

Antileishmanial and Immunomodulatory Activity of *Allium sativum* (Garlic): A Review

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Journal of Evidence-Based
Complementary & Alternative Medicine
2017, Vol. 22(1) 141-155
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DOI: 10.1177/2156587215623126
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Abstract

Leishmaniasis is caused by an obligate intracellular protozoa belonging to *Leishmania* genus. The current drugs for treatment of leishmaniasis possess many disadvantages; therefore, researchers are continuously looking for the more effective and safer drugs. The aim of this study is to review the effectiveness, toxicities, and possible mechanisms of pharmaceutical actions of different garlic extracts and organosulfur compounds isolated from garlic against *Leishmania* spp. in a variety of in vitro, in vivo and clinical trials reports. All relevant databases were searched using the terms “*Allium sativum*,” “Garlic,” “Allicin,” “Ajoene,” “*Leishmania*,” “in vitro,” “in vivo,” and “clinical trial,” alone or in combination from 5 English databases (Web of Science, PubMed, Science Direct, Scopus, Google Scholar) and 3 Persian databases (Scientific Information Database, Iran Medex, and Magiran) from 1990 to 2014. In summary, garlic with immunomodulatory effects and apoptosis induction contributes to the treatment of leishmaniasis.

Keywords

Allium sativum, garlic, *Leishmania*, organosulfur compounds, allicin, ajoene

Received September 21, 2015. Received revised November 10, 2015. Accepted for publication November 16, 2015.

Leishmaniasis is a vector-borne disease that is caused by an obligate intracellular protozoa belonging to *Leishmania* genus. In the Old World and New World it is transmitted by sandfly bites of *Phlebotomus* genus and *Lutzomyia*, respectively. Leishmaniasis is more prevalent in tropical and subtropical regions and based on clinical manifestations, it is responsible for at least 3 types of disease that range from self-healing cutaneous lesions to severe and life-threatening mucocutaneous and visceral forms. According to the World Health Organization reports, leishmaniasis as a major public health concern with 2.4 million disability-adjusted life years is found in 5 continents and about 100 countries. Also, about 12 million people are infected currently and 350 million individuals are at risk for acquiring disease as well. Annually, approximately 0.2 to 0.4 million new cases of visceral leishmaniasis and 0.7 to 1.2 million new cases of cutaneous leishmaniasis occur throughout the world.¹ Pentavalent antimonial compounds like pentostam and glucantime have been used as the most important antileishmanial drugs from the 1940s till now, while unfortunately there is no effective and efficient vaccine against leishmaniasis yet. Also, amphotericin B, pentamidine, paromomycin, and miltefosine are being prescribed as second-choice drugs. Because of some drawbacks even after adjustment of course remedy and dose proportion of these drugs, including painful injection, prolonged treatment course, high cost, drug resistance, toxicity, and different side effects, there is an urgent need for investigators to find cheaper, affordable, safer, and more effective antileishmanial drugs.²

According to a report published by the World Health Organization, about 80% of people have a tendency to use traditional medicine to alleviate their pain and as a treatment for ailments.³ Recently, use of plant-derived and plant extracts has had a rising trend worldwide that is justified by low cost, fewer side effects, easy availability, and wealth of valuable sources of the plants.⁴⁻⁷ In recent years, several researches have been performed by the current authors about efficacy of different plant extracts and other compounds against *Leishmania* spp. viability, including *Allium sativum*,⁸ *Cordia myxa*,⁹ *Crocus sativus*,¹⁰ *Camellia sinensis*,^{11,12} *Satureja khuzestanica*,¹³ and some biurets compounds.¹⁴

Allium sativum (garlic) belongs to Plantae kingdom, Asparagales order, Amaryllidaceae family, Allioideae subfamily and *Allium* genus (<http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?id=4682>). Medicinal benefits

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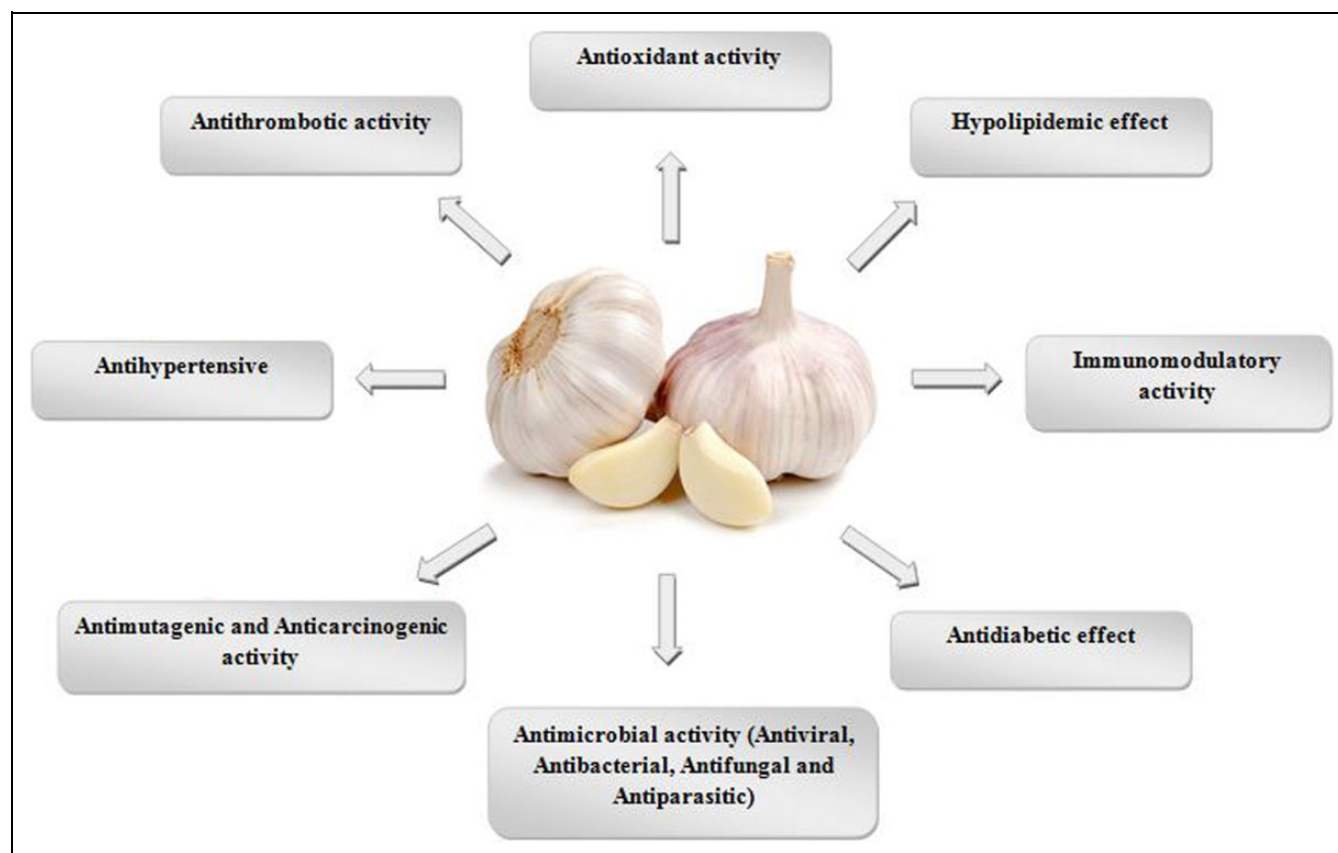


Figure 1. Summary of some therapeutic properties of *Allium sativum* (garlic) bulbs and cloves.

