects and the structure-bioactivity relationship are in progress, which could provide a better understanding of the functional effects about polysaccharide.

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**Original Article**

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# Structural elucidation and *in vitro* antioxidant activities of a new heteropolysaccharide from *Litchi chinensis*

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**Summary** **Two polysaccharides, LCP70S-1 and LCP70W, were isolated from the pulp tissues of *Litchi chinensis* by anion-exchange chromatography and gel-filtration chromatography, while the structure of LCP70S-1 was elucidated and its physico-chemical properties was analyzed. The results demonstrated that LCP70S-1 is composed of L-rhamnose, L-arabinose and D-galactose in the ratio of 1.06:6.39:4.21, and the main chain of the heteropolysaccharide possess (1→3,6)-linked galactopyranosyl branches at O-6. The three branches consist of (1→3)-linked rhamnopyranosyl residues, (1→3,6)-linked galactopyranosyl and (1→5)-linked arabinopyranosyl residues, and terminated with (1→)-linked arabinopyranosyl residues, respectively. The two polysaccharides were further evaluated with 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging activities and their reducing power *in vitro*. The results showed that LCP70W and LCP70S-1 possessed significant antioxidant activities, especially for LCP70S-1. As such, LCP70S-1 could potentially serve as an antioxidant and would value further study for human healthcare.**

***Keywords:*** *Litchi chinensis*, heteropolysaccharide, structural elucidation, antioxidant activity

## 1. Introduction

Natural polysaccharides from different sources have long been studied in medicine and pharmaceutics and has been the core of intense research for the understanding and utilization of bioactive polysaccharides in recent decades (*1*). In fact some of these polysaccharides have already made their way to the market as antitumor, immunostimulating or prophylactic drugs (*2*). More importantly, polysaccharides are promising radical scavengers for the prevention of oxidative stress in living systems owing to their biocompatibility and non-toxicity (*3*).

Finding natural, effective and safe antioxidants is important for healthcare to protect mankind from the attack of free radicals and retard the initiation of many chronic diseases (*4*). Published data have indicated that some plant polysaccharides have strong antioxidant activities and therefore could be developed into supplements or even therapeutic agents (*5,6*). In addition, polysaccharides extracted from *Auricularia auricular* have been regarded as potential health-promoting functional food provided that they possess strong radical scavenging ability (*7*).

Litchi (*Litchi chinensis*), a tropical to subtropical fruit originally from China, is cultivated all over the world in warm climates. Litchi fruit pericarp tissues contain various polysaccharides and proteins in high quantity. Previous investigation by our research group has demonstrated that the polysaccharide from pulp tissues of *L. chinensis* shows strong antioxidant activity which values further research and development as potential antioxidants and health foods (*8*). Recently, Kong has reported that a fraction of water-soluble polysaccharide coded as LFP3 from litchi pulp exhibited higher antioxidant activity (*9*). Meanwhile, another novel water-soluble fraction LCP50W has been

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reported to contain immunomodulatory activity through promoting the proliferation of mouse splenocytes and enhanced cytotoxicity towards NK cells (*10*). Although several bioactive polysaccharide fractions from *Litchi chinensis* have been reported, there are still other fractions value further investigation for food and pharmaceutical industries.

In the current research, crude polysaccharides were extracted from *L. chinensis* to obtain the polysaccharide fraction (LCP70). LCP70 was further purified and two homogenous heteropolysaccharides (LCP70S-1 and LCP70W) were isolated. The apparent molecular weight and monosaccharide composition of the polysaccharides were analyzed. The representative polysaccharide LCP70S-1 was selected to determine its polymer chain structures with physico-chemical and instrumental analyses, such as Fourier-transform infrared spectra (FTIR), GC-MS, $^{1}$H NMR and $^{13}$C NMR spectra which provided systematically valuable and fundamental information on the structure and chain conformation of the polysaccharide. In particular, the *in vitro* antioxidant activities of LCP70S-1 were also evaluated by three methods.

## 2. Materials and Methods

### 2.1. *Plant materials*

The pulp tissues of *Litchi chinensis* were purchased from Guangzhou Qingping Chinese Medicinal Materials Market, China, and identified by Dr. R.M. Yu, Jinan University, China.

### 2.2. *Experimental reagents and materials*

DEAE-52 cellulose and Sephadex G-100 were obtained from Whatman Ltd. Sephacryl S-300 HR was received from Amersham Biosciences. Ascorbic acid (Vitamin C, Vc), hydrogen peroxide ($H_2O_2$) and ferrous sulfate ($FeSO_4$) were obtained from Guangzhou Chemical Reagent Company. All other analytical grade reagents were obtained from Sigma and used as received.

### 2.3. *Extraction, isolation and purification of polysaccharides*

The pulp tissues of *L. chinensis* (300 g) was defatted with ethanol for 8 h and extracted three times with hot water (85°C). The water extract was filtered, concentrated under vacuum and centrifuged at 5,031 g for 15 min. The supernatant was concentrated to one fourth of its original volume and treated with 1 volume of ethanol for precipitation at 4°C. The precipitate was collected and the supernatant was concentrated to a quarter of its original volume for the treatment with 3 volumes of 95% ethanol for further precipitation at 4°C overnight. The precipitate was collected by centrifugation and washed three times with 95% ethanol, anhydrous ethanol and acetone respectively. The resulting precipitate was dialyzed using a cellulose membrane (molecular weight cut off 3500, purchased from Lvniao, China) against distilled water for two successive days. The retained fraction was concentrated and lyophilized. One gram of dried precipitate was dissolved in 20 mL of distilled water, and 5 mL of Sevag reagent (chloroform-n-Butanol, v/v = 4:1) was added three times to degrade any protein component (*11*). The crude polysaccharide was dissolved in distilled water and was fractionated on a DEAE-cellulose-52 column (2.6 × 40 cm, from Whatman) equilibrated by distilled water, with a linear gradient from 0 to 0.5 M NaCl at a flow rate of 0.5 mL/min. All of the fractions were assayed for carbohydrate content by the phenol-sulfuric acid method (*12*), and two sharp peaks were pooled, dialyzed, concentrated, and further purified by a Sephadex G-100 column (1.6 × 70 cm, from Whatman) eluting with distilled water at a flow rate of 0.3 mL/min. The polysaccharide fractions were collected, dialyzed, and lyophilized.

Consequently, two polysaccharides, LCP70W and LCP70S-1, were obtained. LCP70S-1 was used in the subsequent studies on its structure and bioactivity.

### 2.4. *Analyses of chemical composition*

Total sugar content was determined by the phenol-sulfuric acid colorimetric method using glucose as the standard (*12*). Sulfate content was measured according to the literature (*13*). Uronic acid content was evaluated by the carbazole-sulfuric acid method using glucuronic acid as the standard (*14*). The homogeneity and molecular weight of LCP70S-1 were determined by gel permeation chromatography (GPC) on a Sephacryl S-300HR column (1.6 × 70 cm) with standard dextrans (T-4, T-7, T-10, T-70, T-200, and blue dextran) and glucose. The elution volumes were plotted against the logarithm of their respective molecular weights. The elution volume of LCP70S-1 was plotted in the same graph, and the molecular weight was measured (*15*). The monosaccharide composition was analyzed by high-performance anion exchange chromatography (HPAEC) after hydrolization and UV detection, coupled with pulsed amperometric detection (PAD), equipped with a Carbo PAC $^{TM}$PA10 (2.0 × 250 mm) column. The hydrolysate (1 mg) was dissolved in pure water (1 mL). The solution (25 mL) was used for the ionic-chromatographic analysis by HPAEC-PAD of Dionex ICS-2500 system, eluted with a mixture of water and 200 mM NaOH in the volume ratio of 92:8 (*16*).

### 2.5. *Spectroscopic analysis*

Optical rotations were recorded with a Jasco P-1020 polarimeter. IR spectra were recorded with a Tensor 27 Bruker instrument with KBr pellets. The sample was

ground with KBr powder and then pressed into pellets for FTIR measurement at 4,000-500 $cm^{-1}$. $^{13}C$ NMR spectra were recorded with a Bruker 500 instrument and the sample was dissolved in $D_2O$ (*17*).

2.6. *Partial acid hydrolysis*

LCP70S-1 (15 mg) was hydrolyzed with 0.05 M TFA for 6 h at 100°C, and dialyzed with distilled water for 48 h. The fraction out of the sack was collected. After removing TFA by co-distillation with MeOH (1 mL × 3), the fraction was evaporated to dryness (fraction 1). The fraction in the sack was evaporated, and then hydrolyzed with 0.5 M TFA. The hydrolysate was again dialyzed, and the fraction out of the sack (fraction 2) and the fraction in the sack (fraction 3) were collected respectively. Fractions 1-3 were hydrolyzed with 2 M TFA and tested by HPAEC-PAD.

2.7. *Periodate oxidation-Smith degradation*

The polysaccharide (15 mg) was swelled in 5 mL of distilled water, and then 25 mL of 15 mM $NaIO_4$ was added into the solution. Thirty microliters of the solution were withdrawn at 6 h intervals under the dark at 4°C, then diluted to 5 mL with distilled water and measured in a spectrophotometer at 223 nm (*18*). Consumption of $HIO_4$ was determined by a spectrophotometric method (*19*), and formic acid production was measured by titration with 0.061 M NaOH. The nondialysate was concentrated and reduced with $NaBH_4$ (30 mg) for 24 h at room temperature, and neutralized to pH 6.0 with 0.1 M HOAc. After dialyzed and concentrated, the mixture product was hydrolyzed with 2 M TFA (4 mL) at 100°C for 8 h and tested by HAPEC-PAD analysis.

2.8. *Methylation analysis*

Polysaccharide was dried at 30°C under vacuum overnight and was methylated by the method of Hakomori (*20*). The polysaccharide was dispersed in 3 mL of dimethyl sulfoxide and stirred until dissolved after bubbling with $N_2$, then 15 mL of solid anhydrous NaH and 1.5 mL of methyl iodide were added, and the solution was allowed to react in the dark for 2 h. The reaction was terminated by the addition of water, and the permethylated polysaccharide was extracted with chloroform. The permethylated polysaccharide was then further treated with 90% formic acid (3 mL) for 10 h at 100°C in a sealed tube. After removal of the formic acid, the residues were heated with 2 M TFA (2 mL) under the above conditions and the hydrolysate was evaporated to dryness. The methylated sugars were reduced with NaBH4, and then acetylated with acetic anhydride. Alditol acetates were analyzed by GC-MS (*21*). GC-MS was conducted with a Hewlett Packard 5895 instrument, using a fused-silica capillary column (30 × 25 mm) coated with a 0.2 mm film of DB-5. The ionization potential was 70 eV and the temperature of the ion source was 220°C.

2.9. *In vitro antioxidant activity assay*

2.9.1. *Determination of DPPH radical-scavenging activity*

DPPH radical-scavenging activity was determined using the method described by Hua with some modifications (*22*). Briefly, DPPH ethanol solution (190 µL, freshly prepared at a concentration of 0.2 mM) was added to 10 µL of polysaccharide solution of gradient concentrations (0, 25, 50, 100, 200, 400, 800, and 1,600 µg/mL) in water. The absorbance was measured at 517 nm after 30 min. The lower absorbance of the reaction mixture indicated the higher free radical-scavenging activity. The capability to scavenge DPPH radical was calculated by using the following equation:

$$\text{Scavenging ability (\%)} = [(A_0 - A_1)]/A_0 \times 100\%$$

where $A_0$ was the absorbance of control (without sample) and $A_1$ was the absorbance of sample (*23*).

2.9.2. *Determination of hydroxyl radical-scavenging activity*

The scavenging activity for hydroxyl radicals was measured with Fenton reaction. The reaction was started by adding $H_2O_2$ and incubating at room temperature for 60 min. The absorbance of the mixture at 510 nm was measured. The hydroxyl radical-scavenging activity was calculated according to the following equation:

$$\text{Scavenging ability (\%)} = [(A_2 - A_1)]/[(A_0 - A_1)] \times 100\%$$

where $A_0$ was the absorbance of the control (blank, without $H_2O_2$), $A_1$ was the absorbance in the absence of sample, and $A_2$ was the absorbance in the presence of sample (*16*).

