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Research progress on natural products from traditional Chinese medicine in treatment of Alzheimer's disease

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ABSTRACT: Alzheimer's disease (AD) is a severe condition in aging societies. Although research on this disease is advancing rapidly, thus far few very effective drugs are available for AD patients. The currently widely used medicines such as donepezil and galantamine transiently improve the symptoms of patients with mild to moderate AD. They are hardly capable of preventing, halting or reversing the progression of this disease. In the long history of development of traditional Chinese medicine, many herbs have been discovered and employed to treat dementia diseases in clinics in China. In recent decades, a number of agents were isolated from these herbs and their efficacies against AD were tested. Some flavonoids, alkaloids, phenylpropanoids, triterpenoid saponins, and polysaccharides were demonstrated to have potential efficacies against AD *via* targeting multiple pathological changes of this disease. In this article, we reviewed research progress on the efficacies and underlying mechanisms of these agents.

Keywords: Flavonoids, alkaloids, phenylpropanoids, triterpenoid saponins, polysaccharides

1. Introduction

Alzheimer's disease (AD) is characterized by progressive deterioration in intellect including memory and cognitive functions. It is the most common type of dementia among older people, accounting for 50-75% of all dementia cases (*1*). The number of AD patients was estimated at 36 million in 2010 and will triple in the world by 2050 (*2*). In China, this figure is estimated at 9 million currently and the prevalence rate of AD in the population over the age of 60 years is 2.43% (*3,4*). Proportionate increases over the next forty years in the number of people with AD will be much steeper in China since it is witnessing the aging of a society in which the population over the age of 60 years will account for approximately 31% (about 400 million calculated on the current population base) of the whole population by the year of 2050 (*5*). These epidemiological data have painted a less than optimistic outlook in prevention and treatment of this disease in the world, especially in those countries with a rapidly aging society such as China.

The currently approved drugs for treatment of AD, *e.g.* donepezil, rivastigmine, galantamine, and memantine, aim to either inhibit acetylcholine esterase to increase the levels of the neurotransmitter acetylcholine, or antagonize *N*-methyl-D-aspartic acid (NMDA)-type glutamate receptors to prevent aberrant neuronal stimulation (*6,7*). These medicines, however, exhibit modest and transient effects in improving disease manifestation and could hardly prevent, halt, or reverse the disease (*2*). The typical course of AD lasts for a decade or so, from the mildest stage when the symptoms like memory problems appear to the most severe stage when the patients must depend on others for basic activities of daily living and finally die in a completely helpless state. The long duration of AD and shortage of effective or curative treatments bring an enormous emotional and financial burden on patients, their families and society.

Exploration of natural active ingredients from medicinal herbs for treatment of AD has attracted substantial attention worldwide. Thus far drugs, including galantamine and huperzine A which originated from traditional Chinese herbs have been developed and used in clinics to treat mild to moderate AD (*8,9*). In addition to that, various natural agents isolated from traditional Chinese medicines were reported to have anti-AD efficacies through diverse mechanisms and require further investigation. In this article, we give a retrospective view of the research progress on natural products isolated from traditional Chinese medicine in treatment of AD and their underlying mechanisms.

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2. Etiology of AD

AD is related with, but shows intrinsic differences from normal aging (*2*). The underlying mechanisms of onset of this disease have not been thoroughly clarified thus far. Postmortem AD patients demonstrated atrophy of cerebral tissue, especially loss of neurons in hippocampus and the base of the forebrain (*2*). The most evident characteristics of pathological changes in the brain of AD patients are extracellular deposits of β amyloid protein (Aβ) and an intracellular presence of neurofibrillary tangles (NFTs). Analyses of genes in familial AD patients, which probably accounts for less than 1% of AD cases, have brought important research progress regarding the mechanisms of onset of this disease (*10*). Studies indicated that the deposits of Aβ is correlated with gene variations of amyloid precursor protein (APP) and/or an abnormal transformation process (*2*). APP is encoded by the *APP* gene located at chromosome 21 and is transformed to Aβ by cleavage with β-secretase. Knockdown of the gene encoding β-secretase, *i.e*., *β-site APP cleaving enzyme 1* (*BACE1*), leads to reduction of Aβ production (*11*). The aggregation and accumulation of Aβ may result from increased production of Aβ, decreased degradation by Aβ-degrading enzymes, or reduced clearance across the blood-brain barrier. The neurotoxic activities of Aβ are exerted through mechanisms of cell apoptosis and/or inflammation in brain tissue. These research findings suggested promising targets for drug design. However, since the onset of familial AD is not prevalent, other hypotheses of pathogenesis, including hyperphosphorylation of Tau protein, cerebral ischemia, glutamate excitotoxicity, oxidative stress, mitochondria damage, and disequilibrium of calcium homeostasis have also attracted attention and have become acceptable targets (*12-16*).

3. Natural active ingredients against AD

In the past several decades, much research has been done to evaluate the anti-AD effects of natural agents isolated from traditional Chinese medicines from perspectives such as scavenging free radicals, inhibiting lipid peroxidation, suppressing neuronal apoptosis, enhancing the function of cholinergic neurons, and/or improving behavioral abnormalities in experimental animal models. Some flavonoids, alkaloids, phenylpropanoids, triterpenoid saponins, and polysaccharides were demonstrated to have potential efficacies against AD.

3.1. *Flavonoids*

Flavonoids are a series of compounds that are spread widely in higher plants and ferns and have attracted much attention due to their various biological actions (*17*). The characteristic chemical structures of these compounds is two benzene rings with hydroxyl groups linked by a three-carbon chain. The most commonly known biological action of flavonoids is their antioxidant activity, which could be understood from the reduction properties of phenol hydroxyls in the chemical structures. That said, compounds of this type exhibit various pharmacological effects and clinical efficacies that may not be solely related to their anti-oxidative activities, such as effects on the vascular system, inflammatory response, and estrogen-like effects (*17*). These actions of flavonoids constitute the underlying basis for their anti-AD effects. Thus far, flavonoids including ginkgo flavonoids, soy isoflavones, puerarin, total flavonoids of Baical Skullcap stem and leaf, apigenin, rhodosin, hyperoside, and liquiritin were reported to have potent effects against AD (Table 1).

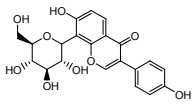
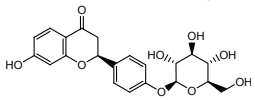
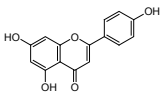
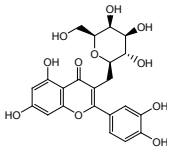
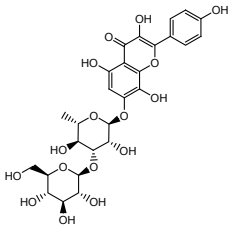
3.1.1. *Ginkgo flavonoids*

Ginkgo flavonoids are the main constituents in the extract of Ginkgo biloba (EGB). Ginkgo flavonoids consist mainly of flavonols such as quercetin, kaempferol, and isorhamnetin and biflavonoids like ginkgetin, isoginkgetin, and amentoflavone (*18,19*). These ginkgo flavonoids have free radical scavenging effects and could inhibit lipid peroxidation. Studies demonstrated that mitochondrial DNA from the brain of old rats exhibited oxidative damage that is significantly higher than that from young rats (*20*). In addition, mitochondrial glutathione was more oxidized and peroxide formation in mitochondria was higher in old than in young rats (*20*). Treatment with EGB could partially prevent the indices of oxidative damage in brain from old animals (*20*). Other studies demonstrated that ginkgo flavonoids exhibited neuroprotective effects *via* antioxidant activity in brain damaged mice caused by ischemia-reperfusion (*21*). One randomized, double-blind, placebo-controlled, and multicenter clinical trial indicated that EGB was safe and capable of stabilizing and improving the cognitive performance and the social functioning of AD patients for 6 months to 1 year (*22*). Currently, EGB is used in clinics as a medical drug for treatment of AD in China, France, and Germany.

3.1.2. *Soy isoflavones*

Soy isoflavones including daidzin daidzin, daidzein, genistin, genistein, glycitin, and glycitein (*23,24*) attracted much interest in recent years due to its estrogen-like effects and role in influencing sex hormone metabolism. Estrogen exerts anti-AD effects through several mechanisms such as reducing Aβ production (*25*), antagonizing the toxicities of Aβ (*26*), promoting synaptic growth and expressions of nerve growth factor (NGF) and its receptor (*27*), *etc*. Although estrogen exhibits the various above potential actions, its application in clinics for treatment of AD is dismal since

Table 1. Flavonoids isolated from traditional Chinese medicine in treatment of AD

Agents	Structures or contents	Typical origin	Reference
Gingko flavonoids	Mixture: mainly including quercetin, kaempferol, isorhamnetin, and biflavonoids like ginkgetin, isoginkgetin, and amentoflavone	*Ginkgo biloba* L.	*18,19*
Soy isoflavones	Mixture: mainly including daidzin, daidzein, genistin, genistein, and glycitin, glycitein	*Glycine max*	*23,24*
Total flavonoids of Baical Skullcap stem and leaf	Mixture: mainly including scutellarin, baicalin, and chrysin	*Scutellaria baicalensis* Georgi	*45,46*
Puerarin		*Radix puerariae*	*40*
Liquiritin		*Glycyrrhiza uralensis* Fisch.	*49*
Apigenin		*Apium graveolens*	*52*
Hyperin		*Hypericum perforatum* L.	*57*
Rhodosin		*Rhodiola rosea*	*59*

it also causes side effects to non-neuronal cells, such as increasing the incidence of breast and endometrial cancer (*28-30*). Studies demonstrated that phytoestrogens such as genistein, one of the main ingredients of soy isoflavones, exerted pharmacological effects in a tissue specific manner (*31*). They selectively act on non-reproductive tissues to a certain degree and thus reduce the risk of side effects.

Animal studies indicated that soy isoflavones were capable of improving learning and memory abilities through influencing the brain cholinergic system and reducing age-related neuron loss especially in female rats (*32-34*). A clinical study demonstrated that postmenopausal women who undertook estrogen-replacement therapy had a significantly lower risk for the onset of AD than women who did not (*35*). Another randomized, double-blind, cross-over, and placebo-controlled trial revealed that soy isoflavones were safe and had positive effects on cognitive function, especially verbal memory, in postmenopausal women (*36*). The underlying mechanisms of favorable effects of soy isoflavones on cognitive function were thought to relate to their potential to mimic the actions and functions of estrogens in the brain (*37*), and promote the synthesis of acetylcholine and neurotrophic factors such as brain-derived neurotrophic factor (BDNF) and nerve growth factor (NGF) in the hippocampus and frontal cortex (*38,39*). These studies provided evidence for the potential usefulness of soy isoflavones in treatment of AD patients.

3.1.3. *Puerarin*

Puerarin is an isoflavanone glycoside extracted from species in the family Leguminosae such as *Radix puerariae* and is currently used to treat ischemic cerebrovascular disease and other vascular dysfunctions in China (*40*). Studies found that puerarin had potent effects in improving learning and memory disorders induced by scopolamine or D-galactose in a mouse model (*41*). Yan *et al*. reported that puerarin protected neurons against apoptosis in the cortex and hippocampus of AD rats caused by $A\beta_{25-35}$ through downregulating $A\beta_{1-40}$ and Bax expression in brain tissues, therefore alleviating the spatial learning and memory impairment of diseased animals (*42*). The anti-AD effects of puerarin were also suggested to be related to its abilities to decrease lipid peroxidase levels and increase superoxide dismutase levels in brain tissues, enhancing cerebral blood flow, and improving brain microcirculation (*43,44*).

3.1.4. *Total flavonoids of Baical Skullcap stem and leaf*

Baical Skullcap is a frequently used traditional Chinese medicine. Studies on its active ingredients revealed that the total flavonoids extracted from the stem and leaf, mainly including scutellarin, baicalin, and chrysin, exhibited a series of pharmacological effects such as anti-inflammation, prevention from myocardial damage induced by ischemia-reperfusion, and improved cerebral ischemia (*45,46*). Regarding its effects against AD, Zuo *et al*. found that total flavonoids of Baical Skullcap stem and leaf were capable of protecting hippocampal neurons against damage induced by injection of $A\beta_{25\text{-}35}$ into hippocampus in rat (*47*). The underlying mechanisms were related to its actions of decreasing the accumulation of lipid peroxide and proliferation of glial cells induced by $A\beta_{25\text{-}35}$ (*47*). Another study conducted by Ye *et al*. demonstrated that the total flavonoids alleviated memory and learning injury and protected hippocampal neurons from morphological changes in AD rats induced by $A\beta_{25\text{-}35}$ injection (*48*). These studies suggested the potential efficacies of total flavonoids of Baical Skullcap stem and leaf against AD.

3.1.5. *Liquiritin*

Liquiritin is an extract from the root of *Glycyrrhiza uralensis* Fisch. (*49*). Yang *et al*. investigated the protective effects of liquiritin on primary cultured rat hippocampal neurons (*50*). They found that pre-treatment with liquirtin for 6 h decreased the elevated levels of intracellular Ca^{2+} concentration and neuron apoptosis caused by $A\beta_{25\text{-}35}$. Liquirtin is also capable of enhancing the effects of nerve growth factor in extending neuroaxons (*50*). It is worth noting that liquirtin could also specifically inhibit the activity of acetylcholinesterase and promote the differentiation of neuronal stem cells into cholinergic neurons (*50,51*). The neuroprotective and neurotrophic effects make liquirtin a promising agent against AD.

3.1.6. *Apigenin*

Apigenin is a flavone usually obtained from *Apium graveolens* (*52*). It is a potent chelating agent that could decrease the metal ions participating in radical reactions and therefore reduce the creation of free radicals (*53*). In addition, apigenin could serve as an antioxidant to scavenge free radicals such as oxygen, nitric oxide (NO), and superoxide anion. On the other hand, apigenin possesses estrogen-like effects which are similar to the actions of estradiol (*54*). Due to these biological actions, apigenin was reported to protect human neuroblastoma cells SH-SY5Y against apoptosis induced by oxidative stress *in vitro* (*55*). *In vivo*, apigenin was found to improve the memory and learning disorders of aging mice induced by D-galactose (*56*).

3.1.7. *Other flavonoids*

Hyperoside is a flavonol isolated from species of *Hypericum* (*57*). In the mouse ischemia-reperfusion injury model, hyperoside was shown capable of inhibiting lactate dehydrogenase activity decline in brain tissues and obviously improve memory and learning disorders of model mice (*58*). Rhodosin is also a flavonol obtained from the root of *Rhodiola rosea* (*59*). Rhodosin functions as an antioxidant which scavenges free radicals, reduces the content of lipid peroxide, and inhibits degeneration of mitochondria in cerebrum cells and hippocampal pyramidal cells (*44*). Administration of rhodosin was reported to be capable of improving the memory and learning abilities of aging or AD mice (*60*).

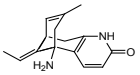
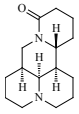
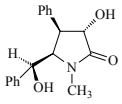
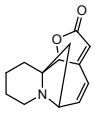
3.2. *Alkaloids*

Alkaloids are a group of naturally occurring cyclic compounds with a number of bioactivities that contain negative oxidation state nitrogen atoms. Compounds in this category exert anti-AD effects mainly through increasing the activity of the cholinergic system, suppressing inflammation, and/or exciting the central nervous system. Galantamine, an alkaloid isolated from plants in species of *Lycoris*, has been widely accepted as an effective drug for treatment of AD worldwide. Other alkaloids including huperzine A, sophocarpidine, clausenamide, arecoline, and securinine are either locally used as an anti-AD agent in clinics or still at the stage of studies (Table 2).

3.2.1. *Huperzine A*

Huperzine A is a reversible and selective cholinesterase inhibitor isolated from Chinese herb *Huperzia serrate* (*61*). It is also an NMDA receptor antagonist which may reduce glutamate induced damage in brain (*62*). It is highly lipid soluble and thus easy to pass through the blood-brain barrier and distribute to the brain after oral administration. Animal studies found that it is capable of enhancing the memory functions of rats (*63*). Clinical trials in China have shown that it is similarly effective compared to galantamine and donepezil, and may even be safer in terms of side effects (*62*). It has been approved and widely used to treat dysmnesia of elder people, amnesia or AD patients in China since 1994. In recent years, huperzine A has attracted increasing attention in the US and European countries for its potential anti-AD efficacies. A multi-center, double-blind, placebo-controlled phase II trial, which enrolled 177 participants with mild to moderate AD, was completed in the US in November 2007 (*64*). Results of this trial demonstrated that cognition and activities of daily living were mildly improved in patients who received 400 μg of huperzine A twice daily for 16 weeks. However, no significant changes were noted in overall disease change

Table 2. Alkaloids isolated from traditional Chinese medicine in treatment of AD

Agents	Structures	Typical origin	Reference
Huperzine A		*Huperzia serrate*	*61*
Sophocarpidine		*Sophora flavescens*	*65*
L-Clausenamide		*Clausena lansium (lour) Skeels*	*67*
MA9701	Arecoline derivative	---	*69*
Securinine		*Securinega suffruticosa*	*70*

or in psychiatric ratings according to the AD Assessment Scale-Cognitive (ADAS-Cog) scale. Currently, huperzine A is used as a dietary supplement for memory support in the US. That said, a Cochrane Database review reported in 2009, including four randomized, controlled trials in China involving 474 patients who received 300-500 μg of huperzine A daily for 8-24 weeks, that huperzine A is a well-tolerated drug that could significantly improve cognitive performance and activities on a daily living scale in patients with AD (*62*). Given this discrepancy, further clarifications on the efficacy of huperzine A against AD are still needed.

3.2.2. *Sophocarpidine*

Sophocarpidine is isolated from the root of *Sophora flavescens* (*65*). Studies found that sophocarpidine decreased the expression levels of interleukin-1β in cerebral cortex and hippocampus and alleviated injury of mitochondria of hippocampal neuronal cells in an AD rat model which was established by injection of ibotenic acid into hippocampus (*66*). Therefore, the anti-AD effects of sophocarpidine may be ascribed to its actions in mitigating inflammation through suppressing the release of inflammatory cytokines in the brain, thereby improving the status of injured neuronal cells and reducing neuron apoptosis.

3.2.3. *Clausenamide*

Clausenamide is isolated from the leaves of *Clausena lansium* (lour) Skeels in the family of Rutaceae [67]. Animal studies demonstrated that L-clausenamide is capable of improving the spatial discrimination disorders of rats induced by Aβ *via* enhancing the activities of cholineacetyltransferase of the cortex (*68*). Further, L-clausenamide promoted the release of glutamic acid from synaptosomes of cerebellum, enhanced the amplitude of long term potentiation (LTP) in the hippocampus CA1 zone, and increased cerebral cortex thickness, thereby improving learning and memory ability of rats (*68*). It was demonstrated that the nootropic effect of L-clausenamide is more potent than that of piracetam (*44*).

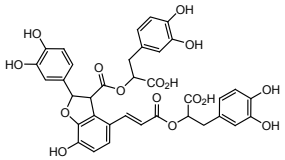
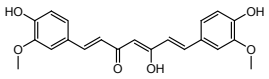
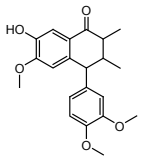
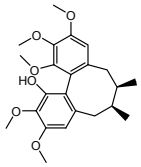
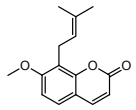
3.2.4. *Other alkaloids*

Compound MA9701 is synthesized based on the structure of arecoline which is an alkaloid isolated from the seeds of *Areca catechu*. It obviously improved the learning and memory disorder of mice which was induced by administration of ethanol or scopolamine (*69*). These effects of MA9701 were regarded to be related to its activities in agonizing the M acetylcholine receptor in the cortex and hippocampus. Securinine is an alkaloid isolated from the leaves of *Securinega suffruticosa* (*70*). It was demonstrated that securinine is capable of exciting the central nervous system and antagonizing the γ-aminobutyric acid (GABA) receptor. Administration of securinine significantly ameliorated the memory hypo-reproducibility of mice caused by 40% alcohol (*71*).

3.3. *Phenylpropanoids*

The phenylpropanoids are a diverse family of organic compounds that are synthesized by plants from the amino acid phenylalanine. The characteristics of the chemical structures of these compounds is that they consist of one or more structural units of C_6-C_3. Compounds in this category that have anti-AD potentials include salvianolic acid B (SAB), curcumin, schisandrone, schisanhenol, and osthole (Table 3).

Table 3. Phenylpropanoids isolated from traditional Chinese medicine in treatment of AD

Agents	Structures	Typical origin	Reference
Salvianolic acid B		*Salvia miltiorrhiza*	*72*
Curcumin		*Curcuma longa L.*	*76*
Schisandrone		*Schisandra chinensis*	*84*
Schisanhenol		*Schisandra chinensis*	*84*
Osthole		*Cnidium monnieri*	*86*

3.3.1. *SAB*

SAB, isolated from the root of *Salvia miltiorrhiza*, is a representative compound in this category (*72*). Its anti-AD effects and mechanisms have been extensively explored by Zhang and his colleagues (*73*). They demonstrated a series of results supporting that SAB is a promising agent in treatment of neurodegenerative diseases. First, SAB is a potent natural antioxidant, which could scavenge superoxide anion and hydroxyl radicals and inhibit lipid peroxidation. Studies demonstrated that it significantly decreased the levels of malondialdehyde (MDA), a product of lipid peroxidation, in the brain tissue of rats which were treated with FeSO4-cysteine or vitamin C-NADPH (*74*). Second, SAB protects the mitochondria of neurons from being damaged and suppresses neuronal apoptosis caused by a cerebral ischemia-reperfusion operation. The investigators indicated that cell apoptosis occurred in the ischemic area after occluding the cerebral artery for 1 h ischemia followed by 24 h reperfusion (*73,75*). Further studies revealed that this cerebral ischemia-reperfusion damaged mitochondrial membrane structure and decreased membrane potential, thus inducing the release of cytochrome *c* and causing elevated expression of caspase-3 (*73,75*). Pretreatment of rats with 10 mg/kg SAB effectively prevented those alternations of mitochondria and blocked the apoptosis of brain cells (*73*). Third, SAB suppresses the accumulation of $A\beta_{1\text{-}40}$, prevents the mitochondria of neuronal cell line PC12 being injured by $A\beta_{1\text{-}40}$, thereby reducing neuronal apoptosis. It was observed under the microscope that $A\beta_{1\text{-}40}$ at a concentration of 100 mg/L started to assemble and form fibrils when incubated at 25°C for 30 h (*73*). SAB at a concentration of 10 nmol/L almost entirely suppressed fibril formation of $A\beta_{1\text{-}40}$ (*73*). Further studies found that SAB at a concentration range of 0.01-1 $\times 10^{-6}$ M inhibited the apoptosis of PC12 cells caused by $A\beta_{1\text{-}40}$ (*73*). In addition, the assembled $A\beta_{25\text{-}35}$ was found to be toxic to PC12 cells after 48 h treatment, which could be significantly alleviated by 1 μmol/L SAB (*73*). Fourth, SAB is capable of suppressing the increase of intracellular calcium and reactive oxygen species (ROS) caused by $A\beta_{1\text{-}40}$. Studies demonstrated that the intracellular concentration of calcium increased from 188 to 326 mmol/L in neuronal cells PC12PS2N1411 after 24 h treatment with $A\beta_{1\text{-}40}$ (*73*). The levels of intracellular calcium were reduced to 249 and 233 mmol/L when incubated with 0.1 $\times 10^{-6}$ and 1 $\times 10^{-6}$ mmol/L SAB, respectively. The $A\beta_{1\text{-}40}$ caused elevated levels of ROS in mitochondria were also significantly decreased by the same concentrations of SAB (*73*). These studies suggested the potential value of SAB in treatment of AD. However, clinical trials are still required to investigate its safety and anti-AD efficacy.

3.3.2. *Curcumin*

Curcumin, isolated from the root of *Curcuma longa* L., is another representative compound in this category

(*76*). Studies in recent years demonstrated that it has a series of bioactivities such as anti-AD, antitumor, anti-inflammation, anti-oxidative, and anti-HIV effects. The underlying mechanisms of anti-AD effects of curcumin were revealed to include the following aspects. First, curcumin suppresses the formation of amyloid plaques. It was found that curcumin is capable of not only interfering with Aβ aggregation which leads to formation of Aβ fibrils, but also destabilizing preformed Aβ fibrils (*77*). In addition, it was reported that curcumin suppressed the up-regulation of APP and β-secretase mRNA levels caused by copper or manganese ions in a time- and dose-dependent manner (*78*). Second, curcumin inhibits Aβ induced inflammation. Giri *et al*. demonstrated that curcumin at a concentration range of 12.5-25 μM reduced the expression of cytokines TNFα and IL-1β and chemokines MIP-1β, MCP-1, and IL-8 in monocytes by suppressing the interaction of early growth response-1 (Egr-1) with $A\beta_{1-40}$ or fibrillar $A\beta_{1-42}$ (*79*). Third, curcumin possesses potent antioxidative effects. Kim *et al*. showed that curcumin protected neuronal cells PC12 and human umbilical vein endothelial cells from being injured by $A\beta_{42}$ due to its strong antioxidant properties (*80*). Another study showed that pretreatment of PC12 cells with 10 μg/mL curcumin decreased the level of antioxidant enzyme and DNA damage caused by $A\beta_{25-35}$ (*81*). Fourth, curcumin inhibits acetylcholinesterase activity. In an *in vitro* study, curcumin inhibited the activity of acetylcholinesterase with an IC_{50} value of 67.69 μM (*82*). Given the above activities of curcumin, thus far at least 6 clinical trials have been implemented to evaluate the efficacies of curcumin alone or in combination with other medications in treatment of AD or cognition impaired diseases (*83*). Among these studies, two were completed, one was terminated for various reasons, and three are under way. The disclosed results demonstrate no significant differences in cognitive function between placebo and curcumin groups. Results of clinical trials currently being conducted are expected and required to further testify to the efficacies of curcumin in treatment of AD.

3.3.3. *Schisandrone and schisanhenol*

Schisandrone is a linan isolated from the fruit of Schisandra chinensis (*84*). Studies found that schisandrone is capable of scavenging superoxide anion free radical and other ROS including H_2O_2 and ·OH generated by the xanthine-xanthine oxidase system and reducing the production of MDA in the process of lipid peroxidation (*44*). In addition, schisandrone significantly suppressed the oxidative stress and inflammatory response induced by Aβ. Furthermore, schisandrone suppressed the elevation of intracellular calcium induced by Aβ, thereby maintaining the intracellular calcium homeostasis equilibrium and protecting neurons from apoptosis (*85*). Schisanhenol is another active ingredient isolated from the fruit of *S. chinensis*. It was found to have effects in protecting rat brain synapses and mitochondria from ROS insult (*44*). Further animal studies are required to investigate the anti-AD effects of schisandrone and schisanhenol.

3.3.4. *Osthole*

Osthole is a coumarin isolated from plants in the Umbelliferae family such as *Cnidium monnieri* (*86*). Studies demonstrated that it significantly improved mouse spatial discrimination and memory disorders (*87*). This effect of osthole is thought to be related to its properties of suppressing lipid peroxidation and acetylcholinesterase activity in brain tissue of rats. It was also reported that osthole improved memory impairments in $AlCl_3$-induced senescence-accelerated mice, which was ascribed to its actions of enhancing activities of glutathione peroxidase (GSH-PX) and superoxide dismutase (SOD) and thus mitigating ROS-induced neuron injury (*88*). Previous studies on osthole mainly focused on its effects in antihypertension, antiarrhythmia, immunoenhancement, and anti-infection. Its potential application in treating dysmnesia related diseases such as AD requires additional investigation in the future.

3.4. *Triterpenoid saponins*

Triterpenoid saponin is composed of triterpene sapogenin and saccharide. Triterpenes include a large group of compounds mostly arranged in a four or five ring configuration of 30 carbons with several oxygens attached. Compounds in this category including Panax notoginseng saponins (PNS), ginsenoside, and gypenosides were found to exhibit potential anti-AD activities (Table 4).

Table 4. Triterpenoid saponins isolated from traditional Chinese medicine in treatment of AD

Agents	Contents	Typical origin	Reference
Panax notoginseng saponins	Mainly including sapoin monomers Rb1, Rd, and Rg1	*Panax notoginseng*	*89*
Ginsenosides	Mainly including saponin monomers Rb1, Rb2, Rc, Rd, Rg1, Re, Rf, and Rg2	*Panax ginseng C. A. Mey.*	*92*
Gypenosides	Including more than 100 dammarane-type triterpenoid saponins	*Gynostemma pentaphyllum*	*95*

3.4.1. *PNS*

These saponins belonging to the dammarane type are the main effective components of *Panax notoginseng* (*89*). PNS exhibits broad biological activities including anti-inflammation, anti-fibrosis, scavenging free radicals, anti-aging, *etc*. Guo *et al*. reported that PNS increased learning and memory ability in rat dementia caused by injection of ibotenic acid into the nucleus basalis of Meynert (NBM) (*90*). It was found that PNS alleviated the neuron injury by $A\beta_{25\text{-}35}$, thereby reducing neuron apoptosis (*44*). In addition, PNS protected nerve cells NG108-15 from apoptosis caused by Aβ *via* stabilizing the membrane of nerve cells (*44*). Furthermore, PNS also promoted the growth of nerve cells, extended the length of axons, and enhanced synaptic plasticity (*44*). Besides those effects, PNS prevented the reduction of choline acetyltransferase and thus enhanced the functions of cholinergic neurons in AD model rats which were established by intra-peritoneal injection of D-galactose combined with excitatory neurotoxin ibotenic acid injection into bilateral NBM (*91*). These activities of PNS may constitute the pharmacological basis responsible for its actions against AD.