and therapeutic properties of garlic are ascribed to presence of organosulfur compounds (OSCs).^{7,15} Historically, garlic has possessed medicinal and dietary importance in different cultures for more than 4000 years. In addition, there is evidence that proves garlic has been consumed for various purposes over the centuries. For instance, during the Greek Olympics for stamina enhancement in athletes, during the Second World War as an antibacterial agent for prevention of gangrene in soldiers, in India as an antiseptic lotion in order to wash ulcers, and in China as a remedy of headache, fever, dysentery, and cholera.¹⁶ Numerous therapeutic properties for *A. sativum* have been mentioned and are summarized in Figure 1. Interestingly, garlic acts as a natural antibiotic but has no damaging effect on friendly bacterial flora.¹⁵ Antiparasitic effects of *A. sativum* have previously been confirmed against *Entamoeba*,¹⁷ *Cryptosporidium*,¹⁸ *Giardia*,^{19,20} *Eimeria*,²¹ *Spironucleus*,²² *Plasmodium*,²³ *Leishmania*,^{8,24} *Trypanosoma*,²⁵ *Trichomonas*,²⁶ *Schistosoma*,²⁷ *Hymenolepis*,¹⁹ *Angiostrongylus*,²⁸ and *Trichuris*.²⁸

It has previously been reported that garlic components possess immunomodulatory effects both in vitro and in vivo such as shifting the secretion pattern of cytokines from TH₂ to TH₁ (T helper cell type 2 and 1, respectively), interferon-gamma (IFN- γ), and nitric oxide (NO) production,^{29,30} although under certain conditions garlic extract may act as immunosuppressive for downregulation of pro-inflammatory responses.³¹

Immunity Against Leishmaniasis

Leishmania life cycle in human body begins after inoculation of metacyclic promastigote forms by female sandflies during blood-feeding process. Shortly after injection, promastigotes are taken up by neutrophils and macrophages (MQs). Promastigotes after entrance to MQs, are quickly converted to a non-motile forms known as amastigote inside the phagolysosome. Then these immobile forms are divided repeatedly using binary fission within MQs and finally lead to bursting and infecting other cells. *Leishmania* spp. are obligatory intracellular parasites; hence, they need MQs for differentiation, survival and multiplication (Figure 2A). When the infection starts, MQs and neutrophils are deployed to the site of infection in order to interact with parasite. MQs are considered as the main effector cells to eliminate intracellular protozoa; accordingly, their proper activation seems critical in order to destroy the *Leishmania* parasites. Overall, MQs activation is classified into 2 categories: alternative and classical activation. The first is promoted by TH₂ cytokines like interleukin (IL-4), IL-10, and IL-13, while the latter is induced using TH₁ cytokines such as IFN- γ . IL-10 restrict secretion of some pro-inflammatory cytokines such as tumor necrosis factor alpha (TNF- α), IL-1, IL-12, and IFN- γ . IL-12 is an important cytokine produced by antigen-presenting cells such as dendritic cells (DCs) and MQs that are necessary for expansion of TH₁ and IFN- γ production. It is worth noting that

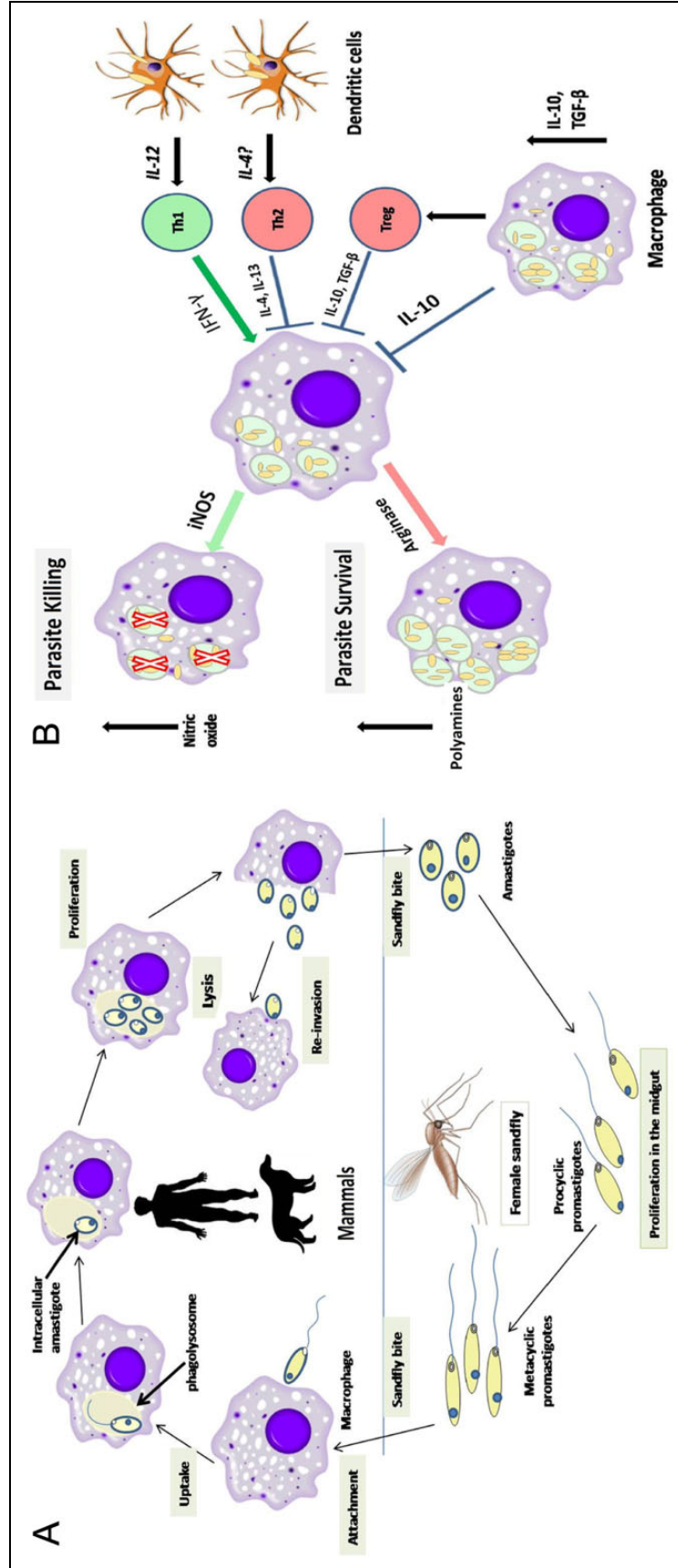


Figure 2. (A) Schematic diagram of the *Leishmania* life cycle. (B) Role of dendritic cells and macrophages in leishmaniasis outcome. Abbreviations: IFN- γ , interferon-gamma; IL, interleukin; iNOS, inducible nitric oxide synthase; TGF- β , transforming growth factor beta; TH₁ and TH₂, T helper cell type 1 and 2, respectively; Treg, T regulatory cell.

