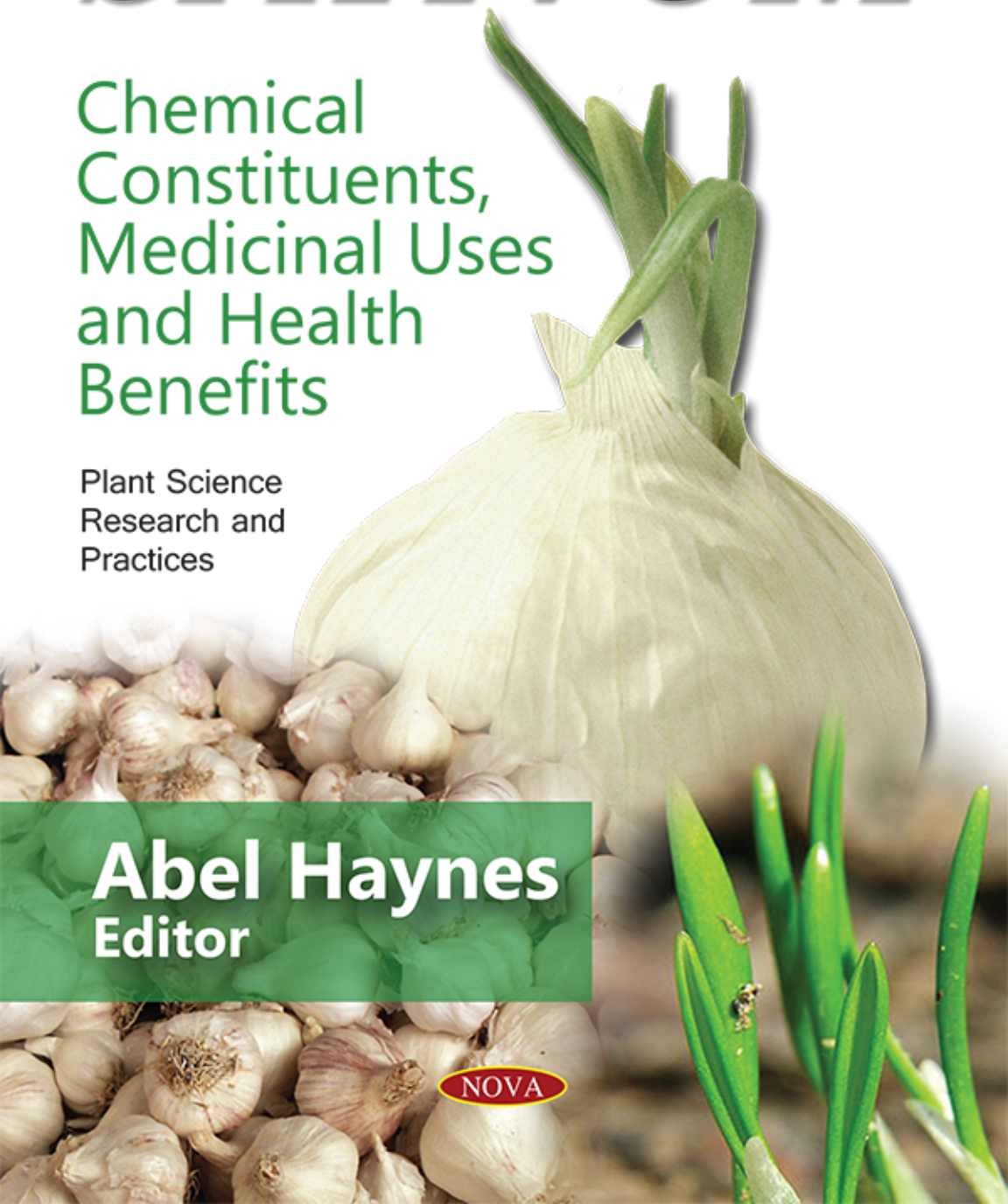
Allium Sativum

Chemical Constituents, Medicinal Uses and Health Benefits

Plant Science Research and Practices



Abel Haynes
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ABEL HAYNES
EDITOR

nova publishers
New York

NOTICE TO THE READER

Library of Congress Cataloging-in-Publication Data

Names: Haynes, Abel, editor.
Title: *Allium sativum* : chemical constituents, medicinal uses and health benefits / editor: Abel Haynes.
Other titles: Plant science research and practices.
Description: Hauppauge, New York : Nova Science Publishers, Inc., [2016] | Series: Plant science research and practices | Includes index.
Identifiers: LCCN 2015043897 (print) | LCCN 2015047901 (ebook) | ISBN 9781634842648 (Hardcover) | ISBN 9781634842655 (eBook)
Subjects: LCSH: Garlic--Therapeutic use. | Garlic--Health aspects. | Garlic--Composition.
Classification: LCC RM666.G15 A756 2016 (print) | LCC RM666.G15 (ebook) | DDC 615.3/2433--dc23
LC record available at http://lccn.loc.gov/2015043897

Published by Nova Science Publishers, Inc. † New York

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PREFACE

The use of *Allium sativum* (garlic) for medicinal purposes has origin in antiquity and is still included in the traditional medicine of many cultures. Oral tradition and recorded history show that garlic is one of the earliest examples of plants used extensively since the existence of man. Historically, there has been great interest in the role and potential benefits of garlic in the management of diseases and maintenance of health. This book provides research on the chemical constituents, medicinal uses and health benefits of *Allium sativum*. The first chapter provides a historical perspective and folkloric applications of garlic in ancient cultures, the supposed role and benefits perceived/claimed to have played in health and disease and the substantive validation made so far by modern science. The next chapter provides a comparative review of *Allium sativum* extract and bioactive constituents. Chapter three reviews recent progresses in facilitating the *in situ* generated allicin methodology and its possible medicinal applications. Chapter four evaluates the antimicrobial activity, *in vitro*, of fresh *Allium sativum Liliaceae* against Staphylococcus aureus (Sa) and Escherichia coli (E. coli). The last chapter's main objective is the development of a preliminary mechanical system for the culture of garlic.

In: *Allium sativum*
Editor: Abel Haynes
ISBN: 978-1-63484-264-8

Chapter 1

HISTORICAL PERSPECTIVE AND FOLKLORIC USE OF GARLIC

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ABSTRACT

The use of garlic for medicinal purposes has origin in antiquity and is still included in the traditional medicine of many cultures. Oral tradition and recorded history show that garlic is one of the earliest examples of plants used extensively since the existence of man. Historically, there has been great interest in the role and potential benefits of garlic in the management of diseases and maintenance of health. Holy writings, recorded history and ancient medical texts especially from Mediterranean and Asiatic regions (Egypt, Greece, Rome, China and India) either made references and/or prescribed the use of garlic in health and disease. Modern science is committed to confirming folkloric claims on its preventive/treatment characteristics especially in cardiovascular disease, blood lipid/glucose regulation, anti-microbial/parasitic activities, immunostimulatory, antitumoral and antioxidant activities. This review will focus attention on the historical perspective and folkloric applications of garlic in ancient cultures, the supposed role and benefits

perceived/claimed to have played in health and disease and the substantive validation made so far by modern science.

INTRODUCTION

The use of garlic for medicinal purposes dates back to antiquity, to the year 1550 BC, when it was already a valuable component of food (Majewski 2014). Garlic has attracted particular attention because of its widespread use around the world and the cherished beliefs many have had that it has kept them healthy, helped them to ward off illnesses and given them more vigor (Rivlin 2001). Today, garlic is still included in the traditional medicine of many cultures and as such it is grown almost everywhere with far more than three hundred varieties known. Garlic is believed to have a complex action such as antimicrobial, antitoxin, antioxidant and diuretic among others. Consequently, it was used for managing cardiac and pulmonary conditions, headaches, abnormal growths, to improve the sexual condition and to cure everything from hemorrhoids to snake bites. In early times, it was believed that having exhibited antimicrobial and antitoxin properties couple with its characteristic pungency, anecdote or marketing value, it was often tried for many other conditions at different times and places. While not all of these usages/claims are supported/validated by reliable or credible contemporary science (Petrovska and Cekovska 2010), folk wisdom should not be ignored/neglected because it has many valuable lessons; some already learned and others yet to be learned (Rivlin 2001).

Scientists today have been validating many of these ancient claims about garlic and its derivatives, trying to identify the bioactive constituents, establish their mechanisms of action and decipher their ultimate role in the prevention and treatment of disease (Rivlin 2001). Also, attempts are being made to investigate interactions of garlic and constituents with other active agents from foods and drugs as well as experimenting ways to improve processing methods in order to enhance bioavailability of bioactive constituents under ordinary conditions of use (Majewski 2014).

In this brief review of folkloric use of garlic in ancient history, it is interesting to learn how diverse cultures at different times and places came to similar conclusions about the use of garlic in the management of ailments. Among these include microbial infection, pulmonary and respiratory complaints. Its efficacy in dropsy and some abnormal growths is compatible with known cardiovascular functions and anticancer properties. Contemporary

research is in the course of validating many of these folkloric claims on the medicinal use of garlic. Another recurring theme is the use of garlic for laborers, soldiers and athletes to improve their strength and stamina.

ORAL TRADITION AND RECORDED HISTORY INFORMATION ON THE USE OF GARLIC

For centuries, garlic has been used in different ways throughout the world. Garlic is one of the earliest documented examples of herbs used for maintenance of health and treatment of disease (Block 1985; Kahn 1996). Most of the information on folkloric use of garlic was mainly transmitted via oral tradition. However, there are few outstanding recorded histories with valuable information on the use of garlic as food and medicine in ancient cultures.

Ancient Egypt and Biblical Records

Archaeological evidence indicates that long before Jacob and his family arrived in Egypt, the Egyptians were cultivating wheat, flax, barley, cucumbers, watermelons, leeks, onions, garlic, and other products (Moyers 1996). The fact that garlic was extensively cultivated in ancient Egypt suggests that prior to the Israelites exodus from Egypt, the Israelites had been well acquainted with garlic cultivation (Ex 9:25, 26, 31, 32; De 11:10). How did the Israelites acquire a taste for garlic? During their 215-year extended stay in Egypt, garlic was part of their diet. Greek historian Herodotus (II, 125) tells of an inscription that listed garlic as one of the foods Egyptian authorities purchased in enormous quantities to feed their pyramid-building slaves. This diet, heavy on garlic, seemed to increase the workers' strength and stamina thereby enabling them to work harder and be more productive (Moyers 1996).

As for the Egyptian elites, it is less certain if they consumed garlic to the same degree but there are indications that they at least made use of garlic. Archaeological findings show that when the Egyptians buried Pharaoh Tutankhamen, they left many valuable objects in his tomb, including garlic. Drawings of garlic were found 3700 years BC in Egyptian tombs. At that time, garlic had such a high commercial value, that it was even considered as a

valuable exchange resource. Thus, this indicates that garlic was in use at the time (Green and Polydoris 1993; Kahn 1996).

More so, the medical text of the era, *Codex Ebers*, prescribed garlic for the treatment of circulatory ailments and several others such as general malaise, infestations with insects and abnormal growths, which may likely represent malignancies and/or abscesses (Bergner 1996; Lawson 1998).

In the wilderness of Sinai, the mixed crowd and the Israelites longed for the garlic they used to eat in Egypt and they said:"How fondly we remember the fish that we used to eat without cost in Egypt, also the cucumbers, the watermelons, the leeks, the onions, and the garlic!" (Nu 11:4, 5). The Jews took such a liking to it that according to Mishnah (Nedarim 3:10), they referred to themselves as garlic-eaters. Also, garlic use was recommended by the Talmud to promote marital relations, perhaps as an aid to fecundity (Moyers 1996). Garlic is still widely used medicinally by the inhabitants of Mediterranean areas as a digestive stimulant, as an antibiotic, and as an antispasmodic.

Ancient Greece

The use of diet, heavy on garlic, to increase workers' strength and stamina was common in ancient Greece. Garlic formed an important part of the military dietary regimen, particularly for soldiers preparing for battle field. Little wonder then that those athletes who competed in the earliest Olympics in Greece made use of garlic as performance enhancing agents (Green and Polydoris 1993; Lawson 1998). Archeological findings have unearthed well-preserved garlic in ancient Greek temples and the palace of Knossos in Crete (Moyers 1996).

The use of garlic was highly promoted by the Greek physicians, Dioscorides and Hippocrates, widely regarded as the father of Medicine. They recommended it for digestive problems, leprosy, cancer, wounds, infections, and heart trouble (Moyers 1996).

Ancient Rome

Roman authorities perceived garlic as an aid to strength and endurance as it was fed to both soldiers and sailors (Green and Polydris 1993). In fact, Roman workers and soldiers chewed garlic before battle, and the Slavs,

claimed it to protect against snakebites. Dioscorides (a Greek), who served as the chief physician for Emperor Nero's army recommended garlic because he believed it "cleans the arteries" (Bergner, 1996; Riddle 1996). Pliny the Elder, was another Greek physician who greatly influenced medicine in Rome. He wrote in his five-volume *Historica Naturalis*, twenty three uses of garlic for a variety of ailments (Bergner 1996; Moyers 1996). Among these was that garlic offers protection against toxins and infections, a finding that is well corroborated by contemporary investigations (Block 1985; Pinto and Rivlin 1999). Garlic was also recommended for disorders of the gastrointestinal tract and for alleviation of joint disease and seizures (Block 1985; Pinto and Rivlin 1999).

Ancient Asia

The use of garlic as part of the daily diet and folkloric medicine has origins in ancient Asia. In ancient China, regular consumption of garlic was a norm though in small quantities. Notably, garlic was consumed together with raw meat and used as a food preservative (Kahn 1996; Moyers 1996). Garlic was prescribed in the management of digestion and respiration related ailments, fatigue, headache, insomnia, male potency as well as emotional problems such as sadness or depression (Kahn 1996; Woodward 1996; Rivlin 2001). The use of garlic in Chinese folkloric medicine has been predominantly in combination with other herb to form a healing tonic. It is believed that garlic was introduced in Japan later than in China, probably 2000 years ago (Kahn 1996; Rivlin 2001).

In ancient India, garlic has been associated with the healing process among three medical traditions; Tibbi, Unani and Auryvedic. They made extensive use of garlic as a central part of the healing efficacy of plants (Moyers 1996). The surviving medical texts, Charaka-Samhita and Bower manuscript, recommended and promoted garlic for the treatment of heart disease and arthritis, infections, infestations and worms, weakness and fatigue, and a variety of digestive disturbances (Woodward 1996; Rivlin 2001). Garlic was a perceived aphrodisiac of plant source (Kahn 1996) and was observed to elicit diuretic effects, possibly causing the mobilization of fluid from the extravascular space with an attendant improvement in cardiovascular health (Rivlin 2001). Some studies have reported that appropriate use of garlic resulted in reduction blood pressure (Steiner et al., 1996), improved elevated serum cholesterol (Rivlin 2001), decreased platelet aggregation (Steiner and

Lin 1999) and offered protection of vascular endothelial cells against damage by LDL (Ide and Lau 1997).

Middle Ages

During the Middle Ages, herbs with therapeutic potentials were especially grown in the monasteries and monks were responsible for transmitting the knowledge of their therapeutic use, including garlic, to surrounding population (Moyers 1996; Rivlin 2001). To Poland, it came in the Middle Ages, with a caravan of merchants from the East. Garlic was used to alleviate constipation when consumed with beverages. The use of garlic to increase workers' strength and stamina seemed to be common during the medieval times. Garlic was perceived and classified as a "hot food" and as such consumed during the winter to protect against the development of respiratory disorders (Moyers 1996). During summer, outdoor workers were advised to consume garlic to prevent heat stroke (Khan 1996; Moyers 1996). Garlic was also utilized against massive debilitation and later in the Great Plagues (Bergner 1996; Woodward 1996). Some leading physician during the later part of the 12th century believed that raw garlic was more effective than cooked garlic, perhaps because the latter has less pungency than the former (Bergner 1996; Kahn 1996).

The Renaissance and Prior to Contemporary Times

During the renaissance period, the medical use of herbs gained increasing attention in Europe. In Europe, it was believed that garlic is able to ward off vampires, demons and evil spirits and have other magical properties. Garlic was one of the major plants grown for medicinal purposes in the so-called "physic" gardens of some leading universities. A leading physician of the 16th Century, Pietro Mattioli of Siena, prescribed garlic for digestive disorders, infestations with worms and renal disorders, as well as to help mothers during difficult childbirth (Moyers 1996).

During this time, many of the ruling classes in Continental Europe began to adopt garlic. For example, King Henry IV of France in the late 16th and early 17th centuries underwent water baptism in a pool of water containing garlic to protect him from evil spirits and from disease. In England, garlic remained the food of the working and the elite classes. It was recommended

for management of constipation, toothache, dropsy, animal bites and the plague. Its purported beneficial effects in treating dropsy suggest that it was thought to improve cardiovascular function, mechanisms of which are only now under study. Doctors carried cloves of garlic with them at all times to protect themselves from the odor of disease (Moyers 1996; Rivlin 2001).