3.4.2. *Ginsenosides*

Ginsenosides, isolated from *Panax ginseng* C. A. Mey., are saponins with broad biological properties (*92*). Wang *et al*. reported that ginsenoside Rg1 improved the learning and memory disorders in AD model mice induced by Aβ (*93*). Mechanisms underlying the anti-AD effects of ginsenoside include boosting the levels of acetylcholine in the synaptic cleft, increasing the numbers of cholinergic receptors, and promoting the synthesis of nucleic acids and proteins. *In vitro* studies demonstrated that ginsenoside Rg1 alleviated ROS injury on nerve cells and suppressed apoptosis of rat nerve cells (*94*). Animal studies demonstrated that ginsenoside Rg1 promoted the development of cranial nerves and increased the number of synapses and the density of muscarinic receptors in mice (*94*). Currently, Radix Ginseng, as a traditional Chinese medicine, is widely used to treat dementia diseases including AD in China. The above studies provide scientific evidence for using Radix Ginseng as an anti-AD medicine.

3.4.3. *Gypenosides*

Gypenosides are a series of saponins isolated from *Gynostemma pentaphyllum* (*95*). They exhibit strong antioxidative properties manifesting as scavenging free radicals and increasing the levels of SOD in brain tissues. In addition, gypenosides are capable of stabilizing the membrane of neurons. *In vivo* studies showed that gypenosides reversed the degeneration of learning and memory abilities induced by Aβ, which may be ascribed to their effects in suppressing the abnormal expression of cyclins and rectifying the disequilibrium of calcium homeostasis in hippocampal neurons (*96*). This evidence supports the use of Herba Gynostemmatis Pentaphylli in treatment of AD patients in clinics.

3.5. *Polysaccharides*

Currently, studies on the anti-AD effects of polysaccharides mainly focus on their activities in immunoregulation, antioxidation, and life extension. For example, sprulina polysaccharides are capable of improving the symptoms of aging mice caused by D-galactose (*97*). Polysaccharides from *Cistanche deserticola* increased the hypoxia tolerance and anti-oxidation action of aging mice (*98*). Polysaccharides from *Ganoderma lucidum* reduced the level of MDA through enhancing SOD activities in hippocampus of rats and suppressing the activation of astrocytes caused by $A\beta_{25\text{-}35}$ *via* immunoregulatory mechanisms (*99*). *Rehmannia glutinosa* oligosaccharides (RGOs) dose-dependently enhanced learning and memory ability in the rat insulted by cerebral ischemia-reperfusion *via* reducing the level of glutamic acid in hippocampus and increasing the levels of phosphorylated extracellular signal-regulated kinase (ERK) and acetylcholine (*100*). In addition, RGOs protected hippocampal neurons from being injured by glutamic acid, which is related to their effects in suppressing the excessive intake of glucose by neurocytes (*100*).

4. Conclusion and prospects

In the long history of development of traditional Chinese medicine, many herbs have been discovered and employed to treat dementia diseases in China. In recent decades, with the advancement of chromatographic and spectroscopic techniques, a number of agents have been isolated from these herbs and their efficacies against AD have been tested both *in vitro* and *in vivo*. The endeavors, on one hand, illustrated the principle of evidence-based medicine to clinically use these medicines to treat AD, and, on the other hand, discovered many monomer compositions as promising drugs or lead compounds for drug design in treatment of AD.

The currently used medications for treatment of AD are mainly symptom-management drugs. Although they do improve symptoms such as memory disorders and play a key role in treatment of AD at present, these drugs are not capable of reversing the progress of AD. In light of the pathogenic complexities of AD, it is probably unlikely that single-target drugs will achieve satisfactory curative effects. Some agents in categories of flavonoids and phenylpropanoids exhibit multiple biological properties that aim to eradicate the root causes of AD onset and may represent the future direction of new drug development.

Some issues also exist in the research and development of natural agents as anti-AD medicines. The results of experiments are sometimes difficult to repeat or compare because it varies for the same herb in methods of isolation or purification and the contents of effective constituents in different studies. In addition, effectiveness is hard to define due to the lack of positive control drugs in some cases. Research on natural products in prevention and treatment of AD started late and currently mostly has stayed at the stage of *in vitro* and animal studies. In the long run, efforts should be paid to screen and select optimal crude drugs, establish regulatory standards, and normalize the evaluation principles of drug efficacy to open new ways for AD drug research and development.

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References

1. World Alzheimer Report 2009. Alzheimer's Disease International. *http://www.alz.co.uk/research/files/WorldAlzheimerReport.pdf*
2. Huang Y, Mucke L. Alzheimer mechanisms and therapeutic strategies. Cell. 2012; 148:1204-1222.
3. News. Alzheimer's Disease Chinese (ADC). *http://www.adc.org.cn/html/news/qqzx_1268.shtm*l (accessed September 21, 2012).
4. Chen CF, He CL, Chen HX, Sun YF, Pan X. A summary of dementia studies in China. Journal of Ningbo University (Educational Science Edition). 2012; 34:45-50.
5. News. *http://news.xinhuanet.com/life/2010-08/20/c_12465497.htm* (accessed September 21, 2012).
6. Cummings JL. Alzheimer's disease. N Engl J Med. 2004; 351:56-67.
7. Sun XT, Jin L, Ling PX. Review of drugs for Alzheimer's disease. Drug Discov Ther. 2012; 6:285-290.
8. Wang R, Yan H, Tang XC. Progress in studies of huperzine A, a natural cholinesterase inhibitor from Chinese herbal medicine. Acta Pharmacol Sin. 2006; 27:1-26.
9. Takata K, Kitamura Y, Saeki M, *et al*. Galantamine-induced amyloid-{beta} clearance mediated *via* stimulation of microglial nicotinic acetylcholine receptors. J Biol Chem. 2010; 285:40180-40191.
10. Bertram L, Lill CM, Tanzi RE. The genetics of Alzheimer disease: Back to the future. Neuron. 2010; 68:270-281.
11. Chen JG. Drugs in treatment of Alzheimer's disease. In: Pharmacology: Volume 17, Drugs in treatment of neurodegenerative diseases of the central nervous system (Yang BF, Su DF, eds.). 6th ed., People's Medical Publishing House, Beijing, China, 2007; pp. 151-155.
12. Behl C, Davis JB, Lesley R, Schubert D. Hydrogen peroxide mediates amyloid beta protein toxicity. Cell. 1994; 77:817-827.
13. Mark RJ, Lovell MA, Markesbery WR, Uchida K, Mattson MP. A role for 4-hydroxynonenal, an aldehydic product of lipid peroxidation, in disruption of ion homeostasis and neuronal death induced by amyloid beta-peptide. J Neurochem. 1997; 68:255-264.
14. Rottkamp CA, Raina AK, Zhu X, *et al*. Redox-active iron mediates amyloid-beta toxicity. Free Radic Biol Med. 2001; 30:447-450.
15. Wei W, Wang X, Kusiak JW. Signaling events in amyloid beta-peptide-induced neuronal death and insulin-like growth factor I protection. J Biol Chem. 2002; 277:17649-17656.
16. Mattson MP, Cheng B, Davis D, Bryant K, Lieberburg I, Rydel RE. beta-Amyloid peptides destabilize calcium homeostasis and render human cortical neurons vulnerable to excitotoxicity. J Neurosci. 1992; 12:376-389.
17. Wu LJ. Flavonoids. In: Natural Medicine Chemistry (Wu LJ, ed.). 4th ed., People's Medical Publishing House, Beijing, China, 2003; p. 173.
18. Wang Y, Cao J, Weng JH, Zeng S. Simultaneous determination of quercetin, kaempferol and isorhamnetin accumulated human breast cancer cells, by high-performance liquid chromatography. J Pharm Biomed Anal. 2005; 39:328-333.
19. Hyun SK, Kang SS, Son KH, Chung HY, Choi JS. Biflavone glucosides from *Ginkgo biloba* yellow leaves. Chem Pharm Bull (Tokyo). 2005; 53:1200-1201.
20. Sastre J, Millan A, Garcia de la Asuncion J, *et al*. A Ginkgo biloba extract (EGb 761) prevents mitochondrial aging by protecting against oxidative stress. Free Radic Biol Med. 1998; 24:298-304.
21. Zhu JT, Choi RC, Chu GK, Cheung AW, Gao QT, Li J. Flavonoids possess neuroprotective effects on cultured pheochromocytoma PC12 cells: a comparison of different flavonoids in activating estrogenic effect and in preventing beta-amyloid-induced cell death. J Agric Food Chem. 2007; 55:2438-2445.
22. Le Bars PL, Katz MM, Berman N, Itil TM, Freedman AM, Schatzberg AF. A placebo-controlled, double-blind, randomized trial of an extract of Ginkgo biloba for dementia. North American EGb Study Group. JAMA. 1997; 278:1327-1332.
23. Manjanatha MG, Shelton S, Bishop ME, Lyn-Cook LE, Aidoo A. Dietary effects of soy isoflavones daidzein and genistein on 7,12-dimethylbenz[a]anthracene-induced mammary mutagenesis and carcinogenesis in ovariectomized Big Blue transgenic rats. Carcinogenesis. 2006; 27:2555-2564.
24. Wang CE, Liu SY. The components, contents and characteristics of soy isoflavones. Food Science. 1998; 19:39-43.
25. Yue X, Lu M, Lancaster T, Cao P, Honda S, Staufenbiel M, Harada N, Zhong Z, Shen Y, Li R. Brain estrogen deficiency accelerates Abeta plaque formation in an Alzheimer's disease animal model. Proc Natl Acad Sci U S A. 2005; 102:19198-19203.
26. Yao M, Nguyen TV, Pike CJ. Estrogen regulates Bcl-w and Bim expression: Role in protection against beta-amyloid peptide-induced neuronal death. J Neurosci. 2007; 27:1422-1433.
27. Heldring N, Pike A, Andersson S, Matthews J, Cheng G, Hartman J, Tujague M, Strom A, Treuter E, Warner M, Gustafsson JA. Estrogen receptors: How do they signal and what are their targets. Physiol Rev. 2007; 87:905-931.
28. Breast cancer and hormone replacement therapy: Collaborative reanalysis of data from 51 epidemiological studies of 52,705 women with breast cancer and 108,411

women without breast cancer. Collaborative Group on Hormonal Factors in Breast Cancer. Lancet. 1997; 350:1047-1059.
29. Beresford SA, Weiss NS, Voigt LF, McKnight B. Risk of endometrial cancer in relation to use of oestrogen combined with cyclic progestagen therapy in postmenopausal women. Lancet. 1997; 349:458-461.
30. Ravnikar VA. Compliance with hormone replacement therapy: Are women receiving the full impact of hormone replacement therapy preventive health benefits? Womens Health Issues. 1992; 2:75-80; discussion -2.
31. Escande A, Pillon A, Servant N, Cravedi JP, Larrea F, Muhn P, Nicolas JC, Cavailles V, Balaguer P. Evaluation of ligand selectivity using reporter cell lines stably expressing estrogen receptor alpha or beta. Biochem Pharmacol. 2006; 71:1459-1469.
32. Lee YB, Lee HJ, Won MH, Hwang IK, Kang TC, Lee JY, Nam SY, Kim KS, Kim E, Cheon SH, Sohn HS. Soy isoflavones improve spatial delayed matching-to-place performance and reduce cholinergic neuron loss in elderly male rats. J Nutr. 2004; 134:1827-1831.
33. Lund TD, West TW, Tian LY, Bu LH, Simmons DL, Setchell KD, Adlercreutz H, Lephart ED. Visual spatial memory is enhanced in female rats (but inhibited in males) by dietary soy phytoestrogens. BMC Neurosci. 2001; 2:20.
34. Pan Y, Anthony M, Watson S, Clarkson TB. Soy phytoestrogens improve radial arm maze performance in ovariectomized retired breeder rats and do not attenuate benefits of 17beta-estradiol treatment. Menopause. 2000; 7:230-235.
35. Henderson VW. Estrogen-containing hormone therapy and Alzheimer's disease risk: Understanding discrepant inferences from observational and experimental research. Neuroscience. 2006; 138:1031-1039.
36. Casini ML, Marelli G, Papaleo E, Ferrari A, D'Ambrosio F, Unfer V. Psychological assessment of the effects of treatment with phytoestrogens on postmenopausal women: A randomized, double-blind, crossover, placebo-controlled study. Fertil Steril. 2006; 85:972-978.
37. Birge SJ. Is there a role for estrogen replacement therapy in the prevention and treatment of dementia? J Am Geriatr Soc. 1996; 44:865-870.
38. Pan Y, Anthony M, Clarkson TB. Effect of estradiol and soy phytoestrogens on choline acetyltransferase and nerve growth factor mRNAs in the frontal cortex and hippocampus of female rats. Proc Soc Exp Biol Med. 1999; 221:118-125.
39. Pan Y, Anthony M, Clarkson TB. Evidence for up-regulation of brain-derived neurotrophic factor mRNA by soy phytoestrogens in the frontal cortex of retired breeder female rats. Neurosci Lett. 1999; 261:17-20.
40. Yeung DK, Leung SW, Xu YC, Vanhoutte PM, Man RY. Puerarin, an isoflavonoid derived from *Radix puerariae*, potentiates endothelium-independent relaxation *via* the cyclic AMP pathway in porcine coronary artery. Eur J Pharmacol. 2006; 552:105-111.
41. Xu XH. Effects of puerarin on fatty superoxide in aged mice induced by D-galactose. China Journal of Chinese Materia Medica. 2003; 28:66-69.
42. Yan FL, Lu G, Wang YQ, Hong Z. Effect of puerarin on the expression of Aβ1-40 and Bax in brain of AD rats induced by Aβ25-35. Chin J Neuromed. 2006; 5:158-161.
43. Jiang B, Liu JH, Bao YM, An LJ. Hydrogen peroxide-induced apoptosis in pc12 cells and the protective effect of puerarin. Cell Biol Int. 2003; 27:1025-1031.
44. Zhang JJ, Zhong XM. Natural drug in senile dementia treatment: Research progress. Journal of Liaoning University of TCM. 2009; 11:47-49.
45. Li XL, Tong L. Research progresses on chemical components and pharmacological effects of Baical Skullcap stem and leaf. Journal of Chengde Medical College. 2006; 23:284-286.
46. Zhao SM, Liu S, Yang HG, Kong XY, Song CJ, Liu YP. Protective effect of scutellaria baicalensis stem-leaf total flavonoid on lipid peroxidation induced by myocardial ischemia reperfusion in rats. Chinese Journal of Anatomy. 2006; 29:450-452.
47. Zuo YZ, Guan LH, Wang RT. Protective effects of SSTF on injury of hippocampus neurons induced by injection Aβ25-35. Journal of Chengde Medical College. 2009; 26:5-7.
48. Ye H, Wang RT, Zuo YZ, Guan LH, Shen XB. The effect of total flavonoid of Scutellarea Baicalensis stem-leaf against learning and memory deficit induced by Abeta25-35 injection in rat hippocampus. Lishizhen Medicine and Materia Medica Research. 2009; 20:879-880.
49. Sun YX, Tang Y, Wu AL, Liu T, Dai XL, Zheng QS, Wang ZB. Neuroprotective effect of liquiritin against focal cerebral ischemia/reperfusion in mice via its antioxidant and antiapoptosis properties. J Asian Nat Prod Res. 2010; 12:1051-1060.
50. Yang Y, Bian GX, Lv QJ. Neuroprotection and neurontrophism effects of liquiritin on primary cultured hippocampal cells. China Journal of Chinese Materia Medica. 2008; 33:931-935.
51. Liu RT, Bian GX, Zou LB, Huang XW, Lv QJ. Neuroprotective effects of liquiritin and its inhibitory actions on cholinesterase activity. Chinese Journal of New Drugs. 2008; 17:574-581.
52. Ko FN, Huang TF, Teng CM. Vasodilatory action mechanisms of apigenin isolated from Apium graveolens in rat thoracic aorta. Biochim Biophys Acta. 1991; 1115:69-74.
53. Sugihara N, Arakawa T, Ohnishi M, Furuno K. Anti- and pro-oxidative effects of flavonoids on metal-induced lipid hydroperoxide-dependent lipid peroxidation in cultured hepatocytes loaded with alpha-linolenic acid. Free Radic Biol Med. 1999; 27:1313-1323.
54. Zhao YH, Chen WQ, Luo SH, Yang H. Effect of apigenin on learning and memory behavior in mice with alzheimer's disease induced with D-galactose. Journal of Guangdong College of Pharmacy. 2005; 21:292-294.
55. Kang SS, Lee JY, Choi YK, Kim GS, Han BH. Neuroprotective effects of flavones on hydrogen peroxide-induced apoptosis in SH-SY5Y neuroblostoma cells. Bioorg Med Chem Lett. 2004; 14:2261-2264.
56. Zhou MM, Xie HS, Zhang J, Zhang Q. The anti-aging effect of apigenin on aging mice induced by D-galactose. Acad J Sec Mil Med Univ. 2007; 28:452-453.
57. Wu Y, Zhou SD, Li P. Determination of flavonoids in Hypericum perforatum by HPLC analysis. Yao Xue Xue Bao. 2002; 37:280-282.
58. Liu XH, Lou HX. Natural agents in treatment of dementia. Qilu Pharmaceutical Affairs. 2004; 23:42-43.
59. Wu L, Cheng SY, Wang Q, Chen YB. Advances in study on the pharmacological effects of active components of Chinese herbs on Alzheimer's disease. Zhongguo Zhong Yao Za Zhi. 2004; 29:387-389.

60. Zhu AQ, Li QX, Zhang XS, Teng CQ, Chu YD, Masters CL, George A, Cardamong T, Evin G. Effects of Rhodiola on Alzheimer pathology and open field activity in APP-C100 transgenic mice. Chinese Journal of Gerontology. 2004; 24:530-533.
61. Ma T, Gong K, Yan Y, Zhang L, Tang P, Zhang X, Gong Y. Huperzine A promotes hippocampal neurogenesis *in vitro* and *in vivo*. Brain Res. 2013; 1506:35-43.
62. Wang BS, Wang H, Wei ZH, Song YY, Zhang L, Chen HZ. Efficacy and safety of natural acetylcholinesterase inhibitor huperzine A in the treatment of Alzheimer's disease: An updated meta-analysis. Journal of neural transmission. 2009; 116:457-465.
63. Wang J, Zhang HY, Tang XC. Huperzine a improves chronic inflammation and cognitive decline in rats with cerebral hypoperfusion. J Neurosci Res. 2010; 88:807-815.
64. Rafii MS, Walsh S, Little JT, Behan K, Reynolds B, Ward C, Jin S, Thomas R, Aisen PS, Alzheimer's Disease Cooperative S. A phase II trial of huperzine A in mild to moderate Alzheimer disease. Neurology. 2011; 76:1389-1394.
65. Liu M, Liu XY, Cheng JF. Advance in the pharmacological research on matrine. Zhongguo Zhong Yao Za Zhi. 2003; 28:801-804.
66. Ni JJ, Wu ZM, Ling SC, Zhu X, Lu PP. Effect of Matrine injection on IL-beta level and ultrastructural changes of hippocampal neuron in Alzheimer disease rat. Chinese Journal of Anatomy. 2006; 29:608-611.
67. Ning N, Hu JF, Sun JD, Han N, Zhang JT, Chen NH. (-)Clausenamide facilitates synaptic transmission at hippocampal Schaffer collateral-CA1 synapses. Eur J Pharmacol. 2012; 682:50-55.
68. Zhu XZ, Li XY, Liu J. Recent pharmacological studies on natural products in China. Eur J Pharmacol. 2004; 500:221-230.
69. Cui YY, Feng JM, Liu HZ, Zhu L, Rong ZX, Lu Y, Chen HZ. Effect of a novel muscarinic receptor agonist MA9701 on cognitive improvement. Chinese Journal of Clinical Pharmacology and Therapeutics. 2003; 8:503-505.
70. Raj D, Luczkiewicz M. Securinega suffruticosa. Fitoterapia. 2008; 79:419-427.
71. Geng HM, Wang YZ, Zhang DQ. Natural medicines in treatment of Alzheimer disease. China Pharmacy. 2006; 17:1019-1021.
72. Li XB, Wang W, Zhou GJ, Li Y, Xie XM, Zhou TS. Production of salvianolic acid B in roots of *Salvia miltiorrhiza* (Danshen) during the post-harvest drying process. Molecules 2012; 17:2388-2407.
73. Zhang JT. Research progresses on salvianolic acid B in treatment of neurodegenerative diseases. Herald of Medicine. 2007; 26:107-110.
74. Huang YS, Zhang JT. Antioxidative effect of three water soluble components isolated from *Salvia miltiorrhiza* in vitro. Acta Pharmaceutica Sinica. 1992; 27:96-100.
75. Chen YH, Du GH, Zhang JT. Salvianolic acid B protects brain against injuries caused by ischemia-reperfusion in rats. Acta Pharmacol Sin. 2000; 21:463-466.
76. Ak T, Gulcin I. Antioxidant and radical scavenging properties of curcumin. Chemico-biological interactions. 2008; 174:27-37.
77. Ono K, Hasegawa K, Naiki H, Yamada M. Curcumin has potent anti-amyloidogenic effects for Alzheimer's beta-amyloid fibrils *in vitro*. J Neurosci Res. 2004; 75:742-750.
78. Lin R, Chen X, Li W, Han Y, Liu P, Pi R. Exposure to metal ions regulates mRNA levels of APP and BACE1 in PC12 cells: Blockage by curcumin. Neurosci Lett. 2008; 440:344-347.
79. Giri RK, Rajagopal V, Kalra VK. Curcumin, the active constituent of turmeric, inhibits amyloid peptide-induced cytochemokine gene expression and CCR5-mediated chemotaxis of THP-1 monocytes by modulating early growth response-1 transcription factor. J Neurochem. 2004; 91:1199-1210.
80. Kim DS, Park SY, Kim JK. Curcuminoids from *Curcuma longa* L. (Zingiberaceae) that protect PC12 rat pheochromocytoma and normal human umbilical vein endothelial cells from betaA(1-42) insult. Neurosci Lett. 2001; 303:57-61.
81. Park SY, Kim HS, Cho EK, Kwon BY, Phark S, Hwang KW, Sul D. Curcumin protected PC12 cells against beta-amyloid-induced toxicity through the inhibition of oxidative damage and tau hyperphosphorylation. Food Chem Toxicol. 2008; 46:2881-2887.
82. Ahmed T, Gilani AH. Inhibitory effect of curcuminoids on acetylcholinesterase activity and attenuation of scopolamine-induced amnesia may explain medicinal use of turmeric in Alzheimer's disease. Pharmacology, biochemistry, and behavior. 2009; 91:554-559.
83. ClinicalTrials.gov. *http://www.clinicaltrials.gov/ct2/results?term=Curcumin++alzheimer&Search=Search* (accessed January 21, 2013).
84. Huyke C, Engel K, Simon-Haarhaus B, Quirin KW, Schempp CM. Composition and biological activity of different extracts from Schisandra sphenanthera and Schisandra chinensis. Planta Med. 2007; 73:1116-1126.
85. Zhu JQ, Tuo XP, Jia LY, Zhou J. Effect of schisandrone on intracellular calcium induced by amyloid-beta protein in cultured hippocampal neurons. Shanghai Med J. 2005; 28:790-792.
86. He Y, Qu S, Wang J, He X, Lin W, Zhen H, Zhang X. Neuroprotective effects of osthole pretreatment against traumatic brain injury in rats. Brain Res. 2012; 1433:127-36.
87. Shen LX, Zhang DS, Zhang L, Ren LM, Chang JQ. Action of osthol on learning and memory and its mechanism analysis. Acta Pharmaceutica Sinica. 1999; 34:1-7.
88. Shen LX, Jin LQ, Zhang DS, Xue GP. Effect of osthol on memory impairment of mice in $AlCl_3$-induced acute senile model. Acta Pharmaceutica Sinica. 2002; 37:178-180.
89. Si YC, Zhang JP, Xie CE, Zhang LJ, Jiang XN. Effects of *Panax notoginseng* saponins on proliferation and differentiation of rat hippocampal neural stem cells. Am J Chin Med. 2011; 39:999-1013.
90. Guo CJ, Wu JX, Li RX. The effects of PNS on Alzheimer disease model of mouse and mechanism of the effects. China Pharmacy. 2004; 15:598-600.
91. Zhong ZG, Qu ZQ, Wang NP, Wang JS, Xie ZG, Zhang FF, Zhang WY, Lu ZP. Protective effects of Panax notoginseng saponins against pathological lesion of cholinergic neuron in rat with Alzheimer disease. Zhong Yao Cai. 2005; 28:119-122.
92. Balaji Raghavendran HR, Rekha S, Cho HK, Jang SS, Son CG. Ginsenoside rich fraction of *Panax ginseng* C.A. Meyer improve feeding behavior following radiation-induced pica in rats. Fitoterapia. 2012; 83:1144-1150.
93. Wang XY, Chen J, Zhang JT. Effect of ginsenoside Rg1 on learning and memory impairment induced by beta-amyloid peptide (25-35) and its mechanism of action.

Acta Pharmaceutica Sinica. 2001; 36:1-4.
94. Liu Y, Li X, Yuan HF. Progress of research on effects of ginsenoside Rg1 in promoting capability of learning and memory. Chinese Journal of Integrative Medicine. 2006; 26:956-960.
95. Zhang GL, Deng JP, Wang BH, Zhao ZW, Li J, Gao L, Liu BL, Xong JR, Guo XD, Yan ZQ, Gao GD. Gypenosides improve cognitive impairment induced by chronic cerebral hypoperfusion in rats by suppressing oxidative stress and astrocytic activation. Behavioural pharmacology. 2011; 22:633-644.
96. Yao BC, Yuan H, Huang X, Zhao JJ. Effects of gypenosides on cell cycle related protein expression and calcium homeostasis in hippocampalneurons of rats treated with beta-amyloid protein fragmant 1-40. Chinese Journal of Pathophysiology. 2006; 22:1618-1622.
97. Zuo SY. Effect of spirulina polysaccharide on the D-galactose induced aging model mice. Chinese Journal of Biochemical Pharmaceutics. 1998; 19:15-18.
98. Xu H, Wei XD, Ou Q, Li Y, Xu XX, Wang JT, Tian GZ. Desertliving cistanche anti-aging action: A comparative study. Heilongjiang Medicine and Pharmacy. 2011; 34:1-2.
99. Guo YJ, Yuan H, Gan SW, Li L. Protective effect of *Ganoderma lucidum* polysaccharide on the brain tissue of rat model of Alzheimer disease induced by beta-amyloid protein fragment (25-35). Chinese Journal of Histochemistry and Cytochemistry. 2006; 15:448-451.
100. Liu WX, Lu YW, Du HT, Wu ZZ. Pharmacological actions of Radix Rehmanniae and its active components: Research advances. Journal of International Pharmaceutical Research. 2009; 36:277-280.

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Brief Report

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Synthesis and antiproliferative assay of 1,3,4-oxadiazole and 1,2,4-triazole derivatives in cancer cells

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ABSTRACT: A series of new 1,3,4-oxadiazole and 1,2,4-triazole derivatives were synthesized. The structures were confirmed by IR, ^{1}H-NMR, and MS. The compounds were evaluated for their antiproliferative activity against K562 (human erythromyeloblastoid leukemia cell line), MDA-MB-231 (human breast adenocarcinoma cell line), HT29 (human colon adenocarcinoma grade II cell line) and HepG2 (human hepatocellular liver carcinoma cell line) *in vitro*. The result showed that 7 compounds displayed inhibitory activities against K562 with the inhibition rate more than 50%. Especially, compound 5f exhibited the most potent activity against K562 with 85% inhibition ratio and could be used as lead compound to search new 1,3,4-oxadiazole derivatives as antiproliferative agent.

Keywords: Synthesis, 1,3,4-oxadiazole, 1,2,4-triazole, antitumor

1. Introduction

It was observed from the literature that 1,3,4-oxadiazole ring is associated with many types of biological properties such as anti-inflammatory (*1*), antibacterial (*2*), antifungal (*3*), antitumor (*4*), antiviral (*5*), hypoglycemic (*6*), anticonvulsant (*7*), analgesic (*8*), herbicidal (*9*), and insecticidal activities (*10*). Another 1,2,4-triazole ring also shows broad-spectrum bioactivities, *e.g.*, preparation their derivatives as agrochemical (*11*), medicinal fungicides (*12*), virucides (*13*), anticancer drugs (*14*), antimicrobial anti-inflammatory (*15*), anticonvulsant (*16*), antihypertensive (*17*), and plant growth regulators (*18*).