IFN- γ is indispensable for upregulation of inducible nitric oxide synthase (iNOS) mRNA expression and eventually leads to NO production, which plays a crucial role in destroying *Leishmania* inside MQs, although some inflammatory cytokines like TNF- α , IL-1, IFN- α , and IFN- β also act alike IFN- γ in iNOS gene expression; while TH₂ cytokines suppress the TH₁ responses and help *Leishmania* survive within MQs.³² Hence if each compound promoted the TH₁-type responses, it would aid in the treatment of leishmaniasis (Figure 2B).²⁹

Phytochemistry of *Allium sativum*

Allium sativum contains many chemical ingredients, including 17 amino acids, more than 33 OSCs, 8 minerals (calcium, potassium, magnesium, germanium, selenium, copper, zinc, and iron), vitamins (A, B₁, B₂, B₃, B₆, B₁₂, C, D, E), and some enzymes (allinase).³³ Allicin as the major biologically active component of fresh garlic is a candidate in antitumor survey. Several studies have been conducted to determine the chemopreventive and anticarcinogenic role of garlic's main component since the in vitro and in vivo studies of Weisberger and Pensky,³⁴ which showed garlic thiosulfinate inhibit the tumor cell growth. Allicin (diallyl thiosulfinate) which is quickly created from alliin using alliinase enzyme in freshly crushed garlic, is considered a very reactive thiosulfinate and the main ingredient responsible for biological functions of garlic. Allicin is metabolized and converted into other OSCs like ajoene, diallyl disulfide (DADS), diallyl trisulfide (DATS), and vinyl dithiines (Figure 3). So allicin is not found in garlic unless it is crushed or damaged. In addition, the pungent flavor of garlic is due to the presence of allicin.¹⁵ Molecular structure of allicin and other OSCs is shown in Table 1. Allicin is a potential anticancer and antimicrobial agent, the activity of which has been the focus of many researchers in past decades.^{7,35,36} Numerous publications have introduced allicin as an effective molecule against infectious agents and this fact has been repeatedly confirmed.^{35,37} Based on reports, bactericidal activity of allicin has been verified against important pathogens, including *Enterococcus* spp, *Bacillus* spp, *Helicobacter pylori*, *Escherichia coli*, *Staphylococcus aureus*, *Salmonella typhimurium*, and *Vibrio cholera*,³⁵ as well as methicillin-resistant *Staphylococcus aureus* strains.³⁸ Also, antifungal effects of allicin against *Epidermophyton* spp, *Trichophyton* spp, *Cryptococcus* spp, and *Candida* spp. are clear.³⁵ Furthermore, it has been reported that allicin possesses antiparasitic activity against *Schistosoma*,³⁹ *Plasmodium*,^{23,40} *Babesia*,⁴¹ *Theileria*,⁴¹ *Trypanosoma*,⁴² *Leishmania*,⁴³ and *Entamoeba*.⁴⁴ In addition, allicin by inhibiting the cysteine proteases of protozoa plays an important role as antitrypanosomal and antiplasmodial agent.⁴² Antimicrobial activity of allicin is justified by its reaction with SH groups of different enzymes such as thioredoxin reductase, alcohol dehydrogenase, cysteine proteinase, and so on.³⁷

Alliin in the amount of 5.3 to 30 mg/g is the highest OSC present in whole garlic and is considered as a derivative of cysteine amino acid. Content of OSCs present in garlic is

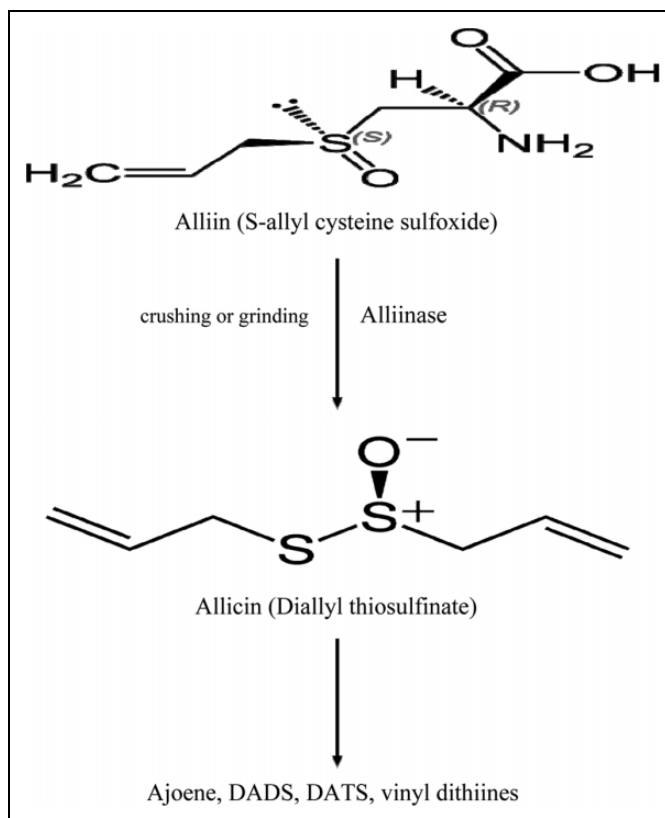
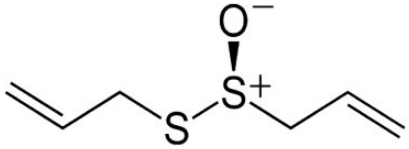
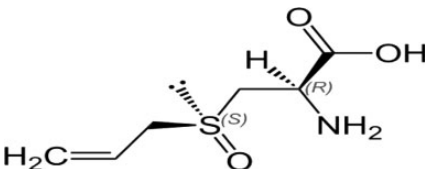
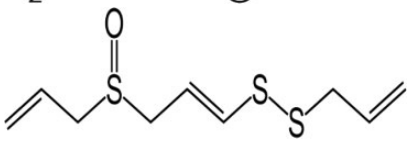
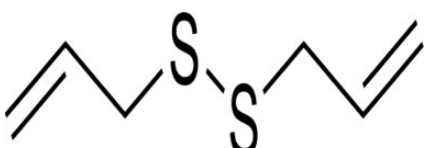
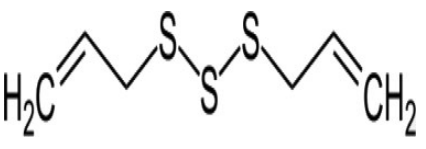
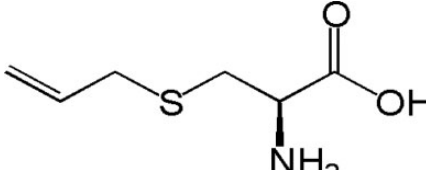
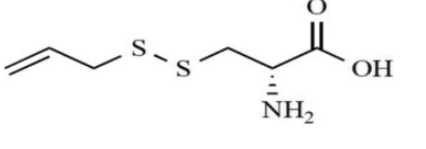


Figure 3. Formation of allicin from alliin, which is catalyzed using alliinase enzyme.

different worldwide. For example, alliin in German fresh garlic was found approximately 5.3 to 9.4 mg/g, while in Korean garlic cloves it ranged from 25 to 30 mg/g. The content of OSCs of garlic is summarized in Table 2.³⁶ Ajoene similar to allicin is a potential antimicrobial agent with wide spectrum effects against both Gram-negative and Gram-positive bacteria, protozoa, and fungi³⁵ while in the study by Weber et al,⁵⁰ ajoene was introduced as the most effective virucidal compound compared with other OSCs in garlic. Ajoene is considered as a *Trypanosoma* trypanothione reductase inhibitor.⁴⁹ Also, it is inhibited from phosphatidylcholine biosynthesis in *Trypanosoma cruzi*.⁶² The antiparasitic and antiproliferative activity of this OSC is attributed to several functional effects on key enzymes of the antioxidant thiol metabolism.⁴⁹ Several studies suggest that garlic because of containing the sulfur compounds like allicin, ajoene, and DATS can delay and prevent growth of parasites such as *Leishmania major*, *Crithidia fasciculata*, *Leptomonas colosoma*, *Giardia lamblia*, *Tetratrichomonas gallinarum*, *Cryptosporidium baileyi*, *Plasmodium berghei*, *Histomonas meleagridis*, and *Trypanosoma* spp.⁴

Phytochemical ingredients and OSCs originated from garlic such as allicin, ajoene, DADS, DATS, S-allyl cysteine (SAC) and S-allylmercaptocysteine (SAMC) induce apoptosis via mitochondrial pathway in numerous cancer cells both in vitro and in vivo.³⁶ For instance, DADS induce apoptosis via caspase-3 activation, which led to cleavage of poly-ADP-

Table 1. Chemical Structure and Function of Some Organosulfur Compounds (OSCs) Present in Garlic.