Close to contemporary times, garlic was brought to the new world by the explorers and sailors from France and Portugal. The Native Americans used garlic in their tea. Later in the 19th century, garlic was part of the Shaker medical armamentarium as a stimulant, expectorant and tonic. Garlic's perceived therapeutic properties were widely accepted by a majority of the population (Moyers 1996).

In the 19th century, French chemist Louis Pasteur studied garlic and described its antiseptic properties. In his book, *Home Book of Health*, John Gunn in 1878 recommended garlic as a diuretic, for treatment of infections, as a general tonic and for asthma and other pulmonary disorders (Moyers 1996). In the early part of the 20th century, in the volume *Health Remedies, a Complete Medical Work and Family Guide*, garlic was promoted for diseases of the lung in children and adults (Moyers 1996; Rivlin 2001). More recently, scientists have studied how the circulatory system benefits from garlic. When Russian military doctors ran short of modern drugs during World War II, they used garlic to treat injured soldiers. Thus, garlic became known as Russian penicillin. In Africa during the 20th century, Albert Schweitzer, a famous missionary-doctor, used garlic to treat amoebic dysentery and other diseases.

FOLKLORIC CLAIMS AND CONTEMPORARY SCIENCE ON THE MEDICINAL USE OF GARLIC: NEED FOR GLOBAL HARMONIZATION

Garlic and Cardiovascular Disease

Cardiovascular disease is by far the greatest killers in modern society. Cardiovascular disease is a complex and multifactorial disease characterized by such factors as high cholesterol, hypertension, reduced fibrinolysis, increased blood-clotting time and increased platelet aggregation. Many studies have demonstrated that normalization of hypertension and abnormal metabolism of lipids and lipoproteins, including cholesterol, improves atherosclerotic coronary artery disease (Kleijnen et al., 1989).

It is established that dietary factors play a key role in the development of some human diseases, including cardiovascular disease. Several epidemiologic studies have indicated that certain diets are associated with low risk of cardiovascular disease and that these diets are rich in fruits, herbs and spices; the common spice among them is garlic (Stavric 1994).

Over the centuries, garlic has acquired a unique position in the folklore of many cultures as a remarkable prophylactic and therapeutic medicinal agent (Rahman 2001). There has been great interest in the role of garlic in reducing cardiovascular risk factors most especially as considerable anecdotal evidence supports the role that garlic has played in the phytotherapy of cardiovascular disease (Bolton et al., 1982). The role of garlic in cardiovascular disease is cited in the *Egyptian Codex Ebers*. Ancient Greek physicians such as Hippocrates and Pliny the Elder, and Indian physician Charak (ca. 3000 BC) the father of Ayurvedic medicine, promoted and recommended the use garlic for heart trouble (Fenwick and Hanley 1985).

Over the last 30 years, this important and exciting role of garlic has been and continues to be confirmed by basic and clinical research reports from around the world. Evidence from numerous studies point to the fact that garlic can bring about the normalization of plasma lipids, enhancement of fibrinolytic activity, inhibition of platelet aggregation and reduction of blood pressure and glucose (Lau et al., 1987; Bordia et al., 1996; Reuter et al., 1996; Steiner et al., 1996; Effendy et al., 1997; Ide et al., 1997). However, some studies have reported no positive result (Morris et al., 1995; Simons et al., 1995; Neil et al., 1996; Isaacsohn et al., 1998). These conflicting reports may be due to the use of different experimental protocols, formulations/ preparations of garlic and different time scales of the studies (Rahman 2001). This situation, therefore, warrant further clinical studies with standardized preparations of garlic with known and established compositions. Such formulations (e.g., Aged Garlic Extract) are now available and are being thoroughly investigated.

Garlic and Cancer

The etiology of cancer is heterogenous and the exact mechanism of cancer process is less understood. Chemo- and radiotherapies are the currently available cancer management procedures but frothed with pain and other side-effects. More so, there is currently no available tangible phytotherapeuticagent to manage this terminal disease. In dilemma scenario where researchers are yet

to lay hold on phytotherapeutic agent to manage cancer, hope becomes the sole sustaining ingredient for possible pyrrhic victory such that a suggestive claim of possible treatment may be seen as being factual.

Evidently, garlic contains several bioactive constituents acting either in isolation and/or in synergy with others to elicit positive functional effects. More so, the biologic activity of garlic and its bioactive constituents may be modified by preparation methods and influenced by dietary components and several physiologic events. Undoubtedly, garlic has commanded a measure of reverence from time immemorial. In recent times, this kingly reverence has appreciatively increase as a result of available compelling scientific evidence tending toward validating the claims that garlic has the propensity to prevent and protect against the incidence of cancer (Fenwick and Hanley 1985; Milner 1996 and 1999; Orekhov and Grunwald 1997; Yoshida et al., 1999). However, data from epidemiologic and experimental studies are tainted with some measure of inconsistency in the much needed compelling evidence to assign garlic and some of its bioactive constituents either or both preventative and protective roles in cancer. A little wonder then why the precise mechanism by which garlic may modulate cancer process is equally less understood albeit several speculations and theories.

Need for Harmonization of Folkloric and Contemporary Health Claims

Herbal medicines continue to play a central role in the healthcare systems of large portions of the world's population (Akerele 1988). This is particularly true in developing countries, where traditional systems of medicine have a long history of use. Contemporary history shows that most of the developed countries had basically ignored the use of herbs with the advent of more potent synthetic drugs in the 1940s, coupled with a lack of clinical data to establish the safety and efficacy of herbs.

However, in the very recent times, herbal medicine has enjoyed a revival in many industrialized countries (Eisenberg et al., 1993 and 1998; MacLennan et al., 1996; Millar 1997). This "herbal renaissance" has been fueled by strong consumer interest in preventative medicine, disappointment with allopathic medicine and the perception that botanicals are safe and free from side effects (Mahady 1998).

In recent times several pre-clinical, clinical and *in vitro* studies on animal models have been conducted to evaluate the protective effect of garlic against

various health related disorders, especially heart disease and cancer. Obviously and outstandingly, AGE exhibits clear and significant biological effects in various conditions such as cardiovascular diseases, cancer, liver problems, improving immune system and in other areas (Majewski 2014). However, the risk of drug interactions with garlic, especially in the elderly and those with chronic diseases, is attracting more and more interest. The available results and intervention studies conducted on humans using other garlic products are not sufficiently consistent so as to warrant further investigations to properly clarify and harmonize claims of real health benefits of garlic and its products. This may partly be due to the inherent chemical complexity of garlic and the use of different processing methods resulting in formulations with varying degrees of efficacy and safety.

These scenarios, therefore, underscore the need to reexamine folkloric claims on the use of garlic and its derivatives and endeavor to evaluate and harmonize available preclinical and clinical results with folkloric claims in the light of credible scientific research procedure. Harmonization of herbal health claims is achievable only for those herbal medicines having sufficient scientific data to support the claims of safety and therapeutic efficacy. However, for others, more scientific information will be required in the form of well-designed, controlled, clinical trials and basic scientific research.

Where possible, there may be a need to fashion out simple but standard experimental protocols to assist the research community, especially in the developing countries where garlic have a long history of use, to carry out preclinical experiments and clinical trials that will truly yield consistent and objective results.

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In: *Allium sativum*
Editor: Abel Haynes
ISBN: 978-1-63484-264-8

Chapter 2

MEDICINAL USE AND HEALTH BENEFITS OF *ALLIUM SATIVUM*: A COMPARATIVE REVIEW OF THE WHOLE EXTRACT VS BIOACTIVE CONSTITUENTS

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ABSTRACT

Garlic (*Allium sativum* Linn) is one of the best-researched, best-proclaimed herbal remedies and commonly used condiment (food and a spice) across various cultures at various times. Garlic constituents include enzymes, sulfur-containing and enzymatically derived compounds as well as non-sulfur compounds such as saponins. Traditionally, experimentally and clinically, either fresh garlic or whole garlic extract and isolated constituent(s) have been employed. The various health benefits of garlic and its products probably arise from a wide variety of constituents, possibly working synergistically, additively or solely. Because of garlic's chemical complexity, variations in processing methods may sometimes impart on preparations with an attendant difference in bioefficacy and safety. Given the emerging trends in functional food science, our understanding of both direct and indirect mechanisms underlying the proclaimed health benefits of fresh garlic bulb, fresh/aged whole extract

and isolated constituent(s) from garlic as well as validated functional health benefits thereof should be high-priority information for the scientific community and the public in general. Therefore, a comparative review and harmonization of proclaimed health benefits may be necessary to clear the air or ascertain any degree of empiricism or science in research information available so far and/or to clarify which of these claims are scientifically valid and traceable (can be pinned) to whole garlic or solely to isolated constituent(s). Although not all of the bioactive ingredients are known, ample research suggests that several bioavailable components likely contribute to the observed beneficial effects of garlic. What though could be the impart of processing/preparation on the health benefits accruable to garlic/extract/constituents? Albeit the limited understanding of the probable mechanisms underlying some of the health benefits of garlic and its derivatives, many mysteries have been uncovered thus allowing for some measure of definitive statements on its applications in relation to efficacy and safety levels.

INTRODUCTION

Garlic (*Allium sativum*) has been used by man in different cultures for hundreds of years for medicinal and flavouring purposes. It has numerous biological activities (Santhosha et al., 2013) that are attributed to its rich content of different volatile organosulfur compounds (OSC) and phytochemicals that work in synergy by combination of mechanism for substance acting on various molecular targets (Prette et al, 2005; Amagase, 2006; Lei et al., 2008).

Clinical and experimental studies on the acclaimed biological activities of garlic has been inconsistent due to different garlic preparations used, unknown active compounds and their bioefficacy, duration of trials among other factors (Rahman and Lowe, 2006). There is also the problem of using whole extract preparations rather than the isolated constituents. It therefore becomes difficult to validate the folkloric use of garlic in the traditional society. This chapter attempts to evaluate the current knowledge on the bioactive constituents of garlic in order to determine how the acclaimed health effects have been pinned down to a particular bioactive component. Garlic substances can be classified depending on the chemistry of the compounds present into thiosulfinates, organosulfur volatiles, vinyldithiins, ajoene and water soluble organosulfur compounds (Santhosha et al., 2013).

GARLIC PRODUCT PROCESSES

Chemical substances abound in fresh, dried garlic or extracts. These substances possibly work solely or synergistically with others to elicit the functional health benefits accruable to garlic. However, it has been shown that the potential health benefit(s) of garlic is largely dependent on the efficacy and safety of the garlic preparations which are also contingent on the processing methods used (Staba et al., 2001). While there are many processed garlic products that are commercially available, they all fall into four major categories: dehydrated garlic powder, garlic oil, garlic oil macerate and aged garlic extract (AGE).

The chemical composition of any garlic product is largely dependent on the potential substrate activity of the enzyme alliinase, the temperature and duration of drying, the type of solvent used for the extraction and the conditions and period of maceration before final extraction (Koch and Lawson, 1996). The major processes utilized are outlined below:

1) *Freeze-Drying.* The freeze-drying of fresh garlic cloves is a type of flash evaporation carried out at low temperature in a partial vacuum. This method results in virtually no changes in chemical composition.
2) *Low Temperature Drying.* This process involves drying sliced fresh cloves at temperatures <50°C for 3 to 4 days. Some allicin is formed due to the slicing process. Allicin is converted to allyl sulphides, which are largely responsible for the typical garlic odour. The final product has many of the attributes of the fresh garlic clove, which include γ-glutamyl cysteine, the precursor to alliin and S-allylcysteine.
3) *Distillation.* Steam-distillation of garlic yields principally allyl sulphides. Allicin is volatile and may be lost or converted to the allyl sulphide degradation compounds. The oil generated may be dissolved in soybean oil or other vegetable oils to form a product.
4) *Maceration in Oil.* Chopped garlic is homogenized and slowly extracted (maceration) in soybean or another vegetable oil. Such products contain vinyldithins, allyl sulphides and ajoene.
5) *Hydroalcoholic Short Extraction.* Fresh or dried garlic is extracted with a hydroethanolic solution for a short maceration time between 12-48hrs. The resulting product is often referred to as a tincture, and may be made in 10, 20 or 30% w/v concentrations in 70% ethanol.

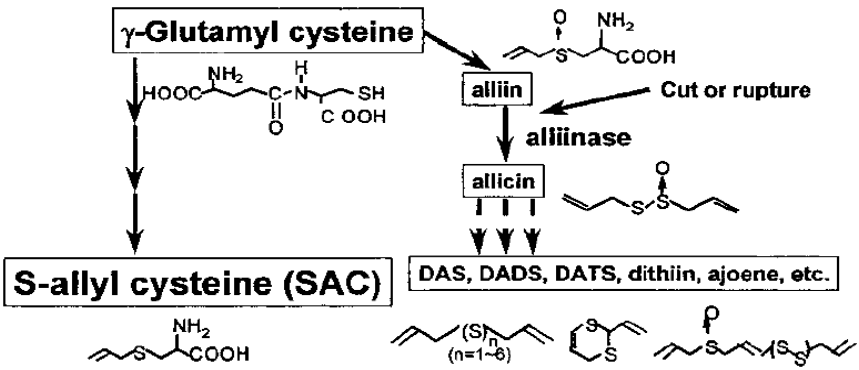
6) *Hydroalchoholic Long Maceration.* Sliced garlic is placed in 20% ethanol and macerated for a long period of time (~ 6 to 20 months), filtered and concentrated. Allicin is completely converted to allyl sulphides, including diallyl disulphides, diallyl trisulphide and allyl methyl trisulphide, which are largely all volatilized or converted (Nakagawa et al.,1980; Sumiyoshi et al., 1984).

THE PRIMARY SULPHUR-CONTAINING CONSTITUENTS OF GARLIC AND THEIR CHEMISTRY

The chemistry of garlic is quite complex and likely developed as a self-protective mechanism against microorganisms and other insults. The primary sulphur-containing constituents in whole, intact garlic are the γ-glutamyl-*S*-alk(en)yl-L-cysteines and *S*-alk(en)yl-L-cysteinesulphoxides, including alliin. Whole garlic typically contains ~1% alliin, together with (+)-*S*-methyl-L-cysteine sulphoxide (methiin) and (+)-*S*-(trans-1-propenyl)-L-cysteine sulphoxide. *S*-(2-carboxy-propyl)glutathione, γ-glutamyl-*S*-allyl-L-cysteine, γ-glutamyl-*S*-(trans-1-propenyl)-L-cysteine and γ-glutamyl-*S*-allyl-mercapto-L-cysteine are also present in garlic cloves (Fenwick and Hanley 1985). During storage of garlic bulbs at cool temperatures, alliin accumulates naturally. On the average, a garlic bulb contains up to 0.9% γ-glutamylcysteines and up to 1.8% alliin. In addition to these main sulphur compounds, intact garlic bulbs also contain a small amount of *S*-allylcysteine (SAC), but no allicin. SAC is formed from γ-glutamyl cysteine catabolism (Figure 1) and has been reported to contribute to the health benefits of some garlic preparations (Amagase et al., 2001).

Garlic is famous for its characteristic odour, arising from allicin and other oil-soluble sulphur components. Once garlic is processed by cutting or crushing, compounds in the intact garlic are converted into hundreds of organosulphur compounds in a short period of time. When garlic is attacked by a microbe, crushed, cut or chewed, or when it is dehydrated, pulverized and then exposed to water, the vacuolar enzyme, alliinase, rapidly lyses the cytosolic cysteine sulphoxides (alliin) to form the cytotoxic and odiferous alkyl alkane-thiosulfinates (allicin) (Figure 1) (Amagase et al., 2001). Typical volatiles in crushed garlic and garlic essential oil include diallyl sulphide (DAS), diallyl disulphide (DADS), diallyl trisulphide, methylallyl disulphide,

methyl allyl trisulphide, 2-vinyl-1,3-dithiin,3-vinyl-1,2-dithiin (Fenwick and Hanley 1985) and *E*,*Z*-ajoene (Block, 1985).



Source: Amagase et al. (2001).

Figure 1. Chemical Change in Garlic.

Allicin is an odorous and extremely transient compound that decomposes to sulfides, including ajoene and dithiins (Freeman and Kodera, 1995; Amagase et al., 2001). Although freshly crushed garlic may contain limited amounts of allicin, no commercially available processed garlic products contain allicin. The acidity of the stomach prevents the conversion of alliin to allicin (Freeman and Kodera, 1995). These findings clearly indicate that allicin does not contribute to the *in vivo* effects of garlic.