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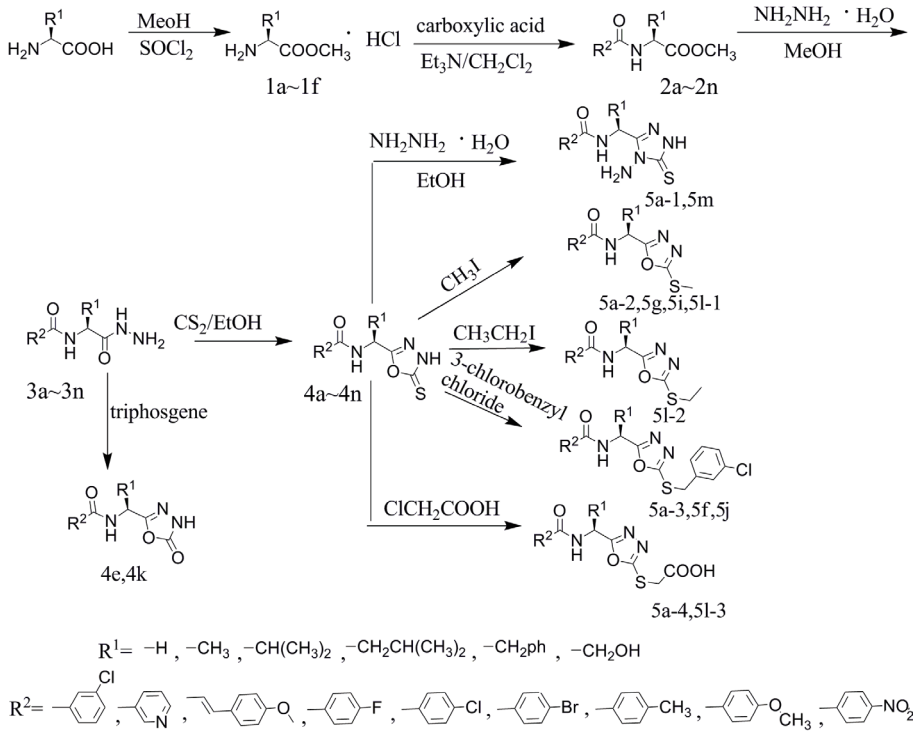
Chronic myelogenous leukaemia (CML) is a haematological malignancy caused by a chromosomal rearrangement that generates a fusion protein, BCR-ABL, with deregulated tyrosine kinase activity. K562 is human erythromyeloblastoid leukemia cell line and can specific express BCR-ABL. Imatinib is an inhibitor of BCR-ABL tyrosine kinase, function through competitive inhibition at the ATP-binding site of the enzyme, which leads to the inhibition of tyrosine phosphorylation of proteins involved in BCR-ABL signal transduction. It shows a high specificity for BCR-ABL, the receptor of platelet-derived growth factor, and c-kit tyrosine kinases, and is well tolerated. Imatinib has significant activity against CML which treatment with α interferon had failed (*19*).

In view of above mentioned facts and an attempt to achieve new potent antitumor agents with good bioavailability and low toxicity, herein, we described the synthesis and the structure-activity relationship (SAR) of a series of new 1,3,4-oxadiazole and 1,2,4-triazole derivatives as potential antitumor agents. Docking simulations were performed using the X-ray crystallographic structure of the ABL complexed with imatinib to explore the binding modes at the active site.

2. Materials and Methods

2.1. *Chemicals*

Target compounds were synthesized *via* the route outlined in Scheme 1. Firstly, amino acids (glycine, L-alanine, L-valine, L-leucine, L-phenylalanine, and L-serine) were reacted with MeOH to yield amino acids methyl ester (*20*), then coupled with carboxylic acids to obtain amide (**2a~2n**) and hydrazinolysised to give hydrazinyl-substituted amide (**3a~3n**) (*21*). (1) Cycling with carbon disulphide yielded the corresponding 1,3,4-oxadiazole derivatives (**4a~4n**) (*22*). (a) Reacting with hydrazine hydrate obtained 1,2,4-triazole derivatives (**5a-1**,**5n**) (*23*). (b) Reacting with methyl iodide, ethyl iodide, 3-chlorobenzyl chloride or chloroacetic acid obtained thioethers (**5a-2**, **5h**, **5j**, **5m-1**; **5m-2**; **5a-3**, **5g**, **5k**). (2) Cycling **3e**, **3k** with



Scheme 1. The synthesis of target compounds.

triphosgene obtained new 1,3,4-oxadiazole derivatives (**4e**, **4k**) (*24*). The structures of the target compounds which were evaluated for antiproliferative activity were confirmed by IR, ^{1}H-NMR, and MS. All of them gave satisfactory analytical and spectroscopic data, which were in full accordance with depicted structures.

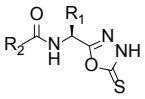
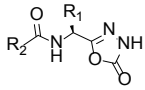
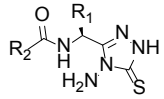
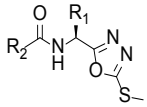
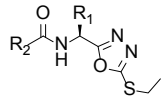
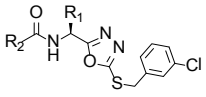
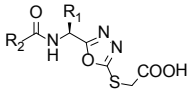
2.2. *Antiproliferation assay (in vitro)*

The antiproliferative activity of 1,3,4-oxadiazole and 1,2,4-triazole derivatives was assessed by means of MTT method (*25*). Imatinib mesylate was used as the positive control. To determine cell proliferation, K562 (human erythromyeloblastoid leukemia cell line), MDA-MB-231 (human breast adenocarcinoma cell line), HT29 (human colon adenocarcinoma grade II cell line), and Hep G2 (human hepatocellular liver carcinoma cell line) were individually plated at density of 1.5×10^4 cells/well, 8.0×10^3 cells/well, 8.0×10^3 cells/well, 4.0×10^3 cells/well, respectively, in 96-well plates at 37°C in 5% CO_2 atmosphere. For MDA-MB-231 cell lines, HT29 cell lines and Hep G2 cell lines, after 24 h of culture, the medium in the wells was replaced with the fresh medium containing compounds of certain concentration (1.0×10^{-4} mol/L). Three wells were tested in parallel for each synthesized compounds. After 48 h, 20 μL of MTT dye solution (5 mg/mL in phosphate buffer, pH7.4) was added to each well and incubated for 4 h at 37°C in 5% CO_2 for exponentially growing cell and 10 min for steady-state confluent cells. The formazan crystals were solubilized with 150 μL of dimethyl sulfoxide (DMSO) and the solution was vigorously mixed to dissolve the reacted dye. For K562 cell lines, the medium containing compounds of certain concentration (1.0×10^{-4} mol/L) was added. After 48 h, 10 μL of MTT dye solution (5 mg/mL in phosphate buffer, pH7.4) was added to each well and incubated for 4 h at 37°C in 5% CO_2 for exponentially growing cell. Then a mixed solution (10% SDS, 5% isobutanol, 0.012 mol/L HCl) was added to each well for 12 h. The absorbance of each well was read on a microplate reader (BTR-600 intruments) at 490 nm. The inhibition ratio on the tumor cells growth by the drugs was determined by the following formula: inhibition ratio on the tumor cells growth = (1 – (experimental group OD value/control group OD value)) × 100%.

3. Results and Discussion

All the synthesized 1,3,4-oxadiazole and 1,2,4-triazole derivatives were evaluated for their antiproliferative activity against K562, MDA-MB-231, HT29, and Hep G2 in Table 1. Preliminary result showed that 7 compounds displayed inhibitory activities against K562 with the inhibition rate more than 50%. Structure-activity relationship of the derivatives inhibition the proliferation of K562 was studied. Comparing 1,2,4-triazole and 5-thioxo-1,3,4-oxadiazole analogues (**5a-1**, **5m**, **4a~4n**), 1,2,4-triazole rings had no significant effect on the antiproliferative activity, while 5-oxo-1,3,4-oxadiazole ring (**4e**, **4k**) leaded to increase in activity. Among alanine analogues (**4a**, **4c**, **4d**, **4n**), compounds **4c** and **4n** showed better activity than others, which indicated that introduction vinylogy and nitro groups to benzene ring might improve the activity. Compounds **5l-1**, **5l-2**, and **5f** displayed excellent antiproliferative activity, which indicated that the substituent attached to

Table 1. Antiproliferative assay of target compounds and imatinib mesylate (rate%)

Structure	Compounds	R_1	R_2	K562	MDA-MB-231	HT29	Hep G2
	4a	$-CH_3$		27	21	15	13
	4b	$-CH(CH_3)_2$		30	17	21	24
	4c	$-CH_3$		57	12	17	15
	4d	$-CH_3$		30	20	11	17
	4f	$-CH_2CH(CH_3)_2$		36	11	15	10
	4g	$-CH_2ph$		42	12	19	13
	4h	$-CH_2OH$		37	18	14	16
	4i	$-CH(CH_3)_2$		32	15	18	20
	4j	$-CH_2CH(CH_3)_2$		57	12	13	12
	4n	$-CH_3$		54	10	16	24
	4e	-H		63	21	23	16
	4k	-H		48	23	25	15
	5a-1	$-CH_3$		40	12	14	12
	5m	$-CH_3$		13	11	9	14
	5a-2	$-CH_3$		12	18	17	7
	5g	$-CH_2ph$		46	16	32	29
	5i	$-CH(CH_3)_2$		36	11	16	20
	5l-1	$-CH(CH_3)_2$		59	20	29	22
	5l-2	$-CH(CH_3)_2$		60	13	17	19
	5a-3	$-CH_3$		17	14	22	23
	5f	$-CH_2CH(CH_3)_2$		85	32	23	12
	5j	$-CH_2CH(CH_3)_2$		34	13	16	14
	5a-4	$-CH_3$		7	12	5	9
	5l-3	$-CH(CH_3)_2$		9	8	11	7
	Imatinib mesylate			93	92	95	94

the 5-position of 1,3,4-oxadiazole ring had significant effect on activity, while carboxyl group made compounds almost lose antiproliferative activity. Compound **5f** was the most potent of all the target compounds with 85% inhibition ratio. Compared with **4i**, compound **4j** with a bulky substituent which connected with chiral carbon was more potent activity. Substituent in benzene ring greatly influenced the activity. In addition, all of target compounds show low activity toward MDA-MB-231, HT29, and Hep G2. In above four types of tumor cells, only the K562 can specific express ABL, and the variant tumor cells showed different sensitivity to anticancer drugs. So the target compounds exhibited K562 selectivity.

In effort to elucidate the possible mechanism which the target compounds displayed antiproliferative activity toward K562 and guide further structure-activity relationship studies, the preferred pharmacophore docking studies were carried out *via* the autodock program. The interaction of **5f** with active site of ABL structure complexed with imatinib (PDB: 3K5V) is shown in Figures 1 and 2. Compounds **5f**, as imatinib, has been shown to recognize efficiently active-site of ABL. The carbonyl group can form hydrogen bond with Asp400 with the distance of 2.81 Å. The nitrogen atom of amide bond forms hydrogen bond with Asp400 and Glu305 with the distance of 2.96 Å and 2.78 Å, respectively. The hydrophobic parts of compound are in contact with nonpolar surface areas of ABL such as Lys290, Ala399, Phe401, Ile312, Lys304, Val308, and Phe378.

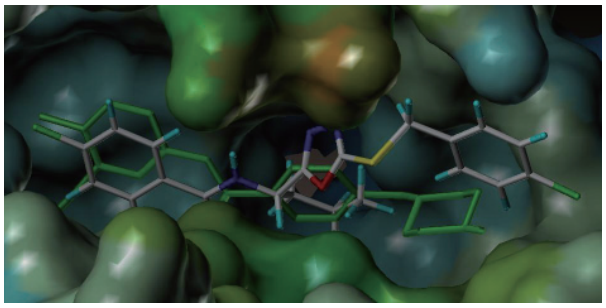


Figure 1. Molecular docking of compound 5f with active-site of ABL. Imatinib is represented as green sticks. Compound **5f** is represented as a tube with colored atoms.

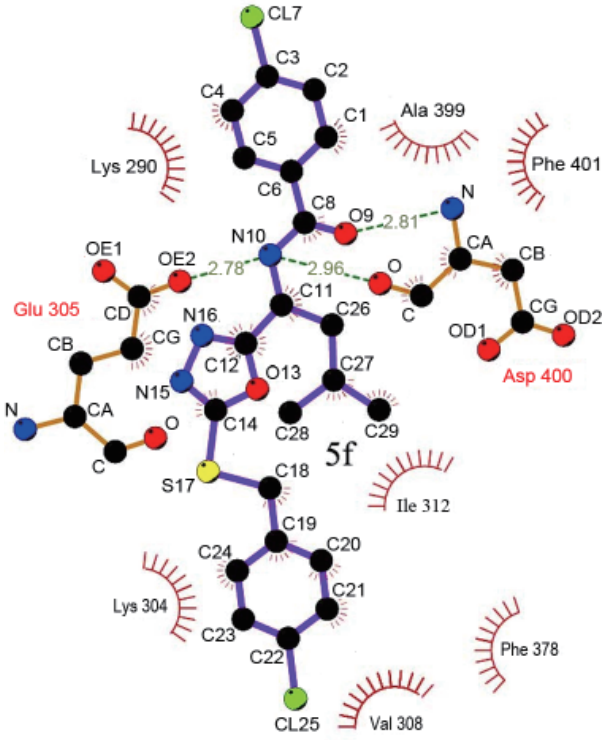


Figure 2. Diagram (LIGPLOT) of the hydrogen bonds and hydrophobic interactions of the compound 5f with active-site residues in ABL.

4. Conclusion

We designed and synthesized a series of new 1,3,4-oxadiazole and 1,2,4-triazole derivatives. Some of them showed moderate potency against K562. Especially, compound **5f** exhibited the best inhibitory activity and could be used as lead compound to search new 1,3,4-oxadiazole derivatives as antiproliferative agents.

Acknowledgements

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References

1. Palaska E, Sahin G, Kelicen P, Durlu NT, Altinok G. Synthesis and anti-inflammatory activity of 1-acylthiosemicarbazides, 1,3,4-oxadiazoles, 1,3,4-thiadiazoles and 1,2,4-triazole-3-thiones. Farmaco. 2002; 57:101-107.
2 Jha KK, Samad A, Kumar Y, Shaharyar M, Khosa RL, Jain J, Kumar V, Singh P. Design, synthesis and biological evaluation of 1,3,4-oxadiazole derivatives. Eur J Med Chem. 2010; 45:4963-4967.
3 Chen CJ, Song BA, Yang S, Xu GF, Bhadury PS, Jin LH, Hu DY, Li QZ, Liu F, Xue W, Lu P, Chen Z. Synthesis and antifungal activities of 5-(3,4,5-trimethoxyphenyl)-2-sulfonyl-1,3,4-thiadiazole and 5-(3,4,5-trimethoxyphenyl)-2-sulfonyl-1,3,4-oxadiazole derivatives. Bioorg Med Chem. 2007; 15:3981-3989.
4 Zheng QZ, Zhang XM, Xu Y, Cheng K, Jiao QC, Zhu HL. Synthesis, biological evaluation, and molecular docking studies of 2-chloropyridine derivatives possessing 1,3,4-oxadiazole moiety as potential antitumor agents. Bioorg Med Chem. 2010; 18:7836-7841.
5 El-Sayed WA, El-Essawy FA, Ali OM, Nasr BS, Abdalla MM, Abdel-Rahman AAH. Synthesis and antiviral evaluation of new 2,5-disubstituted 1,3,4-oxadiazole derivatives and their acyclic nucleoside analogues. Monatsh Chem. 2010; 141:1021-1028.
6 Ladduwahetty T, Baker R, Cascieri MA, *et al*. N-heteroaryl-2-phenyl-3-(benzyloxy)piperidines: a novel class of potent orally active human NK1 antagonists. J Med Chem. 1996; 39:2907-2914.
7 Gupta A, Kashaw SK, Jain N, Rajak H, Soni A, Stables JP. Design and synthesis of some novel 3-[5-(4-substituted) phenyl-1,3,4-oxadiazole-2yl]-2-phenylquinazoline-4(3H)-ones as possible anticonvulsant agent. Med Chem Res. 2011; 20:1638-1642.
8 Ramaprasad GC, Kalluraya B, Kumar BS, Hunnur RK. Synthesis and biological property of some novel 1,3,4-oxadiazoles. Eur J Med Chem. 2010; 45:4587-4593.
9 Kennedy DA, Summers LA. Chemical constitution and activity of bipyridinium herbicides. part XIV. reduction potential and herbicidal activity of 4,4′-(1,3,4-thiadiazole-2,5-diyl)- and 4,4′-(1,3,4-oxadiazole-2,5-diyl)bis(1-methylpyridinium) diiodides. J Heterocyclic Chem. 1981; 18:409-410.
10 Shi W, Qian XH, Song GH, Zhang R, Li RP. Syntheses and insecticidal activities of novel 2-fluorophenyl-5-aryl/cyclopropyl-1,3,4-oxadiazoles. J Fluorine Chem. 2000; 106:173-179.
11 Worthington PA. Synthesis of 1,2,4-triazole compounds related to the fungicides flutriafol and hexaconazole. Pestic Sci. 1991; 31:457-498.
12 Tsumanuma GM, Soares AR, Fancelli AL, Rodrigues MAT, Begliomini E. Effect of herbicides and fungicides on progress curves and damage quantification caused by soybean rust. Cienc Rural. 2010; 40:1485-1491.
13 Vivet-Boudou V, Paillart JC, Burger A, Marquet R. In search of new inhibitors of HIV-1 replication: Synthesis and study of 1-(2′-deoxy-β-d-ribofuranosyl)-1,2,4-triazole-3-carboxamide as a selective viral mutagenic agent. Nucleos Nucleot Nucl. 2007; 26:743-746.
14 Jha A, Murthy YLN, Durga G, Sundari TT. Microwave-assisted synthesis of 3,5-dibenzyl-4-amino-1,2,4-triazole and its diazo ligand, metal complexes along with anticancer

activity. E-Journal of Chemistry. 2010; 7:1571-1577.
15 Al-Omar MA, Al-Abdullah ES, Shehata IA, Habib EE, Ibrahim TM, El-Emam AA. Synthesis, antimicrobial, and anti-inflammatory activities of novel 5-(1-adamantyl)-4-arylideneamino-3-mercapto-1,2,4-triazoles and related derivatives. Molecules. 2010; 15:2526-2550.
16 Siddiqui N, Ahsan W. Triazole incorporated thiazoles as a new class of anticonvulsants: Design, synthesis and *in vivo* screening. Eur J Med Chem. 2010; 45:1536-1543.
17 Eriksson HE, Florvall LG. The preparation and antihypertensive activity of 4-amino-3-(2,6-dichlorobenzylidenehydrazino)-1,2,4-triazole and some related compounds. Acta Pharm Suec. 1976; 13:79-96.
18 Kitahata N, Saito S, Miyazawa Y, Umezawa T, Shimada Y, Min YK, Mizutani M, Hirai N, Shinozaki K, Yoshida S, Asami T. Chemical regulation of abscisic acid catabolism in plants by cytochrome P450 inhibitors. Bioorg Med Chem. 2005; 13:4491-4498.
19 Druker BJ, Talpaz M, Resta DJ, Peng B, Buchdunger E, Ford JM, Lydon NB, Kantarjian H, Capdeville R, Ohno-Jones S, Sawyers CL. Efficacy and safety of a specific inhibitor of the BCR-ABL tyrosine kinase in chronic myeloid leukemia. N Engl J Med. 2001; 344:1031-1037.
20 van Dijk M, Postma TM, Rijkers DTS, Liskamp RMJ, van Nostrum CF, Hennink WE. Synthesis and characterization of tailorable biodegradable thermoresponsive methacryloylamide polymers based on l-serine and l-threonine alkyl esters. Polymer. 2010; 51:2479-2485.
21 Tian B, He M, Tang S, Hewlett I, Tan Z, Li J, Jin Y, Yang M. Synthesis and antiviral activities of novel acylhydrazone derivatives targeting HIV-1 capsid protein. Bioorg Med Chem Lett. 2009; 19:2162-2167.
22 Sahin G, Palaska E, Ekizoğlu M, Ozalp M. Synthesis and antimicrobial activity of some 1,3,4-oxadiazole derivatives. Farmaco. 2002; 57:539-542.
23 Muthal N, Ahirwar J, Ahriwar D, Masih P, Mahmdapure T, Sivakumar T. Synthesis, antimicrobial and anti-inflammatory activity of some 5-substituted-3-pyridine-1,2,4-triazoles. Int J PharmTech Res. 2010; 2:2450-2455.
24 Zampieri D, Mamolo MG, Laurini E, Fermeglia M, Posocco P, Pricl S, Banfi E, Scialino G, Vio L. Antimycobacterial activity of new 3,5-disubstituted 1,3,4-oxadiazol-2(3H)-one derivatives. molecular modeling investigations. Bioorg Med Chem. 2009; 17:4693-4707.
25 Li Z, Gu Z, Yin K, Zhang R, Deng Q, Xiang J. Synthesis of substituted-phenyl-1,2,4-triazol-3-thione analogues with modified d-glucopyranosyl residues and their antiproliferative activities. Eur J Med Chem. 2009; 44:4716-4720.

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Appendix

Chemistry: general procedures

Unless otherwise noted, materials were obtained from commercial suppliers and used without further purification. The solvents of $SOCl_2$, methanol and carbonic dichloride have been distilled before use. All reactions were monitored by thin-layer chromatography on 0.25 mm silica gel plates (60GF-254) and visualized with UV light. Melting points were determined using WRS-1B melting point apparatus and are uncorrected. The IR spectra were recorded by means of KBr pellet technique on a SHIMADZU FTIR-8400 spectrometer. Mass spectra were recorded on a ZQ-4000/2695 equipment in a negative electron spray ionization (ESI) mode. ^{1}H-NMR was recorded on a Bruker AV-600 MHz spectrometer at room temperature, and chemical shifts were measured using TMS as internal standard. Significant ^{1}H-NMR data are reported in the following order: multiplicity (s, singlet; d, doublet; t, triplet; q, quartet; m, multiplet) number of protons.

1. General procedure for compounds 1a-f

The compounds (**1a-f**) were prepared from the corresponding amino acids (glycine, L-alanine, L-valine, L-leucine, L-phenylalanine, L-serine). MeOH (200 mL) was cooled in ice-bath and $SOCl_2$ (18 mL, 0.25 mol) was added dropwise. The reaction mixture was stirred at 0°C for 1 h and then appropriate amino acid (0.2 mol) was added, stirred for another 1 h at 0°C, followed by 24 h at room temperature and then evaporated in vacuum to dryness, recrystallized from appropriate solvent.

*Glycine methyl ester hydrochloride (**1a**)* White solid; yield: 98%; mp: 175.8-176.8°C; IR(KBr) 3,395, 2,885, 2,685, 2,635, 1,747, 1,585, 1,439, 1,261, 880 cm^{-1}; ^{1}H-NMR (600 MHz, DMSO-d_6,) δ 8.504(s, 3H, NH_3Cl), 3.792(s, 2H, CH_2), 3.735(s, 3H, CH_3); ESI-MS: m/z $[M - H]^-$ 122.80.

2. General procedure for compounds 2a-n

To a stirred solution of the compounds **1a-f** (0.02 mol) in CH_2Cl_2 (80 mL) triethylamine (0.04 mol, 5.6 mL) was added, reacted at 0°C for 0.5 h. A solution of carboxylic acid (0.02 mol) in CH_2Cl_2 (40 mL) was added, then DCC (0.022 mol, 4.54 g) in CH_2Cl_2 (20 mL) was added dropwise. The mixture was stirred for 2 h at 0°C, then allowed to warm to room temperature for 48 h. The white precipitate (DCU) was removed. The solution was washed with saturated $NaHCO_3$ (30 mL, ×3), 10% hydrochloric acid (30 mL, ×3), distilled water (30 mL, ×2) in turn, dried with magnesium sulfate anhydrous, filtered. The solvent was evaporated in vacuo, and the residue was dissolved in ethyl acetate, filtered. The ethyl acetate solution was evaporated in vacuo. The residue was recrystallized from appropriate solvent.

*Methyl 2-(4-chlorobenzamido)acetate (**2e**)* White solid; yield: 67%; mp: 110.0-112.0°C; IR (KBr) 3,275, 3,090, 3,028, 2,990, 2,947, 2,851, 1,751, 1,651, 1,435, 853 cm^{-1}; ^{1}H-NMR (600 MHz, DMSO-d_6) δ 9.072(t, J = 5.7 Hz, 1H, NH), 7.893(m, 2H, ArH), 7.578(m, 2H, ArH), 4.02(d, J = 2.7 Hz, 2H, CH_2), 3.659(s, 3H, CH_3); ESI-MS: m/z $[M - H]^-$ 225.33.

3. General procedure for compounds 3a-n

The reaction mixture of compounds **2a-n** (10 mmol) and 80% hydrate hydrazine (20 mmol, 1 mL) in methanol was refluxed at 85°C overnight, then cooled, evaporated in vacuo. The residue was recrystallized from absolute EtOH.

*N-(2-hydrazinyl-2-oxoethyl)-4-chlorobenzamide (**3e**)* White solid; yield: 90%; mp: 214.7-216.5°C; IR (KBr) 3,479, 3,364, 3,302, 3,190, 1,643, 1,547, 1,277, 845 cm^{-1}; ^{1}H-NMR (600 MHz, DMSO-d_6,) δ 9.140(s, 1H, NH), 8.818(t, J = 5.7 Hz, 1H, NH), 7.895(m, 2H, ArH), 7.551(d, J = 8.4 Hz, 2H, ArH), 4.217(s, 2H, CH_2), 3.825(d, J = 6 Hz, 2H, NH_2); ESI-MS: m/z $[M - H]^-$ 225.42.

4. General procedure for compounds 4a-d, 4f-j, 4l-n

The reaction mixture of compounds **3a-d**, **3f-j**, **3l-n** (0.01 mol), KOH (0.01 mol, 0.56 g), carbon disulfide (0.03 mol, 1.8 mL), and ethanol (80 mL) was heated under reflux until the evolution of H_2S ceased (~12 h). Ethanol was distilled off under reduced pressure and the residue was dissolved in water and then acidified with dilute hydrochloric acid (10%). The precipitate was filtered, washed with water, dried, and recrystallized from ethanol.

*(S)-N-[1-(5-thioxo-4,5-dihydro-1,3,4-oxadiazol-2-yl)ethyl]-3-chlorobenzamide (**4a**)* White solid; yield: 85%; mp: 163.1-163.3°C; IR (KBr) 3,244, 3,059, 2,943, 2,770, 2,723, 2,554, 1,643, 1,508, 1,061, 806, 725, 683, 656 cm^{-1}; ^{1}H-NMR (600 MHz, DMSO-d_6) δ 14.539(br, 1H, oxadiazole NH), 9.206(d, J = 7.8 Hz, 1H, NH), 7.94(d, J = 1.8Hz, 1H, ArH), 7.856(d, J = 1.8 Hz, 1H, ArH), 7.661(m, 1H, ArH), 7.550(t, J = 7.8 Hz, 1H, ArH), 5.252(m, 1H, CH), 1.546(d, J = 7.2 Hz, 3H, CH_3); ESI-MS: m/z $[M - H]^-$ 281.32.

*(S)-N-[2-methyl-1-(5-thioxo-4,5-dihydro-1,3,4-oxadiazol-2-yl)propyl]nicotinamide (**4b**)* White solid; yield: 83%; mp: 163.1-163.3°C; IR (KBr) 3,298, 3,271, 2,966, 1,643, 1,539, 1,342, 710 cm^{-1}; ^{1}H-NMR (600 MHz, DMSO-d_6) δ 14.582(br, 1H, oxadiazole NH), 9.227(d, J = 7.8 Hz, 1H, NH), 9.03(d, J = 2.4 Hz, 1H, ArH), 8.747(dd, J_1 = 1.2Hz, J_2 = 3.6Hz, 1H, ArH), 8.229(m, 1H, ArH), 7.544(dd, J_1 = 4.8 Hz, J_2 = 3.0 Hz, 1H, ArH), 4.953(t, J = 8.4 Hz, 1H, CH), 2.308(m, 1H, CH), 1.033(d, J = 6.6 Hz, 3H, CH_3), 0.949(d, J = 6.6 Hz, 3H, CH_3); ESI-MS: m/z $[M - H]^-$ 276.46.

*(S)-N-[1-(5-thioxo-4,5-dihydro-1,3,4-oxadiazol-2-yl) ethyl]-4-methoxylcinnamamide (**4c**)* White solid; yield: 75%, mp: 173.7-175.8°C; IR(KBr) 3,263, 3,071, 2,932, 2,766, 2,341, 1,651, 1,504, 1,173, 826 cm^{-1}; ^{1}H-NMR (600 MHz, DMSO-d_6) δ 14.506(br, 1H, oxadiazole NH), 8.722(d, J = 7.8Hz, 1H, NH), 7.538(d, J = 9.0 Hz, 2H, ArH), 7.44(d, J = 15.6 Hz, 1H, CH), 6.989(d, J = 9.0 Hz, 2H, ArH), 6.48(d, J = 16.2 Hz, 1H, CH), 5.114(t, J = 7.5 Hz, 1H, CH), 3.792(s, 3H, CH_3), 1.466(d, J = 7.2 Hz, 3H, CH_3); ESI-MS: m/z $[M - H]^-$ 303.51.

*(S)-N-[1-(5-thioxo-4,5-dihydro-1,3,4-oxadiazol-2-yl) ethyl]-4-fluorobenzamide (**4d**)* White solid; yield: 80%; mp 172.4-172.6°C; IR (KBr) 3,256, 3,028, 2,978, 2,874, 2,704, 2,611, 1,604, 1,504, 1,150, 814 cm^{-1}; ^{1}H-NMR (600 MHz, DMSO-d_6) δ 14.517(br, 1H, oxadiazole NH), 9.1(d, J = 7.8 Hz, 1H, NH), 7.959(m, 2H, ArH),7.312(m, 2H, ArH), 5.232(t, J = 7.2 Hz, 1H, CH), 1.533(d, J = 7.2 Hz, 3H, CH_3); ESI-MS: m/z $[M - H]^-$ 265.29.