Sulfur Components	Molecular Structure	Function	References
Allicin (diallylthiosulfinate)		Antileishmanial effect on <i>Leishmania infantum</i> and <i>Leishmania donovani</i> promastigotes and amastigotes Antimalarial activity Antiparasitic effect versus <i>Schistosoma mansoni</i> , <i>Trypanosoma brucei</i> , <i>Entamoeba histolytica</i> , and <i>Giardia lamblia</i> Anticancer activity Apoptosis induction in cancer cells Antibacterial, Antifungal and Antiviral activity The most OSC present in whole garlic	43 23, 40 37, 39, 42 45 46, 47 37 36
Alliin (S-allylcysteine sulfoxide)			
Ajoene		Antileishmanial effects on various <i>Leishmania</i> spp. Antitrypanosomal activity The most effective virucidal compound found in garlic Antifungal activity Reduce tumor size using apoptosis induction Inhibit in vitro proliferation of human A549 lung tumor cells Inhibit the growth of human colon tumor cells Apoptosis induction in human leukemia HL-60 cells Antifungal activity Antiparasitic activity against <i>Entamoeba histolytica</i> , <i>Giardia lamblia</i> , and <i>Trypanosoma</i> spp. in vitro Inhibit in vitro proliferation of human A549 lung tumor cells Apoptosis induction in human prostate cancer cells Antifungal activity Anticancer activity	48 49 50 51 52 53 54 55 56, 57 58 53 59 57 36
DADS (diallyl disulfide)			
DATS (diallyl trisulfide)			
SAC (S-allyl cysteine)			
SAMC (S-allylmercaptocysteine)		Antiproliferative effect on colon cancer cells, apoptosis induction by enhancement in caspase3-like activity Apoptosis induction in erythroleukemia cell lines Anticancer activity	60 61 36

ribose polymerase (PARP) in human leukemia HL-60 cells.⁵⁵ Also, ajoene using apoptosis induction led to reduction in basal cell carcinoma tumor size.⁵² In recent years, a new OSC has been isolated from garlic and is known as thiacremonone (2,4-dihydroxy-2,5-dimethyl-thiophene-3-one).⁶³ Since the considerable attention given by the World Health Organization to discover the antileishmanial agents from natural

products, many studies have been carried out in order to introduce effective drug candidates for leishmaniasis treatment worldwide³; thus, the aim of this study is to review the effectiveness, toxicities, and possible mechanisms of pharmaceutical actions of different garlic extracts and organosulfur compounds isolated from garlic against *Leishmania* spp. in a variety of in vitro, in vivo, and clinical trials reports.

Table 2. The Content of Organosulfur Compounds Present in Garlic.^a

Sulfur Components	Contents Range (mg/g)
Allicin	2.3-7.7
Alliin	5.3-30
Ajoene	0.12-0.47
Diallyl disulfide	0.06-0.89
Diallyl trisulfide	0.01-0.39
S-allyl cysteine	0.36-0.60

^a Taken from Yun et al.³⁶

Search Strategy and Study Selection

To evaluate the antileishmanial effects of garlic, all relevant databases were searched using the terms “*Allium sativum*,” “Garlic,” “Allicin,” “Ajoene,” “*Leishmania*,” “in vitro,” “in vivo,” and “clinical trial” alone or in combination from January 1990 up to December 2014. Information of *A sativum* was collected from 5 English databases (Web of Science, PubMed, Science Direct, Scopus, and Google Scholar) and 3 Persian databases (Scientific Information Database, Iran Medex, and Magiran). In order to evaluate the eligibility and inclusion criteria, articles were reviewed by 2 reviewers (MF-R and SK) and discrepancies among studies were unraveled by consensus. Afterward the desired data were extracted carefully using a data extraction form on the basis of title, kind of preparation, type of study (in vitro, in vivo, and clinical trial), parasite strain, and main findings.

Antiproliferative Activity of Garlic Against *Leishmania* spp In Vitro

Antileishmanial activity of drugs and other compounds is usually assessed by 3-(4, 5-methylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) colorimetric method in vitro, which evaluates the mitochondrial succinate dehydrogenase enzyme activity of parasite. MTT assay compared with direct counting method has some advantages, including ease of use, reproducibility and reliability features, excellent precision, high sensitivity, no need for use of radioisotope, and radioactive materials; accordingly, it is extensively utilized for drug responses and has become popular throughout the globe.⁶⁴ Natural compounds of garlic such as alliin and ajoene play a key role against growth inhibition of several species of *Leishmania* in vitro.^{43,48} Furthermore, aqueous garlic extract (AGE) was reported to be effective against *L mexicana* (MNYC/BZ/62/M379 strain).³⁰ Based on the findings of Khademvatan et al,⁸ AGE illustrated a dose-dependent cytotoxic effect against *L major* (MRHO/IR/76/ER) promastigotes and observation by optical microscopic revealed some changes in parasites including: cell shrinkage, cytoplasmic condensation, blebbing of cell membrane, nuclear morphology changes and immobilization approximately 48 hours after treatment.

In the study of Mahmoudvand et al,⁶⁵ antileishmanial effect of *A sativum* extracts on *L tropica* (MHOM/IR/2002/Mash2)

was investigated. IC₅₀ (50% inhibitory concentration) value of aqueous and methanolic extracts was obtained as 19.2 ± 2.51 µg/mL and 12.3 ± 1.15 µg/mL against promastigotes, respectively, while in control group IC₅₀ was 15.4 ± 2.51 µg/mL. Also, findings indicated aqueous and methanolic extracts of *A sativum* possess very low cytotoxicity against murine peritoneal MQ cells with 50% cytotoxic concentration (CC₅₀) 348.2 ± 4.6 µg/mL and 291.4 ± 3.08 µg/mL, respectively, while CC₅₀ in control group was 283.6 ± 4.6 µg/mL. However, methanolic extract against promastigotes demonstrated better efficacy than glucantime, which is currently being used as the first remedy of choice for leishmaniasis. Hence methanolic extract with greater effectiveness compared with aqueous extract and glucantime can be considered as candidate for *L tropica* drug.⁶⁵ Recently, in similar studies, the effectiveness of methanolic extract of garlic against *L major* and *L donovani* promastigotes and amastigotes has been reported. These findings suggest that *A sativum* extract has high potential to control and combat both cutaneous and visceral leishmaniasis agents.^{66,67} The majority of investigations have shown that antileishmanial effects of methanolic extract are more effective than aqueous extract⁶⁵ and usage from methanol solvent is more common than others.⁶⁵⁻⁶⁷ This fact may be due to the greater ability of methanol in isolation and extraction of a wide range of chemical ingredients compared with aqueous extraction. However, in the studies by Kinuthia et al,^{68,69} the results were in contrast, which could be justified by the type of extraction, methodology, and strain of parasite as well as contents of OSC of garlic. Antileishmanial activity of garlic extracts and garlic-derived compounds that have been performed in vitro are summarized in Table 3.