2.9.3. Reducing power assay

The reducing power was evaluated according to the method of Sheng (*24*). Different concentrations of each sample (0.1-20 mg/mL, 2.5 mL) were mixed with phosphate buffer (2.5 mL, 0.2 M, pH 6.6) and 5 mL of potassium ferricyanide $[K_3Fe(CN)_6]$ (1%, w/v). The mixture was incubated at 50°C for 20 min. After 5 mL of trichloroacetic acid (10%, w/v) was added to terminate the reaction, the mixture was centrifuged at 5,000 rpm for 10 min. The upper layer of solution (2.5 mL) was mixed with distilled water (2.5 mL) and $FeCl_3$ (0.5 mL, 0.1%), and the absorbance was measured at 700 nm against a blank. Vitamin C and α-tocopherol were used for comparison.

2.9.4. *Statistical analysis*

All bioassay results were expressed as means ± S.D. The experimental data were subjected to an analysis of variance for a completely random design.

## 3. Results and discussion

3.1. *Extraction, isolation and purification of polysaccharides*

Crude polysaccharide (3.54 g) was obtained from pulp tissues *L. chinensis* with hot water extraction followed by ethanol precipitation. After purification with DEAE-cellulose 52 and Sephadex G-100 column, the profiles of LCP70S-1 and LCP70W appeared as single and symmetrical sharp peaks (Figure 1), which were detected by the phenol-sulfuric acid assay. The yield of LCP70S-1 and LCP70W from crude polysaccharides was 22.4% and 12.6%, respectively. LCP70S-1 did not have any sulfate esters. The uronic acid contents of LCP70S-1 and LCP70W were below the detection limit. The average molecular weight of LCP70S-1 and LCP70W were determined to be as 11.7 kDa and 5.3 kDa respectively, by gel permeation chromatography (GPC). Calibration was performed with dextran molecular weight standards (dextran Mw: $5 \times 10^6$, $2 \times 10^5$, $7 \times 10^4$, $1 \times 10^4$, and $3 \times 10^3$; Pharmacia). HPAEC-PAD analysis showed that LCP70W was composed of glucose, and LCP70S-1 was composed of rhamnose, arabinose, and galactose in a molar ratio of 1.06:6.39:4.21.

3.2. *Structural elucidation of LCP70S-1*

The total carbohydrate content of LCP70S-1 was measured to be 98.2% (w/w) and m-hydroxybiphenyl colorimetric test for uronic acid was shown to be negative. The optical rotation of LCP70S-1 was

$$[\alpha]_D^{20} = -35° \text{ (c 1.0, 23°C, } H_2O\text{)}.$$

The composition of LCP70S-1 is presented in Table 1.

Fractions 1-3 obtained through partial acid hydrolysis were subjected to HAPEC-PAD analysis and the results were shown in Table 1. Fraction 3, the precipitate in the sack, possesses the biggest size among the three fractions. Only galatose could be detected in fraction 3, implying that galactose could be the backbone of the structure of LCP70S-1. The analysis results of fractions 1 and 2 showed that the branched structure of LCP70S-1 was composed of L-arabinose and D-galactose, and terminated with L-arabinose. The periodate-oxidized products were fully hydrolyzed and analyzed by HAPEC-PAD after periodate oxidation. The results shown in Table 1 demonstrated that there was L-rhamnose but no L-arabinose or D-galactose in the oxidation products. It could therefore be inferred that linkages of galactose were (1→), (1→2), (1→6), (1→2,6), (1→4), and (1→4,6), which could be oxidized to produce glycerol and erythritol. The presence of L-rhamnose revealed that some residues of L-rhamnose were (1→3)-linked, (1→2,3)-linked, (1→2,4)-linked, (1→3,4)- linked or (1→2,3,4)-linked, which could not be oxidized, respectively (*25,26*). The fully methylated LCP70S-1 was hydrolyzed with acid and analyzed by GC-MS. The results showed the presence of four components, namely 2,3,5-$Me_3$-Ara, 2,4-$Me_2$-Rha, 2,3-$Me_2$-Ara, 2,4-$Me_2$-Gal in a relative molar ratio of 4.23:0.78:2.17:3.84 (Table 2a). Based on the standard data in the CCRC Spectral Database for partially methylated alditol acetates (PMAA's), the linkage of L-rhamnose and D-galactose were deduced as (1→3), (1→3,6) while the linkages of L-arabinose were deduced as (1→) and (1→5). The results showed a good correlation between terminal and branching points. In addition, these molar ratios also agreed with the overall monosaccharide composition of LCP70S-1 described above.

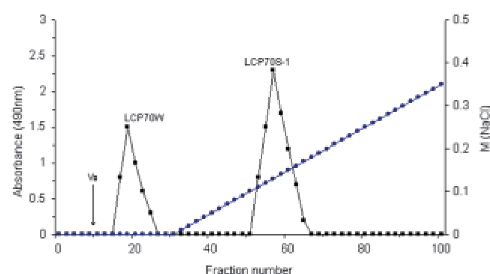


**Figure 1. Profile of LCP70 in DEAE-cellulose 52 column chromatography.** Flow rate, 0.5 mL/min. Fraction size, 1 mL/min. Void volumn, 10 mL.

**Table 1. HAPEC-PAD analysis of acid hydrolysis and Smith degradation products from LCP70S-1**

| Items | Molar ratios | | |
|---|---|---|---|
| | Rhamnose | Rabinose | Galactose |
| LCP70S-1 | 1.06 | 6.39 | 4.21 |
| Fraction 1 | 0.03 | 1.00 | 0.06 |
| Fraction 2 | 0.68 | 1.87 | 0.91 |
| Fraction 3 | n.d. | n.d. | 1.00 |
| Smith degradation | 1.00 | n.d. | n.d. |

n.d., not detected.

3.3. *IR spectroscopy and NMR analysis*

The infrared spectra of the *L. chinensis* LCP70W and LCP70S-1 polysaccharides revealed their major functional groups and the chemical bonds (Figure 2a). The broad peaks at 3,404, 3,436 $cm^{-1}$ were caused by the stretch vibration of O-H. The bands at 2925-2928 $cm^{-1}$ were attributed to the stretch vibration of C-H, and the signals at 1,635 and 1,641 $cm^{-1}$ were due to the bound water. No signal corresponding to sulfate esters was found in FTIR spectra of the two polysaccharides.

**Table 2. GC-MS analysis of partially methylated alditol acetates (PMAA) from LCP70S-1(a) and Assignment of 13C NMR chemical shifts for LCP70S-1(b)**

**(a)**

| Methylation sugar | Molar ratios | Mass fragments (m/z) | Retention time (min) | Linkage type |
|---|---|---|---|---|
| 2,3,5-Me3-Ara | 4.23 | 43, 45, 71, 87, 101, 117, 129, 161 | 13.00 | T→ |
| 2,4-Me2-Rha | 0.78 | 43, 59, 69, 75, 85, 101, 117, 129 | 14.17 | 1→3 |
| 2,3-Me2-Ara | 2.17 | 43, 87, 101, 117, 129, 189 | 15.18 | 1→5 |
| 2,4-Me2-Gal | 3.84 | 43, 71, 101, 129, 161, 189, 233 | 18.97 | 1→3,6 |

**(b)**

| Sugar residue | Chemical shifts (ppm) | | | | | | |
|---|---|---|---|---|---|---|---|
| | C1 | C2 | C3 | C4 | C5 | C6 | $CH_3$ |
| α-L-Ara(1→ | 107.69 | 76.69 | 80.27 | 70.36 | 68.62 | | |
| →3)-α-L-Rha(1→ | 103.33 | 70.08 | 81.08 | 73.58 | 69.40 | | 16.64 |
| →5)-α-L-Ara(1→ | 109.44 | 82.52 | 80.35 | 82.52 | 66.87 | | |
| →3,6)- β-D-Gal(1→ | 107.60 | 70.11 | 84.20 | 68.80 | 76.73 | 61.27 | |

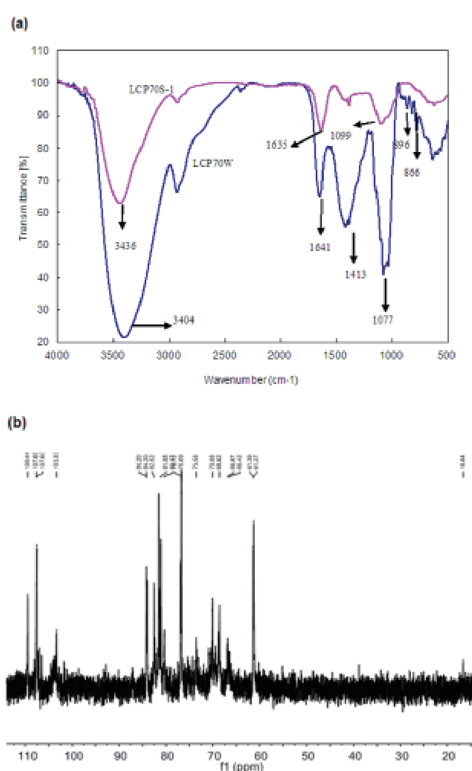


**Figure 2. FTIR (a) and 13C NMR (b) spectra of LCP70S-1.**

In addition, the peaks at 1,077 and 1,099 $cm^{-1}$ were caused by the change of angle vibration of O-H. The characteristic absorption at 896 $cm^{-1}$ indicated the presence of *β*-type glycosidic linkage. There was no absorption at 1,740 $cm^{-1}$, indicating the lack of uronic acid in the polysaccharide structure (*27*). The spectrum of $^{13}C$ NMR of LCP70S-1 was shown in Figure 2b. According to the literatures (*28,29*) the resonances in the region of 103-109 ppm in $^{13}C$ NMR were attributed to the anomeric carbon atoms of L-rhamnose (Rha*p*), D-galactose (Gal*p*) and L-arabinose (Ara*p*). The peaks at 103.33 ppm corresponded to C-1 of (1→3)-linked *α*-L-Rha units, 107.60 ppm corresponded to C-1 of (1→3,6)-linked *β*-D-Gal units, 107.69 ppm

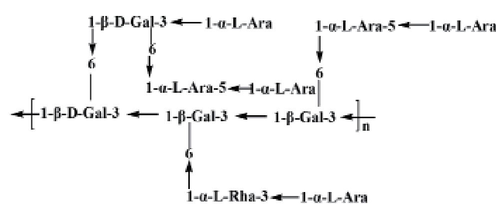


**Figure 3. Predicted structure for LCP70S-1.**

corresponded to C-1 of (1→)-linked *α*-L-Ara units, and 109.44 ppm corresponded to C-1 of (1→5)-linked α-L-Ara units, respectively. The result also indicated that the backbone was composed of (1→3,6)-linked *β*-D-galactopyranosyl residues which branches at O-6. The assignment of the carbon atoms signals was shown in Table 2b.

Based on the results of HPAEC-PAD, GC-MS and $^{13}C$ NMR, the backbone of LCP70S-1 should contain (1→3)-linked galactopyranosyl with branches at O-6. The three branches consisted of (1→3)-linked rhamnopyranosyl residues, (1→3,6)-linked galactopyranosyl and (1→5)-linked arabinofuranosyl residues, and each of them was terminated with a (1→)-linked arabinopyranosyl residues. From the aforementioned results, the repeating structural unit of LCP70S-1 could be illustrated as shown in Figure 3.