*(S)-N-[3-methyl-1-(5-thioxo-4,5-dihydro-1,3,4-oxadiazol-2-yl)butyl]-4-chlorobenzamide (**4f**)* White solid; yield: 85%; mp: 155.8-156.3°C; IR (KBr) 3,251, 3,058, 2,960, 2,930, 2,870, 2,766, 2,615, 1,628, 1,485, 1,169, 1,150, 841 cm^{-1}; ^{1}H-NMR (600 MHz, DMSO-d_6) δ 14.536(br, 1H, oxadiazole NH), 9.111(d, J = 7.8 Hz, 1H, NH), 7.916(d, J = 8.4 Hz, 2H, ArH), 7.589(d, J = 8.4 Hz, 2H, ArH), 5.214(m, 1H, CH), 2.514(t, J = 1.8 Hz, 1H, CH), 1.916(m, 1H, CH_2), 1.763(m, 1H, CH_2), 0.929(dd, J_1 = 6.6Hz, J_2 = 10.2 Hz, 6H, 2 × CH_3); ESI-MS: m/z $[M - H]^-$ 323.54.

*(S)-N-[2-phenyl-1-(5-thioxo-4,5-dihydro-1,3,4-oxadiazol-2-yl)ethyl]-4-chlorobenzamide (**4g**)* White solid; yield: 82%; mp: 224.2-224.3°C; IR (KBr) 3,244, 3,028, 2,920, 2,766, 2,596, 1551, 1,161, 841 cm^{-1}; ^{1}H-NMR(600 MHz, DMSO-d_6) δ 14.569(br, 1H, oxadiazole NH), 9.226(d, J = 7.8 Hz, 1H, NH), 7.822(dd, J_1 = 1.2 Hz, J_2 = 4.8 Hz, 2H, ArH), 7.562(dd, J_1 = 1.2Hz, J_2 = 4.8 Hz, 2H, ArH), 7.295(m, 4H, ArH), 7.211(d, J = 7.8 Hz, 1H, ArH), 5.383(m, 1H, CH), 3.262(m, 2H, CH_2); ESI-MS: m/z $[M - H]^-$ 357.52.

*(S)-N-[2-hydroxy-1-(5-thioxo-4,5-dihydro-1,3,4-oxadiazol-2-yl)ethyl]–4-chlorobenzamide (**4h**)* White solid; yield: 80%; mp: 104.8-105.9°C; IR (KBr) 3,564, 3,422, 3,290, 3,055, 2,874, 2,762, 1,656, 1,481, 1,169, 822 cm^{-1}; ^{1}H-NMR (600 MHz, DMSO-d_6) δ 14.558(br, 1H, oxadiazole NH), 9.08(d, J = 7.8 Hz, 1H, NH), 7.925(m, 2H, ArH), 7.586(m, 2H, ArH), 5.326(br, 1H, OH), 5.15(dd, J_1 = 6.6 Hz, J_2 = 7.2 Hz, 1H, CH), 3.859(ddd, J_1 = 6.6 Hz, J_2 = 4.2 Hz, J_3 = 15 Hz, 2H, CH_2); ESI-MS: m/z $[M - H]^-$ 297.47.

*(S)-N-[2-methyl-1-(5-thioxo-4,5-dihydro-1,3,4-oxadiazol-2-yl)propyl]-4-bromobenzamide (**4i**)* White solid; yield: 87%; mp: 214.5-214.6°C; IR (KBr) 3,263, 3,036, 2,951, 2,909, 2,870, 2,731, 1,651, 1,481, 1,142, 845 cm^{-1}; ^{1}H-NMR (600 MHz, DMSO-d_6) δ 14.573(br, 1H, oxadiazole NH), 9.106(d, J = 8.4 Hz, 1H, NH), 7.842(m, 2H, ArH), 7.723(m, 2H, ArH), 4.923(t, J = 8.1Hz, 1H, CH), 2.312(m, 1H, CH), 1.017(d, J = 6.6 Hz, 3H, CH_3), 0.939(d, J = 6.6 Hz, 3H, CH_3); ESI-MS: m/z $[M - H]^-$ 355.39.

*(S)-N-[3-methyl-1-(5-thioxo-4,5-dihydro-1,3,4-oxadiazol-2-yl)butyl]-4-bromobenzamide (**4j**)* White solid; yield: 88%; mp: 160.8-161.0°C; IR (KBr) 3,260, 3,058, 2,990, 2,870, 2,743, 2,619, 1,628, 1,497, 1,169, 837 cm^{-1}; ^{1}H-NMR (600 MHz, DMSO-d_6) δ 14.537(br, 1H, oxadiazole NH), 9.115(d, J = 8.4 Hz, 1H, NH), 7.844(d, J = 8.4 Hz, 2H, ArH), 7.73(d, J = 9.0 Hz, 2H, ArH), 5.215(m, 1H, CH), 1.905(m, 1H, CH), 1.759(m, 1H, CH_2), 1.684(d, J = 6.0 Hz, 1H, CH_2), 0.929(dd, J_1 =

6.6 Hz, J_2 = 10.2 Hz, 6H, 2 × CH_3); ESI-MS: m/z [M − H]$^-$ 369.42.

*(S)-N-[1-(5-thioxo-4,5-dihydro-1,3,4-oxadiazol-2-yl)ethyl]-4-nitrobenzamide (**4n**)* Yellow solid; yield: 81%; mp: 189.6-190.0°C; IR (KBr) 3,344, 3,098, 2,951, 2,746, 1,647, 1,489, 1,150, 845 cm^{-1}; ^{1}H-NMR(600 MHz, DMSO-d_6) δ 14.557(br, 1H, oxadiazole NH), 9.429(d, *J* = 7.8 Hz, 1H, NH), 8.378(m, 2H, ArH), 8.126(m, 2H, ArH), 5.278(t, *J* = 7.2 Hz, 1H, CH), 1.559(d, *J* = 7.2 Hz, 3H, CH_3); ESI-MS: m/z [M − H]$^-$ 292.41.

5. General procedure for compounds 4e, 4k

To an ice-bath solution of compounds **3e**, **3k** (5 mmol) in 20 mL of water, a solution of triphosgene (5.5 mmol, 1.63 g) in 5 mL of toluene was added dropwise. The mixture was reacted for 12 h at 25°C. White solid was formed which was dissolved by adding dropwise of NaOH (10%). The organic phase was eliminated and the water phase was acidified by dilute hydrochloric acid. The white solid formed was filtered, washed with water, dried and recrystallied from EtOH.

*N-[(5-oxo-4,5-dihydro-1,3,4-oxadiazol-2-yl)methyl]-4-chlorobenzamide (**4e**)* White solid; yield: 90%; mp: 208.6-208.8°C; IR (KBr) 3,283, 3,047, 2,990, 1,747, 1,643, 1,535, 1,092, 845, 760 cm^{-1}; ^{1}H-NMR(600 MHz, DMSO-d_6) δ 12.272(br, 1H, oxadiazole NH), 9.188(t, *J* = 5.4 Hz, 1H, NH), 7.903(d, *J* = 9.0 Hz, 2H, ArH), 7.582(d, *J* = 9.0 Hz, 2H, ArH), 4.394(d, *J* = 5.4 Hz, 2H, CH_2); ESI-MS: m/z [M − H]$^-$ 251.43.

*N-[(5-oxo-4,5-dihydro-1,3,4-oxadiazol-2-yl)methyl]-4-methylbenzamide (**4k**)* White solid; yield: 90%; mp: 180.0-180.1°C; IR (KBr) 3,325, 3,109, 3,036, 2,970, 2,924, 2,789, 1,778, 1,643, 1,535, 1,215, 837 cm^{-1}; ^{1}H-NMR(600 MHz, DMSO-d_6) δ 12.254(br, 1H, oxadiazole NH), 9.011(s, 1H, NH), 7.786(d, *J* = 8.4 Hz, 2H, ArH), 7.3(d, *J* = 7.8 Hz, 2H, ArH), 4373(d, *J* = 5.4 Hz, 2H, CH_2), 2.359(s, 3H, CH_3); ESI-MS: m/z [M − H]$^-$ 231.40.

6. General procedure for compounds 5a-1,5m

A mixture of compounds **4a**, **4m** (5 mmol) in absolute ethanol (20 mL) and 80% hydrate hydrazine (10 mmol, 0.5 mL) was refluxed at 85°C for 8 h. The mixture was cooled and evaporated in vacuum. The residue was recrystallized from absolute EtOH.

*(S)-N-[1-(4-amino-5-thioxo-4,5-dihydro-1H-1,2,4-triazol-3-yl)ethyl]-3-chlorobenzamide (**5a-1**)* White solid; yield: 85%; mp: 218.2-218.7°C; IR (KBr) 3,275, 1,639, 1,535, 1,123, 895, 779, 756 cm^{-1}; ^{1}H-NMR (600 MHz, DMSO-d_6) δ 13.610(s, 1H, triazole NH), 9.008(d, *J* = 7.2 Hz, 1H, NH), 7.944(t, *J* = 1.8 Hz, 1H, ArH), 7.838(d, *J* = 2.4Hz, 1H, ArH), 7.621(m, 1H, ArH), 7.509(t, *J* = 7.8 Hz, 1H, ArH), 5.593(s, 2H, NH_2), 5.262(t, *J* = 7.2 Hz, 1H, CH), 1.517 (d, *J* = 7.2 Hz, 3H, CH_3); ESI-MS: m/z [M − H]$^-$ 295.64.

*(S)-N-[1-(4-amino-5-thioxo-4,5-dihydro-1H-1,2,4-triazol-3-yl)ethyl]-4-methoxybenzamide (**5m**)* White solid; yield: 77%; mp: 239.6-239.9°C; IR (KBr) 3,279, 3,132, 3,044, 2,955, 1,632, 1,535, 1,177, 845 cm^{-1}; ^{1}H-NMR (600 MHz, DMSO-d_6) δ 13.572(s, 1H, triazole NH), 8.699(d, *J* = 7.2 Hz, 1H, NH), 7.869(d, *J* = 8.4 Hz, 1H, NH), 6.991(d, *J* = 9.0 Hz, 2H, ArH), 5.589(s, 2H, NH_2), 5.251(m, 1H, CH), 3.808(s, 3H, CH_3), 1.506(d, *J* = 7.2 Hz, 3H, CH_3); ESI-MS: m/z [M − H]$^-$ 291.64.

7. General procedure for compounds 5a-2, 5g, 5i, 5l-1, 5l-2, 5a-3, 5f, 5j

To a stirred solution containing the compounds **4a**, **4f**, **4g**, **4i**, **4j**, **4l** (5 mmol) and KOH (5 mmol, 0.28 g) in 50% aqueous ethanol (20 mL), a solution of iodomethane (10 mmol, 0.6223 mL), iodoethane (10 mmol, 0.8 mL) or 3-chlorobenzyl chloride (10 mmol, 1.268 mL) in ethanol (5 mL) was added dropwise. After reacting at room temperature for 8 h, the solid product was collected by filtration, washed with water and recrystallized from EtOH.

*(S)-N-[1-(5-(methylthio)-1,3,4-oxadiazol-2-yl)ethyl]-3-chlorobenzamide (**5a-2**)* White solid; yield: 92%; mp: 120.0-120.2°C; IR (KBr) 3,248, 1,643, 1,539, 1,119, 775, 710, 683 cm^{-1}; ^{1}H-NMR(600 MHz, DMSO-d_6) δ 9.223(d, J=7.8Hz, 1H, NH), 7.934(t, J=2.1Hz, 1H, ArH), 7.845(d, J=7.8Hz, 1H, ArH), 7.649(m, 1H, ArH), 7.536(t, J=7.8Hz, 1H, ArH), 5.378(m, 1H, CH), 2.691(s, 3H, CH_3), 1.586(d, J =7.2Hz, 3H, CH_3); ESI-MS: m/z [M − H]$^-$ 295.36.

*(S)-N-[1-(5-(methylthio)-1,3,4-oxadiazol-2-yl)-2-phenylethyl]-4-chlorobenzamide (**5g**)* White solid; yield: 82%; mp: 146.0-146.2°C; IR (KBr) 3,437, 3,287, 1,643, 1,535, 1,481, 1,323, 1,153, 1,092, 841, 702 cm^{-1}; ^{1}H-NMR(600 MHz, DMSO-d_6) δ 9.249(d, *J* = 7.8 Hz, 1H, NH), 7.809(m, 2H, ArH), 7.55(m, 2H, ArH), 7.31(d, *J* = 7.2 Hz, 2H, ArH), 7.269(t, *J* = 7.8 Hz, 2H, ArH), 7.195(t, *J* = 7.2 Hz, 1H, ArH), 5.51(m, 1H, CH), 3.299(m, 2H, CH_2), 2.679(s, 3H, CH_3); ESI-MS: m/z [M − H]$^-$ 371.55.

*(S)-N-[2-methyl-1-(5-methylthio-1,3,4-oxadiazol-2-yl)propyl]-4-bromobenzamide (**5i**)* White solid; yield: 91%; mp: 161.0-161.7°C; IR (KBr) 3,290, 2,962, 2,874, 2,349, 1,655, 1,531, 1,477, 1,173, 845 cm^{-1}; ^{1}H-NMR (600 MHz, DMSO-d_6) δ 9.107(d, *J* = 8.4 Hz, 1H, NH), 7.83(dd, J_1 = 1.8 Hz, J_2 = 4.8 Hz, 2H, ArH), 7.709(dd, J_1 = 1.8 Hz, J_2 = 4.8 Hz, 2H, ArH), 5.069(t, *J* = 8.4 Hz, 1H, CH), 2.697(s, 3H, CH_3), 2.335(m, 1H, CH), 1.019(d, *J* = 6.6 Hz, 3H, CH_3), 0.901(d, *J* = 6.6 Hz, 3H, CH_3); ESI-MS: m/z [M − H]$^-$ 369.37.

*(S)-N-[2-methyl-1-(5-methylthio-1,3,4-oxadiazol-2-yl)propyl]-4-methylbenzamide (**5l-1**)* White solid; yield: 84%; mp: 122.8-122.8°C; IR (KBr) 3,294, 3,020, 2,955, 2,870, 1,651, 1,531, 1,477, 1,169, 841 cm^{-1}; ^{1}H-NMR (600 MHz, DMSO-d_6) δ 8.925(d, *J* = 7.8 Hz, 1H, NH), 7.798(d, *J* =

8.4 Hz, 2H, ArH), 5.072(t, *J* = 8.4 Hz, 1H, CH), 2.699(s, 3H, CH_3), 2.360(s, 4H, CH, CH_3), 1.023(d, *J* = 6.6 Hz, 3H, CH_3), 0.902(d, *J* = 7.2 Hz, 3H, CH_3); ESI-MS: m/z $[M - H]^-$ 303.48.

*(S)-N-[1-(5-ethylthio-1,3,4-oxadiazol-2-yl)-2-methylpropyl]-4-methylbenzamide (**5l-2**)* White solid; yield: 82%; mp: 111.5-111.7°C; IR (KBr) 3,279, 2,966, 2,928, 1,632, 1,543, 1,474, 1,142, 841 cm^{-1}; ^{1}H-NMR(600 MHz, DMSO-d_6) δ 8.928(d, *J* = 8.4 Hz, 1H, NH), 7.795(d, *J* = 8.4 Hz, 2H, ArH), 7.289(d, *J* = 7.8 Hz, 2H, ArH), 5.076(t, *J* = 8.4 Hz, 1H, CH), 3.233(q, *J* = 7.2 Hz, 2H, CH_2), 2.346(m, 4H, CH, CH_3), 1.365(t, *J* = 7.2 Hz, 3H, CH_3), 1.023(d, *J* = 7.2 Hz, 3H, CH_3), 0.903(d, *J* = 6.6 Hz, 3H, CH_3); ESI-MS: m/z $[M - H]^-$ 317.85.

*(S)-N-[1-(5-(3-chlorobenzylthio)-1,3,4-oxadiazol-2-yl)ethyl]-3-chlorobenzamide (**5a-3**)* White solid; yield: 90%; mp: 146.0-148.0°C; IR (KBr) 3,240, 3,051, 2,993, 2,936, 1,643, 1,543, 1,331, 1,123, 860, 799, 714, 679 cm^{-1}; ^{1}H-NMR(600 MHz, DMSO-d_6) δ 9.111(d, *J* = 7.8 Hz, 1H, NH), 7.934(t, J = 1.8 Hz, 1H, ArH), 7.846(d, *J* = 7.8 Hz, 1H, ArH), 7.65(m, 1H, ArH), 7.541(m, 2H, ArH), 7.389(m, 1H, ArH), 7.316(m, 2H, ArH), 5.384(m, 1H, CH), 4.483(s, 2H, CH_2), 1.577(d, *J* = 7.2 Hz, 3H, CH_3); ESI-MS: m/z $[M - H]^-$ 405.50.

*(S)-N-[1-(5-(3-chlorobenzylthio)-1,3,4-oxadiazol-2-yl)-3-methylbutyl]-4-chlorobenzamide (**5f**)* White solid; yield: 88%; mp: 99.0-99.1°C; IR (KBr) 3,445, 3,275, 3,067, 2,955, 2,924, 2,874, 1,643, 1,473, 1,173, 799, 729, 683 cm^{-1}; ^{1}H-NMR (600 MHz, DMSO-d_6) δ 9.1(d, *J* = 7.8 Hz, 1H, NH), 7.9(dd, J_1 = 1.8 Hz, J_2 = 4.8 Hz, 2H, ArH), 7.572(d, *J* = 7.8 Hz, 2H, ArH), 7.512(d, *J* = 1.8 Hz, 1H, ArH), 7.377(m, 1H, ArH), 7.314(m, 2H, ArH), 5.35(m, 1H, CH), 4.475(s, 2H, CH_2), 1.923(m, 1H, CH), 1.772(m, 1H, CH_2), 1.637(m, 1H, CH_2), 0.917(dd, J_1 = 6.6 Hz, J_2 = 12 Hz, 6H, 2 × CH_3); ESI-MS: m/z $[M - H]^-$ 447.61.

*(S)-N-[1-(5-(3-chlorobenzylthio)-1,3,4-oxadiazol-2-yl)-3-methylbutyl]-4-bromobenzamide (**5j**)* White solid; yield: 88%; mp: 129.4-129.6°C; IR (KBr) 3,329, 2,962, 2,874, 1,651, 1,528, 1,481, 1,142, 845 cm^{-1}; ^{1}H-NMR (600 MHz, DMSO-d_6) δ 9.118(d, *J* = 7.8 Hz, 1H, NH), 7.831(d, *J* = 8.4 Hz, 2H, ArH), 7.715(d, *J* = 8.4 Hz, 2H, ArH), 7.518(s, 1H, ArH), 7.382(d, *J* = 7.2 Hz, 1H, ArH), 7.315(m, 2ArH), 5.353(m, 1H, CH), 4.478(s, 2H, CH_2), 1.929(m, 1H, CH), 1.772(m, 1H, CH_2), 1.645(m, 1H, CH_2), 0.918(dd, J_1 = 6.0 Hz, J_2 = 12 Hz, 6H, 2 × CH_3); ESI-MS: m/z $[M - H]^-$ 493.52.

8. General procedure for compounds 5a-4, 5l-3

The compounds **4a**, **4l** (5 mmol), dissolved in NaOH solution (10%, 10 mL), was added dropwise to a solution of chloroacetic acid (10 mmol, 0.945 g), which was previously neutrallized with saturated Na_2CO_3 solution(10 mL). The mixture was stirred for 12 h at room temperature, then acidified by dilute hydrochloric acid (1 mol/L). The precipitate was filtered, washed with water, dried and recrystallized from MeOH/H_2O.

*(S)-N-[1-(5-carboxymethylthio-1,3,4-oxadiazol-2-yl)-ethyl]-3-chlorobenzamide (**5a-4**)* White solid; yield: 78%; mp: 164.5-166.6°C; IR (KBr) 3,256, 3,113, 2,997, 2,831, 1,778, 1,647, 1,539, 1,335, 706 cm^{-1}; ^{1}H-NMR(600 MHz, DMSO-d_6) δ 12.269(br, 1H, OH), 9.077(d, *J* = 7.8 Hz, 1H, NH), 7.930(t, *J* = 1.8 Hz, 1H, ArH), 7.843(d, *J* = 7.8 Hz, 1H, ArH), 7.645(m, 1H, ArH), 7.535(t, *J* = 7.8 Hz, 1H, ArH), 5.088(m, 1H, CH), 1.475(d, *J* = 7.2 Hz, 3H, CH_3); ESI-MS: m/z $[M + H]^+$ 340.75.

*(S)-N-[1-(5-carboxymethylthio-1,3,4-oxadiazol-2-yl)-2-methylpropyl]-4-methylbenzamide (**5l-3**)* White solid; yield: 76%; mp: 160.0-162.0°C; IR (KBr) 3,287, 3,117, 3,032, 2,966, 2,936, 2,827, 1,774, 1,639, 1,539, 1,381, 1,161, 930, 833cm^{-1}; ^{1}H-NMR(600 MHz, DMSO-d_6) δ 12.291(br, 1H, OH), 8.792(d, *J* = 8.4 Hz, 1H, NH), 7.8(d, *J* = 8.4 Hz, 2H, ArH), 7.289(d, *J* = 8.4 Hz, 2H, ArH), 4.731(t, *J* = 8.7 Hz, 1H, CH), 2.361(s, 3H, CH_3), 2.245(m, 1H, CH), 0.96(dd, J_1 = 6.6 Hz, J_2 = 27 Hz, 6H, 2 × CH_3); ESI-MS: m/z $[M - H]^-$ 347.56.

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Antimicrobial action mechanism of flavonoids from *Dorstenia* species

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ABSTRACT: Naturally occurring flavonoids have been reported to possess antimicrobial activity against a wide range of pathogens. However, the antimicrobial action mechanism of these compounds has not yet been elucidated. This study investigated the mechanism underlying the antibacterial activity of four flavonoids: 6,8-diprenyleriodictyol (1), isobavachalcone (2), 6-prenylapigenin (3) and 4-hydroxylonchocarpin (4). In addition, the toxicity of these compounds was evaluated. Determination of the minimum inhibitory concentrations (MICs) was performed by microbroth dilution method. Radiolabeled thymidine, uridine, and methionine were used to evaluate the effect of the compounds on the biosynthesis of DNA, RNA, and proteins while the sensitive cyanine dye DiS-C3-(5) (3,3'-dipropylthiadicarbocyanine iodide) was used for the effect on membrane potential. Bactericidal/bacteriolysis activities were performed by time-kill kinetic method. In the toxicity study, the numbers of survivors was recorded after injection of compounds into the hemolymph of silkworm larvae. Compounds showed significant antibacterial activity against *Staphylococcus aureus* including methicillin-resistant *S. aureus* (MRSA) strains with MICs values ranged between 0.5-128 µg/mL. Depolarization of membrane and inhibition of DNA, RNA, and proteins synthesis were observed in *S. aureus* when treated with those flavonoids. At 5-fold minimum inhibitory concentration, compounds reduced rapidly the bacterial cell density and caused lysis of *S. aureus*. Compounds 1, 2, and 4 did not show obvious toxic effects in silkworm larvae up to 625 µg/g of body weight. Flavonoids from *Dorstenia* species, 6,8-diprenyleriodictyol, isobavachalcone, and 4-hydroxylonchocarpin are bactericidal compounds. They cause damage of cell membrane, leading to the inhibition of macromolecular synthesis. Taking into account the *in vivo* safety and their significant antimicrobial potency, these flavonoids are promising leads for further drug development.

Keywords: Antimicrobial, flavonoids, membrane potential, macromolecules synthesis, bactericidal/bacteriolysis

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1. Introduction

Staphylococcus aureus is still an important pathogen both in community acquired and hospital associated infections (*1*). Clinical isolates of methicillin-resistant *S. aureus* (MRSA) have become the most common cause of infections among the global pathogenic bacteria and many life-threatening diseases (*2*). This situation has created new challenges in the area of drug discovery and much effort has been undertaken in the area of medicinal plants since plant sources is highly relevant for the identification of lead compounds which can result in the development of novel and safe therapeutic agents. The potential use of higher plants as a source of new drugs is still poorly explored and scientists need to continuously improve the quality and quantity of compounds that enter the drug development phase to keep pace with other drug discovery efforts (*3*). Natural products provide majority of new drug leads for a variety of human diseases, most of the leads from natural products that are currently in development have come from either plant or microbial sources. Earlier publications have pointed out that relatively little of the world's plant biodiversity has been extensively screened for bioactivity and that more extensive collections of plants could provide many novel chemicals for use in drug discovery assays (*4*). Many plants of the genus *Dorstenia* are used in African and South American folk medicine in the treatment of illnesses such as, infectious diseases, snakebite, and rheumaticarthritis (*5*). Extensive phytochemical studies

have been carried out on a large number of *Dorstenia* species, and a variety of compounds of which flavonoids have been found widespread and some were found to exhibit interesting pharmacological activity (*6-9*). It is well known that many plant secondary metabolites such as phenolic acids, flavonoids, quinones, and alkaloids possess remarkable biological activities. Flavonoids are found as ubiquitous secondary metabolites in the plant kingdom. They have been reported to possess many useful properties, including anti-inflammatory activity, oestrogenic activity, enzyme inhibition, antiallergic activity, antioxidant activity, vascular activity and cytotoxic antitumour activity, antifungal and antibacterial activities (*10,11*). The occurrence of chalcones and prenylated flavonoids in *Dorstenia* species and their antimicrobial activity is well documented (*12-14*). Despite the great number of papers devoted to the microbial growth inhibiting effect effects of flavonoids, only a very limited number of studies are pertaining to the understanding of how these compounds exerts their antimicrobial activities. In this study, the activity of four flavonoids, 6,8-diprenyleriodictyol (**1**), isobavachalcone (**2**), 6-prenylapigenin (**3**), and 4-hydroxylonchocarpin (**4**) from *Dorsenia* species were evaluated against clinical isolates of *S. aureus*. The most active compounds (**1**, **2**, and **4**) were further investigated to determine their effect on the membrane potential dissipation and on the biosynthesis of macromolecules (DNA, RNA, and proteins) in *S. aureus*. In addition, the *in vivo* experiments were performed to evaluate the possible toxic effect of these compounds using silkworm, *Bombyx mori*.

2. Materials and Methods

2.1. *Plants and natural compounds*

The natural compounds used in this study were obtained from the chemical bank of the Laboratory of Organic Chemistry, University of Yaoundé I, Cameroon. They were isolated from plants of the genus *Dorstenia*. 6,8-diprenyleriodictyol (**1**) was isolated from the aerial parts of *D. mannii*, isobavachalcone (**2**) and 4-hydroxylonchocarpin (**4**) were isolated from the twigs of *D. barteri* while 6-prenylapigenin (**3**) was isolated from the twigs of *D. dinklagei*. The isolation procedure and the structure elucidation of compounds were performed as previously described (*6-9,13,15,16*). Chemical structures of compounds are shown in Figure 1.

2.2. *Chemicals and antibiotics*

Radiolabelled [methyl-^{3}H]thymidine and [^{3}H]uridine were purchased from Moravek Biochemical (Brea, CA, USA), and [^{35}S]methionine was purchased from the Institute of Isotopes (Budapest, Hungary). DiS-C_3-(5) (3,3'-dipro pylthiadicarbocyanine iodide) dye was purchased from AnaSpec, Inc., Otoole Avenue, USA. Amphotericin

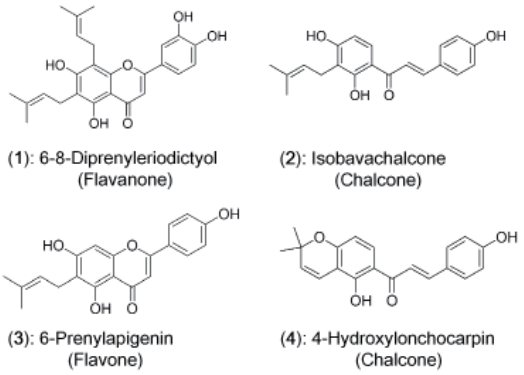

Figure 1. Chemical structure of compounds.

B, gentamicin, vancomycin, and ampicilllin (Wako Pure Chemical Industries, Osaka, Japan); rifampicin and chloramphenicol (Nacalai Tesque, Kyoto, Japan), norfloxacin and nisin (Sigma Aldrich Chemie Gmbh Steinheim, Germany) were used as reference antibiotics.

2.3. *Antimicrobial assays*

2.3.1. *Microorganisms and culture conditions*

Clinical isolates of methicillin sensitive and methicillin-resistant *S. aureus* strains (MSSA1, MRSA3, MRSA4, MRSA6, MRSA8, MRSA9, MRSA11, and MRSA12) and fungi (*Candida albicans* ATCC10231, *Candida tropicalis* pK233, *Cryptococcus neoformans* H99, and *Cryptococcus neoformans* KN99a) were used. They were obtained from the culture collection of the Laboratory of Microbiology, Graduate School of Pharmaceutical Sciences, The University of Tokyo, Japan. Luria Bertani 10 (LB10) agar medium or Muller-Hinton agar (MHA) and Sabouraud dextrose agar (SDA) were used for the activation of bacteria and fungi strains respectively. They were subcultured in appropriated agar plates 24 h prior to any antimicrobial test. The Mueller Hinton broth, Cation-Adjusted with calcium and magnesium ions (CAMHB) and RPMI-1640 were used for the minimum inhibitory concentration (MIC) determination.

2.3.2. *MIC determination*

MIC was determined by broth microdilution method following the guidelines of Clinical and Laboratory Standards Institute (*17,18*).

2.4. *Antimicrobial action mechanisms study with S. aureus*

2.4.1. *Membrane permeabilization assay*

The effect of the compounds on the membrane potential was tested using membrane potential-sensitive dye diS-C3-(5) (*19*). Briefly, *S. aureus* MSSA1 was allowed to grow in LB10 at 37°C to an OD_{600} of 0.5-0.6. The

cell suspension was centrifuged at 10,000 rpm for 10 min and at 4°C, then washed twice in buffer (5 mM HEPES, pH 7.2; 5 mM glucose) and the OD_{600} adjusted to 0.05 with the same buffer. A 2 mL of this suspension was placed in a 1-cm cuvette; stock solution of diS-C3-(5) and compounds were added to give a final concentration of 0.4 µM and 5-fold MIC respectively. Changes in fluorescence were monitored with FP-6200 spectrofluorometer at an excitation wavelength of 622 nm and an emission wavelength of 670 nm.