Various properties of allicin such as anticancer activity and its antimicrobial effects, which are apparent, as well as numerous articles have verified antiproliferative activity of allicin against a broad spectrum of infectious agents.^{35,37} It is noteworthy that mechanism of action of allicin is correlated by interaction with thiol (SH) groups of some enzymes.³⁷ In the study by Jesus Corral-Caridad et al,⁴³ allicin, an OSC isolated from garlic, inhibited the proliferation of *L infantum* and *L donovani* promastigotes effectively in a time- and dose-dependent manner. The dose of 120 µM allicin completely destroyed the promastigotes after 72 hours. In addition, allicin was able to restrict the multiplication of *L. infantum* and *L. donovani* intracellular amastigotes in both J774 cell line and murine peritoneal MQs after 24 hours.⁴³ Allicin with interference in thiol-redox proteins like glutathione and trypanothione/trypanothione reductase led to damaging the *Leishmania* parasites. In addition, microtubule disruption is another target for allicin.⁴³ In other investigations, aqueous and ethanolic extracts of garlic as well as allicin inhibited the growth of *L mexicana* and *L chagasi* in vitro.^{74,75} It is known that allicin by disrupting the activity of cysteine proteases enzyme, is considered as an antiplasmodial and antitrypanosomal agent; therefore, a similar mechanism might be responsible for antileishmanial effect of allicin.⁴²

Ajoene, an OSC isolated from garlic, is a known antimicrobial and anticancer agent. The cytostatic and antiprotozoal activity of

Table 3. Antileishmanial Activity of Garlic Extracts and Garlic-Derived Compounds as Examined In Vitro.

Preparation	Leishmania Strain	Findings	Reference
AGE	<i>L. major</i> (MRHO/IR/76/ER)	IC ₅₀ : 37 mg/mL Inhibit the proliferation and growth of promastigotes Cytotoxic effect against parasite Cell cycle arrest Cell shrinkage Nuclear morphology changes Apoptosis induction by overexpression from PARP and metacaspase genes PS externalization Internucleosomal cleavage of DNA (DNA fragmentation)	8
AGE	<i>L. major</i> (MRHO/IR/76/ER)	IC ₅₀ : 37 mg/mL Overexpression from IFN- γ and iNOS genes in J774 cells infected by parasite	70
AGE	<i>L. major</i> (MRHO/IR/76/ER)	IC ₅₀ : 37 mg/mL \uparrow IL-12 production by infected MQs	71
MGE	<i>L. major</i>	IC ₅₀ : 4.94 μ g/mL Inhibit the proliferation and growth of promastigotes	72
AGE MGE	<i>L. tropica</i> (MHOM/IR/2002/Mash2)	IC ₅₀ : 19.2 \pm 2.51 μ g/mL and CC ₅₀ : 348.2 \pm 4.6 μ g/mL for aqueous extract IC ₅₀ : 12.3 \pm 1.15 μ g/mL and CC ₅₀ : 291.4 \pm 3.08 μ g/mL for methanolic extract Acceptable cytotoxicity against promastigotes specially for methanolic extract Low cytotoxicity against murine MQs for both extracts	65
MGE	<i>L. donovani</i> (MHOM/IN/1998/KE16)	IC ₅₀ : 89 \pm 7 μ g/mL and 67 \pm 5 μ g/mL for promastigotes and amastigotes, respectively Show appreciable activity against both promastigotes and amastigotes forms	67
CSA	<i>L. donovani</i> (MHOM/IN/80/DD8) and 5 other local strains	\downarrow Promastigotes proliferation	73
AGE Ethanol extract	<i>L. mexicana</i> and <i>L. chagasi</i>	Inhibition of <i>L. mexicana</i> and <i>L. chagasi</i> growth	74
Allicin	<i>L. mexicana</i> and <i>L. chagasi</i>	Inhibition of <i>L. mexicana</i> and <i>L. chagasi</i> growth	75
SAC	<i>L. major</i> (MRHO/IR/76/ER)	\uparrow Survival and promastigotes growth in culture medium	76
Ajoene	<i>L. mexicana</i> (Lm: MHOM/VE/80/NR) <i>L. mexicana venezuelensis</i> (Lmv: MHOM/VE/80/H16) <i>L. mexicana amazonensis</i> (Lma: M112, IFLA/BR/67/PH8) <i>L. donovani chagasi</i> (Ldch: MHOM/BR/74/PP75)	IC ₅₀ : 1.83 μ M Leishmanicidal effect against all species Ultrastructural survey showed: morphological changes, nuclear and mitochondrial membrane alterations	48
AGE	<i>L. major</i> (IDUB/KD/94=NLB-144)	IC ₅₀ : 575.75 μ g/mL	69
MGE	<i>L. major</i> (IDUB/KD/94=NLB-144)	IC ₅₀ : 863.12 μ g/mL	68
Allicin	<i>L. infantum</i> and <i>L. donovani</i>	Inhibit the proliferation of <i>L. infantum</i> and <i>L. donovani</i> promastigotes Transmission electron microscope showed: decrease in cytoplasmic electron dense bodies, chromatin condensation of nucleus and kDNA, deteriorate the mitochondria and kinetoplast membranes integrity and cell swelling	43
AGE	<i>L. major</i> (MRHO/IR/76/ER)	Killing amastigotes inside MQs	77
MGE	<i>L. major</i> and <i>L. donovani</i>	IC ₅₀ : 34.22 μ g/mL and 37.41 μ g/mL for <i>L. major</i> and <i>L. donovani</i> promastigotes, respectively Cytotoxic effect against parasite	66
AGE RIO	<i>L. mexicana</i> (MNYC/BZ/62/M379)	\downarrow Proportion of infected MQs and number of parasites per cell	30
Active ingredients derived from garlic	Not mentioned	Cytotoxic effects against parasites in vitro	78

Abbreviations: AGE, aqueous garlic extract; CC₅₀, 50% cytotoxic concentration; CSA, crude soluble antigen; IC₅₀, 50% inhibitory concentration; IFN- γ , interferon-gamma; IL, interleukin; iNOS, inducible nitric oxide synthase; MGE, methanolic garlic extract; MQs, macrophages; PARP, poly ADP-ribose polymerase; PS, phospholipid phosphatidylserine; SAC, S-allyl cysteine.

ajoene may be partially due to several effects on key enzymes which contribute to antioxidant thiol metabolism.^{49,51,52} In the study by Ledezma et al,⁴⁸ antiproliferative effects of ajoene were evaluated on *Leishmania* spp. such as *L. mexicana* (Lm: MHOM/VE/80/NR), *L. mexicana venezuelensis* (Lmv: MHOM/VE/80/H16), *L. mexicana amazonensis* (Lma: M112, IFLA/BR/67/PH8), and *L. donovani chagasi* (Ldch: MHOM/BR/74/PP75). Interestingly, a dose-dependent antiproliferative activity from 0.1 to 10 μ M ajoene was seen in all *Leishmania* spp. Concentrations greater than 1 μ M exhibited cytotoxic activity (IC₅₀: 1.83 μ M), while higher than 10 μ M induced complete destruction and lysis of all parasite species after 96 hours of incubation.⁴⁸ It has been shown that ajoene, by inhibition of phosphatidylcholine biosynthesis and trypanothione reductase enzyme, prevented multiplication of *Trypanosoma cruzi*.^{49,62} Hence antileishmanial activity of ajoene can be justified using the mentioned mechanism in *Trypanosoma* parasites.