### 3.4. *In vitro antioxidant activities*

#### 3.4.1. *Scavenging activity of polysaccharides for DPPH radicals*

DPPH assay has been widely used to evaluate free radical scavenging activity of antioxidants by monitoring their ability to scavenge the stable free radical from DPPH molecule. As shown in Figure 4A, LCP70S-1, LCP70W and vitamin C reacted directly with and quenched DPPH radicals to different degrees with increased activities at higher concentrations. The maximum value of LCP70S-1

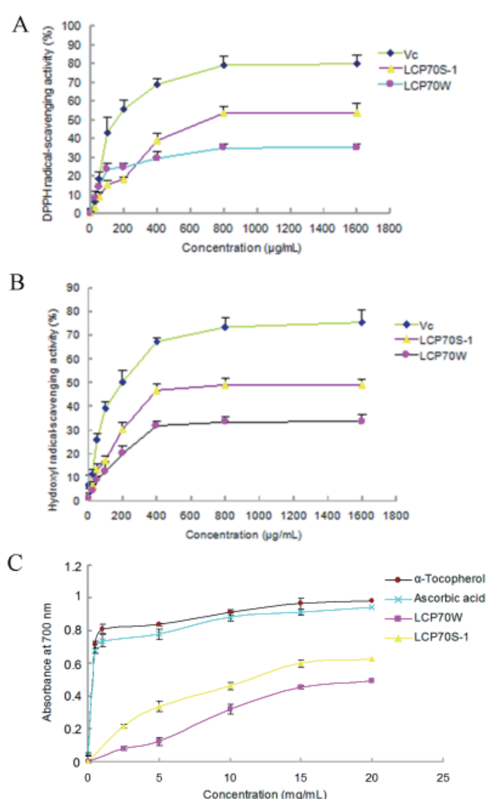


**Figure 4. DPPH radical-scavenging activity (A), hydroxyl radical-scavenging activity (B) and reducing power (C) of LCP70S-1 and LCP70W.** Values are means ± S.D. of three separate experiments.

and LCP70W reached up to 67.4% and 44.1% of that of Vc, respectively.

3.4.2. *Scavenging activity of polysaccharides for hydroxyl radicals*

Hydroxyl radical is very reactive and can be generated in biological cells through the Fenton reaction. As shown in Figure 4B, LCP70S-1, LCP70W and Vc exhibited concentration-dependent scavenging activities against hydroxyl radicals generated in a Fenton reaction system. The maximum value of LCP70S-1 and LCP70W reached to 65.0% and 44.6% of that of Vc, respectively.

3.4.3. *Reducing power*

The reducing properties were generally associated with the presence of reductones, which have been shown to exert antioxidant action by breaking the free-radical chain through donating a hydrogen atom. Figure 4C depicted the reducing power of tested samples. Higher absorbance value means stronger reducing power of samples. In this assay, the reducing power of the tested polysaccharides steadily increased with increasing sample concentration. The reducing abilities of LCP70S-1 and LCP70W at 20.0 mg/mL were 0.63 and 0.47, respectively, which were weaker than those of $\alpha$-tocopherol and Vc. Our data showed that the reducing power of the polysaccharides probably played a role in the antioxidant ability of *L. chinensis*.

In conclusion, this paper described a novel water-soluble polysaccharide (LCP70S-1) isolated from the pulp tissues of *L. chinensis* contained predominantly three monosaccharides, which were found out to be L-rhamnose, L-arabinose and D-galactose. *In vitro* antioxidant activity assays showed that this natural polysaccharide possesses free radical scavenging ability. We further illustrated that natural polysaccharide possesses free radical scavenging ability which could possibly attributed to the curative effect of *L. chinensis* as traditional Chinese medicine. Further investigation on LCP70S-1 could be launched to explore its potential as a potential antioxidant for human healthcare.

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*Original Article*

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# Galactosylation of caffeic acid by an engineered β-galactosidase

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**Summary** **Glycosylation is useful for improving the chemical properties and physiological functions of biologically and pharmacologically important compounds. The glycosylation of phenolic compounds can increase their solubility and stability in water. The addition of galactose residue has special meaning as it facilitates targeted delivery of drugs to the liver cancer cells with abundant galactose acceptors on the cell surface. In this work, the engineered β-galactosidase W980F from *Lactobacillus bulgaricus* L3 was utilized for the glycosylation of caffeic acid, a well-known phenolic phytochemical with broad bioactivities. The reaction was performed by incubation of the enzyme with 200 mM of lactose and 100 mM of caffeic acid at 45°C for 1 h. The product was purified and analyzed by MS and NMR spectra. The MS revealed a signal of $[M-H]^-$ at *m/z* 341.09, suggesting monogalactosylated products of caffeic acid ($M_r$ 342). The NMR spectra further identified the products to be caffeic acid 3'-*O*-β-galactopyranoside and caffeic acid 4'-*O*-β-galactopyranoside in a ratio of 1:3. This was the first discovery that caffeic acid could be galactosylated by the engineered glycosidase.**

***Keywords:*** *Lactobacillus bulgaricus* L3, β-galactosidase, galactosylation, caffeic acid

## 1. Introduction

Caffeic acid, 3,4-dihydroxycinnamic acid, is a well-known phenolic phytochemical present in plants such as coffee and honey. It reportedly has broad bioactivities including anti-oxidant, anti-cancer, anti-inflammatory, immunomodulatory, neuroprotective and tissue reparative effects (*1-10*). The clinical and experimental findings demonstrated anticancer properties of caffeic acid against both ER (estrogen receptor-α) positive and ER negative breast cancer (*1*). Additional data showed it attenuated solar UV-induced skin carcinogenesis (*2*) and reduced the risk of patocellular carcinoma caused by hepatitis C virus (*3*). Also, caffeic acid inhibited the multiplication of viruses such as influenza A virus, herpes simplex virus, and polio virus (*4*), and suppressed the growth of bacteria such as *Pseudomonas aeruginosa* commonly isolated from wound infections (*5,6*). Besides, caffeic acid exhibited an immunomodulatory action in human monocytes with no cytotoxic effects (*7*). Moreover, caffeic acid showed inhibitory activity against α-synuclein fibrillation, which would be helpful for design of novel therapeutic drugs for Parkinson's disease (*8*). More importantly, caffeic acid exerted neuroprotective and antidementia effects, at least in part, by preventing the loss of neural cells and synapses in ischemic brain injury (*9,10*). Despite the broad biological activities, the low stability and poor solubility of caffeic acid reduced its applicability as a pharmaceutical product (*6*).

Glycosylation is considered to be a very useful method for improving the chemical properties and physiological functions of biologically and pharmacologically important compounds (*11-15*). The introduction of the glycosyl residue into the phenolic compounds can increase their solubility and stability in water and protect them from oxidation (*11,12*). Also, glycosylation can improve biological and pharmacological functions, including the decrease of toxicity and side effects, as well as the increase of bioavailability of drugs that need to pass through the blood-brain barrier (*11*). For instance, hydroquinone is toxic while its glucoside arbutin has antibacterial and skin whitening effects (*12*). Similar finding is about the eugenol with function as a hair restorer but liable to sublimate, the glucoside of which can be gradually degraded into eugenol by the indigeneous microorganisms of human skin and acts as a pro-drug

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additive in commercial hair-restorers (*13*). Another example is that the glucosides of quercetin exhibit unexpectedly improved bioavailability (*14*). In a word, new glycosides of phenolic compounds could have novel pharmacological properties (*12*).

Variation of the introduced glycosyl residues shows different impact on the properties of phenolic compounds. The galacosylated hydroquinone is 1.19 times higher antioxidant than the glucoside counterpart, arbutin (*15*). More interestingly, there exist abundant galactose acceptors on the hepatocyte surface that could bind with galactose derivatives (*16*). Thus, the glycosylation with galactose could aid targeted delivery of drugs to liver cancer cells but not to nearby normal cells. For example, the galatosylated lithocholic acid shows high specificity to mouse liver cells *in vivo* (*17*).

Generally, there are two basic classes of methods for the glycosylation (*18*). One is the chemical method that requires laborious protection and deprotection steps to control the stereo- and regio-specificity of products, along with the use of toxic catalysts and solvents. The other is the enzymatic glycosylation that possesses the advantages of high stereo- and regio-selectivity and can be accomplished in one step under mild conditions. Glycosyltransferases (EC 2.4) and glycosidases (EC 3.2.1) are two classes of enzymes responsible for this application (*19*). Typically, glycosyltransferases are effective and catalyze stereo/regio-selective reactions, but they have strict substrate selectivity and require costly glycosyl donors in the one-step reactions. As for large-scale synthesis, the glycosidases exhibit obvious advantages including the readily available enzyme source, the simple and inexpensive donor substrates, as well as the wide acceptor tolerance (*20*).

β-Galactosidases (EC 3.2.1.23) are among the most important glycosidases that can catalyze the galactosylation process (*21*). However, only one natural β-galactosidase from *Kluyveromyces lactis* was reported to be able to galactosylate hydroquinone (*15*). The low nucleophilicity of phenolic hydroxyl groups might be related to the difficulty of their glycosylation by the enzymes. In the previous work, the β-galactosidase from *Lactobacillus bulgaricus* L3 had been engineered to possess high transglycosylation activity toward a series of single phenolic-ring compounds including phenol, hydroquinone, catechol and pyrogallol (*22*). In this work, the enzyme was extendedly used to glycosylate the caffeic acid with a more complex phenolic structure, resulting in caffeic acid galactosides potentially with novel pharmacological properties.

## 2. Materials and Methods

### 2.1. *Strains*

The β-galactosidase gene from *L. bulgaricus* L3 had been submitted to GenBank with accession No. EU734748.1. This gene was inserted into the pET-21b vector (pET-21b-bga) and subjected to site mutation (W980F) in the previous work (*22*). The *Escherichia coli* BL21(DE3) carrying the recombinant wild-type and mutant genes were cultured in LB medium containing 10 g of peptone, 5 g of yeast extract and 5 g of NaCl in 1,000 mL of water (pH 7.5), supplemented with ampicillin at 100 μg/mL. The solid medium additionally included 15 g/L agar.

### 2.2. *Preparation of the W980F enzyme*

The *E. coli* BL21(DE3) carrying the recombinant mutant genes was grown in 30 mL of LB medium containing ampicillin at 37°C for 12 h, and then transferred into 1 L of fresh medium in a 1:100 (v/v) ratio. The enzyme was induced by addition of isopropyl-1-thio-β-D-galactoside (IPTG) when the cell density reached 0.6-1.0 at 600 nm. After continuous cultivation for 3 h, the cells were harvested and disrupted by ultrasonic treatment. The lysate was centrifuged at 12,000 rpm for 30 min at 4°C and the enzyme was purified from the suspension by $Ni^{2+}$ chelation chromatography. The column with 5 mL of Ni Sepharose (GE Healthcare) was equilibrated with phosphate buffer (50 mM sodium phosphate, 500 mM sodium chloride, pH 7.0). The column was loaded with samples and washed with phosphate buffer and subsequent with washing buffer (50 mM sodium phosphate, 500 mM sodium chloride, 50 mM imidazole, pH 7.0). Bound protein was eluted with elution buffer (50 mM sodium phosphate, 500 mM sodium chloride, 300 mM imidazole, pH 7.0). Aliquots of the fractions were run on 10% SDS-PAGE and stained with Coomassie Brilliant Blue to analyze their purity. Fractions containing the pure protein were pooled, dialyzed and stored at -20°C. The wild-type enzyme was prepared with the same procedures.

### 2.3. *Enzyme and protein assays*

The β-galactosidase activity was measured by adding 50 μL of enzyme solution to 450 μL of 2 mM *o*NPGal. The reaction was performed at 37°C for 10 min and then stopped by adding 1 mL of 500 mM $Na_2CO_3$. The amount of *o*-nitrophenol released was measured at 420 nm. One unit of enzyme activity (U) was defined as the amount of enzyme required to liberate 1 μmol of *o*-nitrophenol per minute under the assay conditions. The amount of protein was quantified by the method of Lowry with bovine serum albumin as the standard.

### 2.4. *Transglycosylation reactions*

The transglycosylation reactions were performed at 45°C for 1h in 50 μL mixtures (pH 7.0) containing 0.2 μg of pure enzyme, 200 mM of lactose and 100 mM of caffeic acid. The control reactions followed the same

conditions except for the use of inactivated enzyme. All the reactions were terminated by heating at 100°C for 5 min. Glycoside products were detected by TLC and HPLC as described below.