2.4.2. *Effect on DNA, RNA, and proteins synthesis in S. aureus*

Metabolic incorporation of (methyl-^{3}H)thymidine, (^{3}H) uridine, and (^{35}S)methionine into cellular DNA, RNA, and protein respectively, was used to evaluated the effect of compounds on macromolecules synthesis as previously described by Ferrari and Widholm (1973) (*20*) and modified by Paudel *et al*. (2012) (*21*).

2.4.3. *Bactericidal and bacteriolysis activities*

Bactericidal and bacteriolysis activities were determined by time-kill kinetic method as described by Ooi *et al*. (2009) (*22*), with slight modifications. For bacteriolysis experiment, full growth of *S. aureus* MSSA1 in MHB were diluted 100 times and incubated at 37°C to produce an OD_{600} of 0.8 as starting inoculum. Compounds were added to give a final concentration of 5 × MIC and incubated at 37°C with shaking, then 100 µL were removed from each tube at 0, 15, 30, 60, and 120 min and the optical density measured at 600 nm. For bactericidal activity, the above mentioned bacteria suspension was diluted 100 times and incubated at 37°C to produce an OD_{600} of 0.3, then at different time intervals, 100 µL were removed from each tube; serially tenfold diluted and plated onto MHA plates, to determine the viable number of colony-forming units (cfu) per mL. Samples of 10^2 cfu/mL were below the limit of detection. Vancomycin, gentamicin and nisin were used as positive controls and no-drug tubes were also included.

2.5. *Toxicity study in silkworms*

The toxicity of compounds against silkworms was evaluated as described by Hamamoto *et al*. (2009) (*23*). Briefly, fifth instar silkworm larvae (*Bombyx mori*) were fed on the 1st day and reared overnight at 27°C. The test compounds were dissolved in saline containing 20% DMSO, then 50 µL of various concentrations were injected into the hemolymph (n = 10 per group), affording doses of 125, 250, 375, and 625 µg/g of larvae. After 24 h, the survival rates of silkworms were recorded.

3. Results

3.1. *Antimicrobial activity of flavonoids derived from Dorsenia plants*

Results of the antimicrobial assay are depicted in Table 1. All the compounds showed prominent antimicrobial activities against the tested fungal and bacterial pathogens. Compounds **1**, **2**, and **4** showed significant antibacterial activity against *S. aureus* including MRSA strains, with MICs values ranged between 0.5-16 µg/mL. *S. aureus* MSSA1 exhibited comparable sensitivity towards compound **1** and two reference antibiotics gentamicin and norfloxacin (MIC of 0.5 µg/mL). Remarkable potent antifungal activity was observed against *C. neoformans* H99 strains; MIC of compound **4** against this strain was similar to that of amphotericin B (MIC of 0.5 µg/mL). Taking into account the medical importance of the tested microorganisms, this result can be considered as promising in the perspective of new antibiotic drugs development. For natural compounds that significantly inhibit the growth of microbial pathogens to be an attractive alternative for conventional antimicrobials for

Table 1. Minimal inhibitory concentration (MIC) of compound 1, 2, 3, 4, and reference antibiotics

Microorganisms	MIC (µg/mL)											
	(**1**)	(**2**)	(**3**)	(**4**)	Van	Gen	Nis	Rif	Amp	Chl	Nor	AmB
S. aureus MSSA1	0.5	2	16	4	0.25	0.5	128	0.015	4	8	0.5	nd
S. aureus MSSA3	4	16	32	1	2	2	–	0.06	32	16	1	nd
S. aureus MRSA4	1	4	32	8	2	64	–	0.03	64	64	0.5	nd
S. aureus MRSA6	4	8	64	4	2	–	–	0.12	32	4	2	nd
S. aureus MRSA8	1	4	32	4	2	–	128	0.06	–	16	1	nd
S. aureus MRSA9	4	4	32	4	2	–	128	0.06	64	8	4	nd
S. aureus MRSA11	4	4	32	4	2	1	–	0.12	64	16	2	nd
S. aureus MRSA12	4	4	16	4	1	–	128	0.06	128	8	1	nd
C. albicans ATCC10231	128	–	64	–	nd	nd	nd	nd	nd	nd	nd	0.25
C. tropicalis pK233	–	–	–	–	nd	nd	nd	nd	nd	nd	nd	0.5
C. neoformans H99	1	2	–	0.5	nd	nd	nd	nd	nd	nd	nd	0.5
C. neoformans KN99a	–	–	–	–	nd	nd	nd	nd	nd	nd	nd	0.5

Chl, chloramphenicol; Amp, ampicillin; Van, vancomycin; Nor, norfloxacin; Gen, gentamicin; Nis, nisin; Rif, rifampicin; AmB, amphotericin B; nd, not determined; –, MIC > 128 µg/mL.

their application in therapy, it is important to establish the mechanism of their antimicrobial activity.

3.2. *Membrane permeabilization assay*

Membrane depolarization was determined using potential-sensitive dye diS-C3-(5). As shown in Figure 2, the addition of compounds **1**, **2**, and **4** induced an increase of fluorescence. Cell hyperpolarization results in uptake of the diS-C3-(5) dye molecules by the cells, thus decrease the fluorescence, while depolarization results in the release of dye thus increase the fluorescence. Membrane depolarization occurred was lower compared to nisin used as reference antibiotics.

3.3. *Effect on DNA, RNA, and proteins synthesis in S. aureus*

The metabolic incorporation of isotope-labeled uridine, thymidine, and methionine into the corresponding macromolecules in *S. aureus*, were examined to measure the biosynthesis of nucleic acids and protein. As shown in Figure 3-5, compounds **1**, **2**, and **4** induced substantial inhibiting effect on macromolecules synthesis. A slight amount of protein synthesis was observed in the presence of compound **4**. Compounds inhibited DNA and RNA synthesis to an extent similar to that of the reference antibiotics norfloxacin and rifampicin, respectively. Thus, we speculated that, the membrane damaging induced by compounds lead to the bacterial death.

3.4. *Bactericidal and bacteriolysis activities*

The result of time-kill assay is presented in Figure 6. A perusal of this figure shows a rapid decrease of survival number of *S. aureus* MSSA1 strain in the presence of compounds **1**, **2**, and **4**. Survived number cfu/mL of bacterial cells dropped below the detection limit (100 cfu/mL) after 15 min treatment for compounds **1** and **2**. Compared to initial inoculum, compound **4** showed 2-log reduction in cfu counts after 15 min. In contrast, killing activity by vancomycin and gentamicin was lower, giving less than 1-log reduction from the control

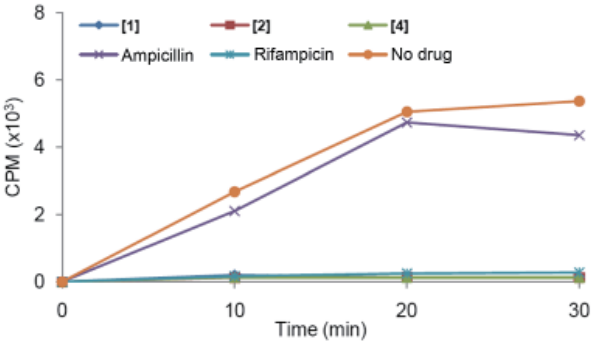

Figure 4. Effect of compounds 1, 2, and 4 on RNA synthesis.

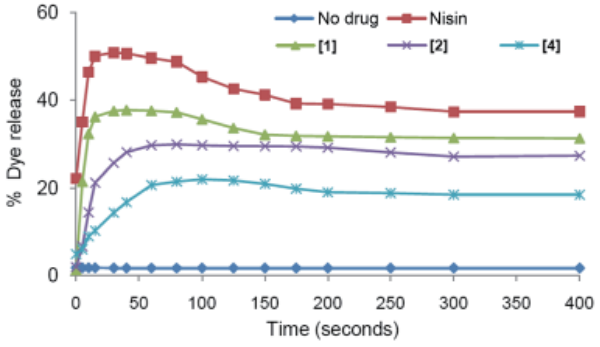

Figure 2. Effect of compounds 1, 2, and 4 on membrane permeabilization.

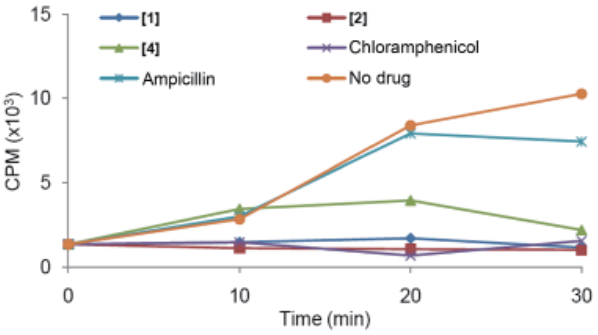

Figure 5. Effect of compounds 1, 2, and 4 on proteins synthesis.

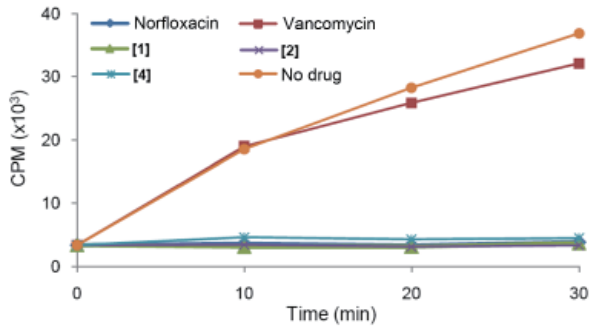

Figure 3. Effect of compounds 1, 2, and 4 on DNA synthesis.

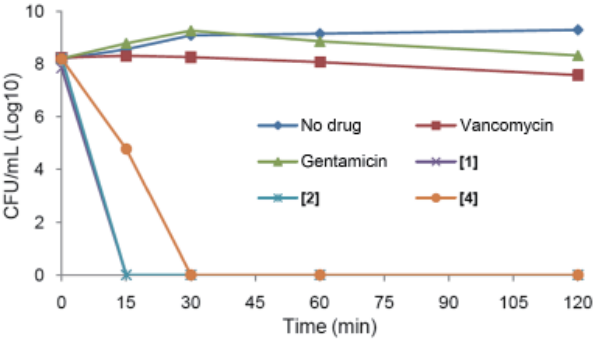

Figure 6. Time-kill curve of compounds 1, 2, and 4 against *S. aureus* MSSA1.

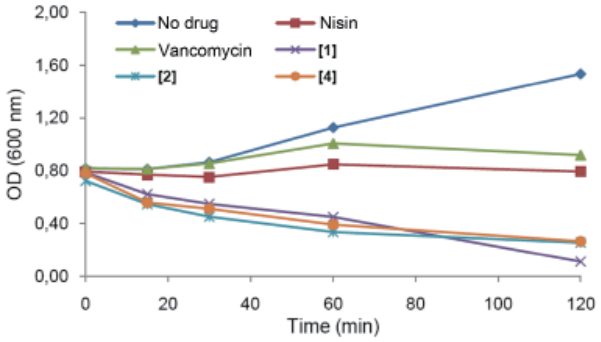

Figure 7. Bacteriolysis activity of compounds 1, 2, and 4 against *S. aureus*.

even at 120 min. The result of bactericidal activity was consistent with that of bacteriolysis activity (Figure 7) which showed a decrease in the optical density of *S. aureus* suspension treated with compounds **1**, **2**, and **4**. After 120 min, compounds **1**, **2**, and **4** induced a decline in cell turbidity of 86%, 64%, and 63%, respectively in bacteria suspension compared to the 0 time value, indicating the lysis of bacteria cells.

3.5. *Toxicity study of compounds in silkworm larvae*

The use of silkworms (*Bombyx mori*) as animal model to evaluate the toxicity of drug candidates has been proposed. Evidence is now accumulating that metabolism pathway in this invertebrate is common to that of mammals (*23*). We evaluate toxicity of compounds **1**, **2**, and **4** by using this model, no death or any obvious toxic effect was observed 24 h after injection of up to 625 µg/g of larvae, of compounds into silkworm. Based on this result, we concluded for LD_{50} values of these compounds were larger than 625 µg/g of larvae.

4. Discussion

The present study showed significant antibacterial activity against *S. aureus* including MRSA strains, this result confirm the antimicrobial potency of four flavonoids from *Dorstenia* species. Although flavonoids have been reported to possess interesting activity against a wide range of microorganisms, no study has been reported on the activity of 6,8-diprenyleriodictyol, isobavachalcone, 6-prenylapigenin, and 4-hydroxyl-onchocarpin against MRSA strains. Although no definite structure-activity relationship could be determined, some structural features that might have influenced the antimicrobial activity can be drawn from the comparison of the chemical structures of compounds with different activities. 6-8-Diprenyleriodictyol (**1**) was the most active, followed by isobavachalcone (**2**), and 4-hydroxylonchocarpin (**4**). 6-Prenylapigenin showed the lowest activity among these four flavonoids. It appears that, in general flavones (compounds **1** and **3**), 2'-hydroxyl group and the isoprenoid moiety play a greater role in increasing the antibacterial activity. 6-8-diprenyleriodictyol was 16× and 32× more active than 6-prenylapigenin against *S. aureus* MSSA1, MRSA4, and MRSA8, respectively. Published literature indicates that, the addition of an isoprenoid moiety renders higher activities in the flavonoid molecule than in the parent compounds from the pharmacological point of view (*24*). One of the proposed reasons for the enhanced biological activities of prenylated flavonoids is that the prenylation of the flavonoid core increases the lipophilicity and the membrane permeability of the compound (*25*). But this assumption was not applicable to chalcones. Athough isobavachalcone carries an isoprenoid moiety, its activity as well as that of 4-hydroxylonchocarpin were highly selective from one microbial strain to another. Nonetheless, previous studies reported that, the antimicrobial inhibitory effect of chalcones was correlated to the substitution patterns of the aromatics rings (*26*).

In membrane depolarization experiment, the membrane potential-sensitive fluorescent probe diS-C3-(5), distributes between the cells and the medium depending on the cytoplasmic membrane potential; hyperpolarization of the cell is accompanied by a decrease in fluorescence, and depolarization produced an increase in fluorescence (*27*). Our work reported here confirms the observations reported by others on the membrane damage effect of flavonoids compounds (*28,29*). When considering the overall results of bacteria viability, lysis, and membrane depolarisation, it seems that, the killing cell process induced by our compounds may be firstly due to the membrane damage, leading to the death of cell. This sequence of events could be confirmed by the loss of biosynthetic activity. DNA, RNA, and proteins synthesis were inhibited in *S. aureus* when treated with the flavonoids **1**, **2**, and **4**. A plausible explanation of this result is that, by damaging cytoplasm membrane, the compounds might interfere with the energetic metabolism depending on respiratory chain (*30*), since membrane potential is required for the active uptake of various metabolites and for the biosynthesis of macromolecules. The membrane potential is fundamental to the survival and growth of bacterial cells, it is essential for bacteria to maintain their capacity for ATP synthesis which is the main energy source for almost all chemical processes in living systems. Therefore, our data represents further evidence that, the loss of membrane potential might affect the overall bacterial metabolic activity, resulting in some biosynthetic pathway inhibition, as demonstrated by the strong inhibition of DNA, RNA, and protein synthesis. Previously, other flavonoids compounds (licochalcone A and a flavanone lonchocarpol A) were reported to inhibit the incorporation of radioactive precursors into macromolecules (*31*). Furthermore, the

licochalcones A and C were found to inhibit NADH-cytochrome c reductase in bacteria (*32*). As stated by O'Grady (1971) (*33*), the main problem in any investigation attempting to elucidate the mechanism of action of an antibacterial agent is that it is difficult to distinguish with certainty the primary event from those that follow. As for most of bactericidal antibiotic agents, a non specific mechanism could be postulated. Therefore we undertook to investigate if the action mode of these flavonoids compounds is bactericidal or bacteriostatic and to assess in lysis could be involved in the death of cells. As shown in Figures 6 and 7 compounds reduced rapidly the bacterial cell density and caused lysis of *S. aureus*. This observation suggested that the action of compounds **1**, **2**, and **4** in *S. aureus* could be bactericidal. Our result is supported by the observation that other flavonoids compounds such as epigallocatechin gallate and galangin induced 3-log reduction or more in viable counts of *S. aureus* (*11,34*). The rapid bactericidal activity of compounds **1**, **2**, and **4** suggested a non-specific action mechanism and that, they might damage bacterial membrane as demonstrated with other reference antibiotics such as daptomycin (*22*).

The toxicity information obtained from this study is useful in choosing doses for repeat-dose study. It has been reported that, flavonoids have low toxicity because they are widely distributed in edible plants and beverages, and have been used in medicine. When used as dietary supplements or as pure compounds in pharmacological doses, flavonoids do not appear to cause unwanted side effect. Even when raised to the level of 10% of total caloric intake, flavonoids supplementation has been shown non-toxic (*35,36*). Since the LD_{50} values are larger than 625 µg/g of larvae, we concluded that flavonoids compounds herein studied is likely to be nontoxic. Interestingly, previous study reported that compounds **1** and **3** were not toxic to normal human cell AML12 hepatocytes (*37*). In view of these observations, our compounds may be considered for preclinical studies for their use as antibacterial drug candidates.

Finally, this study has demonstrated that, flavonoids compounds studied have bactericidal/ bacteriolytic effects on *S. aureus*; DNA, RNA, and proteins synthesis are inhibited in the killing process and membrane permeabilization occurred. On the basis of these results we can suppose that the mechanism of action of these compounds is non-specific. Such information may assist in their optimisation as lead compound, considering their *in vivo* low toxicity evidence here reported. Taking into account the medical importance of MRSA strains, compounds **1**, **2**, and **4** can be considered as promising in the perspective of new antibiotic drugs discovery, with the possibility of making analogues with improved pharmacological or pharmaceutical properties.

Acknowledgements

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References

1. Chung PY, Navaratnam P, Chung LY. Synergistic antimicrobial activity between pentacyclic triterpenoids and antibiotics against *Staphylococcus aureus* strains. Ann Clin Microbiol Antimicrob. 2011; 10:25.
2. Zuo GY, Li Y, Wang T, Han J, Wang GC, Zhang YL, Pan WD. Synergistic antibacterial and antibiotic effects of bisbenzylisoquinoline alkaloids on clinical isolates of methicillin-resistant staphylococcus aureus (MRSA). Molecules. 2011; 16:9819-9826.
3. Rates SNK. Plants as source of drugs. Toxicon. 2001; 39:603-613.
4. Newman DJ, Cragg GM. Natural products as sources of new drugs over the 30 years from 1981 to 2010. J Nat Prod. 2012; 75:311-335.
5. Adjanohoun JE, Aboubakar N, Dramane K, *et al*. Traditional medicine and pharmacopoeia: Contribution to ethnopharmacological and floristic studies in Cameroon. OAU/STRC, Lagos, Nigeria, 1996; p. 299.
6. Ngadjui BT, Abegaz BM, Dongo E, Tamboue H, Fogue K. Geranylated and prenylated flavonoids from the twigs of *Dorstenia mannii*. Phytochemistry. 1998; 48:349-354.
7. Ngadjui BT, Dongo E, Tamboue H, Fogue K, Abegaz BM. Prenylated flavanones from the twigs of *Dorstenia mannii*. Phytochemistry. 1999; 50:1401-1406.
8. Ngadjui BT, Kouam SF, Dongo E, Kapche GW, Abegaz BM. Prenylated flavonoids from the aerial parts of *Dorstenia mannii*. Phytochemistry. 2000; 55:915-919.
9. Abegaz BM, Ngadjui TB, Dongo E, Tamboue H. Prenylated chalcones and flavones from the leaves of *Dorstenia kameruniana*. Phytochemistry. 1998; 49:1147-1150.
10. Harborne JB, Williams CA. Advances in flavonoid research since 1992. Phytochemistry. 2000; 55:481-504.
11. Cushnie TTP, Lamb AJ. Recent advances in understanding the antibacterial properties of flavonoids. Int J Antimicrob Agents. 2011; 38:99-107.
12. Kuete V, Ngameni B, Tangmouo JG, Bolla JM, Alibert FS, Ngadjui TB, Pages JM. Efflux Pumps are involved in the defense of gram-negative bacteria against the natural products isobavachalcone and diospyrone. Antimicrob Agents Chemother. 2010; 54(5): 1749-1752.
13. Mbaveng AT, Ngameni B, Kuete V, Konga SI, Ambassa T, Roy R, Abegaz BM, Etoa FX, Ngadjui BT, Beng VP. Antimicrobial activity of the crude extracts and five flavonoids from the twigs of *Dorstenia barteri* (Moraceae). J Ethnopharmacol. 2008; 116:483-489.
14. Mbaveng AT, Kuete V, Ngameni B, Beng VP, Ngadjuim BT, Meyer JJ, Lall N. Antimicrobial activities of the methanol extract and compounds from the twigs of *Dorstenia mannii* (Moraceae). BMC Complement Altern Med. 2012; 12:83.
15. Ngadjui BT, Dongo E, Abegaz BM, Fotso S, Tamboue H. Dinklagins A , B and C: Three prenylated flavonoids and

other constituents from the twigs of *Dorstenia dinklagei*. Phytochemistry. 2002; 61:99-104.
16. Ngameni B, Ngadjui BT, Folefoc GN, Watchueng J, Abegaz BM. Diprenylated chalcones and other constituents from the twigs of *Dorstenia barteri* Var.*Subtriangularis*. Phytochemistry. 2004; 65:427-432.
17. Clinical and Laboratory Standards Institute. Reference method for broth dilution antifungal susceptibility testing of yeasts. Third Informational Supplement. CLSI Document M27-S3. Clinical and Laboratory Standards Institute, Wayne, PA, USA. 2008.
18. Clinical and Laboratory Standards Institute. Reference method for dilution antimicrobial susceptibility tests for bacteria that grow aerobically. Approved Standard-8th Edition. CLSI Document M07-A8. Clinical and Laboratory Standards Institute, Wayne, PA, USA, 2009.
19. Sims PJ, Waggoner AS, Wang CH, Hoffman JF. Studies on the mechanism by which cyanine dyes measure membrane potential in red blood cells and phosphatidylcholine vesicles. Biochemistry. 1974; 13: 3315-3330.
20. Ferrari ET, Widholm MJ. A simple, rapid, and sensitive method for estimation of DNA, RNA, and protein synthesis in carrot cell cultures. Anal Biochem. 1973; 56:346-352.
21. Paudel A, Hamamoto H, Kobayashi Y, Yokoshima S, Fukuyama T, Sekimizu K. Identification of novel deoxyribofuranosyl indole antimicrobial agents. J Antibiot. 2012; 65:53-57.
22. Ooi N, Miller K, Hobbs J, Rhys-Williams W, Love W, Chopra I. XF-73, a novel antistaphylococcal membrane-active agent with rapid bactericidal activity. J Antimicrob Chemother. 2009; 64:735-740.
23. Hamamoto H, Tonoike A, Narushima K, Horie R, Sekimizu K. Silkworm as a model animal to evaluate drug candidate toxicity and metabolism. Comp Biochem Physiol C Toxicol Pharmacol. 2009; 149:334-339.
24. Tsuchiya H, Sato M, Miyazaki T, *et al*. Comparative study on the antibacterial activity of phytochemical flavanones against methicillin-resistant *Staphylococcus aureus*. J Ethnopharmacol 1996; 50:27-34.
25. Sasaki H, Kashiwada Y, Shibata H, Takaishi Y. Prenylated flavonoids from *Desmodium caudatum* and evaluation of their anti-MRSA activity. Phytochemistry. 2012; 82:136-42.
26. Avila HP, Smania EDFA, Monache FD, Junior AS. Structure-activity relationship of antibacterial chalcones. Bioorg Med Chem. 2008; 16:9790-9794.
27. Patrzykat A, Friedrich LC, Zhang L, Mendoza V, Hancock EWR. Sublethal concentrations of pleurocidin-derived antimicrobial peptides inhibit macromolecular synthesis in *Escherichia coli*. Antimicrob Agents Chemother. 2002; 46:605-614.
28. Cushnie TTP, Lamb JA. Detection of galangin-induced cytoplasmic membrane damage in *Staphylococcus aureus* by measuring potassium loss. J Ethnopharmacol. 2005; 101:243-248.
29. Mirzoeva OK, Grishanin RN, Calder PC. Antimicrobial action of propolis and some of its components: The effects on growth, membrane potential and motility of bacteria. Microbiol Res. 1997; 152:239-246.
30. Vinnikov AI, Oriabinskaia LB, Golodok LP. The effect of antibiotics on staphylococcal respiratory chain enzymes. Ukr Biokhim Zh. 1994, 66:51-58.
31. Mori A, Nishino C, Enoki N, Tawata S. Antibacterial activity and mode of action of plant flavonoids against *Proteus vulgaris* and *Staphylococcus aureus*. Phytochemistry. 1987; 26:2231-2234.
32. Haraguchi H, Tanimoto K, Tamura Y, Mizutani K, Kinoshita T. Mode of antibacterial action of retrochalcones from *Glycyrrhiza inflata*. Phytochemistry. 1998; 48:125-129.
33. O'Grady FW. Mode of action of antibacterial agents. Proc R Soc Med. 1971; 64:529-533.
34. Cushnie TTP, Lamb AJ. Antimicrobial activity of flavonoids. Int J Antimicrob Agents. 2005; 26:343-356.
35. Middleton E, Kandaswami C. Effect of flavonoids on immune and inflammatory cell functions. Biochem Pharmacol. 1992; 43:1167-1179.
36. Middleton JE, Kandaswami C. The impact of plant flavonoids on mammalian biology: Implications for immunity, inflammation and cancer. In Harbone JB (Ed). The flavonoids: Advances in research since 1986. Chapman & Hall, London, 1994; pp. 619-652.
37. Kuete V, Ngameni B, Wiench B, Krusche B, Horwedel C, Ngadjui BT, Efferth T. Cytotoxicity and mode of action of four naturally occuring flavonoids from the genus *Dorstenia*: Gancaonin Q, 4-hydroxylonchocarpin, 6-prenylapigenin, and 6,8-diprenyleriodictyol. Planta Med. 2011; 77:1984-989.

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Original Article

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Differences in the mode of phagocytosis of bacteria between macrophages and testicular Sertoli cells

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ABSTRACT: Sertoli cells, the sole somatic cell type in the seminiferous epithelium, play an essential role in spermatogenesis and spermiogenesis by nursing germ cells for their survival and differentiation as well as physically inhibiting the entrance of harmful substances into the seminiferous tubules. Sertoli cells possess the characteristics of immune cells; they express pattern recognition receptors, secrete antimicrobial proteins, and engulf dead or dying cells. In this study, we determined the mechanism by which Sertoli cells engulf and kill bacteria compared to that of macrophages. When the primary cultured Sertoli cells of rats were incubated with *Staphylococcus aureus*, they produced the mRNA of neutrophil protein 3, an antimicrobial peptide of the α-defensin family, but not superoxide or nitric oxide, in contrast to mouse peritoneal macrophages. Sertoli cells effectively phagocytosed *S. aureus* in a manner that was accompanied by cytoskeleton rearrangement and dependent on phosphatidylinositol 3-kinase. Engulfed bacteria appeared to stay alive in Sertoli cells, while they were rapidly killed in macrophages. These results collectively suggest that Sertoli cells eliminate bacteria that have invaded the seminiferous epithelium without evoking inflammation, unlike macrophages.

Keywords: Bacterial infection, innate immunity, macrophage, phagocytosis, Sertoli cell

1. Introduction

Mammalian spermatogenesis takes place in the luminal part of the seminiferous tubules. A permeability regulation physically protects the seminiferous epithelium from the invasion of substances that may affect tissue homeostasis. This mechanism, called the blood-testis barrier, is composed of inter-Sertoli tight junctions (*1*,*2*). Sertoli cells, a somatic cell type in the seminiferous epithelium, play an essential role in the production of sperm by helping spermatogenic cells to survive and differentiate (*3-6*). We previously showed the existence of immunity in the seminiferous epithelium against invading bacteria (*7*). Sertoli cells possess some of the characteristics of immune cells: they engulf apoptotic spermatogenic cells (*8*), produce antimicrobial proteins (*9-13*), and express pattern recognition receptors such as Toll-like receptors (*14-17*). The phagocytosis of apoptotic spermatogenic cells by Sertoli cells is achieved through the specific binding of phosphatidylserine exposed on the surface of target cells to class B scavenger receptor type I (SR-BI) of Sertoli cells (*18-20*). Scavenger receptors are known to serve as receptors in the phagocytosis of bacteria by mammalian (*21-23*) as well as insect (*24-26*) phagocytes. These findings allowed us to anticipate the direct involvement of Sertoli cells in immune responses against bacteria in the seminiferous epithelium. In the present study, we examined the phagocytic activity of Sertoli cells against bacteria and compared it to that of macrophages.

2. Materials and Methods

2.1. *Materials*

The monoclonal anti-rat SR-BI antibody, clone 3D12, was generated in mice as described previously (*27*). U0126, SB203580, and SP600125, inhibitors of signaling pathways leading to the activation of the mitogen-activated protein kinases (MAPK) ERKI/II, p38, and JNK, respectively, were purchased from Cell Signaling Technology (Beverly, MA, USA). Wortmannin and cytochalasin B, inhibitors of phosphatidylinositol 3-kinase and actin polymerization, respectively, were obtained from Sigma-Aldrich (St. Louis, MO, USA), F12-L15 medium and trypsin were from Invitrogen (Carlsbad, CA, USA), and norepinephrine was from Meiji (Tokyo, Japan).