There is evidence which suggests that some molecules that act anticancer agents could be effective against *Leishmania* spp.^{79,80} SAC has anticancer activity, although in the study by Eslami et al,⁷⁶ SAC (a water-soluble OSC isolated from garlic) increased the survival and *L. major* (MRHO/IR/76/ER) promastigotes growth in culture medium, which found the concentration of 10 mM had the best effect on growth. It seems that SAC protects the parasites from destruction and oxidative damage.⁷⁶ The finding of Eslami et al⁷⁶ is inconsistent with previous studies that were performed by other OSCs isolated from garlic like allicin and ajoene on *Leishmania* spp.^{43,48} To clarify the reason for this issue, further investigation is required.

Antileishmanial Effects of Garlic In Vivo and Clinical Trials Studies

Investigations which have exhibited acceptable and effective results in vitro must be followed as in vivo and clinical trials studies. Based on previous findings, BALB/c mice are considered as a vulnerable experimental animal to *L. major* infection and are afflicted with chronic and non-healing ulcers. These murine strains secreted TH₂-related cytokines, including IL-4, IL-5 and IL-10 during leishmaniasis infection while cellular immune responses are weak.⁸¹ Garlic extract stimulate the TH₁-type responses and augment the engulfment activity of peritoneal MQs in *L. major*-infected vulnerable mice that consequently help to cure the disease.^{29,77} Treatment with AGE simultaneously leads to dramatic reduction in footpad swelling and parasitic—*L. mexicana* (MNYC/BZ/62/M379 strain)—burden in the lesions in mice model while these changes in control group that were treated with glucantime were not statistically significant.³⁰ Also, combination of *Tridax procumbens* and *A. sativum* extracts is more effective against *L. mexicana* (Hd18- (MHET/MX/ 97/Hd18) in mice than when used alone and leads to increase in IgG2a/IgG1 (immunoglobulin G) ratio.²⁴ It seems a combined therapy of *A. sativum* via other compounds by synergistic effects shows better effectiveness in leishmaniasis treatment, which has been repeatedly confirmed in

numerous studies.^{24,29,82} Methanolic garlic extract (MGE) after oral and peritoneal administration led to a dramatic reduction in hamster *L. donovani* and *L. major* burden.⁶⁶ Also, in an in vivo survey conducted by Ghaffarifar et al,⁸³ AGE and R10 fraction were used as ointment in order to evaluate diameter of ulcers. R10 showed better efficacy than AGE in BALB/c, C57BL/6 and outbred Swiss Webster (SW) mice in ulcer size.⁸³ In a few reports, after treatment with garlic extract no effective response was observed although the lesion size in mice models was increased.^{84,85}

Garlic extracts and OSCs isolated from garlic like allicin by intensifying the host pro-inflammatory responses, DCs activation, provoking the expansion of MQs and T cells, lead to decrease in parasitemia and protection against acute murine malaria infection, but it has no effect on IL-10 production,⁴⁰ which is similar to our previous study on *L. major*-infected MQs.⁷¹ This issue is in accordance with the fact that allicin in some circumstances is able to downregulate the inflammatory responses.³¹ There is a similar strategy about usage of garlic in *Leishmania* spp.^{70,77,82} In an investigation performed by Ahmadi-Renani et al,⁸² AGE showed acceptable response in reduction of ulcer size in mice but combination of vitamin A and garlic had better efficacy compared with either garlic or vitamin A alone. Also, a direct correlation was observed between amount of NO production and healing process.⁸² Some surveys that have been conducted on animal models are summarized in Table 4.

Few reports are available about the efficacy of garlic extracts and garlic-derived compounds that were tested as clinical trials studies in patients with leishmaniasis. Although some results may be in conflict with each other, but overall garlic is reported to be an antileishmanial agent in all kinds of studies. In the following we will discuss, in summary, the performed studies.

Recently, a comprehensive investigation was performed by Samdani and Iqbal Choudhary⁷⁸ based on laboratory studies and clinical trials about novel formulation from garlic extract on cutaneous leishmaniasis remedy. In this survey, a topical gel was prepared containing active compounds of garlic in order of next tests. Antileishmanial effects in vitro followed in vivo, in which this topical gel was administered once daily on mice. During six weeks their ulcers healed perfectly. It was also followed in humans as clinical trial. Seventy patients with cutaneous leishmaniasis whose disease was confirmed by smear examination were included in the study. After applying the gel, 57 patients (81.42%) responded to treatment whereas in 13 patients (18.57%) no response was seen. Also, 10 patients and 40 patients perfectly recovered after 6 and 8 weeks of therapy, respectively (Table 5).⁷⁸ In another clinical trial study, MGE was used topically twice a day with concentration of 0.1 g/mL in patients with cutaneous leishmaniasis. All (100%) the patients with cutaneous leishmaniasis responded against MGE treatment,⁸⁶ which is similar to the study by Samdani and Iqbal Choudhary,⁷⁸ whereas in a randomized, double blind survey done on 197 patients with cutaneous leishmaniasis, after the usage of placebo or 5% garlic cream, no significant differences were seen between the 2 groups. The

Table 4. Antileishmanial Activity of Garlic Extracts and Garlic-Derived Compounds as Examined In Vivo.

Preparation	Parasite strain	Findings	Reference
AGE	<i>L major</i> (IDUB/KD/94=NLB-I44)	↓ Lesion size, footpad swelling and parasitic burden	69
MGE	<i>L major</i> (IDUB/KD/94=NLB-I44)	↓ Lesion size, footpad swelling and parasitic burden	68
Allicin	<i>L infantum</i> and <i>L donovani</i>	Inhibit the proliferation of <i>L infantum</i> and <i>L donovani</i> intracellular amastigotes in both J774 cell line and murine peritoneal MQs	43
AGE	<i>L major</i> (MRHO/IR/76/ER)	↑ Peritoneal MQs phagocytic activity and killing amastigotes inside MQs	77
MGE	<i>L major</i> and <i>L donovani</i>	Cytotoxic effect against parasite ↑ NO production Healing effect on ulcer size ↓ Parasite burden	66
AGE	<i>L mexicana</i> (MNYC/BZ/62/M379)	↓ Lesion size, footpad swelling and parasitic burden ↓ Proportion of infected MQs and number of parasites per cell ↑ IFN- γ production by T cells ↑ NO production and killing power of MQs Shifting toward TH ₁ immune responses	30
R10			
AGE	<i>L mexicana</i> Hd18-(MHET/MX/ 97/ Hd18)	↓ Lesion size, footpad swelling and parasitic burden ↑ Provoke production of antibodies (IgG) Combination of <i>Tridax procumbens</i> and <i>Allium sativum</i> extracts lead to increase the IgG ₂ a/IgG ₁ ratio Tendency to TH ₁ immune responses	24
AGE	<i>Leishmania</i> spp.	↓ Diameter of ulcers in mice ↑ NO production Combination of vitamin A and garlic showed better efficacy compared with when used alone	82
AGE	<i>L major</i> (MRHO/IR/76/ER)	↓ Lesions growth in mice and course of disease ↑ DTH responses ↑ Destruction of <i>Leishmania</i> amastigotes in peritoneal MQs ↑ Level of IFN- γ and IL-2 as indicator of TH ₁ -type response ↓ Level of IL-4 and IL-10 as indicator of TH ₂ -type response	29
AGE	<i>L major</i> (MRHO/IR/76/ER)	R10 showed better efficacy than AGE in BALB/c, C57BL/6, and outbred SW mice in diameter of ulcers AGE illustrated dramatically differences with control group in ulcer size of BALB/c mice ($P < .05$) R10 exhibited significantly differences with control group in ulcers size of BALB/c and outbred SW mice ($P < .05$)	83
R10 fraction (use as ointment)			
Hydroalcoholic extract	<i>L major</i> (MRHO/IR/76/ER)	No effective response was observed although the lesion size in mice models was increased	84
Ethanol extract	<i>L major</i> (MRHO/IR/76/ER)	No effective response was observed although the lesion size in mice models was increased	85
Active ingredients derived from garlic as gel form (use as topical gel)	Not mentioned	Curing the ulcers in all experimental mice during 6 weeks	78

Abbreviations: AGE, aqueous garlic extract; DTH, delayed type hypersensitivity; IFN- γ , interferon-gamma; IgG, immunoglobulin G; IL, interleukin; MGE, methanolic garlic extract; MQs, macrophages; NO, nitric oxide; SW mice, Swiss Webster mice; TH₁ and TH₂, T helper cell type 1 and 2, respectively.