In addition to the odoriferous oil-soluble compounds, less odorous water-soluble organosulphur compounds have been shown to be biologically active in various areas e.g S-allyl cysteine (SAC) (Sumiyoshi and Wargovich, 1990; Amagase and Milner, 1993; Imai et al., 1994). Additional constituents of intact garlic include the following: steroidal glycosides, lectins (Kaku et al., 1992), prostaglandins, fructan, pectin, essential oil, adenosine, vitamins B_1, B_2, B_6, C and E, biotin, nicotinic acid, fatty acids, glycolipids, phospholipids, anthocyanins, flavonoids, phenolics and essential amino acids (Fenwick and Hanley, 1985).

HEALTH BENEFITS OF GARLIC

Garlic has been appraised as a remedy for the treatment and prevention of a number of diseases such as cardiovascular and cerebrovascular diseases,

cancer and other metabolic diseases, hyperlipidaemia, hypertension and diabetes. The different functional health effects elicited by garlic are attributable to its bioactive components particularly the sulphur-containing compounds such as S-allyl cysteine (SAC), diallyl disulphide (DADS), and S-methyl cysteine sulphoxide. Various studies have revealed that different garlic preparations elicit different biological health effects with varying degrees of efficacy and safety, and very much dependent on their varied bioactive contents (Borek, 2001).

ANTI-CANCER HEALTH EFFECT-MECHANISM OF ACTION

Cancer is a disease of complex etiology that is classically defined as uncontrolled cellular division. The transformation of normal cells into neoplastic cells involves at least three discernible phases: initiation, promotion and progression (Borek, 1993). Oxidant-induced DNA damage and mutagenesis are determinants in the multistage process of cancer; inhibition of these events by phytochemical antioxidants may reduce the risk of the disease (Borek,1993, 1997). Evidence for the anticancer protection of garlic comes from both epidemiologic and preclinical investigations (Donaldson, 2004; Dorant et al., 1996). Fresh garlic extracts were reported to arrest the growth and also alter the morphology of MCF7 breast cancer cells (Modem et al., 2012).

AGE has been shown to inhibit both early and late stages of carcinogenesis in many tissues, including colon, mammary glands, lungs, skin, stomach and oesophagus (Wargovich et al., 1988; Wattenberg et al., 1989; Sumiyoshi and Wargovich, 1990, Amagase and Milner, 1993, Amagase et al., 1996; Liu et al., 1992; Milner, 1996; Reeve et al., 1993). A summary of anticancer organosulfur compounds in garlic extract is represented in Table 1.

Bioactive Constituents and Anti-Cancer Health Effects

The orgnosulfur constituents have the potential to suppress the growth of cancer cells and block cell cycle which can be linked to regulation of several key elements in cellular signal transduction. The allyl group is paramount in bringing about the growth depression (Pinto et al., 1997) but it should be noted that not all allyl sulfides are equal in their ability to reduce tumor proliferation (Sundaram and Milner, 1993). It has been shown that the water soluble SAC were less effective in retarding the growth of neoplasm compared to DADS

and DATS (Sundaram and Milner, 1993). Diallyl disulfide (DADS) was reported by Nakagawa et al. (2001) to have anticancer effects against breast cancers by interactions with polyunsaturated fatty acids known as modulators of breast cancer growth. Another study demonstrated that DATS could inhibit the growth of cells from human skin, prostrate, colon and lung (Herman-Antosiewicz and Singh, 2004).

Table 1. Anticancer organosulfur compounds in garlic extracts

Organosulfur compounds	Reported anticancer activity	Reference
Diallyl sulfide (DAS)	Inhibits growth, induces apoptosis of human cervical cancer Hela cells *in vitro.*	Wu et al., 2011.
Diallyl sulfide (DAS)	Decreased cell proliferation and induced apoptosis in anaplastic thyroid carcinoma (ATC).	Shin et al., 2010.
Diallyl disulfide (DADS)	Inhibition of human breast cancer cell line.	Nakagawa et al., 2001.
Diallyl disulfide (DADS)	Inhibits proliferation, invasion and angiogenesis of osteosarcoma cells.	Li et al., 2013.
Diallyl disulfide (DADS)	Inhibits the growthof H-ras oncogene transformed tumors in nude mice.	Singh, 2001.
Diallyl disulfide (DADS)	Supprssed7, 17-dimethylbenzo[∂] anthracene (DMBA) induced skin tumorigenesis.	Arora et al., 2006
Diallyl trisulfide (DATS)	Induction of apoptosis in tumor cells.	Li et al., 2006.
Diallyl trisulfide (DATS)	Inhibits human leukemia cell growth.	Nakagawa et al., 2001
Diallyl trisulfide (DATS)	Inhibits growth of PC-3 human prostate cancer xenografts male nude mice.	Kalra et al., 2006
Diallyl sulfide, Diallyl sulfoxide (DASO), Diallyl sulfone ($DASO_2$).	Inhibits chemically induced carcinogenesis and mutagenesis.	Yang et al., 2001.
S-allyl cysteine (SAC).	Inhibit growth of malignant progression of highly metastatic human non-small cell lung carcinoma in nude mice.	Arora et al., 2006.
S-allyl cysteine (SAC) and S-allyl marcaptocysteine (SAMC).	Inhibits proliferation of human prostate cancer (LNCaP) and human breast cell line (MCF-7).	Pinto and Rivlin, 2001.
S-methyl cysteine (SMC).	Chemopreventive agent against hepatocarcinigenesis and colon carcinogenesis.	Fukushima et al., 2001.
Ajoene	Inhibits B16/BL6 melanoma growth and metastasis to lung C57BL/L mice.	Taylor et al., 2006.
Allixin	Inhibits aflatoxin-induced DNA damage and mutagenesis in *Salmonella typhimurium*	Yamasaki et al., 1991

One previous study with osteosarcoma cells showed that DATS can inhibit cell cycle progression, and inducing apoptosis (Li et al., 2009). It has also been reported to inhibit angiogenesis. This it does by down regulation of vascular endothelial growth factor (Li et al., 2006). Hepatocarcinogenesis was inhibited by DAS when administered after the initiating procedure (Wattenberg et al., 1989).

The report of Singh (2001) demonstrated that DADS inhibits the growth of H-ras oncogenes transformed tumors *in vivo* by inhibiting the membrane association of $p21^{H\text{-}ras}$. The report also suggested that the allyl group may be an important determinant in the inhibitory effect of DADS on tumor growth (Singh, 2001).

DATS has been reported to promote apoptosis in human cancer cell lines and to significantly give protection against colorectal cancer in animal model (Yu et al., 2012). Ajoene induced apoptosis in human leukemic cells by the stimulation of peroxide production and activation of nuclear factor KR (Dirsch et al., 2002). DADS was reported to inhibit the growth of H-ras oncogene transformed tumors *in vivo* by inhibiting the membrane association of $p21H^{-ras}$ (Singh, 2001). Allixin is an important flavonoid in AGE and has been shown to prevent tumour promotion, inhibits aflatoxin-induced DNA damage and mutagenesis in *Salmonella typhimurium* (Yamasaki et al., 1991).

The exact mechanisms of the anticancer properties of garlic and its derivatives are still not clearly understood. One of the likely viable modes of action of *allium* derivatives could be via competitive inhibition of the enzyme cytochrome $P_{450}2E1$ which is known to activate xenobiotic substances including carcinogens (Brady et al., 1991; Yang et al., 2001). The mechanism of inhibition of DNA damage by allixin has been reported to be mediated via reduction in the DNA-damaging oxidant by-products that occur during the induction of P450 enzymes (Yamasaki et al., 1991). By this action, garlic and its derivatives may potentially make carcinogen less able to initiate carcinogenic process (Pinto and Rivlin, 2001). Another possible anticancer mechanism of action of garlic and its derivatives is the stimulation of glutathione and enhancement of glutathione peroxidase activity (Perchellet et al., 1986). The ability of garlic to accumulate selenium, which is an integral part of glutathione peroxidase, is one of the attributes that make garlic an anticancer agent (Santhosha et al., 2013). The allyl sulfide helps to regulate the drug metabolizing enzyme activity and thereby promoting the antioxidant enzyme activities (Yin et al., 2002). Summarily, the following molecular mechanism can be suggested – regulation of cell cycle progression, enhanced tumor apoptosis and antioxidant potential, blocking of cell cycle, inhibition of

carcinogen activation, enhanced phase II drug metabolizing enzymes and modulation of immune response.

ANTIOXIDANT HEALTH EFFECTS OF GARLIC: MECHANISM OF ACTION

Oxidative modification of DNA, proteins and lipids by reactive oxygen species (ROS) plays a crucial role in aging and disease processes. The phenolic extract of garlic has been shown to inhibit angiotensin-1 converting enzyme in cisplatin induced lipid peroxidation (Oboh et al., 2013) while whole extract of garlic attenuated oxidative stress and inflammation in fructose-fed rats (Sivaraman et al., 2013). Aged garlic extract (AGE) is ladened with antioxidant phytochemicals that prevent and protect against oxidative damage, acting singly or in synergy (Amagese 1997; Borek, 2001). AGE exhibits antioxidant activity 10 times more than fresh garlic extract (Borek, 2001).

The antioxidative properties of AGE and its bioactive components are monitored by their capacity to scavenge reactive oxygen species (ROS) and prevent the formation of lipid peroxides. A wide variety of mechanisms have been postulated to explain the antioxidant activity of AGE. A summary of some antioxidant health effects of AGE is shown in Table 2.

Scavenging ROS, Inhibiting LDL Oxidation and Lipid Peroxide Formation

The antioxidative actions of AGE and its components are strongly connected to their ROS scavenging capability with an attendant inhibition of lipid peroxides formation (Amagase, 1997; Awazu and Horie, 1997; Horie et al., 1989; Imai et al., 1994; Borek, 2001). Oxidized LDL has been shown to promote vascular dysfunction, which partly contributes to atherosclerosis via its cytotoxic effects on endothelial cells (Ide and Lau, 1997).

Enhancement of Endogenous Cellular Antioxidant Defences

Enhancement of Enzymatic and Non-Enzymatic Antioxidants

Glutathione, an important aspect of non-enzymatic antioxidant defence mechanism in living cells, protects cellular constituents from the damaging effects of peroxides formed in metabolism and other ROS reactions.

Decreased tissue GSH level is associated with cell damage, depressed immunity and the progression of aging, and may increase the risk of cancer development. AGE has been reported to enhance cellular glutathione in a variety of cells, including those in normal liver and mammary tissue (Liu et al., 1992).

Studies in cell cultures of endothelia cells subjected to oxidant stress show that AGE protects endothelial cells from ROS injury by modifying cellular scavenging enzymes. When bovine arterial endothelial cells were exposed to the oxidants hypoxanthine and xanthine oxidase or hydrogen peroxide, the presence of AGE enhanced the levels of superoxide dismutase (SOD), catalase, and glutathione peroxidase with concomitant reduction in the production of superoxide radical and hydrogen peroxide in a dose- and time-related fashion (Wei and Lau, 1998). The experiments show the potential ability of AGE to protect endothelial cells from oxidant injury, which is linked to the development of atherosclerosis and cardiovascular disease (Efendy et al., 1997; Wei and Lau, 1998).

Table 2. Antioxidant health effects of Aged Garlic Extract (AGE)

Property	Reference
Enhancement of glutathione	Liu et al., 1992
Enhancement of scavenging enzymes	Wei and Lau,1998
Reduction of cardiovascular and cerebrovascular disease	Ide and Lau, 1997
Inhibition of oxidant ischemic brain injury.	Borek, 2001
Inhibition of nuclear factor $-_{K}$B activation	Geng et al., 1997
Inhibition of DNA damage and mutagenesis	Ide and Lau, 1997
Inhibition of carcinogenesis	Amagese et al.,1996
Radioprotection against UV-induced suppression of immunity	Reeve et al.,1993
Inhibition of cardiotoxicity by doxorubicin	Awazu and Horie, 1997
Inhibition of oxidant-induced liver toxicity	Wang et al., 1998
Protection against age-related brain atrophy	Richardson, 1993

Bioactive Constitulents: Anti-Oxidant Health Effects

Many other studies have also shown the role of the water soluble constituents in the antioxidant properties of AGE. S-allyl-cysteine (SAC) and allyl-mercapto-L-cysteine (SAMC) were reported to be antioxidative and mainly responsible for antioxidant activity of AGE (Numagami and Ohnishi,

2001; Ide et al., 1996). SAC is reported to inhibit free radical production, lipid peroxidation and neuronal damage in rat brain ischemia (Numagami and Ohnishi, 2001). Ryu et al. (2001) identified Nα-(!-deoxy-D-fructos-1-yl)-L-arginase as another antioxidant compound in AGE. N-trans-coumaroyloctopamine (1) and N-trans-feruloylopamine (2) were recently identified as antioxidant compounds of garlic skin (Wu et al., 2015).

EFFECTS OF GARLIC ON CARDIOVASCULAR AND CEREBROVASCULAR DISEASES

Cardiovascular disease (CVD) has been described as a chronic disease in humans with varied etiology ranging from hypercholesterolemia, diabetes mellitus, hypertension, increased oxidation damage and smoking (Ross, 1999). The oxidative modification of lipids, typically LDL, has been implicated in the development of cardiovascular and cerebrovascular diseases (Cox and Cohen, 1996; Witztum, 1993). Oxidation of lipids modifies membranes and impairs their function. Fluidity is decreased, membrane-bound enzymes and receptors are inactivated, red blood cells are damaged and endothelial cells are injured, increasing blood vessel fragility. Oxidation of LDL accelerates the growth of fatty streaks in blood vessel walls (Efendy et al., 1997) and the formation of plaque (Ide and Lau, 1997). Toxic aldehydes formed in lipid oxidation react with the apoprotein B of the LDL particle to produce a novel epitome that is recognized by macrophage receptors, resulting in the formation of foam cells and atherosclerotic plaques and increased risk of heart disease and stroke (Witztum, 1993).

Garlic is known to be a potent anti-atherogenic supplement (Gorinstein et al., 2007). Several studies have investigated the effectiveness of garlic extracts in protection against CVD. Garlic extracts and fractions could prevent diet-induced hypercholesterolemia in cholesterol-fed rats and mice (Slowing et al., 2001; Mohmoodi et al., 2006; Mohammadi and Oshaghi, 2014) and can attenuate the ratio of serum LDL to HDL (Mohmoodi et al., 2006; Ebrahimi et al., 2015). Garlic powder was reported to reduce total cholesterol and low density lipoprotein (LDL) cholesterol (Sobenin et al., 2010). Garlic extracts have been variously reported to have anti-hypertensive effects by increasing NO synthesis (Al-Qattan et al., 2006), induction of vasodilatation with hydrogen sulphide (Ginter and Simko, 2010), and inhibition of angiotensin converting enzyme activity.

The report of Zahid Ashraf et al. (2005) indicated that garlic extract is a vasorelaxant and may reduce the atherogenic potentials of cholesterol in rats. Garlic oil has been reported to reduce systolic and diastolic blood pressure and oxidized LDL in hypertensive patients (Dhawan and Jain, 2004).

AGE is known to inhibit lipid oxidation and oxidative modification of LDL, thus reducing the amount of circulating oxidized LDL and the subsequent accumulation of cholesterol in macrophages, smooth muscles and blood vessel walls, resulting in the inhibition of atherogenic fatty streaks (Efendy et al.,1997; Ide and Lau, 1997). These effects, coupled with other actions of AGE, increase its potential to lower the risk of cardiovascular and cerebrovascular disease. Other protective actions of AGE include inhibition of platelet aggregation and suppression of prostanoid synthesis with subsequent anti-inflammatory, anti-atherogenic and anti-thrombotic effects (Dimitrov and Bennink, 1997).

The protection of endothelial cell integrity via inhibition of lipid peroxidative injury and reduction in serum cholesterol and other lipids by AGE adds to its functional ability in preventing heart disease and stroke (Borek, 2001; Weiss et al., 2006).

Bioactive Constituents: Cardiovascular and Cerebrovascular Health Effects

Some of the bioactive compounds in garlic and aged garlic extract have been implicated in the cardiovascular effects attributed to garlic (Table 3). Allicin and its derived thiosulfinates are known as the major bioactive compounds responsible for its antithrombotic property (Cavagnaro et al., 2007). Diallyl disulfide and diallyl trisulfide were reported to suppress oxidized LDL-induced vascular cell adhesion (Lei et al., 2008). The water soluble and lipid soluble compounds in garlic inhibited cholesterol synthesis in both human and animal studies (Yeh and Liu, 2001).

Allicin from garlic powder could positively affect two atherosclerosis risk factors (Ali et al., 2000) and also induce hypotension in rabbit eye by acting on the neuroeffector junction (Chu et al., 1993). The report of Fallon et al. (1998) showed that garlic protected rats from hypoxic pulmonary hypertension by employing a mechanism that effect pulmonary arterial rings in the endothelium. Cruz et al. (2007) reported the antihypertensive effects of SAC in nephrectomized rats. They suggested that the antioxidant property of SAC

could be responsible for the observed reduction in hypertension and renal damage.