2.5. *TLC and HPLC analysis*

TLC was performed with Silica gel 60 F254 plates (Merck, Germany). The developing solvent was a mixture of *n*-butanol:ethanol:water (5:3:2, v/v/v). Sugars on the TLC plate were detected by spraying with a solution of 0.5% (w/v) 3,5-dihydroxytoluene dissolved in 20% (v/v) sulfuric acid and subsequent heating at 120°C for 5 min. HPLC was performed by Agilent 1200 series equipped with Agilent Zorbax carbohydrate analysis column (4.6 × 250 mm). The column temperature was maintained at 30°C. Samples were eluted with 85% (v/v) acetonitrile at a flow rate of 1.0 mL/min, through a refractive index detector (G1362A) for the sugar analysis or a UV detector (G1314B) at 254 nm for the analysis of phenolic derivatives. Conversion yield of aromatic product (%): [product concentration (mM)/concentration of lactose used (mM)] × 100 (*15*).

2.6. *Purification of glycoside products*

Ten milliliters of transglycosylation reactions were performed at 45°C for 1 h. The resulting glycosylated products were concentrated by vacuum freeze dehydration. Then, the samples were subjected to a Bio-Gel P2 column (1.5 × 100 cm) and subsequently loaded on preparative Silica gel 60 $F_{254}$ 1-mm plates (Merck, Germany) for purification.

2.7. *MS and NMR analysis*

Mass spectra were recorded on a Shimadzu LCMS-IT-TOF instrument (Kyoto, Japan) equipped with an ESI source in negative ion mode at a resolution of 10,000 full width at half-maximum. $^{1}$H and $^{13}$C NMR spectra were recorded at 26°C with a Bruker DRX Advance-600 spectrometer (Bruker Biospin AG, Fallanden, Switzerland) at 600 MHz for $^{1}$H and 150 MHz for $^{13}$C. Chemical shifts were given in ppm downfield from internal TMS of $D_2O$. Chemical shifts and coupling constants were calculated from a first-order analysis of the spectra. Assignments were fully supported by homo- and hetero- nuclear correlated 2D techniques, including COSY (correlation spectroscopy), HSQC (hetero-nuclear single quantum coherence) and HMBC (hetero-nuclear multiple band correlation) experiments following standard Bruker pulse programs.

## 3. Results and Discussion

The wild-type and mutant (W980F) recombinant β-galactosidases from *L. bulgaricus* L3 were expressed in *E. coli* BL21 (DE3), and subsequently purified to electrophoresis purity through $Ni^{2+}$ chelation chromatography. The ability of their transglycosylation toward caffeic acid were tested by incubation of the enzymes with lactose and caffeic acid at 45°C for 1 h. Then the reactions were detected by TLC. As shown in Figure 1a, an obvious novel product spot appeared in the reaction catalyzed by the mutant W980F when compared with that catalyzed by the wild-type enzyme. In the β-galactosidase-catalyzed reactions, the side products, glucose and galactose, were formed by the enzymatic hydrolysis of lactose, while the prebiotic galactooligosaccharides (GOS) were produced by the enzymatic self-condensation of lactose (*21,22*). The migration distance of the novel product was larger than the monosaccharides resulting from the hydrolysis reaction, consistent with the characteristic of the phenolic glycoside. HPLC analysis of the reaction mixture revealed a 7% conversion yield of the new product.

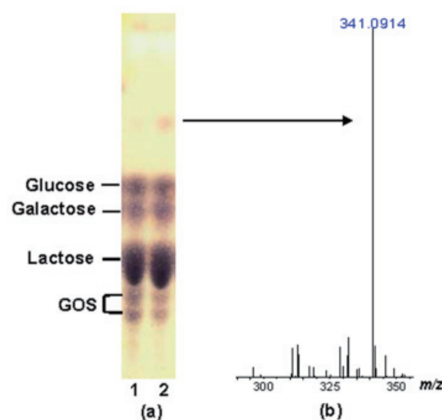


**Figure 1. TLC analysis of the transglycosylation reactions catalyzed by wild-type and mutant enzymes (a) as well as MS analysis of the purified novel product (b).** Lane 1, reaction catalyzed by the wide-type enzyme; Lane 2, reaction catalyzed by the W980F enzyme.

The novel product was purified and analyzed by negative ESI-MS. Figure 1b showed a characteristic signal of $[M-H]^-$ at *m/z* 341.09, confirming that the newly produced glycoside was monogalactoside ($M_r$ 342) of caffeic acid ($M_r$ 180). Although the TLC result revealed one spot that was monoglycosylated as determined by MS, NMR analysis of the derivative displayed signals of two products (Figures S1-S5, *http://www.ddtjournal.com/docindex.php?year=2015&kanno=2*). The signals of galactose were overlapped while those of caffeic-acid residues were separate. In the $^{1}$H NMR spectra, chemical shifts of the galactose protons located at $\delta$ 4.92 to 3.64 ppm while those of caffeic acid were in the range of $\delta$ 7.27 to 6.22 ppm (Figure S1). The anomeric H-1 peaks at 4.92 ppm as well as the coupling constant of H-1 and H-2 ($J$ = 7.8 Hz) confirmed the β-linkage between the sugar and caffeic acid. In $^{13}$C NMR spectrum, chemical shifts of the sugar carbons located at $\delta$ 101.1 to 60.5 ppm while those

from $\delta$ 181.4 to 114.6 ppm belonged to the caffeic-acid residues (Figure S2).

The complete structural characterization was achieved using 2D-NMR analysis, including $^1$H-$^1$H COSY, $^1$H-$^{13}$C HSQC and HMBC experiments, to assign the chemical shifts and configurations. In HMBC, the C-3′ ($\delta$ 144.9) and C-4′ ($\delta$ 145.9) in two separate phenolic rings displayed correlation signals with the anomeric H-1 of the sugar residue, respectively (Figure S5). Based on the above analysis, the chemical structure of the glycoside products were identified to be caffeic acid 3'-*O*-β-galactopyranoside and caffeic acid 4'-*O*-β-galactopyranoside, respectively. According to the peak area of phenolic H-5' in the $^1$H NMR spectra, the ratio of the two products was 1:3. Thus, their structure data were summarized in Table 1.

Figure 2 showed the outline of the biosynthesis of these compounds using the engineered β-galactosidase from *L. bulgaricus* L3. The caffeic acid was galactosylated for the first time *via* glycosidase-mediated catalysis. Although glycosidases have great advantage of relaxed substrate specificity for acceptors in the enzymatic synthesis, the glycosylation of phenolic compounds were still challenging for these enzymes. The low nucleophilicity of phenolic hydroxyl groups might be responsible for the difficulty in the enzymatic glycosylation when compared to the alcoholic hydroxyl nucleophiles that are easily glycosylated by the enzymes. Currently, only a few natural glycosidases were discovered to be able to glycosylate phenolic compounds. They were glucose-transferred glycosidases in most cases, such as α-amylase (EC 3.2.1.1) and α-glucosidase (EC 3.2.1.20) from *Bacillus subtilis*, *Saccharomyces cerevisiae*, *Xanthomonas campestris* and *Xanthomonas maltophilia* (*12,13, 23-25*). Only one natural β-galactosidase was available from *K. lactis* for the galactosylation of simple hydroquinone (*15*).

The β-galactosidases have attracted particular interest due to their production of promising galactose-containing chemicals, including diverse oligosaccharides, alkyl-glycoside, glycoconjugates and others that play important roles in the industries of food additives, cosmetics, and medicines (*21*). However, these enzymes still have limitations in the glycosylation of phenolic compounds that have many physiological functions. The β-galactosidase from *L. bulgaricus* L3 was proved to be a wonderful tool for glycoside synthesis in the previous work. It could efficiently synthesize galacto-oligosaccharides as well as 6'-galactosyl sucralose with combined functions of prebiotics and sweeters (*26-28*). In the recent work, the site-saturation mutagenesis of the β-galactosidase from *L. bulgaricus* L3 generated a W980F mutant with broadened substrate specificity toward pyrogallol. In this work, the W980F was found to be able to glycosylate caffeic acid, suggesting the change of tryptophan into phenylalanine made it possible for glycosylation of more complex polyphenol structure. The acceptor specificity of the enzyme was further expanded. This was a breakthrough in the galactosylation of unusual acceptors by glycosidases. Also there reported other successful examples of glycosidases engineering that optimized enzyme properties including broadening the acceptor specificity. One example was that the nucleophile variant of cellulase (E197S) from *Humicola insolens* displayed transglycosylation activity towards flavonoid acceptors, which were not part of its normal substrate (*29*).

It is also worth noting that glycosylation of the caffeic acid was of great meaning considering that glycosylation of phenolic compounds could bring improved properties such as increased solubility and stability in water (*11-15*). The solubility of caffeic acid in water as well as its stability toward light irradiation

**Table 1. $^1$H and $^{13}$C NMR data assignment for the caffeic-acid glycosides produced by the W980F enzyme**

| C-atom | CA-4'-*O*-β-Gal* | | CA-3'-*O*-β-Gal | |
|---|---|---|---|---|
| | $\delta$C | $\delta$H | $\delta$C | $\delta$H |
| C-1 | 101.1 | 4.92 | 101.1 | 4.92 |
| C-2 | 70.3 | 3.72 | 70.3 | 3.72 |
| C-3 | 72.3 | 3.64 | 72.3 | 3.64 |
| C-4 | 68.3 | 3.86 | 68.3 | 3.86 |
| C-5 | 75.4 | 3.73 | 75.4 | 3.73 |
| C-6 | 60.5 | 3.64 | 60.5 | 3.64 |
| C-1' | 130.9 | - | 127.9 | - |
| C-2' | 114.6 | 7.04 | 114.9 | 7.27 |
| C-3' | 145.6 | - | 144.9 | - |
| C-4' | 145.9 | - | 147.3 | - |
| C-5' | 116.0 | 7.02 | 116.5 | 6.82 |
| C-6' | 120.8 | 7.00 | 123.9 | 7.08 |
| C-7' | 175.9 | - | 181.4 | - |
| C-8' | 122.9 | 6.25 | 121.8 | 6.22 |
| C-9' | 140.0 | 7.14 | 140.4 | 7.16 |

* CA, caffeic acid.

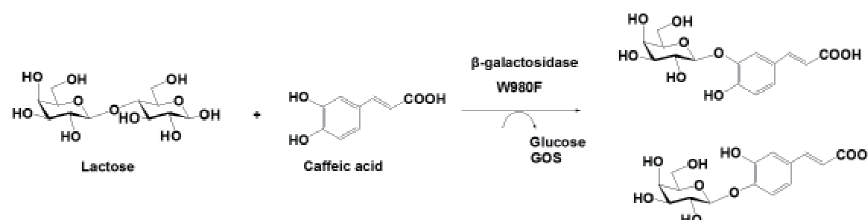


**Figure 2. W980F-catalyzed glycosylation of caffeic acid and chemical structures of the glycoside products.**

had been improved by the glucosylation without the loss of any biological activity, such as its antiozidative and antimutagenic properties (*23*). This confirmed the positive effects of glycosylation on the optimization of phenolic compounds. As properties of glycosides could vary with the type of sugars attached, it is of particular meaning to modify phenolic compounds with novel sugars. The W980F-mediated galactosylation provided an alternative to the existing enzymatic glucosylation method for the modification of caffeic acid. The enzymatic introduction of galactose into the caffeic acid would bring promising properties and widen the scope of their applications such as the design of liver-targeted drugs. Thus, the caffeic acid galactosides obtained in this work not only possess inherent values by themselves but also could act as intermediates for further modification to be highly valuable products.