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2.2. *Culturing cells and bacteria*

Rat Sertoli cells were primary cultured as described previously (*18*,*19*). Briefly, the testes of 20-day-old male Donryu rats were processed to obtain cells contained in the seminiferous tubules, and the resulting dispersed cells, which mostly consisted of spermatogenic cells and Sertoli cells, were maintained with F12-L15 medium supplemented with 10% (v/v) heat-treated fetal bovine serum and norepinephrine for 3 days at 32.5˚C. Spermatogenic cells lightly attached onto the monolayer of Sertoli cells were removed by pipetting, and the remaining Sertoli cells were used in the experiments. Peritoneal macrophages of thioglycollate-treated C57BL/6 female mice were prepared and maintained in cultures as described previously (*28*). The *Staphylococcus atureus* strains, NCTC8325 and Smith, and *Escherichia coli* strain, WN5-2, were grown in Luria-Bertani medium at 37˚C, harvested at full growth, washed with phosphate-buffered saline (PBS), and used in the experiments as described previously (*29*).

2.3. *Reverse transcription-mediated polymerase chain reaction*

Total RNA was extracted from Sertoli cells by a conventional acid-phenol method and subjected to reverse transcription (RT) with oligo d(T) as a primer. The resulting cDNA was used as a template in semi-quantitative polymerase chain reaction (PCR). The oligonucleotides used as primers in PCR were: 5'-AAGAGCGCTGTGTCTCTTGC (forward) and 5'-CAACAGAGTCGGTAGATGCG (reverse) for the mRNA of rat neutrophil peptide (RNP-3), and 5'-TGAAGGTCGGTGTCAACGGTAAAGGC (forward) and 5'-CATGTAGGCCATGAGGTCCACCAC (reverse) for the mRNA of glyceraldehyde-3-phosphate dehydrogenase (G3PDH).

2.4. *Determination of nitric oxide and superoxide, level of phagocytosis of bacteria, and colony-formable bacteria*

S. aureus was added to cultures of Sertoli cells or macrophages (phagocytes:bacteria = 1:1000) for the assay for nitric oxide and superoxide, and the mixtures were incubated at 37˚C for 30 min. Culture supernatants were then collected by centrifugation and subjected to assays for superoxide using Diogenes (National Diagnostics Inc, Atlanta, GA, USA) and nitric acid by the Griess method, as described previously (*29*). In an assay for phagocytosis, Sertoli cells or peritoneal macrophages were incubated with *S. aureus*, which had been surface-labeled with fluorescein isothiocyanate (FITC), at a ratio of phagocytes:bacteria of 1:100 at 32.5˚C (with Sertoli cells) or 37˚C (with macrophages), and were washed with PBS to remove unengulfed bacteria. The remaining cells were treated with 3% (w/v) paraformaldehyde and ice-cold methanol followed by examination under a fluorescence phase-contrast microscope, as described previously (*18*). To determine colony-formable bacteria in phagocytes, Sertoli cells or peritoneal macrophages, which had been incubated with *S. aureus* (phagocytes : bacteria = 1:100) at 37˚C for 30 min and washed, were further maintained and lyzed by incubating with water for 10 min. The lysates were plated on agar-solidified Luria-Bertani medium, incubated at 37˚C overnight, and the number of colonies was determined.

2.5. *Data processing and statistical analysis*

Results from quantitative analyses were expressed as the mean ± SD of the data from at least three independent experiments. Other data were representative of at least three independent experiments that yielded similar results. Statistical analyses were performed using the two-tailed Student's *t*-test, and *p* values of less than 0.05 were considered significant and indicated in the figures.

3. Results

3.1. *Phagocytosis of S. aureus by primary cultured Sertoli cells*

We first examined the phagocytosis of *S. aureus* by primary cultured Sertoli cells. The *S. aureus* strain NCTC8325 surface-labeled with FITC was added to cultures of Sertoli cells, and the mixture was incubated, washed, and cytochemically examined for the occurrence of phagocytosis. An image of fluorescence microscopy showed that Sertoli cells engulfed many bacteria (Figure 1A, left panels). A numerical analysis of the data indicated that Sertoli cell phagocytosis of *S. aureus* took a relatively longer period to occur (Figure 1A, right panel). We obtained similar results with another *S. aureus* strain, Smith (data not shown). Phagocytosis of the strain NCTC8325 was not influenced by an antibody that neutralized SR-BI or the inhibitors of MAPKs, but was reduced in the presence of cytochalasin B and wortmannin, inhibitors of actin polymerization and phosphatidylinositol 3-kinase, respectively (Figure 1B). This suggested the involvement of cytoskeleton rearrangement in the phagocytosis of *S. aureus* by Sertoli cells.

3.2. *Increase in α-defensin mRNA, but not superoxide and nitric oxide in Sertoli cells upon incubation with S. aureus*

We next examined the response of Sertoli cells to bacteria in terms of the production of antimicrobial substances. RNP-3, an antimicrobial peptide of the α-defensin family, has been shown to be produced in mammalian testes (*9-13*). Therefore, we determined the level of RNP-3 mRNA in Sertoli cells before and after incubation with

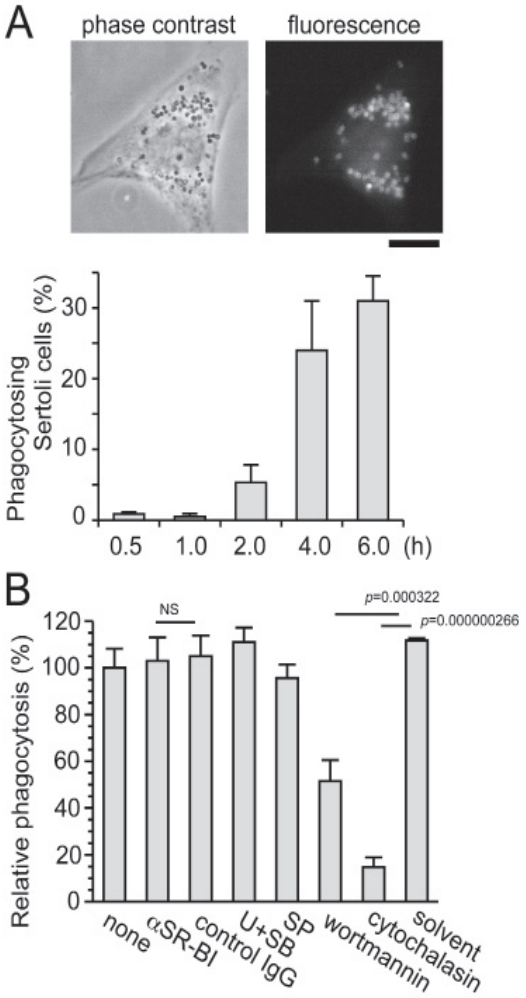

Figure 1. Phagocytosis of *S. aureus* by Sertoli cells. Primary cultured rat Sertoli cells were subjected to an assay for phagocytosis using FITC-labeled live *S. aureus* (strain NCTC8325) as targets. (**A**) Sertoli cells incubated with bacteria for the indicated periods were washed and examined under a fluorescence-phase contrast microscope for the ratio of Sertoli cells that engulfed bacteria. Shown at the top are phase-contrast and fluorescence views of the same microscopic field (scale bar, 10 μm). Results after numerical analysis of the data are shown at the bottom. Representative data from one of three independent experiments that yielded similar results are shown. (**B**) Sertoli cells were pre-incubated with the indicated proteins and chemicals for 30 min prior to being subjected to an assay for phagocytosis for 2 h. The concentrations of the supplements are: 50 μg/mL for anti-SR-BI IgG (αSR-BI) and normal mouse IgG (control); 10 μM for U0126 (U), SB203580 (SB), SP600125 (SP), and cytochalasin B; and 0.1 mM for wortmannin. The solvent for the chemicals was dimethyl sulfoxide. Representative data from one of two independent experiments with similar results are presented. NS, difference not significant.

the *S. aureus* strain NCTC8325 and the *E. coli* strain WN5-2. Total RNA extracted from Sertoli cells was analyzed by RT-mediated PCR for the level of mRNA. The intensity of a signal derived from RNP3 mRNA increased upon incubation with *S. aureus* and, to a lesser extent, with *E. coli*, while G3PDH mRNA, analyzed as an internal control, did not change after incubation with either bacterium (Figure 2A). An increase in the level of RNP-3 mRNA was not influenced when Sertoli cells

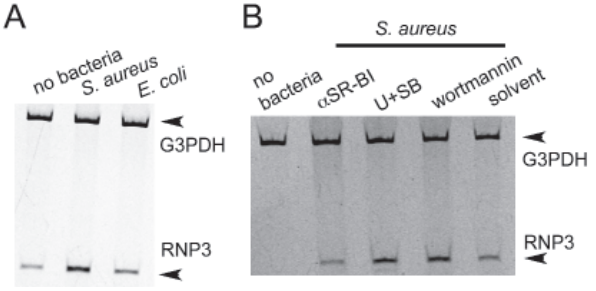

Figure 2. Levels of RNP-3 mRNA in Seroli cells incubated with *S. aureus* and *E. coli.* Primary cultured Sertoli cells were incubated in the presence and absence of *S. aureus* (strain NCTC8325) and *E. coli* (strain WN5-2) at ratios of phagocytes: targets of 1:1000 and 1:3000, respectively, at 32.5°C for 30 min. Total RNA extracted from Sertoli cells was subjected to RT-mediated PCR, and the final products were separated on a polyacrylamide gel followed by staining with ethidium bromide. (**A**) PCR products derived from the mRNA of RNP-3 and G3PDH, analyzed as an internal control, were shown. Data from one of five independent experiments with similar results are presented. (**B**) Incubation of Sertoli cells with *S. aureus* was performed in the presence of the indicated protein and chemicals. The symbols and concentrations of the supplements are the same as those shown in Figure 1B. Representative data from one of two independent experiments that yielded similar results are shown.

were incubated with bacteria in the presence of an anti-SR-BI antibody, the inhibitors of MAPKs, or an inhibitor of phosphatidylinositol 3-kinase (Figure 2B).

We next examined the production of radicals in Sertoli cells after incubation with bacteria. Cultures of Sertoli cells and mouse peritoneal macrophages were supplemented with NCTC8325, and culture supernatants were then examined for the production of nitric oxide and superoxide. We found that the production of either substance could not be detected in Sertoli cell cultures, while both nitric oxide and superoxide were detectable in the culture supernatants of peritoneal macrophages (Figure 3), as we reported previously (*29*). All these phenomena observed with Sertoli cells after the phagocytosis of the *S. aureus* strain NCTC8325 were reproduced when Smith, another *S. aureus* strain, was used as target bacteria (data not shown).

3.3. *Impaired killing of S. aureus in Sertoli cells*

We then examined how soon engulfed bacteria were killed in Sertoli cells. Sertoli cells or peritoneal macrophages were incubated with NCTC8325, washed to remove unengulfed bacteria, and further maintained. The cells were then lyzed and analyzed for the presence of colony-formable bacteria (Figure 4). We found that the number of colonies obtained from the lysates of Sertoli cells was almost constant in the 2-h incubation after washing. Similar results were obtained in the reaction using Smith, another *S. aureus* strain, as target bacteria (data not shown). In contrast, *S. aureus* engulfed by macrophages appeared to be killed in 1 h. These results indicated that *S. aureus* engulfed by Sertoli cells is resistant to killing.

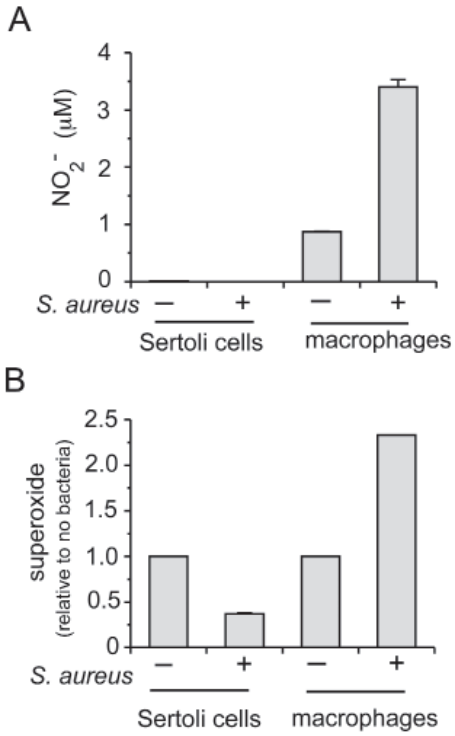

Figure 3. Production of nitric oxide and superoxide by Sertoli cells and macrophages incubated with *S. aureus*. Sertoli cells and peritoneal macrophages were incubated in the presence and absence of *S. aureus* (strain NCTC8325) (phagocytes:targets = 1:1,000) for 30 min, and culture supernatants were analyzed for the levels of nitric oxide and superoxide. Representative data from one of three independent experiments that yielded similar results are shown.

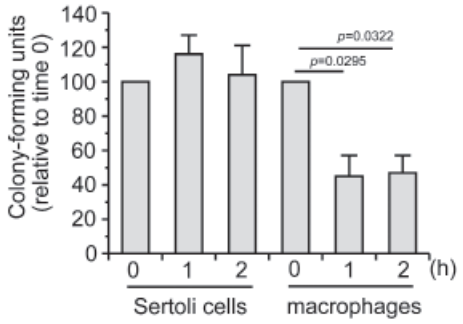

Figure 4. Survival of bacteria after engulfment by Sertoli cells. Sertoli cells and peritoneal macrophages were incubated with *S. aureus* (strain NCTC8325), washed, and further incubated for the indicated periods. The cells were lyzed and examined for the level of colony-formable bacteria. Representative data from one of five (with Sertoli cells) and three (with macrophages) independent experiments that yielded similar results are presented.

4. Discussion

We previously reported the existence of immune responses against invading bacteria in the seminiferous epithelium (*7*). Sertoli cells, the sole somatic cell type in the seminiferous epithelium, possess the characteristics of immune cells. In the present study, we examined the mode of action of Sertoli cells in response to bacteria and compared it to the action of peritoneal macrophages. We found that Sertoli cells isolated from rat testes and maintained in primary cultures effectively engulfed *S. aureus*, as did macrophages, in a manner accompanied by cytoskeleton rearrangement. However, engulfed bacteria were not efficiently killed in Sertoli cells, unlike those engulfed by macrophages. Sertoli cells produced an antimicrobial peptide, and not nitric oxide or superoxide upon incubation with *S. aureus*, while macrophages secreted both radical species. Such actions of Sertoli cells against *S. aureus* should account, at least partly, for immunity in the seminiferous epithelium. Regarding a comparison between the two types of phagocytes, the responses of Sertoli cells to bacteria were less pro-inflammatory than those of macrophages.

Authentic immune cells with phagocytic activity such as macrophages and neutrophils produce radicals when they encounter and phagocytose invading bacteria. Our findings indicate that Sertoli cells, tissue-restricted phagocytes, engulf bacteria, but do not actively kill them. Sertoli cell phagocytosis of bacteria may be interpreted as a defense mechanism by which pathogens are cleared without disturbing homeostasis in the seminiferous epithelium, in which spermatogenesis and spermiogenesis take place. A certain type of defensin plays a role in sperm maturation besides antimicrobial activity (*30*). Therefore, it is possible that RNP-3 is involved in spermatogenesis and/or spermiogenesis rather than killing *S. aureus.*

Sertoli cells retain the apical-basolateral polarity in primary cultures with the apical side toward the medium (*27*). It is thus most likely that Sertoli cells engulfed bacteria at their apical side in our assay for phagocytosis. Sertoli cells are responsible for the phagocytic elimination of spermatogenic cells undergoing apoptosis (*18*), and this action of Sertoli cells is necessary for the effective progress of spermatogenesis (*31*,*32*). Sertoli cell phagocytosis of apoptotic spermatogenic cells takes place in the seminiferous epithelium, and SR-BI serves as a receptor that recognizes phosphatidylserine exposed on the surface of target cells (*18-20*,*32*). However, SR-BI may not be involved in the phagocytosis of *S. aureus* by Sertoli cells because the antibody that neutralized the activity of SR-BI had no effect on phagocytosis. Whatever the receptor for phagocytosis is, it should recognize bacteria with no aid from immunoglobulin as an opsonin in the seminiferous epithelium, a site of immune privilege (*33*).

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References

1. Russell LD, Peterson RN. Sertoli cell junctions: Morphological and functional correlates. Int Rev Cytol. 1985; 94:177-211.
2. Russell LD. Morphological and functional evidence for Sertoli-germ cell relationships. In: The Sertoli cell (Russell LD, Griswold MD, eds.). Cache River Press, Clearwater, USA, 1993; pp. 365-390.
3. Kierszenbaum AL. Mammalian spermatogenesis *in vivo* and *in vitro*: A partnership of spermatogenic and somatic cell linages. Endocr Rev. 1994; 15:116-134.
4. Enders GC. Sertoli-Sertoli and Sertoli-germ cell communications. In: The Sertoli cell (Russell LD, Griswold MD, eds.). Cache River Press, Clearwater, USA, 1993; pp. 447-460.
5. Sharpe RM. Experimental evidence for Sertoli-germ cell and Sertoli-Leydig cell interactions. In: The Sertoli cell (Russell LD, Griswold MD, eds.). Cache River Press, Clearwater, USA, 1993; pp. 391-417.
6. Byers S, Pelletier RM, Suáerz-Quian C. Sertoli-Sertoli cell junctions and the seminiferous epithelium barrier. In: The Sertoli cell (Russell LD, Griswold MD, eds.). Cache River Press, Clearwater, USA, 1993; pp. 431-446.
7. Nagaosa K, Nakashima C, Kishimoto A, Nakanishi, Y. Immune response to bacteria in seminiferous epithelium. Reproduction. 2009; 137:879-888.
8. Nakanishi Y, Shiratsuchi A. Phagocytic removal of apoptotic spermatogenic cells by Sertoli cells: Mechanisms and consequences. Biol Pharm Bull. 2004; 27:13-16.
9. Sang Y, Ortega MT, Blecha F, Prakash O, Melgarejo T. Molecular cloning and characterization of three β-defensins from canine testes. Infect Immun. 2005; 73:2611-2620.
10. Lennartsson A, Pieters K. Vidovic K, Gullberg U. A murine antibacterial ortholog to human bactericidal/permeability-increasing protein (BPI) is expressed in testis, epididymis, and bone marrow. J Leukoc Biol. 2005; 77:369-377.
11. Avellar MC, Honda L, Hamil KG, Yenugu S, Grossman G, Petrusz P, French FS, Hall SH. Differential expression and antibacterial activity of epididymis protein 2 isoforms in the male reproductive tract of human and rhesus monkey (*Macaca mulatta*). Biol Reprod. 2004; 71:1453-1460.
12. Com E, Bourgeon F, Evrard B, Ganz T, Colleu D, Jégou B, Pineau C. Expression of antimicrobial defensins in the male reproductive tract of rats, mice, and humans. Biol Reprod. 2003; 68:95-104.
13. Grandjean V, Vincent S, Martin L, Rassoulzadegan M, Cuzin F. Antimicrobial protection of the mouse testis: Synthesis of defensins of the cryptdin family. Biol Reprod. 1997; 57:1115-1122.
14. Bhushan S, Tchatalbachev S, Klug J, Fijak M, Pineau C, Chakraborty T, Meinhardt A. Uropathogenic *Escherichia coli* block MyD88-dependent and activate MyD88-independent signaling pathways in rat testicular cells. J Immunol. 2008; 180:5537-5547.
15. Bhushan S, Schuppe HC, Fijak M, Meinhardt A. Testicular infection: Microorganisms, clinical implications and host-pathogen interaction. J Reprod Immunol. 2009; 83:164-167.
16. Bhushan S, Schuppe HC, Tchatalbachev S, Fijak M, Weidner W, Chakraborty T, Meinhardt A. Testicular innate immune defense against bacteria. Mol Cell Endocrinol. 2009; 306:37-44.
17. Riccioli A, Starace D, Galli R, Fuso A, Scarpa S, Palombi F, De Cesaris P, Ziparo E, Filippini A. Sertoli cells initiate testicular innate immune responses through TLR activation. J Immunol. 2006; 77:7122-7130.
18. Shiratsuchi A, Umeda M, Ohba Y, Nakanishi Y. Recognition of phosphatidylserine on the surface of apoptotic spermatogenic cells and subsequent phagocytosis by Sertoli cells of the rat. J Biol Chem. 1997; 272:2354-2358.
19. Shiratsuchi A, Kawasaki Y, Ikemoto M, Arai H, Nakanishi, Y. Role of class B scavenger receptor type I in phagocytosis of apoptotic rat spermatogenic cells by Sertoli cells. J Biol Chem. 1999; 274:5901-5908.
20. Kawasaki Y, Nakagawa A, Nagaosa K, Shiratsuchi A, Nakanishi, Y. Phosphatidylserine binding of class B scavenger receptor type I, a phagocytosis receptor of testicular Sertoli cells. J Biol Chem. 2002; 277:27559-27566.
21. Areschoug T, Gordon S. Scavenger receptors: Role in innate immunity and microbial pathogenesis. Cell Microbiol. 2009; 11:1160-1169.
22. Baranova IN, Vishnyakova TG, Bocharov AV, Leelahavanichkul A, Kurlander R, Chen Z, Souza AC, Yuen PS, Star RA, Csako G, Patterson AP, Eggerman TL. Class B scavenger receptor types I and II and CD36 mediate bacterial recognition and proinflammatory signaling induced by *Escherichia coli*, lipopolysaccharide, and cytosolic chaperonin 60. J Immunol. 2012; 188:1371-1380.
23. Leelahavanichkul A, Bocharov AV, Kurlander R, *et al*. Class B scavenger receptor types I and II and CD36 targeting improves sepsis survival and acute outcomes in mice. J Immunol. 2012; 188:2749-2758.
24. Philips JA, Rubin EJ, Perrimon N. Drosophila RNAi screen reveals CD36 family member required for mycrobacterial infection. Science. 2005; 309:1251-1253.
25. Rämet M, Pearson A, Manfruelli P, Li X, Koziel H, Guöbel V, Chung E, Krieger M, Ezekowitz RAB. Drosophila scavenger receptor CI is a pattern recognition receptor for bacteria. Immunity. 2001; 15:1027-1038.
26. Stuart LM, Deng J, Silver JM, Takahashi K, Tseng AA, Hennessy EJ, Ezekowitz RAB, Moore KJ. Response to *Staphylococcus aureus* required CD36-mediated phagocytosis triggered by the COOH-terminal cytoplasmic domain. J Cell Biol. 2005; 170:477-485.
27. Nakagawa A, Nagaosa K, Hirose T, Tsuda K, Hasegawa K, Shiratsuchi A, Nakanishi Y. Expression and function of class B scavenger receptor type I on both apical and basolateral sides of the plasma membrane of polarized testicular Sertoli cells of the rat. Develop Growth Differ. 2004; 46:283-298.
28. Shiratsuchi A, Nakanishi Y. Phosphatidylserine-mediated phagocytosis of anticancer drug-treated cells by macrophages. J Biochem. 1999; 26:1101-1106.
29. Watanabe I, Ichiki M, Shiratsuchi A, Nakankshi Y. TLR2-mediated survival of *Staphylococcus aureus* in macrophages: A novel bacterial strategy against host innate immunity. J Immunol. 2007; 178:4917-4925.
30. Zhou CX, Zhang YL, Xiao L, Zheng M, Leung KM, Chan MY, Lo PS, Tsang LL, Wong HY, Ho LS, Chung YW, Chan HC. An epididymis-specific b-defensin is important for the initiation of sperm maturation. Nat Cell Biol. 2004; 6:458-464.
31. Maeda Y, Shiratsuchi A, Namiki M, Nakanishi Y. Inhibition of sperm production in mice by annexin V microinjected into seminiferous tubules: Possible etiology of phagocytic clearance of apoptotic spermatogenic cells and male infertility. Cell Death Differ. 2002; 9:742-749.
32. Nakagawa A, Shiratsuchi A, Nakanishi Y. *In vivo* analysis of phagocytosis of apoptotic cells by testicular Sertoli cells. Mol Reprod Dev. 2005; 71:166-177.
33. Fijak M, Meinhardt A. The testis in immune privilege. Immunol Rev. 2006; 213:66-81.

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Original Article

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Acetaminophen induced gender-dependent liver injury and the involvement of GCL and GPx

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ABSTRACT: Acetaminophen (AP) is widely used as the antipyretic and analgesic drug in clinic, and it can induce serious liver injury in the case of excessive abuse. The present study showed that AP (400 mg/kg) induced obvious liver injury, while in male mice the hepatotoxicity induced by AP was much more serious than in female mice as indicated by the results of alanine aminotransferase (ALT) activity and reduced glutathione (GSH) amount. Further, the enzymatic activity and protein expression of glutamate-cysteine ligase (GCL) and glutathione peroxidase (GPx) were all higher in female mice liver than in male after the administration of AP (200 mg/kg). Meanwhile, AP (10 mM) decreased GCL and GPx activity in isolated mouse hepatocytes in the time-dependent manner, while the inhibitors of GCL and GPx can augment AP induced-cytotoxicity. Taken together, our results demonstrate the gender-related liver injury induced by AP and the important role of GCL and GPx in regulating such hepatotoxicity.

Keywords: Acetaminophen, gender, hepatotoxicity, GCL, GPx

1. Introduction

Acetaminophen (AP) also known as paracetamol, is widely used in clinical as antipyretic and analgesic drug. AP can induce serious liver injury when it is used overdose, or even sometimes it is used under the recommended dose. As AP belongs to the type of over-the-counter (OTC) drug, so it can be easily purchased by people, and thus leads to the abuse of this drug. The abuse of AP makes the problem about it-induced acute liver failure more frequent and thus becomes a big problem for clinicians. Drug-induced liver injury (DILI) has been the serious medical problem and with significant mortality in clinical situations, among which AP has been the primary cause of drug-induced acute liver failure in American (*1*).

Reduced glutathione (GSH) is an important antioxidant reducing sulfhydryl (-SH) tripeptide, and plays important roles in counteracting with oxidative stress injury and maintains cellular redox balance (*2,3*). Intracellular GSH and its related enzymes such as glutamate-cysteine ligase (GCL), glutathione peroxidase (GPx), glutathione reductase (GR), and glutathione-s-transferase (GST) constitute the key antioxidant system in body to counteract with oxidative stress injury (*4*). AP will be converted into the metabolite *N*-acetyl-*p*-benzoquinoneimine (NAPQI) by liver cytochrome P450s 2E1 (CYP2E1), CYP1A2, CYP2D6, CYP3A, *etc.* (*5*). Generally, NAPQI will be detoxified by GSH, while when GSH is exhausted; it will form covalent binding with macromolecules such as protein or DNA, and thus leads to acute liver failure (*6*). *N*-Acetyl-L-cysteine (NAC), the precursor of GSH biosynthesis, is generally used as the antidote to AP-induced hepatotoxicity in clinic (*7,8*).

Experimental and clinical research demonstrates that gender-dependent difference will lead to the big difference of liver injury caused by exogenous toxins. There is already report that gender is a critical factor involved in AP-induced liver injury, and male animals are more susceptible than female (*9*). Generally, there is the difference in metabolic enzymes between male and female, thus gender is a critical factor affects drug metabolism and pharmacokinetics, which will contribute to the gender-dependent hepatotoxicity. But the previous study already demonstrated that metabolism did not contribute to the gender-dependent liver injury induced by AP (*9*). The present study is designed to observe AP-induced gender-dependent liver injury, and the potential regulating of GSH antioxidant system.

2. Materials and Methods

2.1. *Chemicals and reagents*

β-Actin polyclonal antibody was purchased from Cell

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Signaling Technology (Danvers, MA, USA). Anti-GPx-1, GCLC/GCLM antibodies were the products of Santa Cruz Biotechnology Corporation (Santa Cruz, CA, USA). Peroxidase-conjugated goat anti-Rabbit IgG (H + L) and peroxidase-conjugated goat anti-mouse IgG (H + L) were obtained from Jackson ImmunoResearch (West Grove, PA, USA). Nitrocellulose membranes were obtained from Bio-Rad (Hercules, CA, USA) and enhanced chemiluminescence detection system was from Amersham Life Science (Buckinghamshire, UK). GSH, oxidized glutathione (GSSG), and NADPH were purchased from Roche Diagnostics GmbH (Mannheim, German). AP, 5, 5'-dithio-bis-(2-nitrobenzoic acid) (DTNB), glutathione reductase, D,L-buthionine-(*S*,*R*)-sulfoximine (BSO), mercaptosuccinic acid (MA), and all other reagents unless indicated were obtained from Sigma Chemical Co. (St. Louis, MO, USA).

2.2. *Experimental animals*

Specific pathogen free female and male ICR mice (18-22 g body weight) were purchased from Shanghai Laboratory Animal Center (Shanghai, China). The mice were fed with a standard laboratory diet and given free access to tap water. The animal room was maintained at a temperature of 22 ± 1°C with a 12 h light-dark cycle (6:00-18:00) and 65 ± 5% humidity. All mice were received humane care in compliance with the institutional animal care guidelines approved by the Experimental Animal Ethical Committee of Shanghai University of Traditional Chinese Medicine. Both sexes of mice were divided into 3 groups, vehicle group, the group treated with AP for 200 and 400 mg/kg body weight respectively, and each group contained 8 mice.

2.3. *Treatments of animals*

AP was dissolved in warm saline. Female and male mice were given a single doses of AP (200 and 400 mg/kg, *i.p.*) and solvent control group were treated with warm saline. Animals were killed 4 h after administration.