Table 3. Cardiovascular organosulfur compound in garlic extract

Organosulfur compounds	Reported Cardiovascular activity	Reference
Diallyl disulfide (DADS)	Suppress oxidized LDL-induced vascular cell adhesion	Lei et al., 2008
S-allyl cysteine (SAC).	Antihypertensive and cardioprotective in the presence of captopril	Asdeq and Inamdar, 2010
S-ethyl cysteine (SEC), S-propyl cysteine (SPC)	Inhibition of cholesterol synthesis by 40-60 percent	Yeh and Liu, 2001
γ-glutarmyl-S-allyl cysteine (GSAC), γ-glutarmyl-S-methyl cysteine (GSMC), γ-glutarmyl-S-propyl cysteine (GSPC)	Inhibition of cholesterol synthesis by 40-60 percent	Yeh and Liu, 2001
Diallyl sulfide (DAS), Diallyl disulfide (DADS), Diallyl trisulfide (DATS), dipropyl sulfide, diproyl trisulfide	Inhibition of cholesterol synthesis by 40-60%	Yeh and Liu, 2001
Alliin and allicin.	Inhibition of hepatic hydroxyl methylglutaryl-CoA reductase activity	Sangeetha et al., 2006
Alliin and allicin derived thiosulfinates	Antithrombotic activity	Cavagnaro et al., 2007

Allyl methyl sulfide (AMS) and diallyl sulfide (DAS) were reported to serve as an efficient antioxidant bioactive compound in garlic that can ameliorate structural changes resulting from hypertension (Castro et al., 2010).

There are also several other studies on the anti-atherosclerosis properties of garlic bioactive constituents. Diallyl sulfide and diallyl trisulfide have been reported to be the principal constituents responsible for such property (Jain and Konar, 1978). Allicin could also affect atherosclerosis by acting as antioxidant, inhibiting the uptake of LDL and degradation of macrophages (Gonen et al., 2005). Garlic directly affects atherosclerosis by its ability to decrease arterial cell lipid content and preventing intracellular lipid accumulation (Mikaili et al., 2013) and also through the inhibition of hepatic activity of lipogenic and cholesterogenic enzymes (Mathew and Biju, 2008).

Many *in vitro* and *in vivo* studies have shown that garlic compounds and its extract possess antithrombotic actions. Allicin is a potent anti-platelet constituent of garlic extract (Agrawal, 1996). The report of MacDonald et al.

(2004) demonstrated that aromatic thiosulfonate derived from garlic inhibited platelet aggregation while Choi and Park (2012) reported that diallyl trisulfide (DATS) has antithrombotic activity. Methyl allyl trisulfide (MATS) has been shown to inhibit anti-platelet activity by inhibiting arachidonic acid cascade (Ariga et al., 2000). Ajoene was reported to possess anti-aggregatory effect that is linked to its direct interaction with putative fibrinogen receptors (Apitz-Castro et al., 1986) and irreversibly inhibit platelet aggregation initiated by arachidonic acid (Srivastava and Tyagi, 1993). One study reported that S-ethyl cysteine (SEC) prevented lipid biosynthesis in cultured rat hepatocytes (Yeh and Liu, 2001).

ANTI-AGING EFFECTS OF GARLIC

ROS play a major role in age-related neurodegeneration (Richardson, 1993). Studies of a senile dementia model in mice showed that AGE prevented atrophic changes in the frontal brain, improved learning abilities and memory retention, and increased longevity in the senescence-accelerated mouse (Moriguchi et al., 1997). This suggests that AGE may have anti-aging effects and the potential to prevent age-related deterioration of brain functions that are linked to dementia and Alzheimer's disease (Borek, 2001). The studies also indicate that the antioxidant actions of AGE may have an important role in its anti-aging effects.

HYPOGLYCAEMIC EFFECTS OF GARLIC

Clinical studies on hypoglycaemic effect of garlic are less well defined. Preclinical studies reveal that garlic is effective in reducing blood glucose in streptozotocin-induced and alloxan-induced diabetes mellitus in rats and mice (El-Demerdash et al., 2005). Several mechanisms have been suggested to explain this observation. Augusti and Sheeela (1996) proposed that the antioxidant effect of S-allyl cysteine sulphoxide isolated from garlic may reduce diabetic condition in rats. Jain and Vyas (1975) proposed that garlic can act as an antidiabetic agent by increasing either the pancreatic secretion of insulin from the cells or its release from bound insulin.

EFFECTS OF GARLIC ON THE IMMUNE SYSTEM

Garlic has been shown to be a possible biological response modifier (immunomodulation) (Kyo et al., 2001). Because certain diseases can be caused by immune dysfunction, modification of immune functions by garlic may contribute to the treatment and prevention of diseases. Thus, some pharmacologic effects of garlic are probably mediated through immunomodification (Kyo et al., 2001; Lamm et al., 2001).

Anti-Allergic Effect

Allium vegetables, including garlic, inhibit the release of ß-hexosaminidase, which is correlated with histamine release, in rat basophilic leukaemia cells (RBL-2H3), suggesting an anti-allergic effect. Kyo et al. (1997) also found that aged garlic extract (AGE) has an anti-allergic property.

ANTIBACTERICIDAL EFFECTS OF GARLIC

Garlic exhibits a broad antibiotic spectrum against both gram-positive and gram-negative bacteria (Sivam, 2001). Garlic has been reported to inhibit *Aerobacter*, *Aeromonas*, *Bacillus*, *Citrella*, *Citrobacter*, *Clostridium*, *enterobacter*, *Escherichia*, *Klebsiella*, *Lactobacillus*, *Leuconostoc*, *Micrococcus*, *Mycobacterium*, *Proteus*, *Providencia*, *Pseudomonas*, *Salmonella*, *Serratia*, *Shigella*, *Staphylococcus*, *Streptococcus* and *Vibrio*. Noteworthy among the reported findings are the following:

1) Garlic exhibits a broad antibiotic spectrum against gram-positive and gram-negative bacteria (Sivam, 2001).
2) Garlic is active even against organisms that have become resistant to antibiotics.
3) The combination of garlic extracts with antibiotics leads to partial or total synergism (Didry et al., 1992).
4) A garlic oil preparation showed good anti-tuberculosis activity in guinea pigs (Jain, 1993).
5) As a result of the bactericidal activity of garlic, toxin production by bacteria is also prevented (Sivam, 2001).

Garlic and Helicobacter Pylori

Helicobacter pylorus is a bacterium implicated in the etiology of stomach ulcers and cancer (Fuchs and Mayer, 1995). The incidence of stomach cancer is lower in individuals with a high intake of *allium* vegetables in developed and developing (high risk) countries (Steinmetz and Potter, 1991a, b). Feldberg et al. (1988) showed that allicin exhibits its antimicrobial activity mainly by immediate and total inhibition of RNA synthesis, although DNA and protein syntheses are also partially inhibited, suggesting that RNA is the primary target of allicin action (Sivam et al., 1997, 2001).

Food Preservation

Garlic inhibits the growth of microorganisms as well as toxin production. More research is needed to assess the value of garlic as an alternative to chemical food preservatives, especially in foods in which the garlic flavour would be an added bonus. There is also potential use for garlic by itself or in combination with other herbs and spices to extend the shelf-life of raw meat products (Sivam, 2001).

CHEMO- AND RADIOPROTECTIVE EFFECTS OF GARLIC

In recent times, the use of botanicals in chemo- and radioprotection has been on the rapid increase, mostly because of the supposedly less frequent side effects when compared to synthetic agents. Garlic extracts have been evaluated as antidote for toxicant distribution in different organs (Massadeh et al., 2007), radiation (Lau, 1998) and for its antioxidative properties in curbing chemically induced oxidative insult (Suru, 2008; Ola-Mudathir et al., 2008; Obioha et al., 2009; Ola-Mudathir and Suru, 2015).

Supplementation with AGE may have an important protective role against liver toxicity caused by a variety of medicinal and environmental substances. AGE was recently shown to protect against oxidative damage by inhibiting lipid peroxidation in liver cells exposed to phenobarbital, a sedative and bromobenzene-3, 4-oxide, an environmental toxic agent (Wang et al., 1998). Earlier studies (Tadi et al., 1991) showed that AGE protects against liver toxicity by benzo(a) pyrene and aflatoxin B_1, two potent free radical-producing environmental carcinogens (Borek, 1993,1997). Studies in mice

showed that SAC and SAMC were potent inhibitors of liver toxicity induced by the industrial oxidant carbon tetrachloride and by the commonly used analgesic agent, acetaminophen (Nakagawa et al., 1988).

SAFETY AND TOXIC EFFECTS OF GARLIC

Although garlic has been used safely in cooking as a popular condiment and traditionally for medicinal purposes, it is commonly known that excessive consumption of garlic can cause problems. Significant synergy or antagonism of the garlic substances, or their derivatives, on human physiology exist and vary with an individual's age, pathology, dosage regimen and possible drug, food or metabolite interactions. The long historical experience of garlic consumption has satisfied many individuals and entities that the human risk-to-benefit ratio is beneficial, although occasional human allergic reactions, gastric distress, topical sensitivities and increased blood coagulation times are not uncommon (Staba et al., 2001). Garlic odor on breath and skin are recognized (Mader, 1990). Also observed are decreases in serum protein, calcium, and anaemia (Nakagawa et al., 1980); bronchial asthma, contact dermatitis (Garty, 1993) and inhibition of spermatogenesis (Dixit and Joshi, 1982).

Raw extracts of garlic have been reported to exhibit toxic effects when ingested at certain concentrations. Nakagawa et al. (1980) demonstrated the toxic effects of peroral administration of raw garlic juice (5ml/kg). This dose caused the death of 5 rats with serious stomach injury in 21 days. The body weight of still living rats decreased at the beginning as their food and water intake also decreased. The growth of the rats was retarded and was thought to be caused by the stomach injury due to raw garlic. Also observed, were swellings of the liver, hypertrophy of the spleen and adrenal glands, and decrease of erythrocytes with various morphological changes. But almost these changes were not observed at any time on rats given AGE.

In another study, Banerjeea et al. (2001) examined the effects of chronic garlic intake on various endogenous antioxidant enzymes and lipid peroxidation on two major organs, the liver and kidneys of rats fed with fresh garlic homogenates daily by gavage in three different doses (250, 500 and 1000mg/kg/day) for 30 days. The 1000mg/kg/day dose of garlic caused depressed endogenous antioxidants without altering thiobarbituric acid reactive substances (TBARS), marked histopathological and ultrastructural changes in both liver and kidneys. These changes suggest that garlic, although

has the ability to enhance the endogenous antioxidant states at low doses, a reversal of these effects is associated with higher doses. This indicates the need to identify a safe dose range for garlic (Nakagawa et al., 1980).

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In: *Allium sativum*
Editor: Abel Haynes
ISBN: 978-1-63484-264-8

Chapter 3

MEDICINAL APPLICATIONS OF *IN SITU* GENERATED ALLICIN

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ABSTRACT

Allicin is one of the most bioactive molecules found in nature. It is an organosulfur compound released by crushing garlic cloves and is formed by a reaction between alliin (major organosulfur compound in the cytoplasm of intact garlic cloves) with the enzyme alliinase (compartmentalized in the vacuole of garlic cloves). Allicin degrades quickly in gastric fluid, intestinal fluid, and blood in humans. In addition, allicin penetrates cell membrane easily and reacts rapidly with accessible thiol groups and disappears from circulation within a few minutes after injection. These properties make the pharmaceutical applications of allicin challenging. However, to circumvent the ineffectiveness of allicin, a binary system consisting of alliinase and its substrate alliin has been proposed. In this binary system, the two inert but stable components (alliin and alliinase) generate an active compound (allicin) that exhibit biological activity *in situ*. Here, we review recent progresses in facilitating the *in situ* generated allicin methodology and its possible

medicinal applications. The following topics will be discussed: (a) strategy for mass production of alliinase and diastereo pure alliin; (b) stabilities of alliinase and alliin under various environments; (c) methods of conjugating alliinase to antibody for use in site-directed allicin generation in situ; (c) methods of assessing the efficacy and creation of the in situ generated allicin; and (d) anticancerous/antimicrobial activities of the in situ generated allicin.

INTRODUCTION

Allicin (diallyl thiosulfinate) is naturally formed in crushed garlic by the action of the enzyme alliinase on alliin (S-allyl-L-cysteine sulfoxide). It is a natural defense mechanism for the plant against microorganisms, insects, and pests (Cavallito and Bailey 1944, Miron et al., 2006, Block 2010, Howtopedia 2012, Bhagat et al., 2014). Allicin is reported to have lipid-lowering, antiblood coagulation, antithrombotic, antihypertension, anti-inflammatory, antioxidant, anticancerous, antiviral, antimicrobial, and antiparasitic activities with low toxicity towards human (Koch 1996, Lawson 1998, Block 2010, Lu et al., 2012, Lee et al., 2013a, Borlinghaus et al., 2014, Salama et al., 2014, Ilic et al., 2015). Therefore, not surprisingly, garlic is the bestselling herbal supplement in the United States and allicin has been explored as a potential lead compound for drug design (Tapiero et al., 2004, Blumenthal 2005). Pure allicin can be made by oxidation of diallyl disulfide (DADS), by purifying from aqueous garlic extract (AGE), or by passing a solution of alliin through an immobilized alliinase column (Miron et al., 2006b, Lee et al., 2013a, 2013b). However, allicin, with a characteristic smell of garlic, is unstable and decomposes into biologically less active oil-soluble organosulfur compounds (OSCs) such as diallyl sulfide, DADS, diallyl trisulfide (allitridi), dithiins, and ajoene and water-soluble OSCs such as Sallyl cysteine and S-allyl mercaptocysteine (Block 1985, Ichikawa et al., 2006, Lanzotti 2006, Dethier et al., 2012). Allicin degrades during drying by vacuum and frying/boiling/steaming/baking/roasting /microwaving on cooking (Gorinstein 2005, Lee et al., 2013b, Palermo et al., 2014). In addition, the stability of allicin is affected strongly by pH and temperature (Yu et al., 1989, Lawson and Wang 2005a, Lawson and Gardner 2005b, Fujisawa et al., 2008a, Durairaj et al., 2009, Wang et al., 2015). At room temperature, allicin is most stable at pH 5.5, but degrades quickly at lower or higher pH. It begins degrading in 0.5 h and is not detectable after 2 h when the pH is higher than 11.0 or lower than 1.5. However, pure allicin HPLC fraction (~1.3 mg/mL) is stable for months when

stored in 50% acetonitrile (ACN) aqueous solution with 0.1% trifluoroacetic acid, pH 2 at -20^{o}C (Lee et al., 2013b). Allicin is reported to be more stable in 20% alcohol than in water with a half-life activity of 11 days, but unstable in vegetable oil with half-life activity of 0.8 hours (Fujisawa 2008b). Due to its instability in the acidic environment (pH 1.2) of gastric fluid, it degrades before it reaches blood and other tissues when passing through the stomach, so it is doubtful it contributes to health benefits in the body (Freeman and Kodera 1995, Freeman and Kodera 1997, Fujisawa 2008b). Allicin degrades quickly at temperatures higher than 40^{o}C, with rapid degradation when it reaches 70℃. It is active for minutes at 80^{o}C, hours at 50^{o}C, days at 37^{o}C, weeks at 25^{o}C, months at 4^{o}C, and up to a year at -20^{o}C. Allicin is more stable at high concentration. Pure allicin or allicin in aqueous garlic extract with various concentrations is ineffective in stopping the tumor growth of the *Mus musculus* colon carcinoma CT26.WT cell inoculated BALB/c mouse by different injection routes and durations (Baoshiang Lee, pers. comm.). In this experiment, tumors are generated in BALB/c mice (6 weeks old) by injection of *Mus musculus* colon carcinoma CT26.WT cell (3–5 x 10^{6} cells/mouse) into animals for 2 weeks. Another property of allicin which impedes its effectiveness is that as soon as allicin is in the body, it reacts with accessible thiol groups, penetrating biological membranes and decomposing to other compounds. Therefore, allicin is undetectable after incubating in blood for few minutes and disappears from the circulation in minutes after injection (Freeman and Kodera 1995, Miron et al., 2000, Rabinkov et al., 2000). This is why the majority of the potent anti-cancerous/anti-bacterial activities effects of allicin are demonstrated in vitro and makes pharmaceutical applications of allicin a challenging endeavor (Lee et al., 2013a, Borlinghaus et al., 2014, Ilic et al., 2015).