**Acknowledgements**

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## Commentary

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# Cancer-associated carbohydrate antigens for clinical diagnostic markers – its effectiveness and limitations

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**Summary** **Cancer cells express various aberrant glycoconjugates. Several kinds of carbohydrate antigens have been used for the serological tumor markers. In particular, the serological level of sialylated carbohydrate antigens, which contain the sialic acid residue in their structure, showed effectiveness in diagnosing cancer behavior. Although large number of carbohydrate antigens in serum of cancer patients was elevated broadly in various cancers, each tumor marker has different sensitivity and specificity for each cancer. Therefore, the combined use of several tumor markers which have different characteristics is effective for better sensitivity in diagnosing cancer behavior. The mechanism of synthesizing cancer-associated carbohydrate antigens is not fully understood because it is very complex. In addition, new cancer-associated carbohydrate antigens are also identified by molecular oncological studies. Those investigations are considered to develop more effective tumor markers to diagnose cancer behavior.**

***Keywords:*** Tumor marker, sialylated carbohydrate antigens, clinical diagnosis

Glycoconjugates are important factors for various biological events in an organism. Aberrance of glycoconjugates, *e.g.* the change of structure or expression level, is detected in diseased patients and suggested to associate with the progression of diseases. In cancer, various aberrant glycoconjugates are expressed in cancerous tissues and investigated in relation to cancer behavior such as invasion and metastasis. Several kinds of those overexpressed glycoconjugates are detected in the serum of cancer patients and used as clinical diagnostic markers. The serological tumor markers are considered to be effective tools for screening cancer patients in routine medical care and monitoring the state of cancer patients.

In clinical tumor markers, there are many kinds of cancer associated carbohydrate antigens (Table 1). Each tumor marker has different sensitivity and specificity for various cancers. A large number of those carbohydrate antigens in serum of cancer patients is elevated broadly in several gastrointestinal and gynecological cancers. Carbohydrate antigen (CA) 15-3 has been used for the evaluation of metastasis and recurrence of breast cancer although its sensitivity is poor (*1,2*). CA125, whose epitope is on the tandem repeat peptide in MUC16, is suggested to be effective for longitudinal monitoring of ovarian cancer (*3*). Patients with cardiovascular disease are also suggested to show the elevation of CA125. In a study of patients with congestive heart failure, patients in a more advanced stage showed elevation of CA125 level (*4*). Therefore, CA125 shows a false positive if the patients suffered from those diseases. On the other hand, alpha-fetoprotein (AFP), which is frequently used as the diagnostic marker of hepatocellular carcinoma (HCC), also has carbohydrate structures. Elevation of AFP level also detects in benign liver diseases such as hepatitis and liver cirrhosis. The *Lens culinaris* agglutinin-reactive fraction of AFP (AFP-L3), which receives the structural change of carbohydrate (addition of fucose residue), contributes to an increase in sensitivity in screening HCC patients (*5,6*). The peptide structure or glycoform of the epitope of these antigens have been investigated insufficiently.

Sialylated carbohydrate antigens, which contain sialic acid residues in their structure, frequently overexpress in cancer and are used for diagnosing cancer behavior.

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**Table 1. Carbohydrate antigens that are used for clinical tumor marker**

| Tumor markers | Cancers which show significant elevation | *References* |
|---|---|---|
| CA15-3 | Breast cancer | *(1,2)* |
| CA125 | Ovarian cancer, Uterus cancer | *(3, 27-29)* |
| CA72-4 | Ovarian cancer, Uterus cancer, Gastric cancer | *(17-19)* |
| CA19-9 | Colorectal cancer, Gastric cancer, Pancreatic cancer, Bile duct cancer | *(12-14, 30)* |
| sialyl $Le^x$ | Lung cancer, Gastric cancer, Bile duct cancer | *(31-33)* |
| sialyl Tn | Ovarian cancer, Uterus cancer, Gastric cancer | *(16,32-34)* |
| DUPAN-2 | Pancreatic cancer, Bile duct cancer | *(20-22)* |
| SPan-1 | Pancreatic cancer, Bile duct cancer | *(35,36)* |

Many immunohistochemical studies showed the overexpression of sialylated carbohydrate antigens in cancer tissues by using lectins (*7-10*). Sialylation, the moiety of silalic acid, is considered to play an important role in tumor progression. Although various structures of sialylated carbohydrate are considered to be synthesized in cancer cells, specific types contribute to the clinical diagnosis for each kind of cancer. In the case of gastrointestinal cancers, sialyl Lewis-related antigens such as sialyl Lewis x (sialyl $Le^x$) and sialyl Lewis a (sialyl $Le^a$) antigens are the most often investigated tumor markers as sialylated carbohydrate antigens (*11*). Sialyl-$Le^a$ antigen is also known as CA 19-9 and frequently used in clinical diagnosis combined with Carcinoembryonic antigen (CEA) (*12-14*). The combined evaluation of CEA and CA19-9 is recommended for better sensitivity in diagnosing the prognosis of gastrointestinal cancer patients. Another cancer-associated sialo-oligosaccharide antigen is sialyl Tn antigen. This antigen is barely expressed in normal epithelial tissue and is carried on cancer-associated proteins such as CD44 and mucins (*15,16*). Those antigens are considered to have an important biological role in cancer invasion and metastasis. Serological levels of CA72-4, related to sialyl Tn antigen, are elevated in patients with gastric cancer and ovarian cancer (*17-19*). DUPAN-2, which is identical with sialyl $Le^c$ antigen, can be used for supportive diagnosis or monitoring behavior of pancreatic cancer (*20-22*). This antigen is also elevated in patients with hepato-biliary diseases but not with gastrointestinal cancers. Those sialylated carbohydrate antigens have been suggested to be related to induction of worse cancer behavior such as cancer cell invasion and metastasis while the detailed mechanism is still under investigation.

Mechanisms of overexpression of cancer-associated carbohydrate antigens are very complex. Up-regulation of glycosyltransferases was suggested to induce the aberrant structure of carbohydrate chains. There are many kinds of glycosyltransferases expressed in cancer cells, and therefore differences in up-regulated mechanisms caused by the different kinds of tumor markers for each cancer. In the case of sialic acid-containing carbohydrate antigens, down-regulation of ST6GalNAc VI, which transfers α2,6-linked sialic acid to GlcNAc to synthesize a disialyl Lewis structure, is suggested to induce the elevated levels of sialyl Lewis-related antigens (*23,24*). Biochemical and pathological studies performed by Marcos NT *et al*. suggested the main regulator of sialyl Tn antigen expression was ST6GalNAc-I activity (*25,26*). Investigating the change of expression and activity of glycosyltransferases is considered to contribute to the elucidation of cancer biology including the expression of tumor markers.

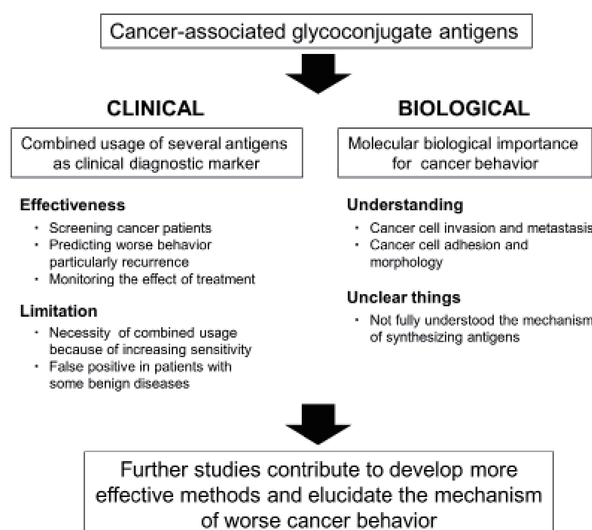


**Figure 1. Current effectiveness and limitation of cancer-associated glycoocnjugate antigens.**

Serological tumor markers have been used for monitoring the effect of surgical treatment or chemotherapy for cancer. If the level of tumor marker is elevated before the patient received the cancer therapy, an altered level of tumor marker is suggested to mean a change of cancer behavior. However, as described above, multiple tumor markers should be evaluated simultaneously to increase the sensitivity of diagnosis. Carbohydrate antigens which have different structures and specificity were identified. Therefore, effective use of those current tumor markers can contribute to a powerful diagnostic method while other imaging techniques are necessary for definitive diagnosis (Figure 1). In addition, new cancer-associated glycoconjugates can also be investigated for specific detection of cancers. Further investigation is expected to develop more sensitive and specific tools to screen cancer patients at an early stage.

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*Mini-Review*

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# Current use of silkworm larvae (*Bombyx mori*) as an animal model in pharmaco-medical research

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**Summary** **We advocate the use of silkworm larvae, *Bombyx mori*, as an animal model for discovery of drug candidates. We have established several disease models using silkworms, which offer technical advantages in drug development and the study of host-pathogen interaction. This mini review briefly describes recent trends in the use of silkworm larvae as a non-mammalian model for drug discovery and it offers suggestions regarding the potential for silkworm use in pharmaceutical-biomedical research.**

***Keywords:*** Animal model, silkworm, drug discovery

## 1. Merits of silkworm models

More often than not, evaluation of drug candidates involves problems deciding which animals to use. These problems can be classified into ethical issues in terms of animal welfare, cost, and ease of maintenance (*1,2*). In Asia and Africa, little attention has been paid to ethical issues. However, research bodies and institutions around the world have committees that oversee experimental use of animals. The guiding rationale behind rules regulating animal use are the potential benefit to human health, minimized pain or distress to animals, and use of timely and humane procedures for euthanasia based on the principles of Replacement, Reduction, and Refinement (*3-5*). Problems with animal use during evaluation of the therapeutic effects of pharmaceuticals can be avoided through use of alternative animal models, and especially non-mammalian models. Non-mammals, such as *Caenorhabditis elegans* and *Drosophila melanogaster*, have been used as models (*6-9*). However, these animals are too small to handle and are thus unsuitable for injecting an accurate volume of a test sample, which is a technical requirement for *in vivo* experiments. Therefore, the use of silkworm larvae, *Bombyx mori*, may represent a way to overcome these problems. Courtesy of sufficient size, silkworm larvae offer a number of advantages, such as facilitation and accuracy of intra-hemolymph injection (corresponding to intravenous injection in mammals), the possibility of intra-midgut injection (corresponding to oral administration in mammals), and the ease of organ isolation for research (*10-13*). Moreover, typical physical changes (like variation in color, a reduction in size or elongation, and slowed movement) are readily apparent when comparing these phenotypes with those of the controls. One need not wait for larvae to die before making reproducible scientific inferences, especially in time or dose-dependent experiments.

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## 2. Evaluation of the therapeutic effect of antibiotics

Silkworm larvae have been used to evaluate the virulence of human pathogenic bacteria. For instance, previous reports have shown that *S. aureus*, *P. aeruginosa*, and *V. cholera*, were lethal to silkworm models (*12,13*). The current authors' research group has used a silkworm model of infection to evaluate S. aureus virulence factors (*14,15*). The agr locus, a hemolysin gene regulator, was found to contribute to the pathogenicity of S. aureus in animal hosts (*15*). Silkworm models have also been used to study other virulence factors, including bacterial toxins and hemolysins (*16*). By extension, this means that silkworm is an effective model organism for the study of host-pathogen interaction and genetic changes and virulence factors of pathogens.

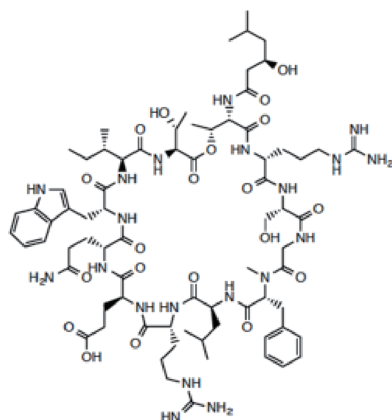

**Figure 1. Structure of lysocin E discovered using a silkworm model of infection** (*20*). A cyclic-lipopeptide, lysocin E is a novel antibiotic that is effective against Gram-positive bacteria including methicillin-resistant *Staphylococcus aureus* (MRSA).