All blood was collected for measurement of serum alanine and aspartate aminotransferases (ALT/AST) activities. Livers were also collected and washed with ice-cold saline and then quick-freezed in liquid nitrogen for further experiments.

2.4. *Measurement of GSH levels*

Liver reduced and oxidized glutathione levels were determined by the DTNB assay according to the previous reported method with a minor modification (*10*). Briefly, mice liver tissues were homogenized in cold metaphosphoric acid (5%) buffer, and then centrifuged at 10,000 g for 10 min and the supernatant was transferred to new tubes for detection of glutathione levels. The reaction mixture contained 1 mM EDTA, NADPH (0.24 mM), glutathione reductase (0.06 units), DTNB (86 μM) and samples. Yellow 5-thio-2-nitrobenzoic acid (TNB) formation was monitored at 412 nm. GSSG was measured by first masking the GSH in the metaphosphoric acid (5%) buffer with 10 mM 2-vinylpyridine in the presence of 6% triethanolamine and derivatized in 37°C overnight as previously described (*11*). Protein concentrations were determined according to the method of Bradford (*12*), using bovine scrum albumin as standard. The levels of glutathione were calculated based on tissue protein concentration and expressed as nM per mg protein.

2.5. *Enzymatic assays*

ALT and AST were determined according to the methods reported previously (*13*). GCL activity was assayed according to the previous reported method (*14*). The activity of GCL was calculated based on tissue protein concentration and expressed as U/mg protein. One unit of GCL activity was equal to the quantity of the oxidation of 1 mM NADPH per min.

GR activity was assayed as described method (*15*). The activity of GR was calculated based on tissue protein concentration and expressed as mU/mg protein. One unit of GR activity is defined as 1 mM GSSG catalyzed per min.

GPx activity was assayed according to the previous reported method (*16*). A unit of enzyme activity is defined as the amount of enzyme catalyzing the formation of 1 μM of GSH per min, and the activity of GPx was calculated based on tissue protein concentration.

GST activity was assayed according to previous reported method (*17*). A unit of GST activity is defined as the amount of enzyme catalyzing the formation of 1 μM of product per min, and GST activity is defined as the units of enzyme activity per mg protein.

2.6. *Western-blot analysis*

Liver tissues were homogenized in lysis buffer containing 50 mM Tris (pH 7.5), 1 mM EDTA, 150 mM NaCl, 20 mM NaF, 0.5% NP-40, 10% glycerol, 1 mM phenylmethylsulfonyl fluoride, 10 μg/mL aprotinin, 10 μg/mL leupeptin, 10 μg/mL pepstatin A and then centrifuged at 3,000 g for 3 min and the supernatant was transferred to new tubes. Protein concentrations were assayed and normalized to equal protein concentration. Proteins were separated by SDS-PAGE and blots were probed with appropriate combination of primary and HRP conjugated secondary antibodies.

2.7. *Mice primary hepatocytes isolation*

Mice primary hepatocytes were isolated by an improved two-step perfusion separation as previously described (*18*). Cell viability, as determined by trypan blue exclusion, was generally > 85%. Freshly isolated hepatocytes were seeded in 60 mm dishes (1×10^6 cells/dish) or 96-well plates (2×10^4 cells/well) in M199 containing 10% fetal

bovine serum, 100 U/mL penicillin/streptomycin, 2 mM glutamine, 10 ng/mL epidermal growth factor (EGF) and 100 nM dexamethasone and cultured at 37°C with 5% CO_2.

2.8. *Determination of cellular GCL and GPx activity*

Mice hepatocytes were incubated with AP (5 mM) for 12 and 24 h, and then the cells were harvested with PBS. As for GCL assay, the cells were lysed with ultrasonication in lysis buffer (50 mM Tris (pH 7.5), 0.25 M sucrose, 0.1 mM EDTA, 0.7 mM β-mercaptoethanol and 1 mM phenylmethylsulfonyl fluoride) on ice and then centrifuged at 10,000 g, 4°C for 60 min. The supernatant of the cells was transferred to new tubes for GCL assay. The method of cellular GCL activity assay was the same as described above in the liver tissue. As for GPx assay, the cells were also lysed with ultrasonication in PBS and then centrifuged at 3,000 g, 4°C for 3 min. The supernatant was transferred to new tubes for cellular GPx activity assay. The method for cellular GPx activity assay was the same as described above in the liver tissue.

2.9. *Cell viability determination*

Mouse hepatocytes were pretreated with BSO (200 μM, pretreated 12 h) or incubated with MA (7 mM) 24 h after AP treatment. After treated with AP for 48 h, cells were further incubated with 500 μg/mL 3-(4,5-dimethyl-thiazol-2-yl) 2,5-diphenyltetrazolium bromide (MTT) for 4 h. The functional mitochondrial dehydrogenases in survival cells can convert MTT to blue formazan, which was dissolved in 10% SDS-5% iso-butanol-0.01 M HCl (*19*). The optical density was measured at 570 nm with 630 nm as a reference and cell viability was normalized as a percentage of control. Each experiment was performed at least three times by using hepatocytes freshly isolated from different mice.

2.10. *Statistical analysis*

The results were expressed as means ± SEM. Differences between groups were assessed by one-way ANOVA with LSD post hoc test. The analyses were carried out using the SPSS 11.5 software package for Windows. $p < 0.05$ was considered as statistically significant.

3. Results

3.1. *AP-induced liver injury in male and female mice*

The elevation of serum ALT and AST activity is the major indicator for the liver injury (*20*). As shown in Figure 1, we can see that AP (400 mg/kg) increased serum ALT and AST activity. Meanwhile, the increase of serum ALT activity in male mice was higher than that in female ($p < 0.01$).

3.2. *Effects of AP on liver GSH and GSSG amounts in male and female mice*

GSH is the liver important antioxidant. As shown in Figure 2, we found that AP (400 mg/kg) decreased liver GSH amount, while increased liver GSSG amounts, and thus led to the damage on the balance of liver GSH and GSSG. Further, we found that there was a difference in the basal GSH and GSSG amounts, and they were

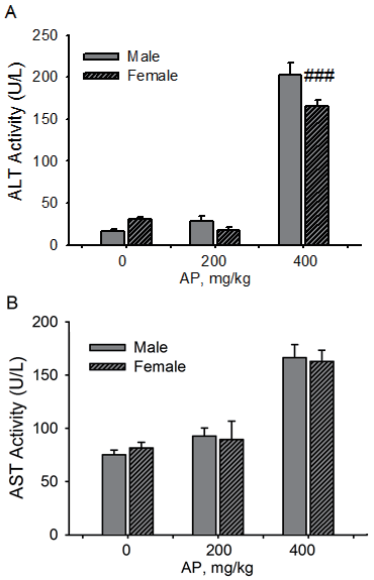

Figure 1. AP-induced liver injury in male and female mice. (A) ALT activity, **(B)** AST activity. Data were expressed as means ± SEM ($n = 8$). $^{###}$ $p < 0.001$ compared to female mice under the same treatment.

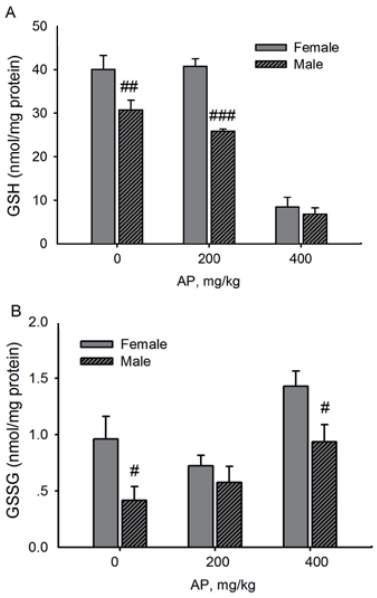

Figure 2. Effects of AP on liver GSH and GSSG amounts in male and female mice. (A) GSH amounts, **(B)** GSSG amounts. Data were expressed as means ± SEM ($n = 8$). $^{#}$ $p < 0.05$, $^{###}$ $p < 0.001$ compared to female mice under the same treatment.

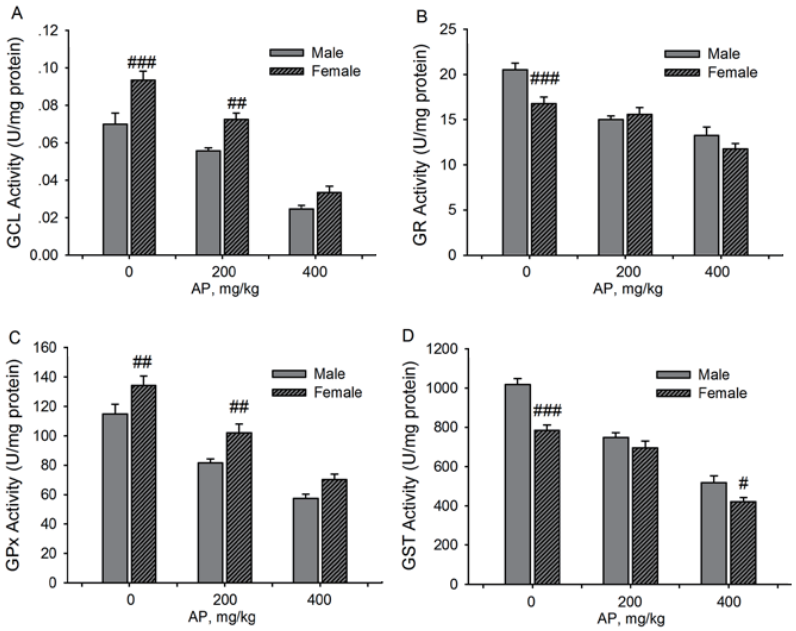

Figure 3. Effects of AP on GCL, GR, GPx and GST activity in male and female mice livers. (A) GCL, **(B)** GR, **(C)** GPx, **(D)** GST activity. Data were expressed as means ± SEM (n = 8). $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$ compared to female mice under the same treatment.

both higher in female than in male ($p < 0.01$, $p < 0.05$). With the administration of AP (200 mg/kg), liver GSH amount was higher in female than in male ($p < 0.001$), while there was no difference in male and female after the administration of AP (400 mg/kg).

3.3. *Effects of AP on GCL, GR, GPx, and GST enzymatic activity in male and female mice*

Next, we observed the effects of AP on the enzymatic activity of GSH-related antioxidant enzymes. The results in Figure 3 showed that AP (200 mg/kg) decreased liver GCL, GR, GPx, and GST activity in male mice, and decreased liver GCL, GPx, and GST activity in female mice. In female mice, GCL and GPx activity were higher than that in male ($p < 0.05$, $p < 0.001$). Further, after the administration of AP (200 mg/kg), GCL and GPx activity were higher in female than that in male ($p < 0.05$, $p < 0.01$).

3.4. *Effects of AP on GCLC and GPx1 protein expression in male and female mice*

Further, we observed the expression of catalytic subunit of GCL (GCLC) and modified subunit of GCL (GCLM), and GPx1 protein. The results of Western blot (Figure 4) showed that GCLC and GPx1 protein expression in female were higher than in male after treated with AP (200 mg/kg).

3.5. *GCL and GPx was involved in AP-induced cytotoxicity in mice hepatocytes*

As shown in Figure 5, we found that AP (5 mM)

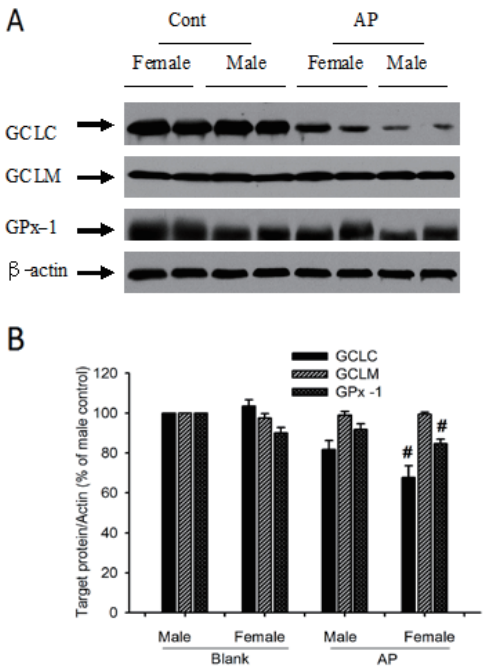

Figure 4. Effects of AP on GCLC and GPx1 protein expression in male and female mice. Twenty μg protein extracts from representative liver tissue were analyzed by SDS-PAGE and immunoblotting by using GCLC, GCLM, GPx-1, and β-actin antibodies, respectively **(A)**. Percentage of target protein/actin was calculated by comparing to the male control **(B)**. Six-eight mice were used in each gender and the result represents one of at least three separate experiments. $^{\#}p < 0.05$ compared to female mice under the same treatment.

obviously decreased cellular GCL and GPx activities in cultured mice hepatocytes ($p < 0.05$, $p < 0.001$). Further, BSO and MA, which are the specific inhibitor of GCL and GPx, both augmented AP-induced cytotoxicity ($p < 0.01$, $p < 0.001$).

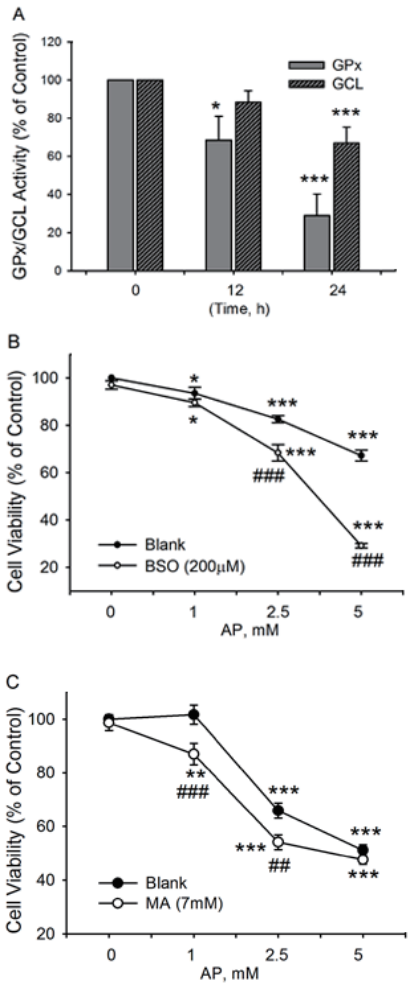

Figure 5. Effects of AP on GCL and GPx activity in isolated mice hepatocytes. (A) GCL and GPx activity. Freshly isolated mice hepatocytes were treated with AP (5 mM) for 24 and 48 h. The cells were collected for cellular GCL and GPx activities assay. The results were expressed in percentage of control and presented as means ± SEM (n = 6), * $p < 0.05$, *** $p < 0.001$ compared to control. **(B)** Effect of BSO on AP-induced cytotoxicity. Mice primary hepatocytes were pretreated with BSO (200 μM) for 12 h, and then incubated with various concentrations of AP (0, 1, 2.5, 5 mM) for 48 h. The survival cells were determined by MTT assay. The results were expressed in percentage of control and presented as means ± SEM (n = 6), * $p < 0.05$, *** $p < 0.001$ compared to control, ### $p < 0.001$ compared with AP alone. **(C)** Effect of MA on AP-induced cytotoxicity. Hepatocytes were treated with various concentrations of AP (0, 1, 2.5, 5 mM) for 24 h, and then incubated with MA (7 mM) in the presence of AP for another 24 h. The survival cells were determined by MTT assay. The results were expressed in percentage of control and presented as means ± SEM (n = 6), ** $p < 0.01$, *** $p < 0.001$ compared with control, ## $p < 0.01$, ### $p < 0.001$ compared to AP alone.

4. Discussion

DILI is the liver damage caused by the parent drug or its metabolites, and now it is a serious problem during drug development and also for the use of many established drugs. It is reported that about 1,000 kinds of drugs can cause liver damage, and the severe cases can cause liver failure and require liver transplantation or even death (*21*). Prevention and treatment of DILI has become the challenging and persistent research topic of international medical community, pharmaceutical industry, and medical institution. Among all the drugs, AP overdose is currently the most frequent cause of acute liver failure in the United States and Great Britain (*22*).

In the present study, we found that the elevation of serum ALT activity induced by AP was higher in male than in female mice (Figure 1). ALT is the sensitive indicator of liver injury, and our results confirmed the previous study that male mice was more sensitive to AP-induced liver injury (*9*).

There are already reports that cellular GSH is important for the detoxification of AP-induced liver injury (*6,7*). In the present study, our results showed that AP (200 mg/kg) had no much effect on GSH amount in female mice, but in male mice GSH amount was obviously decreased, and thus led to the significant difference in GSH amount between male and female mice. Our results indicate that GSH may be a more sensitive indicator than ALT/AST for AP-induced liver injury, as AP of 200 mg/kg can decrease GSH amount in male mice, but had no effect on serum ALT/AST. Our results also demonstrate that AP-induced the damage on GSH redox balance is more serious in male than in female, and thus male mice is inclined to AP-induced hepatotoxicity.

In our results we can see that AP (200 mg/kg) obviously decreased GCL, GPx, GR, and GST enzymatic activity, and the dose is lower than the dose of 400 mg/kg, which induced the elevation of ALT/AST. The result suggests that GCL, GPx, GR, and GST may be the more sensitive indicators than ALT/AST for AP-induced liver injury. Further results showed that AP (200 mg/kg)-induced the decrease of GCL and GPx was higher in male mice than in female. Also, the results of Western-blot demonstrated that the expression of catalytic subunit of GCL (GCLC) and GPx-1 were higher in female than in male after given AP (200 mg/kg), which may contribute to its high activity in female mice. GCL and GPx are both antioxidant enzymes, of which GCL is critical for the biosynthesis of GSH, while GPx counteracts with cellular excessive reactive oxygen species (ROS) *via* utilizing GSH (*23,24*). There is already report that GCL is critical for AP-induced gender-related liver injury (*25,26*), and our present results further suggest that GPx may also be involved in regulating AP-induced gender-related liver injury.

Next, the results demonstrated the decrease of GCL and GPx activity after the administration of AP for the indicated time. Further, BSO and MA, which are the specific inhibitors of GCL and GPx (*26-29*), markedly augmented the cytotoxicity of AP on cultured mouse hepatocytes. These results indicate the important roles of GCL and GPx in regulating AP-induced hepatotoxicity, which may contribute to AP-induced gender-dependent liver injury.

Taken together, our results demonstrated the gender-dependent liver injury induced by AP, further we found that GCL and GPx was critical for regulating gender-related difference in the hepatotoxicity induced by AP. The present study reminds clinic doctors to pay attention to the gender-dependent liver injury induced by AP, and also shall consider the individual differences of liver GSH antioxidant system.

Acknowledgements

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References

1. Ostapowicz G, Fontana RJ, Schiodt FV, Larson A, Davern TJ, Han SH, McCashland TM, Shakil AO, Hay JE, Hynan L, Crippin JS, Blei AT, Samuel G, Reisch J, Lee WM. Results of a prospective study of acute liver failure at 17 tertiary care centers in the United States. Ann Intern Med. 2002; 137:947-954.
2 Meister A, Anderson ME. Glutathione. Annu Rev Biochem. 1983; 52:711-760.
3 DeLeve LD, Kaplowitz N. Glutathione metabolism and its role in hepatotoxicity. Pharmacol Ther. 1991; 52:287-305.
4 Deneke SM, Fanburg BL. Regulation of cellular glutathione. Am J Physiol. 1989; 257:L163-L173.
5 Corcoran GB, Mitchell JR, Vaishnav YN, Horning EC. Evidence that acetaminophen and *N*-hydroxyacetaminophen form a common arylating intermediate, *N*-acetyl-*p*-benzoquinoneimine. Mol Pharmacol. 1980; 18:536-542.
6 James LP, Mayeux PR, Hinson JA. Acetaminophen-induced hepatotoxicity. Drug Metab Dispos. 2003; 31:1499-1506.
7 Sies H. Glutathione and its role in cellular functions. Free Radic Biol Med. 1999; 27:916-921.
8 Kelly GS. Clinical applications of *N*-acetylcysteine. Altern Med Rev. 1998; 3:114-127.
9 Dai G, He L, Chou N, Wan YJ. Acetaminophen metabolism does not contribute to gender difference in its hepatotoxicity in mouse. Toxicol Sci. 2006; 92:33-41.
10 Sies H, Akerboom TP. Glutathione disulfide GSSG efflux from cells and tissues. Methods Enzymol. 1984; 105: 445-451.
11 Griffith OW. Determination of glutathione and glutathione disulfide using glutathione reductase and 2-vinylpyridine. Anal Biochem. 1980; 106:207-212.
12 Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem. 1976; 72:248-254.
13 Reitman S, Frankel S. A colorimetric method for the determination of serum glutamic oxalacetic and glutamic pyruvic transaminases. Am J Clin Pathol. 1957; 28:56-63.
14 Zheng S, Yumei F, Chen A. De novo synthesis of glutathione is a prerequisite for curcumin to inhibit hepatic stellate cell (HSC) activation. Free Radic Biol Med. 2007; 43:444-453.
15 Carlberg I, Mannervik B. Glutathione reductase. Methods Enzymol. 1985; 113:484.
16 Rotruck JT, Pope AL, Ganther HE, Swanson AB, Hafeman DG, Hoekstra WG. Selenium: Biochemical role as a component of glutathione peroxidase. Science. 1973; 179:588.
17 Habig WH, Jakoby WB. Assays for differentiation of glutathione S-transferases. Methods Enzymol. 1981; 77:398-405.
18 Hatano E, Bradham CA, Stark A, Iimuro Y, Lemasters JJ, Brenner DA. The mitochondrial permeability transition augments Fas-induced apoptosis in mouse hepatocytes. J Biol Chem. 2000; 275:11814-11823.
19 Hansen MB, Nielsen SE, Berg K. Re-examination and further development of a precise and rapid dye method for measuring cell growth/cell kill. J Immunol Methods. 1989; 119:203-210.
20 Kamei T, Asano K, Nakamura S. Determination of serum glutamate oxaloacetate transaminase and glutamate pyruvate transaminase by using L-glutamate oxidase. Chem Pharm Bull. 1986; 34:409-412.
21 Kaplowitz N. Drug-induced liver injury. Clin Infect Dis. 2004; 38(Suppl 2):S44-48.
22 Jaeschke H, McGill MR, Ramachandran A. Oxidant stress, mitochondria, and cell death mechanisms in drug-induced liver injury: Lessons learned from acetaminophen hepatotoxicity. Drug Metab Rev. 2012; 44:88-106.
23 Biterova EI, Barycki JJ. Structural basis for feedback and pharmacological inhibition of Saccharomyces cerevisiae glutamate cysteine ligase. J Biol Chem. 2010; 285:14459-14466.
24 Lei XG. *In vivo* antioxidant role of glutathione peroxidase: Evidence from knockout mice. Methods Enzymol. 2002; 347:213-225.
25 McConnachie LA, Mohar I, Hudson FN, Ware CB, Ladiges WC, Fernandez C, Chatterton-Kirchmeier S, White CC, Pierce RH, Kavanagh TJ. Glutamate cysteine ligase modifier subunit deficiency and gender as determinants of acetaminophen-induced hepatotoxicity in mice. Toxicol Sci. 2007; 99:628-636.
26 Masubuchi Y, Nakayama J, Watanabe Y. Sex difference in susceptibility to acetaminophen hepatotoxicity is reversed by buthionine sulfoximine. Toxicology. 2011; 287:54-60.
27 Hoang YD, Avakian AP, Luderer U. Minimal ovarian upregulation of glutamate cysteine ligase expression in response to suppression of glutathione by buthionine sulfoximine. Reprod Toxicol. 2006; 21:186-196.
28 King N, Lin H, Suleiman MS. Cysteine protects freshly isolated cardiomyocytes against oxidative stress by stimulating glutathione peroxidase. Mol Cell Biochem. 2010; 343:125-132.
29 Franco JL, Posser T, Dunkley PR, Dickson PW, Mattos JJ, Martins R, Bainy AC, Marques MR, Dafre AL, Farina M. Methylmercury neurotoxicity is associated with inhibition of the antioxidant enzyme glutathione peroxidase. Free Radic Biol Med. 2009; 47:449-457.

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Evaluation of natural anthracene-derived compounds as antimitotic agents

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ABSTRACT: Plants that contain anthracene-derived compounds such as anthraquinones have been reported to act as anticancer besides their use for millennia to treat constipation, but the mechanism of action is still unfolding. Therefore we pursue this study to explore a new horizon in the anticancer property of these agents with relevance to mitotic arrest. To achieve this goal, the antimitotic activity of a series of naturally occurring anthracene-derived anthraquinones including anthrone, alizarin (1,2-dihydroxyanthraquinone), quinizarin (1,4-dihydroxyanthraquinone), rhein (4,5-dihydroxyanthraquinone-2-carboxylic acid), emodin (1,6,8-trihydroxy-3-methyl-anthraquinone), and aloe emodin (1,8-dihydroxy-3-hydroxymethyl-anthraquinone) were evaluated using *Allium cepa* root tips. Initial results revealed that the mitosis was inhibited after 3, 6, and 24 h, respectively, of incubation with 500, 250, and 125 ppm of each compound in a dose-dependent manner. Furthermore, alizarin at 500 ppm was proved to be the most active compound to arrest the mitosis after 24 h followed by emodin, aloe emodin, rhein, and finally quinizarin. Interestingly, this inhibition of mitosis was irreversible in root tips incubated with each compound at concentration of 500 ppm but not with 250 ppm or 125 ppm, where the roots regained their normal mitotic activity after 96 h post-incubation in water. This re-evaluation of an old remedy suggests that several bioactive anthraquinones possess promising anti-mitotic activity that may have the potential to be lead compounds for the development of a new class of multifaceted natural anticancer/antimitotic agents.

Keywords: Antimitotic, *Allium cepa*, anthraquinones

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1. Introduction

Every year almost 9 million cancer cases are newly diagnosed in developing countries where cancer incidence continues to increase at alarming rates. According to cancer society statistics, at least one third of these individuals are not expected to survive the disease, making cancer the most prevalent cause of death (*1*). Systemic chemotherapy forms the mainstay of cancer treatment, and agents that disrupt mitotic spindle assembly, so called 'anti-mitotics', have emerged as a new and very promising strategy in treating a wide variety of cancers. Traditional anti-mitotic agents include microtubule toxins such as taxol, other taxanes, and the vinca alkaloids, all of which have proven successful in the clinic. However, these compounds act not only on proliferating tumor cells, but exhibit significant side effects on non-proliferating cells including neurons that are highly dependent on intracellular transport processes mediated by microtubules. On top of that, patient response remains highly unpredictable, drug resistance is common, and in addition, toxicity is a problem (*2,3*). To address these problems, the search has intensified for more successful therapies like the classical anti-microtubule drugs, while avoiding some of the adverse side effects. Because there is still no safe anti-mitotic agent, the use of nutritional therapy in the area of drug discovery proved to be an efficient, safe, and economic tool in health care.

During the last decade, it was reported that many herbal formulas-containing anthraquinones were successfully used for treatment of cancer. Emodin, aloe emodin, and rhein, the most extensively studied anthraquinones, have been reported to inhibit proliferation in breast, lung, cervical, colorectal, and prostate cancers cells (*4-7*) with little or no cytotoxic effects in several normal cells, such as human fibroblast-like lung WI-38 cells, rat heart endothelial cells, rat hepatic stellate cells, and rat hepatocytes (*8*). However, the underlying molecular mechanism(s) involved in the anticancer effects of anthraquinones is still unfolding. As such, in the present study, an expansion has been made to explore the new horizon in anticancer properties of certain naturally available anthraquinones (Figure 1) with

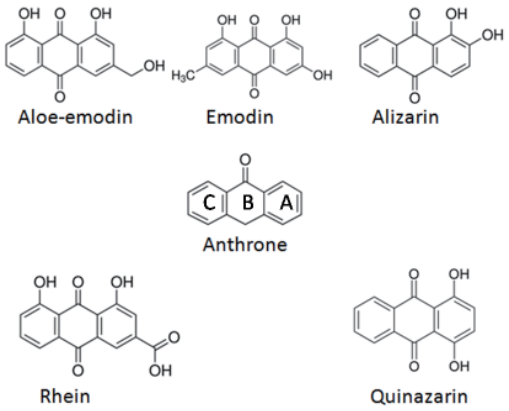

Figure 1. Structural characteristic of natural anthracene-derived compounds, anthrone, aloe emodin, emodin, alizarin, quinizarin, and rhein.

relevance to mitotic arrest. The approach chosen here relies on *in vitro* design since *in vivo* testing to evaluate mitosis is often expensive, time consuming, and requires approval by ethical committees, particularly with mammalian models. Preliminary antimitotic screening in the current study was conducted using the *Allium cepa* root tip assay, a reliable *in vitro* system, which reflects specific effects and allows the selection of promising antimitotic prototypes. This test was initially used by (*9*) in studies on the effect of plant extracts and various chemical compositions on meristematic cells. Up to now, it has been widely used for detection of cytostatic, cytotoxic, and mutagenic properties of different compounds, including anticancer drugs of plant origin (*10,11*). The development and acceptance of such simple bioassays, convenient to use in-house, with a paucity of research funds, are urgent in third world nations, where cultural folk medicines are strongly used safely and effectively to treat human cancers.

2. Materials and Methods

2.1. *Materials*

Anthraquinone, anthrone, alizarin (1,2-dihydroxy-anthraquinone), quinizarin (1,4-dihydroxy-anthraquinone), rhein (4,5-dihydroxy-anthraquinone-2-carboxylic acid), emodin (1,6,8-trihydroxy-3-methyl-anthraquinone), and aloe emodin (1,8-dihydroxy-3-hydroxymethyl-anthraquinone) were kindly provided by Prof. Dr. A. El-Gaml, Pharmacognosy Department, Faculty of Pharmacy, Mansoura University, Mansoura, Egypt.