In recent years, progress has been made in circumventing the instability and short half-life of allicin by using a binary system consisting of the enzyme alliinase and its substrate alliin to generate allicin *in situ* (Miron et al., 2003, Arditti et al., 2005, Fry et al., 2005, Appel et al., 2010, Chhabri et al., 2015, Gupta et al., 2015). In this binary system, the two inert and relatively stable constituents (alliin and alliinase) generate a bioactive compound (allicin) that exhibits biological activity *in situ*. In one proof-of-concept study (Arditti et al., 2005), alliinase is chemically conjugated to an antibody against a tumor marker (ErbB2) and subsequently the alliinase-antibody conjugate is administered in a human N87 tumor cell line xenograft athymic nude mouse for one day to allow the maximum accumulation of conjugate at the tumor site. The tumor growth is successfully inhibited upon addition of alliin while at the

same time the normal tissues are unharmed due to the inert nature of alliin and alliinase and the high clearance rate of allicin. It is therefore possible to initiate biological activity in a controlled way. Apart from circumventing the instability of allicin, this binary system has additional advantages, such as low toxicity toward normal cells, usage of much higher concentration of its individual components and site-directed activation. Furthermore, alliinase is able to react with other S-alk(en)yl-L-cysteine sulfoxides such as dihydroalliin, S-isopropyl-L-cysteine sulfoxide, and S-butyl-L-cysteine sulfoxide (Stoll and Seebeck 1951, Lancaster and Collin 1981, Krest et al., 2000, Shen and Parkin 2000). These results pave the way to a range of possible binary systems with specific chemical and biochemical properties.

In situ allicin has shown promising applications *in vivo*. Recently, progress has been made on reagent preparation and reaction conditions to realize the biomedical potential of the *in situ* allicin approach. These include but are not limited to (a) facial synthesis methods for mass production of diastereo pure alliins to alleviate the need of purchasing expensive alliin; (b) simple and effective method of preparing alliinase; and (c) simple biochemical techniques to assess the efficacy of the *in situ* allicin. In addition, the inactivity of fluorenylmethyloxy-carbonyl (Fmoc) protected alliin, alliin methyl ester, alliin amide, and others in alliinase enzymatic reaction indicate that the amino, carboxyl, and cysteine moieties of alliin are critical for alliinase reaction (Stoll and Seebeck 1951, Gupta et al., 2015, Baoshiang Lee, pers. comm.). Results also indicate that alliin as a substrate is most effective when added to the cancer cell culture within 3 days of adding alliinase and ineffective after 7 days. In addition, alliinase reacts less with (-)-L- alliin than with (+)-L- alliin. Cancer cell viability 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, bacterial plate-diffusion growth inhibition assay, flow cytometry cell cycle analysis, and tumor animal model are effective tools in validating the anticancerous/antipathogen activities of the *in situ* allicin. All these aspects of the in situ allicin system are discussed in this review.

PREPARATIONS OF ALLIIN AND ITS ANALOGS

Alliin, a major organosufur compound in *Allium sativum,* contains sulfur and carbon stereocenters and is the precursor of allicin. One gram of fresh garlic cloves contains 6-14 mg of alliin (0.6-1.4% fresh weight). It is stable at pH 1-12 and at 37^{o}C or below. It retains activity after heating for a day at 90^{o}C or 2 days at 60^{o}C. Alliin has been found to be stable in serum at 37^{o}C for

weeks and in cancer cell culture at 37^{o}C in humid environment containing 5% CO_2 for up to 3 days (Baoshiang Lee, pers. comm.). In addition, alliin is active in organic solvents such as methanol (MeOH), ethanol, isopropanol, dimethylformamide (DMF), dimethyl sulfoxide (DMSO), and ACN. A simple and efficient way of preparing alliin is essential in the *in situ* generated allicin approach. It has been shown that (+)-L-alliin is more reactive with alliinase than (-)-L-alliin. This has motivated development of facile synthesis of diastereo pure (+)-L-alliin, which would help in realizing the biomedical potential of *in situ* binary allicin synthesis. There are many synthesis schemes for making diastereomers of alliin. It can be made from L-cysteine and allyl-bromide with subsequent hydrogen peroxide oxidation, and also asymmetric sulfur oxidation using tetraisopropyl ortho-titannate or oxygenase (Jayathilaka et al., 2015). Solvent extractions, multiple fractional crystallization, or HPLC are used to separate and purify alliin. Fractional crystallization and HPLC have proven to be effective tools in isolation of disasteriomers (Dethier et al., 2012, Jayathilaka et al., 2014). In addition, alliin can be prepared from garlic by extraction with heat, microwave, organic solvent, or sonication deactivation of alliinase (Mallika et al., 2014). The result obtained at optimum extraction conditions recovers 90% of alliin from fresh garlic cloves.

In general, substrates of alliinase reaction contain the following structural features: (a) they must be derivatives of cysteine; (b) the sulfur atom of the cysteine derivative must link to alk(en)yl group; (c) the amino group of the cysteine portion of the compound must not be blocked; and (d) the sulfur atom of the cysteine derivative must be a sulfoxide. Garlic alliinase hydrolyzes (+)-alliin, a naturally occurring substrate for the enzymatic synthesis of allicin, more rapidly than (-)-alliin (Stoll and Seebeck 1951; Lancaster and Collin 1981, Krest et al., 2000; Shalini et al., 2015). The fact that DADS has a significantly lower activity indicates that the thiosulfinate group plays an important role in the activity (Borlinghaus et al., 2014). Small et al. (1947) considered thiosulfinates as a new class of antibacterial compounds and chemically synthesized many different thiosulfinates. The thiosulfinate derivatives differed in the alk(en)yl groups (length of carbon chain; number of branch) that are attached to the thiosulfinate. Twenty different bacteria have been treated with these thiosulfinate. Results show that (a) branching of the alkyl groups resulted in a reduced activity; (b) *n*-pentyl-thiolsulfinate is the most effective thiosulfinate; and (c) the bacteriostatic outcome of thiosulfinates became stronger against gram positive bacteria but weaker against gram negative bacteria when carbon chain length increases.

It is known that the amine group of the alliin is crucial in forming a bond with alliinase cofactor pyridoxal-5'-phosphate (PLP). Fmoc-(+)-alliin and (+)-DL-alliin methyl ester, (+)-alliin-amino acid, and (+)-alliin amide have been synthesized to test the roles of amino and carboxyl groups in alliinase reaction (Baoshiang Lee, pers. comm.). Fmoc-(+)-alliin is synthesized by mixing (+) alliin with N-(9-Fluorenylmethoxycarbonyloxy)succinimide (FmocOSu). (+)-DL-alliin methyl ester is synthesized by mixing (+) alliin with anhydrous methanol in acidic conditions. (+)-alliin-amino acid and (+)-alliin amide are synthesized using Fmoc-(+)-alliin with standard solid phase peptide synthesis procedure. The (+)-alliin methyl ester produced contains both (+)-L-alliin methyl ester and (+)-D-alliin methyl ester due to racemization (Smerdka et al., 2004, Li and Sha 2008). Instead of fractional crystallization in 95% ethanol, RP-HPLC is used to purify Fmoc-(+)-alliin, (+)-DL-alliin methyl ester, (+)-alliin-amino acid, and (+)-alliin amide. The alliinase activity assay results of Fmoc-(+)-alliin, (+)-DL-alliin methyl ester, (+)-alliin-amino acid, and (+)-alliin amide show that all compounds are inactive thus supporting the theory that both amino and carboxyl groups of alliin are essential in alliinase reaction. Alliinase belongs to the class of carbon-sulfur lyases named Salk(en)yl-L-cysteine sulfoxide lyase and catalyzes the cleavage of carbon-sulfur bond of S-allyl-L-cysteine sulfoxide in a beta-elimination/deamination reaction involving an aminoacryl bound PLP-amino acrylate (Schiff's base) reaction intermediate (Nock and Mazelis 1987, Manabe et al., 1998, Shimon et al., 2007, Musah et al., 2009, Ravilious and Jez 2012). Alliinase provides acidic and basic groups at the active site of the enzyme, inducing polarization of the S=O group of the substrate, which initiates the carbon-sulfur bond breakage and produces an acrylamide bound PLP and allyl sulfenic acid. This catalytic mechanism is similar to that of cystathionine β-lyase (Ravilious and Jez 2012). Subsequently, the schiff's base of acrylamide bound PLP is spontaneously hydrolyzed and decomposes to pyruvate and ammonia. Simultaneously, condensation of two allyl sulfenic acids yields allicin. In agreement with this mechanism, it is plausible that the Fmoc group in Fmoc-alliin is blocking PLP cofactor binding and the inability to form carboxylate anion in (+)-alliin methyl ester, (+)-alliin-amino acid, and (+)-alliin amide inhibits the alliinase reaction. It is interesting to note that the optimal pH value for alliinase catalytic activity is 6.5, while the temperature is 33ºC.

Attempts to make a better subtract of alliinase by modifying the cysteine moiety of alliin have proven to be unsuccessful (Baoshiang Lee, pers. comm.). Alliin analogs which contain homologs of cysteine such as S-allyl-L-homocysteine sulfoxide, S-allyl-penicillamine-sulfoxide, S-allyl-3-methyl

cysteine sulfoxide, S-allyl-3-phenyl cysteine sulfoxide, and S-allyl-3-methyl-3-phenyl cysteine sulfoxide are inactive. The synthesis procedure of making alliin is used to make the first two compounds. The last three compounds are made according to the literature (Stanfield et al., 1986). In addition, selenium analog of alliin is not active and unstable (Block et al., 2001, Jayathilaka et al., 2015). For the synthesis of selenium analog of alliin (Baoshiang Lee, pers. comm.) 100 mg of (0.3 mmol) L-selenocysteine is dissolved in 0.5 N NaOH (1.64 ml) and ethanol (0.41 ml) is added. Reaction is cooled to 0°C and NaBH4 (80 mg, 2.1 mmol) is added while stirring. Yellow color disappeared when the reaction mixture is warmed to room temperature after which it is cooled again to 0°C and 2N NaOH (0.81 ml) and allyl bromide (103 µl, 1.2 mmol) is added. The L-selenocysteine and allyl bromide coupling is conducted for 3 h at room temperature. Subsequently, the pH of the reaction solution is adjusted using conc. HCl to 5.0. The resulting clear solution is injected onto a RP-HPLC column. The pure Se-2-propenyl-L-selenocysteine is eluted at 11 min and dried as a while powder (60% yield, >95% pure).

PREPARATIONS OF ALLIINASE

The enzyme responsible for the conversion of alliin to allicin is alliinase, which is present in unusually large amounts in garlic cloves, accounting for at least 10% of the total protein content (10 mg/g fresh weight). The reaction catalyzed by alliinase requires PLP as a cofactor and is categorized as a β-elimination-deamination reaction involving an aminoacryl intermediate bound to PLP. The crucial step in this enzymatic reaction is cleavage of the C-S bond of alliin to yield sulfenic acid and α-aminoacryl acid, which are spontaneously hydrolyzed to allicin, pyruvic acid, and ammonia. Alliinase is active in DMSO and isopropanol, slightly active in MeOH and ethanol, and inactive in ACN, Tetrahydrofuran (THF), and DMF (Baoshiang Lee, pers. comm.). The enzyme is sensitive to temperature, pH, and buffer. At room temperature, alliinase is most stable at pH 6, but losses activity rapidly at pH lower than 3 or higher than 9 getting denatured in the acidic environment of gastric fluid. It degrades quickly when temperature is higher than 40°C, with degradation accelerating at 70°C or higher. Alliinase in 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) buffer containing 10% glycerol, 1 mM $CaCl_2$, 1 mM $MnCl_2$, 1 mM $MgCl_2$, 20 µM PLP, and 0.25 M NaCl at 1g/mL is stable for minutes at 80°C, hours at 50°C, days at 37°C, weeks at 25°C, months at 4°C, and up to several years at -20°C. Alliinase in PBS is not stable, lasting only two weeks at

-20°C, one month at 4°C and 37°C, and two months at 25°C. In serum it loses activity in 8 hours at 37°C. It can be produced from garlic or by recombinant protein method.

Purification of Alliinase from Fresh Garlic Cloves

Garlic extract is prepared from fresh garlic cloves (Lee et al., 2013a, 2013b). Aqueous garlic extract (AGE) is prepared from peeled garlic cloves in hepes buffer (50 mM, pH 7.2) containing 10% glycerol, 1 mM $CaCl_2$, 1 mM $MnCl_2$, 1 mM $MgCl_2$, 20 µM PLP, and 0.25 M NaCl at 1g/mL (Lee et al., 2013a, 2013b). A 900 W high-torque motor blender is used to crush and pulverize the garlic cloves using three 5-second pulses with 2-minute breaks between pulses at 4ºC. The garlic puree is strained through 3 layers of cheesecloth. The filtrate is kept overnight at -20ºC. The following day, AGE is spun at 9878 x g for 4 minutes at 4ºC. Finally, the supernatant is filtered through a sterilized 0.22µm syringe driven filter to remove any residual precipitate and to sterilize the AGE. The clear supernatant is aliquoted and stored at -80ºC until use.

Many protocols have been used to prepare alliinase from AGE using gel-filtration, ion exchange, hydroxyapatite, and/or ConA Sepharose 4B affinity columns with varying enzymatic activities and protein purities obtained (Stoll and Seebeck 1951, Rabinkov et al., 1995, Miron et al., 1998, Kuettner *et al.*, 2002, Musah et al., 2009, Gupta et al., 2015). In one protocol (Gupta et al., 2015), the enzyme alliinase is purified to at least 95% homogeneity using PD10 and gel-filtration columns. Briefly, 2.5 mL of AGE is loaded onto a PD10 column. High-molecular weight (>5000 Da) biomolecules eluted into the first 3.5 mL fraction and low-molecular weight compounds into next 5 mL fraction with hepes buffer (50 mM, pH 7.2) containing 10% glycerol, 1 mM $CaCl_2$, 1 mM $MnCl_2$, 1 mM $MgCl_2$, 20 µM PLP, and 0.25 M NaCl. The addition of bivalent cations $Mg^{2+,}$ Mn^{2+}, and Ca^{2+} help stimulate the reaction rate. Subsequently, 2 mL of the first 3.5 mL PD10 protein fraction is injected onto Phenomenex Yarra 3 µm gel-filtration 300 x 7.8 mm HPLC column and the column is developed using hepes buffer at a flow rate of 1 mL/min and detection at 280 nm. Pure alliinase fraction is collected at 8 min which is verified by alliinase enzyme activity assay and mass spectrometry. The enzyme activity assay of alliinase is determined by the spectrophotometric assay using 4-mercaptopyridine (4-MP) method in a cell free system (Miron et al., 2002). Alternatively, a RP-C18 HPLC column can be used with buffer A:

25 mM triethylamine acetate (TEAA) at pH 7 and Buffer B:isopropanol. Concentration of alliinase is measured by 1-D sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) band intensity using BSA as standard. The alliinase solution displayed a single protein band on 1-D SDS-PAGE containing 4-12% (w/v) polyacrylamide with 2-(N-morpholino) ethanesulfonic acid (MES) running buffer. Fourier transform LC/MS data on trypsin digestion of the protein band and on protein solution have confirmed that the protein band is the enzyme alliinase with a molecular weight of 51917.47 Da. This method produces 5 mg alliinase/mL/g garlic cloves AGE solution, 2 mg alliinase/ml PD10 column solution, 1 mg alliinase/mL gel filtration column fraction. Eighty percent of the alliinase in AGE is recovered with an activity of 12 units per mg.

Alternatively, alliinase is purified from fresh garlic bulbs using another protocol (Stoll and Seebeck 1951, Rabinkov et al., 1995, Miron et al., 1998, Kuettner et al., 2002, Mallika et al., 2014). Alliinase is prepared using different buffers and column steps at 4°C. Peeled bulbs are homogenized in Buffer A (pH 6.5, 20 mM of Sodium phosphate buffer, 5 mM EDTA, 5% w/v NaCl, 10% v/v glycerol and 20 μM PLP). The inclusion of PLP and glycerol in buffers is to stabilize the enzyme during purification. The homogenate is squeezed through four layers of cheese cloth followed by centrifugation at 10,000 rpm for 30 min. The protein precipitating between 20%-25% polyethylene glycol (PEG) 6000 is collected and dissolved in Buffer B (15% (w/v) Sucrose, 1% NaCl). The supernatant is obtained through centrifugation at 10,000 rpm for 30 min and dialyzed for18 h at 4°C using the Buffer C (1% NaCl, 15% Sucrose). The obtained supernatant is freeze dried to obtain the partially purified enzyme. Alternately, the supernatant is further purified using ConA Sepharose column. The sodium phosphate buffer with the addition of EDTA, NaCl and Glycerol had highest stability and activity. The optimum enzyme activity of alliinase is at temperature of 31°C and pH of 6.1. The stability of the alliinase is inversely proportional to the temperature.