Hamamoto *et al*. using silkworm models to quantitatively evaluated the therapeutic effects of known antibiotics (including vancomycin, kanamycin, chloramphenicol, flomoxef, teicoplanin, tetracycline, minocycline, arbekacin, and linezolid), and they reported that in the model drugs had an $ED_{50}$ against human pathogenic bacteria (*Staphylococcus aureus and Stenotrophomonas maltophilia*) and true fungi (*Candida albicans* and *Candida tropicalis*) that was consistent with the $ED_{50}$ when those drugs were administered to mice (*11*). In a silkworm model of infection, orally administered antibiotics displayed potency similar to that displayed in larger animals. A study also noted that the silkworm model was suitable for evaluation of the toxicity and metabolism of therapeutic drug candidates (*17*). Silkworm models, therefore, offer a more economic means of testing the effectiveness of antibiotics. Nosokomycin, a derivative of moenomycin A from *Streptomyces sp*. K04-0144, was discovered in 2010 in a silkworm model of MRSA infection (18). More recently, lysocin E (Figure 1), a bactericidal antibiotic from the cultured supernatant of the soil bacterium *Lysobacter sp*. RH2180-5, was discovered in a silkworm model of infection and was found to target menaquinone, a component of the electron-transport chain in the membrane of Gram-positive bacteria (*19*). Furthermore, a silkworm model of infection is an excellent model with which to exclude non-therapeutic molecules.

## 3. Models to evaluate anti-diabetic drugs

During the development of hyperglycaemic silkworm model for anti-diabetic drug screening, Matsumoto *et al*. (*20,21*) reported an immediate rise in the hemolymph sugar level (corresponding to blood glucose in mammals) in larvae fed a high glucose diet. Larvae fed a high glucose diet were found to suffer from hyperglycemia-related disorders, including reduced body size (Figure 2). Much like mammals, silkworms have been shown to possess a regulatory system for hemolymph sugar homeostasis (*22*). The anti-hyperglycemic effect of known anti-diabetic drugs was successfully evaluated using hyperglycemic silkworm model (*20*). The administration of recombinant human insulin led to a decrease in hemolymph sugar levels in larvae fed a high glucose diet and consequently restored growth (*20*). The AMPK-signaling pathway plays an important role in maintaining systemic glucose levels in larger animals, and this pathway was also found to function in hyperglycemic silkworms (*20*).

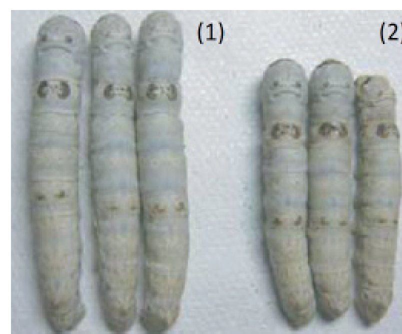


**Figure 2. Hyperglycemic silkworms show growth defects** (*21*). $5^{th}$ instar larvae fed a normal diet and injected with **(1)** saline or **(2)** glucose solution.

'Humanized silkworms', which refer to transgenic silkworms expressing human insulin receptors, have also been used to evaluate therapeutically active insulin receptor agonists (*21*). Administration of human insulin to humanized silkworms decreased hemolymph sugar levels and facilitated phosphorylation of *Akt*, which is a key factor of insulin-signaling pathway, in the fat body. Therefore, silkworms are not only useful animals but also are also simpler, cheaper, and easier to handle when evaluating anti-diabetic drug candidates. Moreover, silkworm models could be used to study other human metabolic disorders, such as obesity and hypertension, given the similarities in the patho-physiological mechanisms of silkworm and larger animals.

## 4. Prospects for the future and potential challenges

Silkworm larvae represent a satisfactory model for discovery and evaluation of therapeutic substances. The aforementioned findings demonstrate that silkworms compare favorably to other animal models. However, certain areas of pharmaco-medical research, such as those involving neurological disorders and aging, have yet to embrace the use of silkworm larvae as animal models. One argument against the use of silkworms in such studies is because larvae do not contract the same genetic diseases as humans. Therefore, genetic engineering techniques (biotechnology procedures to manipulate the silkworm genome using gene knock-

outs and knock-ins) have been established (*10*), and silkworm genes could be manipulated to induce such disease states. Thus, diseases not naturally contracted by insects could be successfully studied using genetically engineered silkworms. In this way, silkworm via genetic modification present scientists with models for addressing mechanistic questions and molecular targets in drug discovery research.

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*Original Article*

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# Comparison and combination effects on antioxidant power of curcumin with gallic acid, ascorbic acid, and xanthone

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**Summary** **Curcumin has been extensively reported as a potential natural antioxidant. However, there was no data on activity comparison as well as the biological interactions of curcumin with other natural antioxidants. The aim of the present study was to investigate the antioxidant power of curcumin in comparison with three important natural antioxidants; gallic acid, ascorbic acid, and xanthone on free radical scavenging action and their combination effects on this activity. The results indicated that the activities of these compounds were dose-dependent. The 50% effective concentration ($EC_{50}$) of curcumin was found to be 11 µg/mL. Curcumin showed significantly higher antioxidant activity than ascorbic acid and xanthone but less than gallic acid. Interestingly, curcumin revealed synergistic antioxidant effect when combined with gallic acid whereas the antagonistic effect occurred in curcumin combination with ascorbic acid or xanthone. These results suggest that curcumin-gallic acid combination is the potential antioxidant mixture to be used in place of the individual substance whereas using of curcumin in combination with ascorbic acid or xanthone should be avoid.**

***Keywords:*** Antioxidant, curcumin, gallic acid, synergistic effect, antagonistic effect

## 1. Introduction

There are abundant studies showing the role of antioxidants on reduction of radical oxygen species (ROS) in the maintenance of human health and prevention as well as treatment of various diseases caused by the excessive ROS. Antioxidants from bio-resources have received much attention because of their points of safety and natural aspects. Most plants in the world contain various kinds of phytochemical compounds such as phenolic acids, curcuminoids, carotenoids, and flavonoids which are the major sources of natural antioxidants (*1*).

Curcumin is a well-known natural antioxidant existing in various kinds of plants, especially in *Curcuma longa* L. (turmeric). Curcumin in its crude form has been used for many centuries as spice and dietary supplement as well as component of many traditional Asian medicines. It has been reported that curcumin has a wide range of pharmacological activities against many chronic diseases including type II diabetes, rheumatoid arthritis, multiple sclerosis, Alzheimer's disease and atherosclerosis (*2-6*). Further, curcumin inhibits platelet aggregation, suppresses thrombosis, inhibits human immune deficiency virus (HIV) replication, enhances wound healing and prevents liver injury, cataract formation, pulmonary toxicity and fibrosis (*7-9*). Many reports revealed that curcumin has these pharmacological activities because of its basic beneficial antioxidant, anti-inflammatory, antibacterial, and anticancer activities (*10,11*). Regarding to the antioxidant role, curcumin has been demonstrated to have an effective scavenging property on various harmful free radicals which not only ROS but also nitrogen dioxide radicals, superoxide anions and hydroxyl radicals (*12*). Curcumin also displayed the high binding affinity on metal ions that related to the ROS formation especially on the iron chelating ability (*13*). Moreover, curcumin can inhibit free radicals from mediating lipid peroxidation of membranes or oxidative DNA damage which are the important initiator for carcinogenesis (*14,15*).

Three other natural compounds that are generally classified as potential antioxidants are gallic acid, ascorbic acid, and xanthone. They are the antioxidants found in many kinds of plants. Gallic acid is a phenolic antioxidant commonly found in fruit and flowering

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plants, for example, grapes, berries, lemons, oak barks as well as in wine (*16,17*). It has been reported to have anti-inflammatory, anticancer, and anti-hyperglycemic activities (*18*). Ascorbic acid or vitamin C is an antioxidant exists in many kinds of fruits. It has been effectively used as a standard antioxidant for free radical scavenging evaluation. The previous study demonstrated that ascorbic acid involved in many biological functions such as a cofactor of enzymatic reaction and played a role on cancer and chronic diseases through an antioxidant activity (*19*). Xanthone is one of the important natural antioxidants. It has been studied for recent years and reported that xanthone possesses anticancer, anti-inflammatory, antibacterial, and cardioprotective activities (*20,21*).

Even the antioxidant activity of curcumin has been extensively reported, there was only the action of the individual compound. Taken together of curcumin with gallic acid, ascorbic acid, or xanthone, it is assumed to get the higher beneficial antioxidant effects of free radical scavenging property. However, to our knowledge so far there are no studies on these biological effects of curcumin in combination with these compounds. The present study explores not only the combination effects but also the comparative antioxidant power of curcumin in comparison with gallic acid, ascorbic acid and xanthone individually.

## 2. Materials and Methods

### 2.1. *Materials*

Analytical grade curcumin, gallic acid, ascorbic acid, 6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid (Trolox) and 2-diphenyl-1-picrylhydrazyl (DPPH) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Xanthone was extracted from *Garcinia mangostana* fruit peels (*22*). Absolute ethanol was purchased from Merck (Darmstadt, Germany). Other reagents and chemicals were of the highest grade available.

### 2.2. *Samples preparation*

Stock solution of curcumin, gallic acid, ascorbic acid, and xanthone were prepared by dissolving in ethanol and diluted to get the concentrations of 1000, 500, 250, 125, and 62.5 μg/mL. Stock standard Trolox solution was prepared and diluted to have the concentration series of 125, 100, 75, 50, 25, and 12.5 μg/mL.

### 2.3. *Effect of reaction time on antioxidant activity*

The DPPH free radical-scavenging method performed by Okonogi *et al*. (*23*) with some modification was used to measure the free radical scavenging activity of the samples. Briefly, 20 μL of the ethanolic solution of curcumin, gallic acid, ascorbic acid, or xanthone at a concentration of 50 μg/mL was pipetted into a microtiter plate. Then 180 μL of $10^{-4}$ M DPPH in ethanol or 180 μL of absolute ethanol as a negative control was added and mixed rapidly. The absorbance of the samples was measured at 540 nm by using microtiter plate reader (Biorad 680, Hercules, CA, USA) every 10 min over 60 min time period.

### 2.4. *Determination of antioxidant activity of a single compound and a combination*

In this study, the determination of antioxidant activity of the test samples was performed as described in section 2.3, however the absorbance was recorded at 30 min of reaction and the concentration of the test substances was varied as followings. For the antioxidant comparative test of the individual compound, 20 μL of the ethanolic solution of curcumin, gallic acid, ascorbic acid, or xanthone at the concentration ranging from 6.25 μg/mL to 100 μg/mL was added into a microtiter plate. Trolox was used as a positive control. For the combination test samples, 10 μL of curcumin were firstly mixed with 10 μL of 0.14 μg/mL gallic acid or 25 μg/mL of ascorbic acid or 25 μg/mL of xanthone. Then 180 μL of $10^{-4}$ M DPPH in ethanol was added. For a negative control, 180 μL of absolute ethanol was added instead of $10^{-4}$ M DPPH ethanolic solution. The mixtures were kept for 30 min then the absorbance of each sample was measured at 540 nm by using microtiter plate reader. The antioxidant activity was expressed as the 50% effective concentration ($EC_{50}$) which 50% free radicals was scavenged by this concentration and as Trolox equivalent antioxidant capacity (TEAC) which obtained from 1 mg of the test sample. The lower the $EC_{50}$ value or the higher the TEAC value, the higher the antioxidant power of the compound.

### 2.5. *Calculation of antioxidant activity and combination index*

The antioxidant activity of the single compound and mixture was calculated by following equation; % Antioxidant activity = $[(Abs_{control} - Abs_{sample})/Abs_{control}] \times 100$, where $Abs_{control}$ is the absorbance value of DPPH solution and $Abs_{sample}$ is the absorbance value of the test sample plus DPPH solution.

To evaluate the interaction biological activity of the mixture, the experimental data were transformed into the combination index (CI) parameter (*24*). This parameter was calculated with the following equation; $CI = (MC_a/SC_a) + MC_b/SC_b)$, where $MC_a$ and $MC_b$ are the concentration of compound A and compound B in the mixture to achieve 50% of antioxidant activity. $SC_a$ and $SC_b$ are the $EC_{50}$ of the single compound A and the single compound B, respectively. The CI value

indicates a synergistic, additive, or antagonistic effect if it is < 1, = 1, or > 1, respectively.

2.6. *Statistical analysis*

The experiments were done in triplicate and the results are expressed as mean ± SD. Statistical analysis was done by using ANOVA and $p$ value at a level of 95% confidence limit.