Table 5. Antileishmanial Activity of Garlic Extracts and Garlic-Derived Compounds as Examined in Clinical Trials

Preparation	Findings	Reference
Active ingredients derived from garlic as gel form (use as topical gel)	57 (81.42%) out of 70 CL patients responded to treatment	78
MGE (used topically)	100% of CL patients responded against MGE treatment 91.6% and 8.4% of CL patients cured during 15-22 and 30 days, respectively	86
5% garlic cream	No significant differences were seen between 2 groups ($P = .9865$)	87

Abbreviations: CL, cutaneous leishmaniasis; MGE, methanolic garlic extract.

authors recommended concentration higher than 5% and different combinations of garlic should be used in other studies.⁸⁷ The discrepancy between different findings might be due to study population, immune system condition of patients, severity of illness, and kind of *Leishmania* spp. agents. The studies by Samdani and Iqbal Choudhary⁷⁸ and Khalid et al⁸⁶ confirmed that garlic is an excellent source for treatment of cutaneous leishmaniasis and for better understanding of its therapeutic applications, further studies are required. Since the amount of OSCs found in garlic varies in different regions (Table 2)³⁶ the results obtained by authors might also differ from one another.

Garlic Extract and Garlic-Derived Compounds Induce Apoptosis

Programmed cell death (PCD), also called apoptosis, is characterized using several features including: activation of caspases cascade, change of nucleus morphology, chromatin condensation, cell shrinkage, blebbing of plasma membrane, formation of apoptotic bodies, loss of mitochondrial functions, phospholipid phosphatidylserine (PS) externalization and DNA fragmentation.⁸⁸ There are numerous evidences that confirmed apoptosis induction by different garlic-derived and garlic extracts occurring in various cells, especially cancerous cells.^{46,47,52,89} Initially, it was believed that apoptosis happens exclusively in multicellular animals; however, recently it has been revealed that this phenomenon also occurs in unicellular eukaryotes such as *Plasmodium*, *Leishmania*, *Trypanosoma*, *Toxoplasma*, *Trichomonas*, *Entamoeba*, *Blastocystis*, and *Giardia*.^{79,88,90} So, any compound that is able to induce PCD process in *Leishmania* spp, could be considered as an appropriate candidate drug for the treatment.

In our recent study, PS externalization was measured after AGE treatment using annexin-V FLUOS staining kit. PS exposure is a hallmark of early stage PCD that translocated from inner cell membrane to outer membrane during this process. Also, annexin is a dye that binds with high affinity to PS. Percentage of annexin-positive *L major* promastigotes 36 and 48 hours after AGE treatment (IC₅₀: 37 mg/mL) was 65.55% and 86.11%, respectively; while in control group it was 3.9% in both time points. DNA fragmentation is another technique used in order to differentiate between necrosis and apoptosis and is considered a hallmark of late-stage PCD in which during this phenomenon internucleosomal cleavage of DNA (approximately between 180-200 bp) happens, whereas in necrosis DNA cleavage is random. In this survey, after 24 hours fragmented DNA in agarose gel electrophoresis confirmed the PCD process, which indicates that *A sativum* extract is able to induce apoptosis. In addition, overexpression of metacaspase and PARP genes was detected 4 hours after *A sativum* extract treatment using reverse transcriptase-polymerase chain reaction (RT-PCR); therefore, aqueous *A sativum* extract is able to induce apoptosis by several mechanisms and this fact was verified using flow cytometry (PS externalization), DNA fragmentation assay (DNA cleavage) and RT-PCR (overexpression of

PARP and metacaspase gene). PCD induction by AGE in *L major* promastigotes might be due to the presence of water-soluble OSCs such as SAC and SAMC⁸ that in other researches have shown similar activity in cancer cells.^{15,36,60,61} AGE-induced apoptosis is caspase-independent in *L major* promastigotes,⁸ while apoptosis induction using SAMC in SW-480 and HT-29 cell lines, correlated with enhancement in caspase3-like activity.⁶⁰ Apoptosis induction by garlic extract has been shown in cultured human nonneoplastic (MRC-5) and neoplastic (A549) lung cancer cells,⁵³ in human colon HT-29 and SW-480 cancer cells,⁶⁰ on human colon cancer cells,⁹¹ on breast cancer,⁹² and on murine transitional cell carcinoma.⁹³ A proteomic study showed a strong correlation between organosulfur-sensitive proteins and apoptotic pathways that supports previous surveys.⁹⁴

Allicin initiates and induces the apoptosis in cancerous cells whose mechanisms contribute to mitochondrial pathway of Bcl-2/Bax and antioxidant enzyme systems.⁴⁷ Since *L major*, due to possessing the mitochondria has similarities with the multicellular animals, it can be assumed that a similar mechanism is involved in apoptosis induction of *Leishmania* spp. In the investigation of Jesus Corral-Caridad et al,⁴³ allicin was treated with *L infantum* and *L. donovani* promastigotes and then examined by transmission electron microscope. Resulting changes included: decrease in cytoplasmic electron-dense bodies, vacuolization, cell swelling, deterioration of the mitochondria and kinetoplast membranes integrity, chromatin condensation of nucleus and kDNA.⁴³ In another ultrastructural study, time- and dose-dependent morphological changes such as nuclear and mitochondrial membrane alterations were seen in *L mexicana amazonensis* (Lma: M112, IFLA/BR/67/PH8) amastigotes treated with ajoene.⁴⁸

Leishmanicidal Activities of Garlic by Immunomodulatory Effects

In the literature, it has been reported that garlic shows immunomodulatory activity such as alteration of cytokine production, antibody secretion, lymphocyte proliferation, MQs and DCs activation and phagocytosis promotion.^{29,30,77} It is assumed that garlic derivatives and garlic extracts augment cellular immune response and concurrently reduce the course of *Leishmania* spp infection and burden of parasites in mice.^{24,30,66} NO acts as a key component in eradication and growth inhibition of *Leishmania*, killing the parasites within MQs, its production is stimulated by IFN- γ cytokine.³² Furthermore, it has been reported some cytokines like IFN- γ and GM-CSF (granulocyte macrophage colony-stimulating factor) alone or in combination with chemotherapy have been effective in limiting the leishmaniasis. Treatment with AGE enhances the IFN- γ production by T-cell in mice, indicating tendency toward TH₁ immune response in order to improve a strong cell mediated response for better control against leishmaniasis, while in control groups, glucantime effect is not associated with IFN- γ and NO production, thus antileishmanial effect of garlic extract due to immunomodulatory feature is significantly higher than

glucantime both in vitro and in vivo.³⁰ In garlic-treated groups, a regression in lesion development was reported with shifting from TH₂ to TH₁ immune response and cytokine pattern, which finally reduced the course of infection in BALB/c mice.²⁹ In addition, a single dose of *A sativum* extract in mouse model could lead to control of infection as a result of increasing phagocytosis and killing power of *L major* using MQs.⁷⁷ Garlic extracts and garlic-derived compounds may change lipid trafficking and protein in the surface of cell membrane.⁹⁵ According to these findings, increased phagocytosis using garlic extract could be explained by changing the protein content and probably enhancing the receptor expression in MQs surface cell membrane.⁷⁷