Preparation of Alliinase by Recombinant Protein Expresion

Alliinase has been cloned and expressed by several groups (Van Damme et al., 1992, Rabinkov et al., 1994, Manabe et al., 1998, Weik et al., 1998, Wu et al., 2012). Basically, total mRNA is prepared from garlic cloves. A cDNA library is synthesized with reverse transcriptase, and subsequently the gene coding alliinase is amplified with PCR using published primers. The gene is

introduced in expression vectors of *Escherichia coli*, *Saccharomyces cerevisiae* or *Pichia pastoris* The alliinase is then purified and characterized. In one protocol (Wu et al., 2012), a eukaryote expression plasmid of the alliinase gene from the garlic bulb is constructed for expressing in *Pichia pastoris* system. The alliinase gene is cloned from the garlic bulb by RT-PCR and the eukaryote expression plasmid of alliinase is constructed with the pPIczαC vector. The recombinant plasmid is transformed into *Pichia pastoris* X-33 by electroporation. The positive clones are screened and are induced by methanol. Supernatants after induction are analyzed by SDS-PAGE and western blotting. The activities of the recombinant protein and the alliinase purified from garlic cloves are measured by the pyruvic acid method. The amounts of alliinase are determined by Lowry method. Results show that the alliinase gene is successfully cloned from the garlic bulb and express in the supernatant of *Pichia pastoris* with the correct molecular weight using this protocol. However, the recombinant alliinase has half the enzyme activity compared to the alliinase purified from AGE.

GENERATION OF ALLICIN *IN SITU*

One problem exacerbating a clinical application of allicin is its high clearance rate in human body. As soon as allicin is in the human body, it reacts with accessible thiols, especially with the high amounts of glutathione and is decomposed to other compounds. This makes the pharmaceutical application of allicin challenging. Nevertheless the health benefits of allicin mentioned above are still an attractive prospect and an effective approach to circumvent the instability problem of allicin has been reported (Miron et al., 2003). In this approach, alliinase and alliin are delivered separately *in situ*, thus allowing production of allicin *in situ*. This *in situ* generated allicin approach can be done in two ways. Injecting alliin first into the biosystem at certain concentration for certain time duration followed by delivery of alliinase at certain concentration. Allicin forms in seconds when alliinase comes in contact with alliin but loses the effectiveness quickly due to its extreme instability and its reactivity with accessible thiols. Alternatively, allicin can be generated *in situ* by injecting the alliinase first at certain concentration for particular time duration and then alliin at certain concentration is delivered to the biosystem. Alliin has been tested to be stable in serum at 37^{o}C for weeks and for up to 3 days in cancer cell culture at 37^{o}C in a humid environment containing 5% CO_2. In addition, large amount of alliin is safe to be used.

However, alliinase in serum at 37^{o}C loses activity in 8 hours. In general, if alliinase is added first to the biosystem, alliin is subsequently added after 30 to 60 min. However, if alliin is added first to the biosystem, alliinase is added after 1 day. At an alliin concentration of 11.24 mM, 0.58 µM of alliinase is needed to inhibit 50% of the culture *Mus musculus* colon carcinoma CT26.WT cells (Gupta et al., 2015).

Site-Directed Allicin Generation *In Situ*

In a sophisticated and powerful approach to circumvent the instability of allicin, strides have been made by coupling alliinase to a target specific delivery carrier and subsequently delivering high amounts of the stable substrate alliin, thus allowing production of allicin *in situ* at the site of interest. Antibodies are routinely used in a variety of biomedical fields including biotechnology, medicine, immunotherapy, and diagnostics. With their high specificity and binding ability (the typical equilibrium dissociation constant of an antibody-antigen complex is ~ 10^{-6} - 10^{-12} M), antibodies are mainly used for the biomolecule recognition (Howard And Kaser 2007, Walker 2009, Schwartzbach and Osafune 2015). This makes them a perfect target specific delivery carrier, which allows accumulation of alliinase-antibody conjugate at the target. Despite its short half-life, allicin generated near the target has proven effective. In addition, the normal tissues are unharmed due to the inert nature of alliin and alliinase and the high clearance rate of allicin. Recently several groups (Miron et al., 2003, 2005, Appel et al., 2010, Chhabri et al., 2015) have published reports on successfully shrinking tumors and killing pathogens by attaching alliinase to antibodies against biomarkers of cancer cells or microorganism and site-directed generation of allicin *in situ*.

Preparation of Alliinase-Antibody Conjugation

Two alliinase-antibody conjugation methods have been reported. In one protocol (Appel et al., 2010), conjugation of antibody with alliinase is performed in three steps: (1) thiolation of the antibody with iminothiolane (Lambert et al., 1985); (2) derivatization of alliinase with an amine-reactive crosslinker N-hydroxysulfosuccinimide-polyethyloxy-maleimide (NHS-PEO4-maleimide), and (3) sulfhydryl-directed conjugation of the two modified proteins according to the manufacturer's protocol (Thermo Scientific Pierce). The molar ratio of antibody/alliinase used for conjugation is 1:3. The conjugates are separated from free alliinase by size exclusion chromatography

on a Superdex 200 column. Alliinase activity of the fractions is determined using the NTB (2-nitro-5-thiobenzoic acid) (Miron et al., 1998). Fractions that contained antibody and possessed the highest levels of alliinase activity are pooled and used. The chemical conjugation did not impair alliinase activity.

Alternatively, 3-(2-Pyridyldithio) propionic acid N-hydroxysuccinimide ester (SPDP) is used to prepare alliinase-antibody conjugate (Chhabri et al., 2015). In this protocol, 50 mM SPDP is added to 100 µg of alliinase in 25 mM sodium phosphate buffer (pH 6.5), containing 50% glycerol, and incubated for 1 h at room temperature. Gel filtration column is used to remove excess SPDP. The number of SPDP residues on the modified alliinase is 2.4 measured by the method described by Carlsson et al. (1978). Same procedure is used to modify the antibody with SPDP except that 100 mM SPDP is used. The number of SPDP residues on the modified antibody is 3.8. SPDP-antibody is reduced with 5 mM Dithiothreitol (DTT) for 30 min. Antibody-SH and DTT are separated by desalting column. Antibody-SH containing fraction is immediately combined with SPDP-alliinase at a molar ratio of SPDP-alliinase/antibody-SH (1.1/1). The reaction mixture is incubated for 1 h at 24^{o}C with 20% sucrose. The alliinase-antibody and non-conjugated proteins are separated by size-exclusion chromatography on Superdex G200 with 50 mM phosphate buffer (pH 6.5), containing 10% glycerol and 2 mM PLP.

4.1.2. Method of Adminstration of Alliinase and Alliin In Vivo

It is determined that the maximal accumulation of the alliinase-antibody conjugate in tumor cells occurred after 24 h, with its half-life being about 72 h (Miron et al., 2003). However, the half-life of the conjugate in the blood is less than 8 h. Alliinase by itself does not accumulate in the tumor. Two to three weeks after tumor implantation, mice are injected intravenous (i.v.) with the conjugate. All the mice are constantly supplemented with pyridoxine (vitamin B6) in their drinking water (100 mg/L). PLP (0.2 ml, 20 mM in PBS/mouse) is injected intraperitoneal (i.p.) 30 min before each i.v. injection of the alliinase conjugate or controls. Generally, animal is injected with conjugate first followed one day later by alliin. The half-life of alliin in the blood is about 4-5 h, therefore, it is administered twice a day. Mouse is injected with maximum of 100 µl of 30 mg/mL alliin in PBS. In case of microorganism infection, animal is injected with conjugate first and 30 min to 1h later by alliin. It is found that intrathecal (i.t.) administration of 50 nmol conjugate in 50 µl PBS followed 30 min later by 750 µg alliin in 25 µl PBS is well tolerated by the mice (Appel et al., 2010). Mice are generally divided into four treatment groups each consisting of at least six mice treated as follows: control (PBS),

antibody (80 µg per mouse), alliinase (80 µg per mouse) and alliinase-antibody conjugate (80 µg per mouse) followed 1 day later by alliin (3 mg per mouse, twice a day, during 3 days). Injection routes like i.t., i.v. and i.p. have been used.

ANTI-CANCER ACTIVITY OF *IN SITU* ALLICIN

Several groups have reported anticancer activity of *in situ* generated allicin. In the study by Miron et al. (2003), a site-directed generation of allicin *in situ* is used to inhibit N87 and CB2 tumor growth *in vitro* and *in vivo*. Alliinase from garlic is chemically conjugated to an antibody directed against a specific tumor marker, ErbB2. The procedure consisted of a two-step process including one that targets alliinase-antibody conjugate to the surface of tumor cell, followed by another, that made use of the inert stable compound, alliin. In the presence of alliin, tumor-localized alliinase produced allicin, which effectively killed both ErbB2-expressing N87 and CB2 cells in vitro, whereas 32D cells (a murine hematopoietic progenitor non- ErbB2 expressing cell) are not affected. Therefore, using N87 tumor inoculated athymic nude mice, a high antitumor activity of allicin that is produced *in situ* by the conjugate and alliin administered *in vivo* has been achieved. Alliin injection imitates the reaction occurring in the crushed garlic clove. Alliin undergoes conversion into allicin *in situ*, at the location of the antibody-alliinase conjugate. However, at the same time other tissues are unharmed due to the inert nature of alliin and the high clearance rate of allicin. Because mammalian cells do not produce alliinase, alliin is converted into allicin only by the localized conjugate. The effect of the treatment on tumor growth arrest became significant two weeks after its commencement, and it continued to rise, reaching highly significant inhibition a week later. Ten days after the treatment is stopped, tumor growth inhibition still remained.

In the study by Miron et al. (2005), a site-directed generation of alliin *in situ* is used to inhibit B chronic lymphocytic leukemia (B-CLL) and other B-cell lymphomas tumor growth *in vitro* and *in vivo*. Alliinase is conjugated to the monoclonal antibody rituxiantibody, which recognizes the CD20 antigen, and the resulting conjugate is targeted to CD20 positive B-CLL and other B-cell lymphomas. Upon addition of alliin, allicin is formed in situ, killing the CD20 positive tumor B cells via apoptosis. Following a 72-hour treatment, 85% and 96% reduction is observed in the number of viable B-CLL and EBV-transformed B cells, respectively. Using the human/mouse radiation chimera

for the evaluation of allicin targeting in a preclinical animal model, a significant reduction in the number of recovered B-CLL, mantle cell lymphoma, or EBV-transformed B cells is demonstrated. Results exhibit that this system offers a potent therapy for B-CLL and other B-cell malignancies with less toxicity to the surrounding cells. The main advantages of the alliinase-antibody conjugate over antibody-directed enzyme prodrug therapies, immunotoxins, or drug-conjugated antibodies are (a) the very low concentrations of alliinase-antibody conjugate needed (cytotoxic allicin molecules are continuously generated near target cells following the administrations of alliin); (b) the ability to administer large amounts of the inert alliin; and (c) the effectiveness of allicin killing mechanism.

In the study by Chhabri et al. (2015), a site-directed generation of allicin *in situ* is used to inhibit MIA PaCa-2 pancreatic cancer cell growth *in vitro*. Alliinase is chemically conjugated using SPDP to an antibody which is directed against a specific pancreatic cancer marker, CA19-9. Pancreatic cancer cells express a distinct carbohydrate tumor antigen CA19-9, it has a couple of advantages over conventional protein epitopes with respect to targeting. The first is that CA19-9 is present at the cell membrane such that antibody can easily access to it. Profusion of the epitope provides another benefit. After the alliinase-antibody conjugate is bound to MIA PaCa-2 pancreatic cancer cells, on addition of alliin, the growth of MIA PaCa-2 cells are effectively hampered. The extent of MIA PaCa-2 cell proliferation, in the presence of conjugate (4.7 units) and increasing concentrations of substrate alliin (20–200 µM) is assessed for 24 h. Half maximal inhibitory concentration (IC_{50})of alliin is found to be 69 µM. However, CA19-9 antigen negative OAW 42, HDF, HepG2, MCF-7 and PC3 cancer cells are not inhibited by the same treatment.

In the study by Gupta et al. (2015), an *in situ* generated allicin is used to inhibit growth of *Mus musculus* colon carcinoma CT26.WT cancer cell *in vitro*. Results from *Mus musculus* colon carcinoma CT26.WT cancer cell viability assay MTT assay and flow cytometry cell cycle analysis confirm that the *in situ* allicin is as active as allicin purified from aqueous garlic extract or allicin synthesized chemically in a dose-dependent manner. The data show a notable increase in sub-G1 populations of the cell cycle on the cells treated with allicin, indicating the induction of apoptosis. Cancer cell viability assay MTT assay and flow cytometry cell cycle analysis have been used to demonstrate the anticancerous activities of the *in situ* allicin. The *in situ* allicin converted from alliin by alliinase is very active. The results indicate that (+)-L-alliin is more reactive toward alliinase than (-)-L-alliin, and both amino and

carboxyl groups of the cysteine portion of alliin are critical in alliinase enzymatic reaction using Fmoc protected alliin and alliin methyl ester. In addition, facile pathways to synthesize diastereomerically pure alliins and isolate alliinase have been demonstrated. Results suggest alliin is most effective when added to the cancer cell culture within 3 days of adding alliinase and ineffective after 7 days as a substrate of the enzymatic reaction of alliinase. The data obtained in this study provide useful information on the design of the *in situ* allicin approach.

ANTIMICROBIAL ACTIVITY OF *IN SITU* ALLICIN

Several groups have reported antimicrobial activity of *in situ* generated allicin. In the study by Fry et al. (2005), an *in situ* generated allicin is used to inhibit growth of the highly damaging rice blast fungus Magnaporthe grisea *in vitro*. Since the effectiveness of allicin against a range of otherwise drug-resistant microorganisms has been shown, it has becoming a potential lead compound for drug design and the development of a benign agricultural pesticides. A 100 μL drop of a conidial suspension (10^5 conidia/mL) with the alliin or alliinase is placed on the surface of a plastic coverslip and left in a humid environment at 24°C overnight. Subsequently, either the alliinase (in case of alliin pretreatment) or the alliin (in case of alliinase pretreatment) is added to the conidial suspension for 30 min. The frequency of appressorium formation is determined by the number of appressoria from counting 300 conidia. The efficacy of the alliin, alliinase, and the binary system is determined by incubating 550 μL of a conidia suspension of *M. grisea* containing 10^6 conidia/mL with 200 μL of 75 mM alliin or 100 μL of alliinase (264 μM) or water (control). After an hour, the conidia are recovered by centrifugation and resuspended with 1 mL water. The conidia are than spread onto the surface of a complete medium agar plate. The plate cultures are incubated at 24°C for 4 days and the number of conidial colonies is counted. The in vitro findings conclude that while the alliin (500 μM) or alliinase (12 μM) controls did not stop germination and appressorium formation, addition of alliinase to the alliin/conidia incubation at 0, 0.5, 1.5, and 4 h almost completely inhibited fungal growth. This in situ generated allicin antifungal system based on alliin and alliinase might be superior to the application of conventional fungicides or allicin itself. Although higher concentrations of this antifungal system might have to be used, its intrinsically low toxicity toward

humans and high biodegradability make it an exciting alternative to current agents.

In the study by Appel et al. (2010), a site-directed generation of allicin *in situ* is used to inhibit growth of an opportunistic fungal pathogen *Aspergillus fumigatus in vitro* and *in vivo*. An antibody against *A. fumigatus* is produced and chemically ligated to the enzyme alliinase using iminothiolane and NHS-PEO4-maleimide. The purified alliinase-antibody conjugate bound to conidia and hyphae of *A. fumigatus* at nanomolar concentrations. The *in vitro* antifungal activity of alliinase-antibody conjugates is determined according to the conditions of Clinical and Laboratory Standards Institute (CLSI) document M38-A2 (Canton et al., 2009). Resting conidia (3×10^4 conidia/well) are seeded in 96-well plates and incubated for 4 h at 37^oC with remel RPMI 1640 Agar w/MOPS and 2% Glucose plated medium (100 µl). Conjugate alliinase-antibody is applied in serial twofold dilutions in triplicate, incubated for 30 min at 37°C, and then washed four times, followed by the addition of alliin (0.5 mg/ml). Hyphal growth is monitored by microscopic observation. Minimal inhibition concentration (MIC) readings are taken after 24, 48, and 72 h of incubation with alliin. No changes in the MICs are noted after 24 h. The wells in which no fungal germination is observed are scraped and plated on Sabouraud dextrose agar plates. Subsequently, the number of colonies is counted after 72 h to determine MIC. As a control, the antifungal activity is also determined by incubating *A. fumigatus* with alliinase or with conjugates consisting of the nonspecific antibody and alliinase. *In vivo* therapeutical testing of the conjugate is carried out in immunosuppressed mice infected intranasally with conidia of *A. fumigatus.* Intratracheal (i.t.) instillation of the alliinase-antibody and alliin (four treatments) resulted in 80 to 85% animal survival, with almost complete fungal clearance. Repetitive i.t. administration of the alliinase-antibody and alliin is also effective when treatments are initiated at a more advanced stage of infection. The fungi are eliminated without damaging the lung tissue or manifesting discomfort to the animals. Although i.t. instillation of the alliinase-antibody without alliin or the unconjugated antibody significantly delayed the death of the infected mice, only 20% of the animals survived.