## 3. Results and Discussion

3.1. *Effect of reaction time on antioxidant activity*

The detection of antioxidant activity based on free radical scavenging mechanism investigated by DPPH method was according to the color changes of the DPPH free radicals. The purple radical DPPH solution was converted to the yellow non-radical DPPH by the antioxidant having electron donating activity. The rate of this reaction was found to be depended on the different chemical structure of a substance (Figure 1). At the fixed concentration of 50 µg/mL, curcumin and gallic acid reached the maximum action within 10 min, whereas ascorbic acid achieved the maximum activity within 20 min (Figure 2). Xanthone showed significant lowest rate of scavenging activity ($p < 0.05$) by using 30 min for reaching the maximum activity. After that, the antioxidant activity of all compounds did not increase until the end of the test period. Therefore, the scavenging reaction time of 30 min was selected for the further study.

3.2. *Determination of antioxidant activity of a single compound*

The free radical scavenging activity of various concentrations of individual curcumin, gallic acid, ascorbic acid, and xanthone at a reaction time of 30 min was compared. It was found that all substances showed a concentration dependent activity (Figure 3). At the highest concentration of 100 µg/mL, all substances exhibited the highest antioxidant activity with more than 90% free radical inhibition. The results in Table 1 confirmed that the antioxidant power of the compounds were significantly different. The $EC_{50}$ of curcumin (10.6 ± 0.4 µg/mL) was significantly lower than that of ascorbic acid (24.7 ± 1.4 µg/mL) and xanthone (32.9 ± 0.9 µg/mL) indicating that curcumin has more powerful in antioxidant activity than ascorbic acid and xanthone. This was confirmed by the TEAC value of these compounds that the value of curcumin was significantly higher than these two compounds. However, in comparison with gallic acid, it was found that curcumin has lower antioxidant power than this compound. The $EC_{50}$ of gallic acid (1.2 ± 0.1 µg/mL) obtained from the present study was in the agreement of that from the previous study (0.011 mM or 1.8 µg/

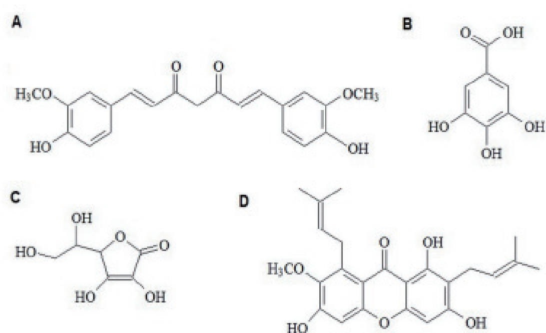


**Figure 1. Chemical structure of curcumin (A), gallic acid (B), ascorbic acid (C), and xanthone (D).**

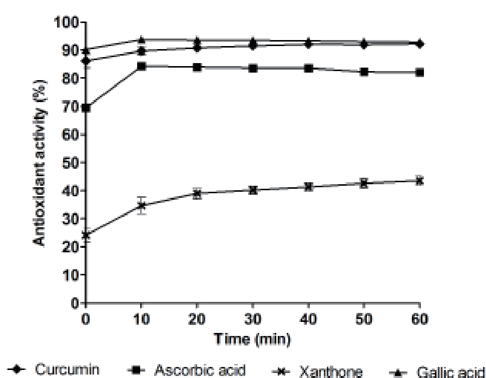


**Figure 2. Antioxidant activity of single compound at different time points ($n$ = 3).**

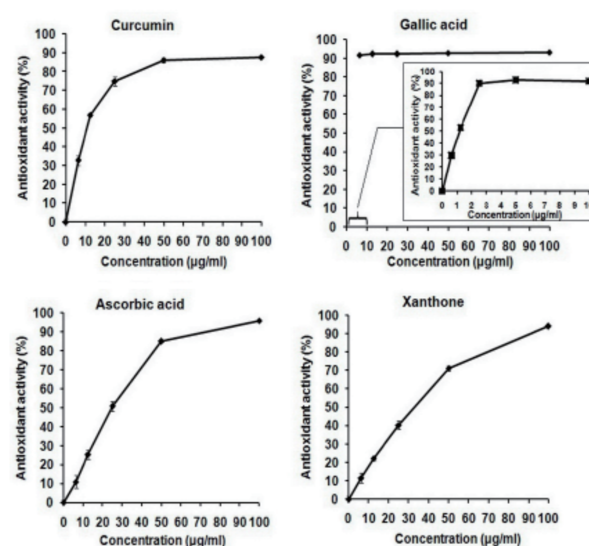


**Figure 3. Antioxidant activity of single compound at different concentrations ($n$ = 3).**

**Table 1. The antioxidant activity of a single compound expressed as 50% effective concentration ($EC_{50}$) and Trolox equivalent antioxidant capacity (TEAC)**

| Compound | $EC_{50}$ (µg/mL) | TEAC (µM/mg) |
|---|---|---|
| Curcumin | 10.6 ± 0.4 | 13.1 ± 0.6 |
| Gallic acid | 1.2 ± 0.1 | 26.4 ± 0.1 |
| Ascorbic acid | 24.7 ± 1.4 | 5.8 ± 0.7 |
| Xanthone | 32.9 ± 0.9 | 5.1 ± 0.4 |

mL) (*25*). The $EC_{50}$ values of curcumin, ascorbic acid, and xanthone in this study were comparable to the previous studies which reported that of 29 µM or 11 µg/mL (*26*), 138 µM or 24 µg/mL (*27*), and 33 µg/mL (*28*), respectively. From these results, it was considered that the power of antioxidant potency of these four compounds was gallic acid > curcumin > ascorbic acid > xanthone. The different in antioxidant power reflected the number of reduced DPPH radicals involved in the free radical scavenging reaction. It was reported that gallic acid, curcumin, and ascorbic acid reduced 6.9, 3.7, and 2.9 molecules of the radical DPPH, respectively (*29*), whereas the ability of xanthone on this action was still unclear. It was reported that α-mangostin, the major compound existing > 90% in xanthone played the most important role on the antioxidant activity of xanthone. The antioxidant potency of α-mangostin was noted by the hydroxyl group that related to free radical scavenging activity (*30*). It was considered that the hydroxyl groups of xanthone might be hindered by the steric effect that made xanthone hardly accessed to the radical site of DPPH.

### 3.3. *Antioxidant activity of curcumin mixture*

The mixtures of curcumin with other antioxidant compounds were studied to examine the combination effects. The CI value was calculated based on the obtained data from the single compounds and the mixtures. Figure 4 shows the antioxidant activities of each mixture compared with that of each single compound. Interestingly, it was found that the mixture of curcumin at various concentrations with gallic acid (the $EC_{50}$ of the mixture = 4.7 ± 1.8 µg/mL) revealed the extremely higher antioxidant activity than that of curcumin or gallic acid alone or in addition. This was confirmed by the calculated CI value presented in Table 2 that the value of this mixture is less than 1. This result indicated that the mixture of curcumin and gallic acid possessed the synergistic antioxidant effect. On the contrary, the mixtures of curcumin and ascorbic acid or xanthone did not display the synergistic effect. Moreover, the calculated CI values of both mixtures demonstrated the value of more than 1 indicating the antagonistic effect. The synergism might be hypothesized by the regeneration mechanism or sacrificial oxidation or exertion of different mechanism of action or combination of different mechanisms (*31*). The interaction of curcumin and gallic acid might be regenerated from their oxidized form by the phenolic hydroxyl group. It was also possible for the sacrificial oxidation that curcumin or gallic acid protected the other one by radical scavenging resulting in the increase antioxidant effect from the non-oxidized form. It was still unclear for the explanation of the antagonism of curcumin and ascorbic acid or xanthone. One possible reason was considered to be according

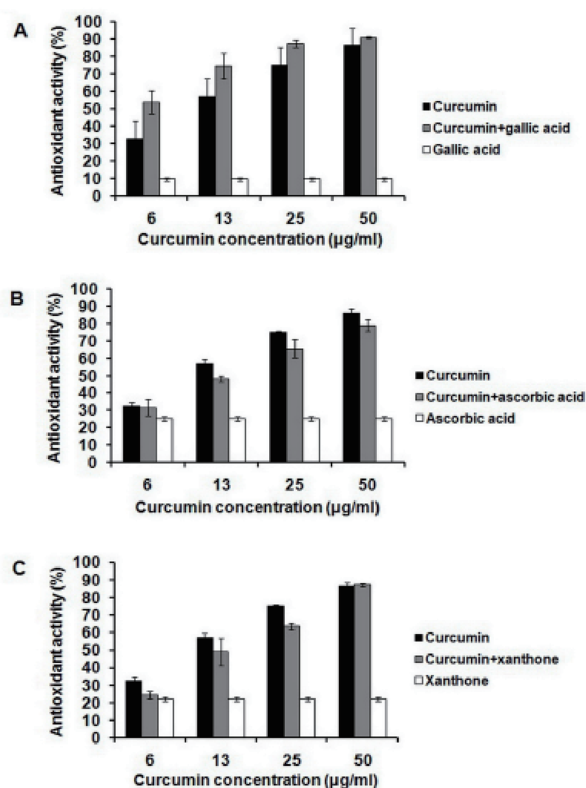


**Figure 4. Antioxidant activity of curcumin in combination with gallic acid (A), ascorbic acid (B), and xanthone (C) in comparison with the anitioxidant activity of the respective individual compound.**

**Table 2. The antioxidant activity of curcumin mixed with other antioxidant compounds expressed as 50% effective concentration ($EC_{50}$) and combination index (CI)**

| Mixtures | $EC_{50}$ (µg/mL) | CI |
|---|---|---|
| Curcumin + Gallic acid | 4.7 ± 1.8 | 0.5 |
| Curcumin + Ascorbic acid | 13.8 ± 2.2 | 1.9 |
| Curcumin + Xanthone | 13.2 ± 0.9 | 2.0 |

to the interference of each other that could provide the reduction of their individual activity (*32*). Also, for xanthone, the hydroxyl group was protected from the steric effect that might obstruct the scavenging ability and decrease the activity when combined with curcumin.

## 4. Conclusion

The present study explores the comparative antioxidant power of curcumin with the other three natural antioxidants; gallic acid, ascorbic acid, and xanthone. It was found that the antioxidant activity of all compounds was dose and time dependent free radical scavenging. The antioxidant power of curcumin was higher than that of ascorbic acid and xanthone, respectively but lower than gallic acid. Interestingly, curcumin and a low dose of gallic acid showed the synergism on antioxidant activity while curcumin with ascorbic acid or xanthone did not act synergistically but agonistically. Therefore,

the combination of curcumin and gallic acid is a promising mixture for the highest antioxidant activity.

**Acknowledgements**

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**Case Report**

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# Crizotinib-associated erythema multiforme in a lung cancer patient

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**Summary** **Crizotinib is an oral small-molecule anaplastic lymphoma kinase (ALK) tyrosine-kinase inhibitor for the treatment of ALK-positive non-small-cell lung cancer (NSCLC). A 63-year old woman with postoperative relapsed ALK-positive NSCLC was treated with crizotinib. Erythema multiforme (EM) occurred one week after initiation of crizotinib therapy. Skin biopsy specimen showed compatible drug eruption. The discontinuation of crizotinib improved her eruption within one week. This report presented the first case of crizotinib-associated EM, which is the preclinical stage of Stevens-Johnson syndrome. Although crizotinib is clinically available, we should be aware of its potential severe skin adverse event.**

***Keywords:*** Lung cancer, crizotinib, drug eruption

## 1. Introduction

Crizotinib is an oral small-molecule anaplastic lymphoma kinase (ALK) tyrosine-kinase inhibitor for the treatment of non-small-cell lung cancer (NSCLC) patients with echinoderm microtubule-associated protein-like 4 (EML4)/ALK rearrangements (*1,2*). ALK is a validated tyrosine kinase target in several cancers including NSCLC, and ALK rearrangements are found in approximately 5% of cases of NSCLC (3). Crizotinib is superior to standard chemotherapy in patients with ALK-positive NSCLC (*3*).