Garlic extract may enhance lectin-induced lymphocyte proliferation and natural killer cells activity.⁹⁶ Furthermore, garlic extract at dose of 20 mg/kg enhance the delayed type hypersensitivity response in mice and histologically lead to hypertrophy and hyperplasia both of the paracortical zone in lymph nodes and periarteriolar sheaths of spleen. The immunopotential and immunomodulatory properties of garlic in enhancement of delayed type hypersensitivity and natural killer cells activity can be an excellent mechanism for cell-mediated immunotherapy.⁹⁷ Some studies have demonstrated garlic extract and its OSCs might contribute to the decrease of NO production in MQ cell lines,⁹⁸⁻¹⁰⁰ whereas in other researches, enhancement of NO production by MQs was seen.^{30,70,82,101} Gained results from the survey by Gharavi et al⁷⁰ illustrated that AGE increases the overexpression of iNOS gene in J774 cell lines that finally lead to NO production. Discrepancy between authors may be associated with type of study (in vitro and in vivo), methodology, kind of extracts, and its concentrations. Antileishmanial activity of garlic extracts and garlic-derived compounds by immunomodulatory effects are summarized in Tables 3 and 4.

Cytokines play a crucial role in adopting the appropriate immune response. TH₁-associated cytokines such as IL-2 and IFN- γ stimulate tightly cellular immune response while TH₂-associated cytokines such as IL-4, IL-5, and IL-10 play a role in humoral immunity. High level of IL-10 suppresses the TH₁ immune responses and cause long-term persistence of *Leishmania* infection in both experimental animals and human while increased production of IL-12 and IFN- γ cytokines, that is essential for healing process leads to a decrease in both duration of infection and burden of protozoa concurrently (Figure 2B).³² Garlic extract could provoke indirectly or directly IFN- γ production by TH₁ cells in *L mexicana*-infected mice, and consequently stimulate the NO production inside MQs. Also, the effect of R10 fraction of garlic on NO production was evaluated and R10 was shown to increase IFN- γ and subsequently NO value³⁰; hence, immunomodulatory activity of garlic was ascribed to a low molecular weight 14 kDa (kilodalton) glycoprotein known as R10⁹⁷ and saponin content.³⁵ In fact, saponin is identified as vaccine adjuvant, especially in antileishmanial vaccines.¹⁰² According to results from the study by Gamboa-León et al,³⁰ antileishmanial effects of garlic extract both in vitro and in vivo was mediated by several

mechanisms associated with TH₁ and MQ activation by which immunomodulatory feature of R10 fraction and other ingredients of garlic lead to a shift toward TH₁ response. Then, IFN- γ secreted from TH₁ affects MQs and enhances their activities like killing power and over production of NO. As was mentioned before, increase in NO level directly correlated with leishmanicidal activity inside MQs.³⁰

In the study by Gharavi et al,⁷¹ the effect of AGE on IL-12 and IL-10 levels in *L major* infected MQs were assessed. Results illustrated IC₅₀ dose (37 mg/mL) of AGE lead to increased secretion of IL-12 from infected MQs while no changes were observed in IL-10 production.⁷¹ Also, AGE over 48-hour incubation destroyed *L major* amastigotes using over expression of IFN- γ and iNOS genes in J774 MQ cell lines.⁷⁰ Thus IL-12, IFN- γ , and subsequent NO production were identified as a crucial factor against *Leishmania* amastigotes defense inside MQs; whereas IL-10 delay improving the *Leishmania* infection.^{70,71} Also, Miltefosine (an anticancer drug which is used as an alternative drug in leishmaniasis) demonstrated similar effect with AGE on IFN- γ and iNOS mRNA expression, IL-12 and IL-10 production in *L major*-infected J774 cell lines.⁸⁰

In *L. major*-infected BALB/c mice that were treated with AGE, ulcer growth was significantly inhibited, whereas in glucantime treated mice a retardation in ulcer expansion was seen in comparison with control groups, but was unable to suppress the lesions growth. A combined therapy with both glucantime and garlic extract illustrated more effectiveness against lesions growth in mice models than when used alone. In AGE-treated mice, high level of IFN- γ and IL-2 and low level of IL-4 and IL-10 were seen that was indicative of a cell-mediated immune response while in control groups results were in contrast. Also, in glucantime-treated mice, TH₂-type response was observed with low level of IFN- γ and IL-2 and high level of IL-4 and IL-10.²⁹ These findings are supported by other reports, which suggest that treatment with glucantime possesses insufficient efficacy in human and experimental animal models.¹⁰³ Combined therapy with both garlic and glucantime induce a TH₁ immune response similar to that stimulated by garlic alone.²⁹ Also, AGE induces production of IgG antibodies in treated mice.²⁴ Finally, in order to achieve successful treatment in leishmaniasis, and additionally to decrease parasite burden, development of strong cell-mediated immune response by IL-2, IL-12, and IFN- γ is required.^{29,71} In recent years, some researchers succeeded in treating the chronic non healing cutaneous leishmaniasis by combined therapy of IFN- γ and IL-2 or anti IL-4 plus pentostam drug.^{104,105} Recently, many studies for leishmaniasis treatment have been performed based on immunotherapy methods that have acceptable perspectives for future.^{106,107}

Conclusion and Future Perspective

Garlic as an important traditional medicine in many studies has been more effective than glucantime. In summary, different garlic extracts and garlic-derived compounds, especially OSCs showed cytotoxic activity against several *Leishmania* species

in a variety of in vitro, in vivo and clinical trials reports. *A sativum* contains potential antimicrobial agents like OSCs such as allicin, ajoene, DADS, and DATS. In addition, apoptosis phenomenon by these ingredients occurs in different cells and pathogen agents. Also, garlic, due to possessing the immunomodulatory activity such as antibody secretion, increase in lymphocytes proliferation, MQs and DCs activation, phagocytosis promotion and by creating a shift in cytokine production pattern from TH₂ to TH₁, leads to expansion of a strong cell-mediated immunity and reduces the course of treatment. It seems combined therapy of garlic via other extracts, vitamins, and so on leads to increasing their efficacy using synergistic effects. In order to improve the efficiency, initially garlic compounds should be purified and then used as complementary medicine in leishmaniasis treatment. It seems that further bioinformatic and in silico studies are needed to predict major biologically active components as potential antileishmanial derivations.

Acknowledgments

The authors would like to thank all staff of Department of Medical Parasitology of Urmia University of Medical Sciences. The authors would like to thank Dr Alborz Eskandari for his help in preparation of figures.

Author Contributions

MF-R and SK contributed to the concept, design, searching the literature, and writing of the manuscript. KHT contributed to manuscript review and manuscript editing.

Declaration of Conflicting Interests

The authors declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

Funding

The authors received no financial support for the research, authorship, and/or publication of this article.

Ethical Approval

As this review did not involve any human or animal subjects, ethical approval was not required.

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