In the study by Gupta et al. (2015), an *in situ* generated allicin is used to inhibit growth of gram-positive *Staphylococcus aureus* and gram-negative *Escherichia coli in vitro*. A bacterial plate-diffusion growth inhibition assay is used to assess the efficacy of *in situ* allicin on eliminating microorganisms. Gram positive *Staphylococcus aureus subsp. Aureus Rosenbach* (ATCC® 6538™) and gram-negative *Escherichia coli (Migula) Castellani and*

Chalmers (ATCC® 8739™) are used as test microorganisms. Bacteria in log phase growth in LB medium are grown at 37ºC oven to an OD_{560} = 0.18 and diluted 10 times with LB medium. 0.5mL of the bacterium solution is spread on a 10 cm diameter petri dish with agar, which is prepared with 15 mL of a sterilized LB agar aqueous solution (40g/liter). Ten mm diameter holes (wells) are punched out and filled with100 μL PBS solution containing 20μL of various concentrations of alliin, alliinase, allicin, AGE, a mixture of alliin and alliinase, or PBS. Plates are then incubated overnight at 37ºC and the diameter of the inhibition zone is measured. A dilution series of the *in situ* allicin is used to establish the proportionality of the relationship between the amount of active allicin and diameter of inhibition zone. Data show that the maximum zone of inhibition (39mm) is observed in *Staphylococcus aureus* and the minimum (17mm) was observed for Escherichia coli. PBS was used as a negative control. The lowest concentration at which there is no growth was reported as minimum inhibitory concentration (MIC). Results from the dose–response study of allicin (data not shown) show that MICs are in the ranges of 56–140 and 280–560μM in *Staphylococcus aureus* and Escherichia coli, respectively. The *in-situ* allicin is as active as allicin purified from AGE or allicin synthesized chemically in a dose-dependent manner.

CONCLUDING COMMENTS AND FUTURE RESEARCH

Beside fat and fiber, the general view of reducing cancer occurrence in term of nutrition and diet is the idea of trace compounds, non-nutrients in vegetables and fruits that work against tumor formation. Garlic is on top of the list as a source of these compounds. It (*Allium sativum*) is among the oldest cultivated crop, and has been used for both culinary and medicinal purposes for thousands of years. Garlic contains a high concentration of sulfur compounds which are responsible both for its pungent odor and medicinal effects. Allicin, the most active constituent of freshly crushed garlic, is produced upon reaction of substrate alliin with the enzyme alliinase. Allicin possesses medicinal properties such as anticancerous, antimicrobial, antivirus, antiparasite**,** *inhibition of platelet aggregation*, antithrombotic, antioxidant, antietiological, cholesterol reduction, wound healing, immunomodulation, anti-inflammatory, and apoptosis. Unfortunately, certain properties of the compound, such as chemical instability, and fast clearance rate in the body, have hampered its practical uses in the past. Recently, it has been shown that it is possible to use a binary system consisting of the plant enzyme alliinase and its substrate alliin

to generate allicin, and hence bioactivity, *in situ*. During application, the two inactive components generate compounds that inhibit growth of cancer cells and microorganisms. It is therefore possible to initiate biological activity in a controlled, yet effective manner. In addition, by conjugating alliinase to an antibody, it is possible to generate allicin in a site-directed modus *in situ*. Apart from circumventing many of the drawbacks of allicin, this binary system has additional important advantages, such as low toxicity of its individual components and selective activation. Moreover, alliinase is also able to use different substrates, therefore paving the way to a range of novel, binary antimicrobial systems with custom-made chemical and biochemical properties. In addition to growth inhibition of many types of cancer cells, the site-directed *in situ* generated allicin principle can be used to eliminate pathogens. The only requirement is the availability of highly specific antibodies, or other kinds of target-specific carriers. Ligation of such carriers to alliinase can be done by using chemical or recombinant fusion methodology. We expect developments in finding good site specific delivery carriers.

Pultz et al. (2014) have identified promising 15 biomarkers for breast cancer. For example, osteopontin and fibroblast growth factor receptor 2 are associated with tumorigenesis, tumor invasion and metastasis. Crawford et al. (2014) have reported that a number of new, exciting biomarkers have emerged recently and these prostate cancer biomarkers hold tremendous promise for providing more selective therapy for patients. Estrada et al. (2015) have detected many biomarkers such as recombinant soluble glycoprotein (rsGP) for Zaire Ebola (ZEBOV), angola recombinant glycoprotein (rGP) and dengue nonstructural protein I (NS1) for Marburg virus (MARV), and couple of malaria antigens. In the light of recent progresses in biomarker discovery, more studies are anticipated on developing new antibodies against specific cancers or pathogens. In addition, more investigation is expected in the use of other substrates of alliinase beside alliin such as thiosulfinate derivatives differing in the alkyl groups that are attached to the thiosulfinate group in chain length as well as in branching mentioned in section 2. Simpler and easier ways of producing alliinase, alliin, conjugates and other routes and methods of drug delivery are additional areas where more research is needed.

ACKNOWLEDGMENTS

Financial support from the Research Resources Center at UIC is gratefully acknowledged.

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In: *Allium sativum*
Editor: Abel Haynes
ISBN: 978-1-63484-264-8

Chapter 4

EVALUATION OF THE ANTIMICROBIAL ACTIVITY OF THE FRESH *ALLIUM SATIVUM LILIACEAE* (GARLIC)

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ABSTRACT

This study aimed at evaluating the antimicrobial activity, *in vitro*, of fresh *Allium sativum Liliaceae* (garlic) against *Staphylococcus aureus* (Sa) and *Escherichia coli* (E. coli). The experiment was conducted at the Microbiology Laboratory at the Adventist University Center of Sao Paulo, between April and June, 2013. The antibacterial activity was evaluated in standardized strains of *Staphylococcus aureus* (ATCC#6538) and *Escherichia coli* (ATCC#8739). Garlic (*Allium sativum L.*) was peeled and sliced in round slices of approximately 2 mm thick, 10 mm in diameter and with 0.094 g weight. As positive controls, were used commercial antimicrobial discs: penicillin G (10 IU) and amikacin (30 ug) and, as a negative controls, filter paper discs. The experiment was done *in vitro*, using the Agar diffusion method. After seeding the bacterial inoculum at a concentration of 0.5 McFarland (1.5 x 108 CFU/ ml), garlic disks were placed on the disks with the aid of sterile forceps. All plates were incubated in culture chamber at 37°C for 48 hours. At the end of this period, it was observed the formation of zones of inhibition of bacterial growth (halos), measured in millimeters using a millimeter ruler. Garlic extract showed an inhibitory halo of 21.0 ± 2.0 mm (n = 12) compared to Sa, while the positive control penicillin G (10 IU) showed an inhibitory halo of 36.0 ± 0.9 mm (n = 12). As to *E. coli*, an inhibition zone was 19.8 mm ± 1.3 (n = 12), while the positive control amikacin (30mcg) showed an inhibitory halo of 25.3 ± 2.1 mm (n = 12). We concluded that *in vitro* fresh *Allium sativum* L. (garlic) inhibited the growth of *E. coli* and Sa, but such inhibition was not statistically significant. However, we suggest further study of a larger sample and also other bacterial strains, since its action in clinical practice has been effective.

INTRODUCTION

Information and research on plants and their benefits have been more and more common. Humans' constant search for curing disease with alternative treatment, as with the use of phytotherapy, may have intensified over the years for its fewer side effects, lower cost and ease of access, especially in Brazil (Santos et al., 2011a; OMS, 2000).

In the past, plants were acknowledged for their medicinal properties. Later on, the arrival of synthetic drugs drove them to the background. Nowadays, they ceased being used only in the food industry and started being thoroughly explored in their therapeutic potential (Louis et al., 2012).

Healthy natural products, including inorganic compounds and an array of plant extracts, are known for their disease control capabilities. Countless researches have been carried out showing the effectiveness and trustworthiness of plants (Hofling et al., 2010; Costa et al., 2010; Carvalho et al., 2011; Feitosa et al., 2011; Badke et al., 2011), especially the many species of garlic, such as *Allium sativum, Allium porrum* and *Allium tuberosum* (Araújo et al., 2009; Rodrigues et al., 2011).

Garlic (*Allium sativum* L.) is an herbaceous plant characterized by a bulb (head) divided in cloves (bulbils). It originated in Central Asia and has been utilized as food or medicine since ancient times (Mota et al., 2005).

It has been noted that the use of medicinal plants has taken great importance in people's day-to-day lives (Badke et al., 2011). Garlic still is often researched as to its nourishing and therapeutic qualities (Mota et al., 2005), being a functional food rich in allicin, which has antiviral, antifungical and antibiotical actions. Besides these actions, it also has hypotensive, hypoglycemic, hypocholesterolemic and platelet antiaggregant activities (Corzo-Martínez et al., 2007; Botelho et al., 2009; Venturoso et al., 2011; Arzanlou et al., 2011).

Ajoene, one of garlic's active agents, currently is one of the most important and available fungicides. It is a therapeutic agent that can be used topically, when treating fungal skin infections for short periods, showing high effectiveness and low recurrence, reducing the risks for systemic toxicity (Ledezma & Apitz-Castro, 2006).

Vaginal leukorrhea is one of the most common feminine conditions during childbearing age, affecting one third of all women, and markedly one half of pregnant women (Naud et al., 2004). When present during pregnancy, it is associated with a higher risk for abortion, amniotic cavity infection, preterm premature rupture of membranes, premature parturition, and low newborn weight (Guerra et al., 2006).

Staphylococcus aureus is responsible for more than 30% of all hospital infections and the infections caused by this bacteria show elevated morbidity and mortality indexes, both in hospital and home occurrences. It is a highly resilient organism that presents accentuated gene polymorphism between strains, which makes infection control difficult and turns previously utilized antibiotics not so effective (Fagundes and Oliveira, 2004; Santos et al., 2007).

This bacteria belongs to the gram positive cocos group, found in healthy people, mainly on the skin and the nasal cavity. However, it may cause skin infections, besides severe infections such as pneumonia, vaginitis, meningitis,

endocarditis, sepsis, and even systemic infections, which may lead to death (Santos et al., 2007).

Escherichia coli (E. coli) is a microorganism that causes most of abdominal, pancreatic and urinary tract infections, and amnionitis (Brasil, 2004). Urinary tract infection is the second most common cause of pregnancy clinical intercurrence, being *E. coli* the main infectious agent, responsible for 75 to 80% of cases (Brasil, 2012).

Both the vaginal leukorrhea and the urinary tract infections are the main causes leading to medical consultations (Naud et al., 2004), usually treated with antibiotics or fungicides, of systemic or topic uses. However, self-medication misuse may have facilitated microorganism resistance, compromising treatment effectiveness (Moraes, et al., 2014; Costa et al., 2006).

Given the increase of bacteria resistance to antibiotics, there exists the need to develop new bacteriostatic means and bactericides which collaborate to the therapeutic course in infected individuals. Thus, the goal of this study is to evaluate the antimicrobial activity found in garlic, *in natura*, when facing *Staphylococcus aureus* and *Escherichia coli.*

MATERIAL AND METHOD

This study is about an experimental research with quantitative approach on the evaluation of garlic's antimicrobial activity (*Allium sativum, Liliaceae*), *in natura*, when facing *Staphylococcus aureus* and *Escherichia coli* standardized strains.

The experiment was conducted at the Microbiology Laboratory at the Adventist University Center of Sao Paulo (UNASP-SP), in the period from April to June, 2013.

Vegetable Material

We used garlic, *in natura*, by the brand "Alimentos Calebe," packed on April 02, 2013, with expiration date after 180 days. The garlic was peeled and sliced with approximately 2 mm thick with the aid of a round glass tube of 10 mm in diameter, cut in disks. The mean *Allium sativum* L. disk weight was 0,099 ± 0,017 g.

Preparation of the Microbial Inoculums

When evaluating the antimicrobial activity of garlic, we used strains of *Staphylococcus aureus (*ATCC #6538) and *Escherichia coli* (ATCC #8739).

The microbial inoculum was diluted in a nutrient broth, corresponding to 0,5 in the McFarland standards (1,5 x 108 UFC/ml) for bacteria, being this concentration reached with the DENSIMAT-BIOMERIEUX densitometer. For this procedure, we used microorganisms extracted from inoculated cultures, approximately 24 hours before.

Preparation of the Culture Medium

The Petri dishes were sealed with brown and white Kraft paper and were autoclaved (autoclave by Phoenix), at 120^0C for 30 minutes for sterilization. The Müller-Hinton agar growth medium was prepared according to specification by the manufacturer, CULTICON-CECON, registered under the Health Ministry nº10000600134, lot: 3650. The dishes with the growth medium were let at rest for 24 hours at 37ºC for sterility control. They were then placed in a refrigerator at 15ºC until the moment of use, for no longer than a three-day period.

Experimental Method – Agar Diffusion Test

Before sowing, the dishes were let at rest until they reached room temperature. The microbial inoculum was evenly distributed over the dishes surface for approximately three minutes, at room temperature, until the solution was absorbed by the growth medium.

Twelve disks of *Allium sativum* L., *in natura*, were applied directly to the dish with the aid of sterile tweezers. As negative controls, we utilized blank disks sterilized with gamma radiation, with no impregnation, measuring 6 mm in diameter. As positive controls, we used commercial Penicillin G (10 UI) disks (SENSIBIODISC-CECON), for *Staphylococcus aureus* and Amicacina (30mcg), for *Escherichia coli*. They were placed over the dishes and softly pressed for full contact with the surface. All dishes were incubated in FANEM LTDA autoclaves, model 002 CB, at 37ºC, during 48 hours.

After this time, growth inhibition halos were measured in millimeters, aided by a millimeter ruler.

Data Analysis

The analysis of data was done by the inhibition zone (halo) diameter measurement descriptive statistics in millimeters (mm), shown by samples and controls. The sample number was equal to 12 and results were expressed in millimeters by the arithmetic inhibition halo diameter mean formed around the disks. Results were expressed as mean (M) ± standard error average and evaluated significance by the analysis of variance ANOVA (GraphPad Prism 5), followed by the Tukey test, which does multiple comparisons, considering $p < 0,05$ as significant difference.

RESULTS

Evaluation of *Allium sativum*, *in natura*, by the Agar Diffusion Method

Garlic (disks with 10 mm diameter and 0,099 ± 0,017g.), presented an inhibition halo of 21,0 ± 2,0 mm (n=12), in comparison to *Staphylococcus aureus* (ATCC #6538), while the penicillin G (10 UI) positive control presented an inhibition halo of 36,0 ± 0,9 mm (n=12). As to the *Escherichia coli* (ATCC #8739), garlic showed an inhibition halo of 19,8 ± 1,3 mm (n=12), while the positive control amicacin (30mcg) presented an inhibition halo of 25,3 ± 2,1 mm (n=12), Tables 1. *Allium sativum* L. (garlic), *in natura*, inhibited the growth of *Staphylococcus aureus* (ATCC #6538) and of *Escherichia coli* (ATCC #8739), even though this inhibition was not statistically significant.

Table 1. Diameter of inhibition halo of the *Allium sativum, in natura,* through Agar Diffusion Test. São Paulo, 2013

Microorganisms tested	***Allium sativum* L. *(Garlic)***	**Positive Control**	**p*****
Staphylococcus aureus[1]	21,0±2,0 mm (n=12)	Penicillin G (10UI) 36,5 ± 0,9 mm (n=12)	NS
Escherichia coli[2]	19,8 ± 1,3 mm (n=12)	Amicacin (30mcg) 25,3 ± 2,1 mm (n=12)	NS

[1]ATCC #6538; [2](ATCC #8739); ***ANOVA: (NS) - Not significant Tukey test.

DISCUSSION

Urinary tract infections affect people all around the world and *Escherichia coli* plays a major role in those infections (Beraldo-Massoli, et al., 2012).

In a study that selected 510 positive urocultures with the goal of assessing the prevalence of uropathogens and their sensitivity profiles, a high *E. coli* resistance rate to first choice antimicrobials in treating urinary infections was found, such as ampicillin (57,9%), pipemidic acid (50,5%), nalidixic acid (48,6%), sulfazotrim (44,8%), cefalexin (43,1%), ciprofloxacin (30,2%), amikacin (20,2%), etc. As such, there are difficulties in finding antibiotics to be promoted for this treatment. Authors conclude that the prevalence and the resistance to antimicrobials must be considered when choosing the appropriate course of action (Moraes et al., 2014).