Common adverse events were visual dysfunction, gastrointestinal disorders, and pitting edema. While the incidence of skin complaints is 11% (*2*), there is no detailed case report on the progress of the drug eruption. We report the first case of erythema multiforme (EM) associated with crizotinib.

## 2. Case Report

A 63-year old woman with ALK-positive NSCLC underwent right upper lobectomy in 2009. After four years of her surgery, computed tomography showed right upper mediastinal lymphadenopathy and multiple nodular shadows in both lung fields. Thus, because of postoperative recurrence, she was treated with crizotinib (250 mg twice a day). One week after the initiation of crizotinib therapy, she was aware of asymptomatic cutaneous lesions. Physical examination revealed erythema multiforme on the trunk and extremities (Figure 1). There was no enanthema on the oral mucosa. She had no obvious symptoms including fever, vision disorder, and general fatigue. Laboratory examinations revealed that the percentage of eosinophils was increased (15.3%) although white blood cell count was normal (3,600/μL). Skin biopsy specimen showed spongiosis (Figure 2A) and perivascular lymphocytic/eosinophilic infiltrates (Figure 2B) in the upper dermis. According to these results, she was diagnosed as having crizotinib-associated EM. EM has the possibility that develops into Stevens Johnson syndrome, one of the severe drug eruptions. Therefore, crizotinib was discontinued and topical clobetasol ointment was undertaken. Her eruption almost improved within one week.

## 3. Discussion

Crizotinib is well tolerated, and severe adverse event

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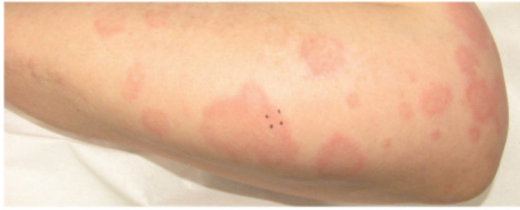

**Figure 1. Erythema multiforme were detected on the left arm.**

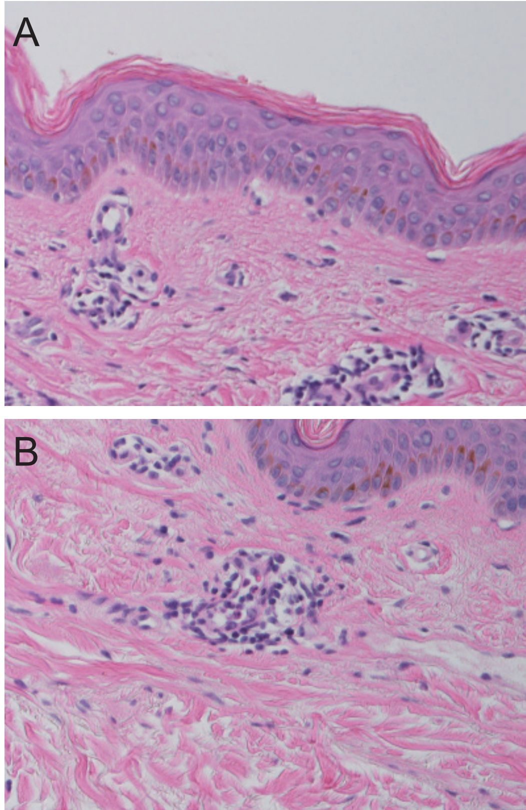


**Figure 2. Histopathological findings. (A)** Spongiosis and **(B)** perivascular lymphocytic/ eosinophilic infiltlates in the upper dermis (original magnification, ×400).

is comparatively rare. In 172 ALK-positive NSCLC patients, the common adverse events were mild (grade 1-2) gastrointestinal disorders (nausea (55%), vomiting (47%), diarrhea (60%), or constipation (42%)), vision disorder (60%), and edema (31%) (*3*). Regarding severe adverse events associated with crizotinib, there were diffuse alveolar damage (*4*) and esophageal ulcer (*5*). To our knowledge, our case is the first report of crizotinib-associated EM.

EM has a varied etiology including drug and infection. In this case, she had no symptom such as an indication of infection. In fact, from the initiation of crizotinib treatment, her eruption became progressively worse. And discontinuation of crizotinib resulted in improvement of EM. She didn't take newly started drug except crizotinib. On ground of such clinical courses, we diagnosed her with EM induced by crizotinib. EM sometimes develops into Stevens Johnson syndrome, which has the possibility of critical organ damage and potentially deadly risk. We should carefully examine the patient developing EM caused by novel agents whose adverse events have never been reported.

In conclusion, although crizotinib is a clinically available ALK inhibitor, we should be aware of its potential skin adverse event. Further accumulation of adverse events is necessary in future.

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Letter

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# The study of forensic toxicology should not be neglected in Japanese universities

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**Summary** **Forensic toxicology is aimed at identifying the relationship between drugs or poison and the cause of death or crime. In the authors' toxicology laboratory at Chiba University, the authors analyze almost every body for drugs and poisons. A simple inspection kit was used in an attempt to ascertain drug abuse. A mass spectrometer is used to perform highly accurate screening. When a poison is detected, quantitative analyses are required. A recent topic of interest is new psychoactive substances (NPS). Although NPS-related deaths may be decreasing, use of NPS as a cause of death is difficult to ascertain. Forensic institutes have recently begun to perform drug and poison tests on corpses. However, this approach presents several problems, as are discussed here. The hope is that highly accurate analyses of drugs and poisons will be performed throughout the country.**

***Keywords:*** Forensic toxicology, new psychoactive substances, forensic institutes

## 1. Introduction

Forensic toxicology is aimed at identifying the relationship between drugs or poison and the cause of death or crime using screening and quantitative analyses of various substances such as drugs, poisons, and alcohol in the blood, urine, saliva, other bodily fluids, or hair of a corpse. In the past, many pharmacy schools gave forensic chemistry lectures and they conducted education and research on drugs and poisons. Only a few colleges have such lectures, but they are returning at some schools of pharmacology. Thus, forensic toxicology is usually restricted to departments of legal medicine in universities. In Japan, many departments of legal medicine study deceased individuals, including analyses of drugs and poisons in the blood and other bodily fluids. Institutes of forensic medicine in foreign countries analyze use of alcohol and antihypnotic substances in living subjects, and some departments of legal medicine in Japan deal with clinical forensic medicine.

Toxicology examines the effects of drugs or poison on the functions of living individuals, and a drug that may be useful at treating an illness may be harmful if ingested at high doses. Such toxicity can directly or indirectly cause death, and death can occur due to accidents, homicides, suicides, or in various other ways. For example, mortality can occur due to ingestion of a fatal dose of an antihypnotic or due to an aneurysm in a cerebral blood vessel. A small quantity of a drug may result in increased blood pressure and cause disruption of an arterial aneurysm, resulting in a subarachnoid hemorrhage.

In Chiba Prefecture, approximately 52,000 people die every year, and approximately 400 bodies are autopsied. The autopsy rate is 0.7%, which is much lower than that in other advanced countries and regions. Based on these statistics, deaths are not thoroughly investigated in Japan. Although the authors' toxicology laboratory performs few autopsies, all bodies except those in skeleton form (from which bodily fluid samples cannot be obtained) are analyzed for drugs and poisons. The substances analyzed include alcohol, carbon monoxide, narcotics such as opium, antihypnotics,

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cannabis, psychotropic drugs, cyanide, various legal drugs including prescription medicines, and a new type of drug known as new psychoactive substances (NPS). In Japan, NPS are referred to as "dangerous drugs."

## 2. The current state of the study of forensic toxicology in the authors' laboratory

### 2.1. *Equipment for analysis*

Initially, a simple inspection kit known as "Triage" was used in an attempt to ascertain whether there is evidence of drug abuse in blood or urine samples. This kit can be used to screen for several classes of drugs such as phencyclidine, amphetamines, methamphetamines, barbiturates, benzodiazepines, cocaine, THC-COOH, tricyclic antidepressants, and opiates. Drug abuse can be detected within 15 min. However, this kit has a rather low level of reliability and it tests only for common drugs, so it is unable to facilitate a more thorough investigation.

A mass spectrometer is used at Chiba University to perform highly accurate drug and poison screening. Gas chromatography-mass spectrometry (GC/MS), liquid chromatography-tandem mass spectrometry (LC/MS/MS), and liquid chromatography quadrupole time-of-flight mass spectrometry (LC/QTOFMS) are performed to analyze substances that are specified in software libraries or other substances such as NPS. When a poison is detected, quantitative analyses are required. If a certain substance is detected, a quantitative analysis is required, and the fatal dose of the drug should be determined in order to examine the relationship between the drug and mortality. Quantitative analyses are performed at the authors' laboratory using the techniques mentioned above.

### 2.2. *Postmortem changes*

The results of quantitative analyses differ in living and dead subjects due to postmortem changes. For example, ethanol is produced posthumously depending on the individual's condition. In instances of fatal accidents while driving where ethanol is detected, drunk driving cannot be directly inferred as the cause of the accident. However, such misjudgments have been made in the past. Tricyclic antidepressants and selective serotonin reuptake inhibitors are known to easily alter the blood ethanol concentrations. Additionally, increases and decreases in blood sugar levels hamper efforts to ascertain the blood sugar level before death. Various parameters are estimated in living individuals in an attempt to determine the cause of death.

### 2.3. *NPS*

A recent topic of interest is "dangerous drugs" (NPS). The authors have encountered several cases involving NPS.

Since March 2013, many drugs with similar chemical structures have been classified as NPS, and the police and other government agencies have begun to monitor their use (*1*). The possession and use of NPS as well as their production, distribution, and sales of NPS have been prohibited since April 2014 (*2*). Although purchasing NPS in a store is difficult, NPS may be sold underground as antihypnotics. As a result of strict controls, NPS-related deaths may decrease, but use of NPS as a cause of death is difficult to ascertain. The structures of NPS are changing constantly, and even the name of the drug can be determined, a standard sample of the drug may not be available in a timely manner.

## 3. Current issues related to the study of forensic toxicology in Japan

*Differences between forensic institutes and departments of legal medicine* Forensic institutes within every prefectural police department have recently obtained equipment such as mass chromatograph spectrometers and they are testing for drugs and poisons. In addition, similar examinations are performed by the National Institute of Health Sciences, the health bureau in every prefecture, and the Narcotics Division of the Ministry of Health, Labor, and Welfare. Drug tests are performed by a department of legal medicine to investigate the cause of death, while other institutes test for drugs to investigate crimes that are directly or indirectly related to drugs. Despite their reputation, forensic institutes do not perform academic studies but instead perform practical tasks. As an example, police and narcotics agents may investigate whether an antihypnotic is consumed or not, determine whether the person was involved in a criminal act, investigate the sale of the drug, and try to ascertain the method of production, distribution, possession, and use of the antihypnotic. However, death investigations by departments of legal medicine examine antihypnotics, measure the quantity of amphetamines and other ingredients, and determine the cause of death. The dangers of antihypnotics should be publicized and the use of these substances should be halted. The aims of a forensic institute are to investigate crime and facilitate the prosecution of criminals, while the aims of a department of legal medicine are to determine the cause and manner of death and prevent its recurrence.

Recently, forensic institutes have begun testing corpses for drugs and poisons. The police consider these tests to be less expensive than the tests conducted by the departments of legal medicine at universities. However, two problems may arise. The first is the objectivity of the decision reached. The police investigate a crime and forensic institutes test for drugs and poison during a criminal investigation. To prove that a murder has

occurred, for example, an objective determination of that fact is indispensable for the court. Therefore, there is considerable debate as to whether forensic institutes under police supervision are capable of making such determinations. A second problem is that police may determine whether an individual's death was associated with illegal activity based on use of a simple inspection kit. If police determine that a death is not related to a crime, they may not perform the required tests. As a result, important cases in terms of public safety may be overlooked. Moreover, the crime itself may be overlooked.

Further research into forensic toxicology is currently underway. Methods of drug testing and testing rates differ among university laboratories. In addition, employing technicians and scientists to analyze drugs and poisons is an expensive proposition. The hope is that highly accurate analyses of drugs and poisons will be performed throughout the country.

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