Moreover, the need for new treatments, which show the same antibiotics effectiveness, has become clear, with lesser bacterial resistance levels, such as natural antimicrobials that have repeatedly shown their effectiveness and trustworthiness (Araújo et al., 2009; Costa et al., 2010; Guimarães et al., 2010; Carvalho et al., 2011; Feitosa et al., 2011; Badke et al., 2011; Rodrigues et al., 2011).

The garlic antimicrobial activity has been known since ancient times and its antibacterial action has been proved efficient both in laboratorial studies and in humans and animals studies. Evidence shows that garlic is therapeutically efficient in preventing and treating several pathologies, be it in extracts, essential oils, and *in natura* (Lima et al., 2011; Santos, et al., 2011b; Venturoso et al., 2011; Silva et al., 2012).

In a study which tested 32 plants used as condiment, 12 of them showed some antibacterial activity, and the nirá garlic and leek, which belong to the same family as *Allium sativum* L., and salvia, were the only ones that successfully inactivate *Escherichia coli* (Carvalho et al., 2005; Pires et al., 2007; Lo et al., 2013).

Garlic's antibacterial, antifungal, antiviral, antiprotozoa, anthelmintic action is based on several studies, and can be ascribed to the allicin affect, *Allium sativum L.*'s active substance, which carries a vast array of gamma substances (Lima et al., 2011; Venturoso et al., 2011; Mantawy et al., 2011; Casella et al., 2012; Leite et al., 2012).

The antibacterial effect found in garlic has also been shown in bacteria such as *Salmonella cholerasuis, Staphylococcus aureus*, and *Escherichia coli*, supporting the present research (Santos, et al., 2011b; Venâncio, 2010)

Penicillin G is the medical therapy most widely adopted when dealing with *Staphylococcus aureus*, with a 96, 4% proneness (Carmona et al., 2012). It must be highlighted that infection-causing microorganisms, treated with penicillin, may become resistant or resilient to such drug, or even present side effects (Neme, 2006). Because of that, it is necessary to investigate new drugs or alternative therapeutical courses to treat several diseases.

The antibacterial activity of *Allium tuberosum Rottler ex Spreng - Liliaceae* -"nirá" garlic has been evaluated towards standardized inoculums of *Staphylococcus aureus* (ATCC#25.923), *Enterococcus faecalis* (ATCC # 19.433), *Salmonella enteritidis* (ATCC # 11.076), and *Escherichia coli* (ATCC#11.229). Tests showed selective antibacterial activity over different Gram-negative inoculums, which reached maximum and permanent inhibition and inactivation for *Salmonella* after 48 hours, and, for *Escherichia coli*, after 72 hours exposition. Gram-positive, *Staphylococcus*, and *Enterococcus* bacteria showed total resistance when facing ethanolic extracts (Araújo et al., 2009).

The results of the present study antagonize with the previously cited study, for it shows that the *Allium sativum* L. possesses an antimicrobial growth inhibition effect, in compliance with other studies (Venturoso et al., 2011; Silva et al., 2012; Casella et al., 2012; Fonseca et al., 2014). Therefore, *Allium sativum* L. must be regarded in treating infections caused by different microorganisms.

CONCLUSION

Allium sativum L., *in natura*, inhibited the growth of *Staphylococcus aureus* (ATCC #6538) and *Escherichia coli* (ATCC # 8739).

These results show that garlic, *in natura*, may usher a new line of investigation to treat bacterial diseases with natural produce. Further studies, *in vitro* and *in vivo*, are fundamental in assuring garlic's antimicrobial activity, so that one can encourage, or not, its good clinical practice.

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In: *Allium sativum*
Editor: Abel Haynes
ISBN: 978-1-63484-264-8

Chapter 5

PRODUCT DEVELOPMENT: MECHANIZED SYSTEM FOR CULTURE OF GARLIC (*ALLIUM SATIVUM* L.)

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ABSTRACT

This work has the main objective to development of a preliminary mechanical system for the culture of garlic (*Allium sativum* L.). It starts with an argument that shows the necessity of new technologies in the production system of garlic. Using the conceptions of projects methodology, a functional solution was created for the planting machine, with some new mechanical creations. As follows, there is a presentation of the prototype of this machinery in detail and the evaluation in laboratory and field tests. It was found that the prototype has relative complexity in their construction in some parts, requiring an infrastructure with casting, turning, among others. However, it can be said that the manufacturing machine is relatively simple because the processes used in their construction are already widely known in the field of metal construction - mechanics. Still show a median efficiency that can be improved through of updates. Soon, through the making of this machine is realized a product innovation that with appropriate corrections can assist in planting garlic.

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INTRODUCTION

Silva et al. (2010) mentions that because of the process of globalization of the economy and to the competitiveness of agricultural products it becomes essential the adoption of new methods and production techniques for successful agricultural activities. The use of agricultural mechanization in the several field operations is one of the greatest tools that increased world grain production, bringing to farmers numerous benefits, among these, cost reduction and speed in carrying out field operations (Oliveira et al., 2007). In addition, according to Arcoverde et al. (2011), with the advent of mechanization, various agricultural activities have become simpler and more practical than when the work was predominantly done manually or with the help of animal traction. Therefore, the increased use of mechanization in agriculture has required new investments in machinery with greater feeding and embedded technology, for meeting the several demands of agricultural activities (Piacentini et al., 2012). Thus, the aim of this paper is to present the results of a mechanized system for the garlic crop.

Teixeira et al. (2009) identified the major machinery used by farmers in Rio Grande do Sul. The following machines were mentioned: bean harvester, corn harvester, peanut grinder, straw chopper, limestone distributor, rotary tiller, brush cutter for two-wheeled tractor, pump spray, mower, mower for vegetables (between lines), oranges juicer, wheat mill and potatoes harvester, vegetable seed sower. The small seed sower and the peanut shelling machine, the two-wheel tractor and sower for corn and beans were also cited by the author (Teixeira, 2009). Note the lack of machinery used for the culture of garlic.

The economic importance of garlic cultivation has increased significantly in recent years, not only for its use as a spice, but also because of some therapeutic qualities attributed to it. In Brazil, garlic has great economic and social importance, once it is a vegetable which is grown in the vast majority, by small producers, with intensive use of Embrapa workforce (2004), which is cited by (Queiroz, 2010). Brazil, traditional garlic importer, has been reducing its foreign dependency through the development of new technologies and the expansion of noble garlic cultivation (Feitosa et al., 2009).

The mechanization of the garlic is mainly used for soil preparation. Threshing, planting, sorting, processing and harvesting are usually done manually by the farmer. The use of workforce in threshing is due to the inadequacy of the machines. Besides, the use of sieves in machines for the classification of bulbils, for size and shape, is not efficient because it can only

establish a correlation between these factors and the weight of cloves when it comes to the cultivation with well defined “teeth” forms. For that reason, such classification system does not meet the main requirement established by it, which is the selection of bulbils by weight. Therefore, in this work the results of the tests performed on the machine will be presented.

MATERIALS AND METHODS

The beginning of the project occurs through the direction for identifying which are the needs of the consumer. From the answers of the consumer, the project requirements were established. In the determination of these requirements, the environments that will be interacting with the product are considered, as well as the restrictions they may impose during the several stages of the production-consumption cycle.

After the definition of the environments, it is determined how they can restrict or influence the product to be developed. For the mechanized system, which consists of a set of machines, the restrictions must be identified for each one of them; it is expected compatibility among them.

At the project stage of the machines of the system, the garlic and the consumer were the considered elements. The Ishikawa diagram (Fishbone) was used to analyze the restrictions implemented by the influential elements of the product during the project phase. Through the use of this diagram, it is possible to identify the elements that might influence at the manufacturing, use and maintenance stages.

Considering the requirements established as a starting point, the stages of the conceptual project, the preliminary project and the detailed project of this conception were developed. This was followed by the methodology proposed by Pahl et al. (2007), in which, after the description of the requirements, the structure of functions is created, which describes the basic tasks that must be performed by the machine. Next, by using the method of morphological matrix, alternative conceptions to solve the proposed problem are generated. Making use of the criterion function, the most appropriate solution to the desired requirements is identified. After the conception is chosen, the preliminary project with the use of mathematical models was started, being useful in the sizing process of the subsystems that comprise the machine. So, when the preliminary project is concluded, the next step was detail it, and then building the prototype. Finally, the tests were carried out in order to know the performance and the efficiency of the prototype.

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RESULTS AND DISCUSSION

The Test Results

The tests are aimed at checking the operation of feeding sets, customization, positioning and planting bulbils. In addition, the tests are useful to detect possible failures presented in these sets, in order to be able to offer solutions for improving their functioning. In the first phase, there were checks in laboratory for testing the subsystems individually and then with the planter machine in the field. It was considered as suitable seed for planting, the one that left the considered subsystem, or machine, without showing damage. The seeds used in the tests were taken along to the outputs of the joint and afterwards placed in plastic bags for being assessed the damage in them.

In the first individual tests of the set, an unsatisfactory performance of the machine components was observed, once the specified functions – feeding, individualization, positioning and planting - were not performed satisfactorily, according to the following problems:

1. Feeding Set: excessive restriction created by the flow regulator element of the container to the passage of seeds, which causes some damages to the conveyor belt feeding. Still, there was excessive removal of seeds located in the spaces between the teeth of the conveyor belt by the extractor rotor elements.
2. Individualization Set: direct passage of the seeds of the conveyor belt to the lower container, due to the high length of stay of the blade of the individualizing device in the upper position. There was the retention of the bulbils on the blade individualizing device, due to the lack of exhaust among them with the lower container.
3. Positioning Set: retention of bulbils, at the apex, in the holes of the cavities of one of the positioners discs. It also occurred the shearing of the bulbils.

4. Set planting: Retention of bulbils, by tipping in planting wheel holes.

After completing relevant modifications in clusters, there was a new battery of assessments on the machine.

This second stage of laboratory tests has the objective of verifying the repeatability provided by new devices, and the influence of different working speeds on machine performance and feeding sets, individualization, positioning and planting.

It was found that the variable "speed," in the feeding set (Table 1), within the considered parameters (270-330 m/h), did not significantly influence the rate of supplying seeds. The kneading type was the most of the damage occurred in the seeds, and they happened during the passage of these by the extractor rotor.

Table 1. Summary of the data obtained in the test of the feeding set - Sample = 100 seeds/20 observations

Speed (m/h)	Considered number of spaces of the conveyer	Mean number of spaces of the conveyor that transported one or more suitable seeds	Mean number of damaged seeds		Mean number of seeds per space of the conveyer	Collection (%)
			Nº	%		
270	36	33.4	0.8	1.9	1.24	92.7
300	36	32.1	0.7	1.7	1.26	89.1
330	36	32.3	0.9	2.2	1.22	89.7
General mean	-	32.7	0.8	1.9	1.24	90.5

Source: Author.

It was noticed a high percentage of damaged seeds in the individualizing set (Table 2). They are of the shear damage type, which was caused by the blade of the individualizing device.

The positioning set with 100 seed (Table 3) samples was tested, observed 20 times, introduced not randomly in the vertical position with the apex upwards (VAU) or vertical with the apex downwards (VAD) (10 observations in each position) in the discharge channel the individualizing device.

The aspect that stands out in this group, as in the previous one, is the high percentage of damaged seeds. It is verified that, with the increase in speed, also increases the number of damaged bulbils. Such damage occurred on the cover of the discs positioners and they were of the shear type.

Table 2. Summary of the data obtained in the test of set individualization

Speed (m/h)	Number of introduced seeds	Mean number of individual suitable seeds	Mean number of damaged seeds	Mean number of damaged seeds	Efficiency (%)
270	100	52.4	3.6	23.2	52.4
300	100	53.1	5.1	24.6	53.1
330	100	52.8	4.4	22.8	52.8
General mean	-	52.7	4.3	23.5	52.7

Source: Author.

Table 3. Summary of the data obtained in the test of set position

Speed (m/h)	Number of introduced seeds	Mean number of positioned seeds	Mean number of damaged seeds	Efficiency (%)
270	100	56.4	8.8	56.4
300	100	59.4	8.8	59.4
330	100	56.7	9.1	56.7
General mean	-	57.5	8.9	57.5

Source: Author.

Table 4. Summary of the data obtained in the test of set planting – sample = 100 seeds/20 observations

Speed (m/h)	Number of introduced seeds	Mean number of positioned stored suitable seeds	Mean number of damaged seeds	Efficiency (%)
270	100	73.2	0.5	73.2
300	100	71.7	0.3	71.7
330	100	70.5	0.7	70.5
General mean	-	71.8	0.5	71.8

Source: Author.

The test of planting set (Table 4) aims to determine the seed position with regard to the opening of the tip. The used procedure consisted of introducing the bulbils in the upright position with the apex peaking up through the top of the tip and the subsequent verification of this opening. The depth of deposition was not studied. The manufacturing process of the tips, through welded plates, was not the most appropriate, by the presence of non-rounded surfaces, which compromised the slipping of the seeds, damaging the efficiency of the device.

Table 5. Summary of the data obtained in the test of planter machine

Speed (m/h)	Expected number of seeds per run	Mean number of seeds per run		Mean number of positioned seeds		Mean number of suitable positioned seeds		Mean number of damaged positioned seeds	
		Nº	%	Nº	%	Nº	%	Nº	%
270	36	12.6	35.0	8.1	22.5	7.1	19.7	1.0	12.3
300	36	12.2	33.9	7.9	21.9	7.0	19.4	0.9	11.4
330	36	13.4	37.2	7.5	20.8	6.6	18.3	0.9	12.0
General mean	-	12.7	35.8	7.8	21.7	6.9	19.1	0.9	11.9

Source: Author.

Finally, the test of the planter machine was carried out at the laboratory. For such test, it was considered a sample of 36 seeds in running of 2.19 meters (planting wheel circumference), being observed 20 times. The test was carried out with the introduction of 36 bulbils in the upper container and, thereafter, it was verified the number of released ones by the tops, then, being positioned and suitable for planting, in other words, without damage (Table 5).

It was verified the high number of machine failures that occurred, which caused an expected number of 36 seeds per run and an increase of an average of 12.7 bulbils, through 20 observations. These failures were due to overlaps and damage the bulbils. From 12.7 seeds released, only 6.9 were positioned and suitable to be planted, in other words, 19.1% of the expected bulbils.

Field Tests

It was analyzed the performance of the machine sets in working speeds higher to the specified one (300 m /h). The first verified problems were lifting the machine by the plow in areas with shallow soils, and the tumbling of seeds within tips. The solution to the first problem was to change the shape of the furrow, with the removal of soil subcompacting function. For the tips, the solution adopted was to adopt manufacturing processes that allow the rounding of its internal surfaces. The Table 6 presents the results of the tests which were carried out.

Table 6. Field test results

Sample	Number of seeds stored in holes		Number of suitable seeds which were stored in the holes in the VAU position		Number of suitable seeds which were stored in the holes in the VAU position and in depth of 60 to 80 mm	
	Nº	%	Nº	%	Nº	%
1	12	33.3	6	16.6	6	16.6
2	10	27.8	5	13.9	4	11.1
3	11	30.5	5	13.9	5	13.9
4	10	27.8	5	13.9	4	11.1
5	11	30.5	6	16.6	6	16.6
6	14	38.9	8	22.2	7	19.4
7	12	33.3	7	19.4	7	19.4
8	10	27.8	6	16.6	6	16.6
9	9	25.0	4	11.1	4	11.1
10	12	33.3	6	16.6	5	13.9
Mean	11,1	30.83	5.8	16.1	5.4	14.5

Source: Author - Sample = 36 seeds/run = 2,19 M.

The high number of failures occurred in the sets reduces the expected number of seeds from 36 to 11.1, on average, through 10 observations. Of these 11.1 seeds, only 5.4 are suitable for planting, the VAU position and within the required depth of 60 to 80 mm range, resulting in 15% of efficiency for the planter machine. This efficiency is below the one achieved by Sixto & Serwatowski (1996), who obtained in a pneumatic planter machine with advancement speed not less than 1 m /s, an efficiency of seed deposition in the soil of approximately 90%.

CONCLUSION

The requirement established for the machine - operating in small and medium national tractors, could not be respected in its completeness. The operation of developed mechanisms limits the speed of operation at approximately 300 m/hour. The problem that occurred in the machine in much higher speeds than the nominal shear was the damage of the seed in the positioner device (mean of 8.9%). Due to the relatively low weight (300 kg with 5 modules) the implement could be operated by two men. The removal of the modules attached to the support bar by screws facilitates the access to different parts of the set. A verification on the prototype leads to the conclusion that he has a few pieces of relative complexity in their buildings,

requiring an infrastructure with molding processes, turning, etc. However, it can be said that the machine is relatively simple to manufacture, since the processes used in their construction are already widespread in the metal-mechanics building area.

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