2.2. *Methods*

In this study, a modification of Podbielkowska's assay (*12*) was introduced as a simple method to evaluate the effect of drugs on the mitotic process. In order to eliminate any significant differences during sampling, a zero time sampling for all bulbs was examined under the same conditions. Therefore no individual variation was assumed for each experiment. *Allium cepa* L. root tips were selected as a model because their chromosomes are small in number (16 pairs), large in size, and easily examined using the light microscope. The roots were fixed and stained with acetocarmine 1% according to the method mentioned above. Acetocarmine stained both DNA and RNA so nearly all parts of the cell were colored but the chromosomes were stained most heavily because some of the RNA had been leached out by HCL. Mitotic percent was calculated in treated roots and statistically compared with that of controls.

2.2.1. *Growing A. cepa L. bulbs*

A. cepa L. bulbs were grown in 250 mL dark jars filled with tap water at room temperature. The water in the jars were continuously aerated and changed daily until the roots reached 2-3 cm.

2.2.2. *Incubation of compounds*

Three bulbs were transferred to the control solution (tap water only) and four others were transferred to each test solution for 24 h. Compounds in this study were dissolved in 1 mL of dimethyl sulfoxide (DMSO) and the volume was made up to 1 L with tap water to produce three concentrations; 500, 250, and 125 ppm. Control solution was prepared by mixing 1 mL of DMSO with tap water to produce 1 L of this solution. All prepared solutions were protected from light during this study. Five roots of three analogous onions were collected after 3, 6, 12, and 24 h incubation. Postincubation of *A. cepa* L. bulbs in tap water was continued for the next 24 and 96 h in the dark to monitor for possible cell recovery.

2.2.3. *Microscopical examination*

The roots were collected, fixed in 95% ethanol:acetic acid (3:1, v/v) for 6-12 h and washed with 70% alcohol. Then they were immersed in ethyl alcohol 95%:1 N hydrochloric acid (1:1, v/v), fixed again for 5 min, squashed and finally stained with 1% acetocarmine.

2.2.4. *Calculations*

For each root sample, the number of meristematic and total cells present in 5 fields was counted using high power (×100) light microscopy. Results for 30 readings were tabulated at different concentrations and times of incubation. The partial mitotic index is given as the number of cells in the course of division as a percentage of all meristematic cells (*13*). The percent mitosis (the number of mitotic cells per one hundred total cells) was calculated for both control and examined samples and statistically compared.

2.3. *Statistical analysis*

The results were expressed as mean ± SE. All analyses were performed with the GraphPad Prism 3 package. Intergroup comparison was made by an ANOVA test. Thereafter, data were checked for skewedness, and an unpaired *t*-test was performed if the distribution of the values was Gaussian. If the distribution was not normal, a Mann-Whitney test was used. *p*-values less than 0.05 were considered to be statistically significant.

3. Results

The results revealed that all tested anthraquinones markedly caused a retardation and/or an inhibition of mitotic activity observed in the root tips of *A.cepa*. This decrease in the mitotic activity was directly proportional to the increase of the compound concentration or prolongation of incubation time. Generally, the inhibition of mitosis was significantly ($p < 0.05$) observed after 3 h incubation with 500 ppm, 6 h incubation with 250 ppm, and after 24 h incubation with 125 ppm. Furthermore, cell recovery did not occur with 500 ppm but occured with 250 ppm and 125 ppm after 96 h post-incubation in water.

Individually, Figure 2 shows the mean mitotic index (counted as percentage of control, 0 ppm) in root tip meristems of *A. cepa* during 24 h incubation in the different concentrations of anthrone, which generally exists in the fresh plant and then is oxidized by an anthrone oxidase to anthraquinon. This 24 h-incubation was followed by 96 h post-incubation in water. As shown in Figure 2, anthrone at 500 ppm caused ~43% inhibition of mitotic activity after 24 h incubation, while 125 ppm of anthrone solution showed the least retardation effect, 22% inhibition of mitotic activity. In addition, it was observed that 500 ppm anthrone solution prevented formation of new healthy roots while this effect decreased gradually in 250 ppm and 125 ppm.

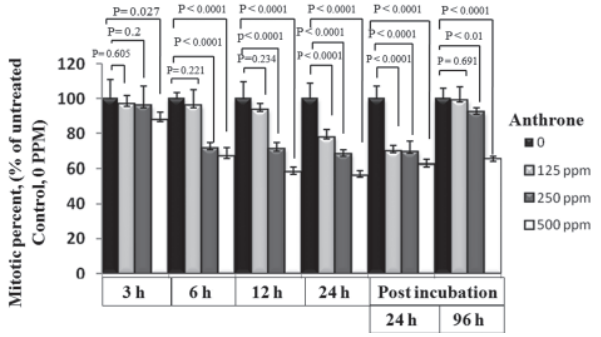

Figure 2. Mean mitotic index of *A. cepa* meristem cells in control roots (water) and during 24 h incubation followed by 48 h post-incubation in different concentrations (125, 250, and 500 ppm) of anthrone, counted as percentage of untreated control, 0 PPM). Five roots of three analogous onions were taken. Data shown are the mean ± SE.

In light of anthron's antimitotic effect, we next sought to determine whether the addition of functional groups would modify this antimitotic behavior of anthraone. Moving over the molecular structure of the selected anthraquinones, we found that introduction of a hydroxyl group in ring A of oxidized anthrone significantly enhanced the anti-mitotic effect of anthraquinones as depicted in Figures 3-5 for aloemodin, emodin, and

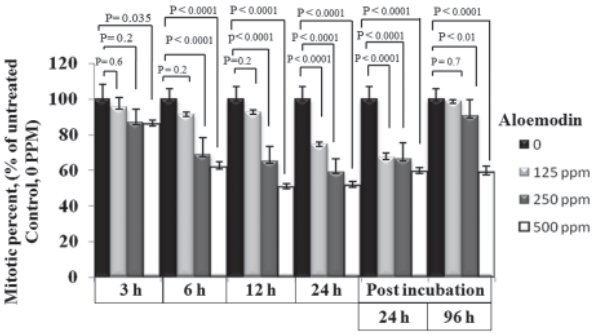

Figure 3. Mean mitotic index of *A. cepa* meristem cells in the control roots (water) and during 24 h incubation followed by 48 h post-incubation in different concentrations (125, 250, and 500 ppm) of aloe emodin, counted as percentage of untreated control, 0 PPM). Five roots of three analogous onions were taken. Data shown are the mean ± SE.

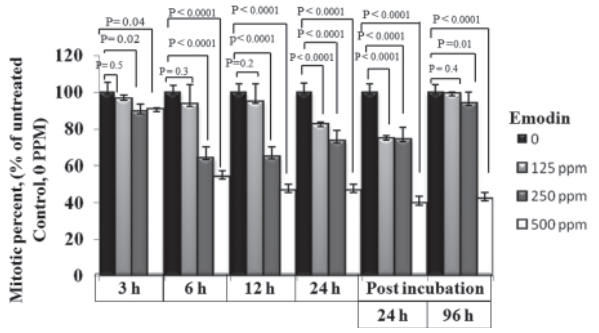

Figure 4. Mean mitotic index of *A. cepa* meristem cells in control roots (water) and during 24 h incubation followed by 48 h post-incubation in different concentrations (125, 250, and 500 ppm) of emodin, counted as percentage of untreated control, 0 PPM). Five roots of three analogous onions were taken. Data shown are the mean ± SE.

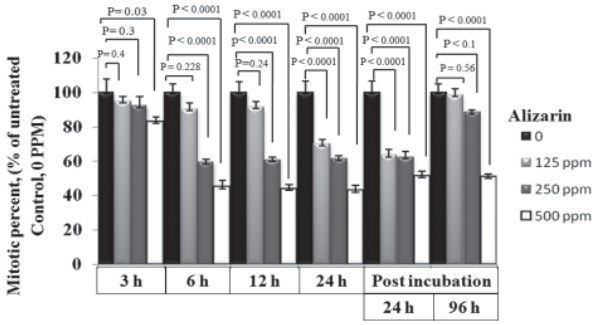

Figure 5. Mean mitotic index of *A. cepa* meristem cells in control roots (water) and during 24 h incubation followed by 48 h post-incubation in different concentrations (125, 250, and 500 ppm) of alizarin, counted as percentage of untreated control, 0 PPM). Five roots of three analogous onions were taken. Data shown are the mean ± SE.

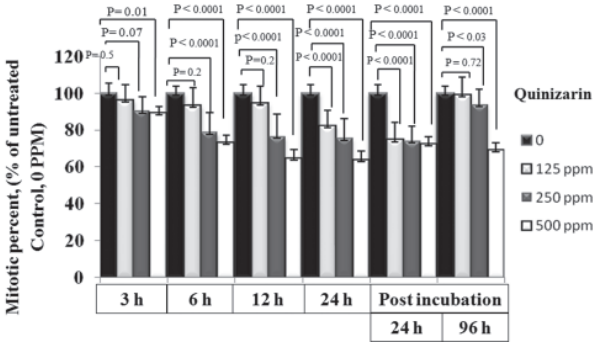

Figure 6 Mean mitotic index of *A. cepa* meristem cells in control roots (water) and during 24 h incubation followed by 48 h post-incubation in different concentrations (125, 250, and 500 ppm) of quinizarin, counted as percentage of untreated control, 0 PPM). Five roots of three analogous onions were taken. Data shown are the mean ± SE.

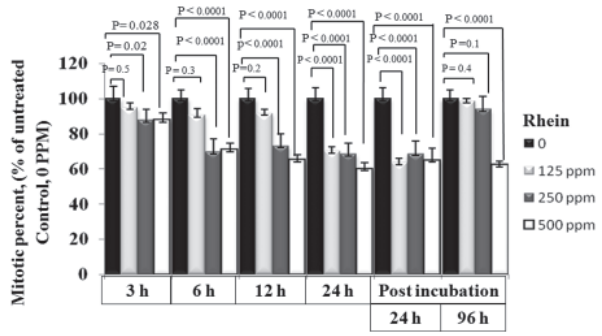

Figure 7. Mean mitotic index of *A. cepa* meristem cells in control roots (water) and during 24 h incubation followed by 48 h post-incubation in different concentrations (125, 250, and 500 ppm) of rhein, counted as percentage of untreated control, 0 PPM). Five roots of three analogous onions were taken. Data shown are the mean ± SE.

alizarin, respectively. In Figure 3, aloe emodin, which has one OH group in ring A, caused ~49% inhibition of mitotic activity of root tip meristems after 24 h incubation at a concentration of 500 ppm. Proceeding further, the addition of a second OH group in ring A resulted in a substantial increase in anti-mitotic activity as illustrated by emodin, which showed ~53% inhibition of mitotic activity of root tip meristems after 24 h incubation at a concentration of 500 ppm (Figure 4). Furthermore, the addition of a second OH in an ortho-substitution had a greater influence on the anti-mitotic effect than substitution in the *meta*-position. This is clearly manifested in alizarin in which the antimitotic activity was raised to 56% after 24 h at a concentration of 500 ppm (Figure 5). However, the addition of a second OH in a *para*-substitution (quinizarin) caused a dramatic decrease in the antimitotic activity to 35% (Figure 6). Likewise, introducing a carboxylic group into ring A also reduced the anti-mitotic effect to 40% as manifested in rhein (Figure 7). One-way ANOVA was used to test for statistical differences in the anti-mitotic effect of 500 ppm after 24 h, and the means were significantly different across the samples ($p < 0.05$) (Figure 8).

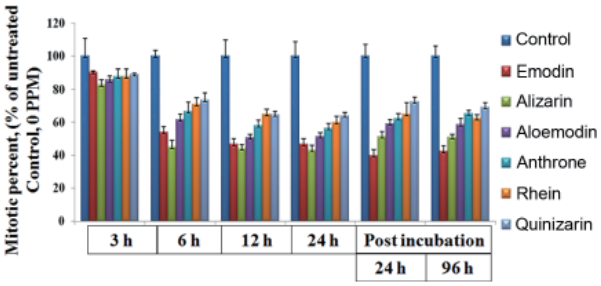

Figure 8. Mean mitotic index of *A. cepa* meristem cells in control roots (water) and during 24 h incubation followed by 48 h post-incubation in the different concentration (125, 250, and 500 ppm) of anthrone, aloe emodin, emodin, alizarin, quinizarin, and rhein, respectively, counted as percentage of untreated control, 0 PPM). Five roots of three analogous onions were taken. Data shown are the mean ± SE.

4. Conclusion

For many years, laboratories from all over the world have been working on finding effective therapies for cancer, a disease of our civilization. Drugs that interfere with the normal progression of mitosis belong to the most successful chemotherapeutic compounds currently used for anti-cancer treatment. In spite of their clinical success, some limitations remain, such as high peripheral neurotoxicity, low bioavailability, poor solubility, complicated synthesis procedures, and drug resistance conferred by multidrug resistance transporters and tubulin mutations (*14-16*). Therefore, there has been great interest in identifying novel antimitotic agents with a new mode of action and improved pharmacology profiles especially concerning reduced toxicity. The reemphasis of natural products in drug discovery has been the subject of recent research in cancer drug discovery and development, and has proved to be a valuable, effective, and inexpensive approach (*17*). In the current study, we put forward a new concept pertaining to the use of natural anthracene-derived anthraquinones as potential anti-mitotic agents.

Anthraquinones are quinone derivatives of anthracene found in many species of medicinal plants that possess a plethora of biological and pharmacological properties. For years, much interest has been placed in the development and use of anthraquinones and its derivatives for the prevention and treatment of cancer. Emodin and aloe emodin, the most abundant anthraquinones in rhubarb, are capable of inhibiting cellular proliferation, induction of apoptosis, and prevention of metastasis. However, both components were found to be able to potentiate the anti-proliferation of various chemotherapeutic agents (*18*). To extend knowledge of the antineoplastic role of anthraquinones, the current study opens a new horizon of understanding their properties in fighting cancer by shedding light on their ability to interfere with mitosis. The anti-mitotic activity was evaluated using the *A. cepa* root meristem model, commonly known as the allium assay. This assay

is a rapid, highly sensitive, and reproducible bioassay for detecting antimitotic events (*19*).

Among anthraquinone derivatives tested here hydroxylanthraquinones are also a common structural moiety of adriamycin and mitoxantrone (common antitumor drugs). It is known that hydroxy- anthraquinones containing a poly-aromatic ring structure can bind to DNA by intercalation and stacking between the base pairs of DNA double helices (*20*). Given these properties, hydroxyanthraquinones represent promising anticancer agents. This input has originated partly from a study showing that alizarin, 1,2-dihydroxy-9,10-anthraquinone, inhibited the growth of syngeneic tumors in treated recipient animals (*21*). These initial observations have been supported by the *in vitro* and *in vivo* antineuroectodermal tumor activity of aloe emodin, a hydroxyanthraquinone present in *Aloe vera* leaves, while no appreciable toxic effects were observed on the animals (*22*). Moreover, these findings were reinforced by recent studies showing that emodin, 6-methyl-1,3,8-trihydroxy-anthraquinone, has a potential anti-tumor effect on pancreatic cancer *via* its dual role in promotion of apoptosis as well as its suppression of angiogenesis, through regulating the expression of NF-κB-regulated angiogenesis-associated factors (*23*). All of this seemingly overwhelming evidence has shaped the concept of hydroxyanthraquinones as antineoplastic agents and several mechanisms have been proposed regarding their anticancer activity. One refers to the ability of the drug to intercalate DNA (*20*) and inhibit DNA topoisomerase II (*24*). Another refers to the ability of the drug to produce free radicals and consequently to cleave DNA (*25*). Nevertheless, this study is the first to demonstrate the antimitotic activity of this group of natural products, suggesting an additional mode of action for hydroxyanthraquinones.

It is noteworthy to mention that although the basic chemical structures of various anthraquinones are similar, the specific functional groups attached at specific positions, particularly for the hydroxyl group, can confer remarkably different bioactivities to the resulting compounds. This notion is exemplified here by reporting that alizarin, which is the only hydroxyquinone having a hydroxyl group in a β position, to be the most active compound to arrest mitosis followed by emodin, aloe emodin, rhein, and finally quinzarin. To the contrary, 1-hydroxyanthraquinone has been reported to be carcinogenic (*26*). Recently it has been reported that lucidin, a hydroxyanthraquinone derivative present in this plant, is mutagenic in bacteria and mammalian cells. In addition, formation of DNA adducts in tissue culture and mice after treatment with this compound has been documented (*27*).

In the current study, analysis of changes in the mitotic index when treated with the selected series of anthraquinones showed that depending on their concentration, the mitotic activity was reduced or inhibited. These observations are supported in existing results concerning animal cells as well as in several human cancer cells (*e.g.*, hepatocellular, lung, breast, esophagus, and gastric) *in vitro* (*4-7*). They induce apoptosis, have an anti-angiogenesis effect, and inhibit the invasion and metastasis of tumor cells (*23,28-30*). Moreover, the antitumor activity of anthraquinone was compared to that of daunorubicin, which is structurally different from anthraquinone but also contains a quinone moiety (*24*).

In conclusion, with the continuing need for novel drug-like lead compounds against the increasing number of ever-more-challenging molecular cancer targets, the chemical diversity derived from natural products will be increasingly relevant for the future of drug discovery. Therefore, the activity of the studied naturally anthracene-derived series in inhibiting mitosis followed by lack of recovery in post-incubation make them potential leads for antimitotic agents.

References

1. WHO, 1 in 2 countries unprepared to prevent and manage cancers, says WHO survey, in, 2013.
2. McGrogan BT, Gilmartin B, Carney DN, McCann A. Taxanes, microtubules and chemoresistant breast cancer. Biochim Biophys Acta. 2008; 1785:96-132.
3. Hill CS, Jr. The barriers to adequate pain management with opioid analgesics. Semin Oncol. 1993; 20:1-5.
4. Chang CJ, Ashendel CL, Geahlen RL, McLaughlin JL, Waters DJ. Oncogene signal transduction inhibitors from medicinal plants. In Vivo. 1996; 10:185-190.
5. Zhang L, Chang CJ, Bacus SS, Hung MC. Suppressed transformation and induced differentiation of HER-2/neu-overexpressing breast cancer cells by emodin. Cancer Res. 1995; 55:3890-3896.
6. Cha TL, Qiu L, Chen CT, Wen Y, Hung MC. Emodin down-regulates androgen receptor and inhibits prostate cancer cell growth. Cancer Res. 2005; 65:2287-2295.
7. Kuo YC, Sun CM, Ou JC, Tsai WJ. A tumor cell growth inhibitor from *Polygonum hypoleucum* Ohwi. Life Sci. 1997; 61:2335-2344.
8. Su YT, Chang HL, Shyue SK, Hsu SL. Emodin induces apoptosis in human lung adenocarcinoma cells through a reactive oxygen species-dependent mitochondrial signaling pathway. Biochem Pharmacol. 2005; 70:229-241.
9. Levan. The effect of colchicines on root mitoses in Allium. Hereditas. 1938; 24:471-486.
10. Kuras M, Nowakowska J, Sliwinska E, Pilarski R, Ilasz R, Tykarska T, Zobel A, Gulewicz K. Changes in chromosome structure, mitotic activity and nuclear DNA content from cells of Allium Test induced by bark water extract of *Uncaria tomentosa* (Willd.) DC. J Ethnopharmacol. 2006; 107:211-221.
11. Ray S, Kundu LM, Goswami S, Roy GC, Chatterjee S, Dutta S, Chaudhuri A, Chakrabarti CS. Metaphase arrest and delay in cell cycle kinetics of root apical meristems and mouse bone marrow cells treated with leaf aqueous extract of *Clerodendrum viscosum* Vent. Cell Prolif. 2013; 46:109-117.
12. Podbielkowska E, Kupidlowska M, Waleza K, Dobrzynska S, Louis A, Zobel A. Coumarins as antimitotics. International Journal of Pharmacognosy. 1994; 32:1-12.
13. Lopez-Saez JF, Fernandez-Gomez E. Partial mitotic index and phase indices. Experientia. 1965; 21:591-592.

14. Malik B, Stillman M. Chemotherapy-induced peripheral neuropathy. Curr Neurol Neurosci Rep. 2008; 8:56-65.
15. Hamel E. Natural products which interact with tubulin in the vinca domain: Maytansine, rhizoxin, phomopsin A, dolastatins 10 and 15 and halichondrin B. Pharmacol Ther. 1992; 55:31-51.
16. Verrills NM, Kavallaris M. Improving the targeting of tubulin-binding agents: Lessons from drug resistance studies. Curr Pharm Des. 2005; 11:1719-1733.
17. Koehn FE. Natural products and cancer drug discovery. Humana Press, New York, 2013.
18. Huang Q, Lu G, Shen HM, Chung MC, Ong CN. Anti-cancer properties of anthraquinones from rhubarb. Med Res Rev. 2007; 27:609-630.
19. Fiskesjo G. The Allium test – an alternative in environmental studies: The relative toxicity of metal ions. Mutat Res. 1988; 197:243-260.
20. Hsiao CJ, Li TK, Chan YL, Hsin LW, Liao CH, Lee CH, Lyu PC, Guh JH. WRC-213, an l-methionine-conjugated mitoxantrone derivative, displays anticancer activity with reduced cardiotoxicity and drug resistance: Identification of topoisomerase II inhibition and apoptotic machinery in prostate cancers. Biochem Pharmacol. 2008; 75:847-856.
21. Hilgert I, Cudlin J, Steinerova N, Vanek Z. Antitumour and immunosuppressive activity of hydroxyanthraquinones and their glucosides. Folia Biol (Praha). 1977; 23:99-109.
22. Pecere T, Gazzola MV, Mucignat C, Parolin C, Vecchia FD, Cavaggioni A, Basso G, Diaspro A, Salvato B, Carli M, Palu G. Aloe-emodin is a new type of anticancer agent with selective activity against neuroectodermal tumors. Cancer Res. 2000; 60:2800-2804.
23. Lin SZ, Wei WT, Chen H, Chen KJ, Tong HF, Wang ZH, Ni ZL, Liu HB, Guo HC, Liu DL. Antitumor activity of emodin against pancreatic cancer depends on its dual role: Promotion of apoptosis and suppression of angiogenesis. PLoS One. 2012; 7:e42146.
24. Perchellet EM, Magill MJ, Huang X, Dalke DM, Hua DH, Perchellet JP. 1,4-Anthraquinone: An anticancer drug that blocks nucleoside transport, inhibits macromolecule synthesis, induces DNA fragmentation, and decreases the growth and viability of L1210 leukemic cells in the same nanomolar range as daunorubicin *in vitro*. Anticancer Drugs. 2000; 11:339-352.
25. Fisher GR, Gutierrez PL, Oldcorne MA, Patterson LH. NAD(P)H (quinone acceptor) oxidoreductase (DT-diaphorase)-mediated two-electron reduction of anthraquinone-based antitumour agents and generation of hydroxyl radicals. Biochem Pharmacol. 1992; 43:575-585.
26. Mori H, Yoshimi N, Iwata H, Mori Y, Hara A, Tanaka T, Kawai K. Carcinogenicity of naturally occurring 1-hydroxyanthraquinone in rats: Induction of large bowel, liver and stomach neoplasms. Carcinogenesis. 1990; 11:799-802.
27. Ishii Y, Inoue K, Takasu S, Jin M, Matsushita K, Kuroda K, Fukuhara K, Nishikawa A, Umemura T. Determination of lucidin-specific DNA adducts by liquid chromatography with tandem mass spectrometry in the livers and kidneys of rats given lucidin-3-*O*-primeveroside. Chem Res Toxicol. 2012; 25:1112-1118.
28. Sun ZH, Bu P. Downregulation of phosphatase of regenerating liver-3 is involved in the inhibition of proliferation and apoptosis induced by emodin in the SGC-7901 human gastric carcinoma cell line. Exp Ther Med. 2012; 3:1077-1081.
29. Popadic D, Savic E, Ramic Z, Djordjevic V, Trajkovic V, Medenica L, Popadic S. Aloe-emodin inhibits proliferation of adult human keratinocytes *in vitro*. J Cosmet Sci. 2012; 63:297-302.
30. Liu A, Sha L, Shen Y, Huang L, Tang X, Lin S. Experimental study on anti-metastasis effect of emodin on human pancreatic cancer. Zhongguo Zhong Yao Za Zhi. 2012; 36:3167-3171

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Original Article

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Preparation and physicochemical properties of surfactant-free emulsions using electrolytic-reduction ion water containing lithium magnesium sodium silicate

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ABSTRACT: Surfactant-free emulsions by adding jojoba oil, squalane, olive oil, or glyceryl trioctanoate (medium chain fatty acid triglycerides, MCT) to electrolytic-reduction ion water containing lithium magnesium sodium silicate (GE-100) were prepared, and their physiochemical properties (thixotropy, zeta potential, and mean particle diameter) were evaluated. At an oil concentration of 10%, the zeta potential was –22.3 – –26.8 mV, showing no marked differences among the emulsions of various types of oil, but the mean particle diameters in the olive oil emulsion (327 nm) and MCT emulsion (295 nm) were smaller than those in the other oil emulsions (452-471 nm). In addition, measurement of the hysteresis loop area of each type of emulsion revealed extremely high thixotropy of the emulsion containing MCT at a low concentration and the olive emulsion. Based on these results, since surfactants and antiseptic agents markedly damage sensitive skin tissue such as that with atopic dermatitis, surfactant- and antiseptic-free emulsions are expected to be new bases for drugs for external use.

Keywords: Surfactant-free emulsions, electrolytic-reduction ion water, lithium magnesium sodium silicate, thixotropy, rheology

1. Introduction

Magnesium aluminum silicate is a laminar clay material and saponite that belongs to the smectite group, markedly increases viscosity, and shows high thixotropy when dispersed in various solvents (*1*). Using these properties, smectite such as magnesium aluminum silicate or lithium magnesium sodium silicate is widely used as a texture enhancer in cosmetics, color antisettling agent in paints, catalyst in specific reactions, adsorbent in functional films, and an anti-dripping agent in adhesives (*2*).

Electrolytic-reduction ion water (ERI) is ion water with physically excess electrons that is obtained by electrolysis of natural water using a special diaphragm system. Due to its special alkalinity and negatively charged ions, ERI removes dirt and bacteria as sources of odor by its detachment action, and, therefore, has cleansing, deodorizing, bactericidal, and anti-dust effects (*3,4*). Unlike synthetic surfactants, ERI does not burden the environment, and, therefore, is mainly used as a cleansing agent for various industrial products.

We previously prepared magnesium aluminum silicate solutions using various solvents including ERI, and reported their rheology characteristics by measuring oscillation employing a stress control-type rheometer (*5*). Using ERI as a dispersion medium, conversion from gel to sol occurred at a low frequency (external factor). In addition, gel was prepared using ERI containing magnesium aluminum silicate as smectite, and gel characteristics for various types of salt were evaluated in detail (*6*). As a result, the presence of Ca^{2+} made the system unstable, but uni- and multi-valent cations excluding Ca^{2+} markedly improved gel stability.

On the other hand, emulsions for medicine such as creams are generally prepared with lecithin as an amphoteric surfactant or polysorbate as a nonionic surfactant. Some surfactants excluding lecithin are known to induce stimulant dermatitis, and may sometimes cause allergic symptoms (*7*). Considering biological safety, surfactant-free emulsions are optimal. However, without using surfactants, emulsification itself is difficult, and temporal stability is poor. Therefore, using the cleansing effects of ERI, surfactant-free stable emulsions were prepared, and their functionality was reported (*8*). Surfactant-free emulsions prepared with ERI were more stable and showed a smaller mean diameter than those containing egg yolk lecithin as a surfactant. These results suggested that skin-irritating reactions and allergic symptoms due to surfactants can be reduced by preparing

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surfactant-free stable emulsions using the emulsification effect of ERI. Therefore, we prepared smectite gel using ERI as a dispersion medium, and reported its physiochemical characteristics (*5,6*).

In addition, to evaluate application to medical drugs, we evaluated the healing-promoting effects of ERI on burns and atopic dermatitis, and observed its effectiveness and alleviation of these symptoms (*9-12*).

Based on the above reports, ERI compared with purified water can allow the preparation of drugs with a drug delivery system (DDS) that can maintain the gel state. ERI with emulsification characteristics and a gelling drug maintaining function are useful for preparing controlled-release transdermal therapeutic drugs.

In this study, we prepared surfactant-free emulsions by adding various types of oil to ERI containing lithium magnesium sodium silicate as smectite, and evaluated their physiochemical characteristics.

2. Materials and Methods

2.1. *Materials*

ERI containing lithium magnesium sodium silicate (*13*) (GE-100: A. I. System Product Corp., Aichi, Japan), jojoba oil (Wako Pure Chemical Industries, Ltd., Osaka, Japan), squalane and olive oil (Kanto Chemical Co., Inc., Tokyo, Japan), were used as purchased and glyceryl trioctanoate (medium chain fatty acid triglycerides, MCT, Panacet® 800 were kindly provided by NOF Corporation (Tokyo, Japan). All the other reagents were of analytical grade.

2.2. *Preparation of surfactant-free emulsions*

ERI containing 3% lithium magnesium sodium silicate (GE-100) was mixed with 0.1, 1, and 10% oil (jojoba oil, squalane, olive oil, or MCT), and samples of various emulsions (100 g) were prepared using an ultrasonic homogenizer (UH-300: SMT Co., Ltd., Tokyo, Japan) under the following emulsification conditions: standard horn, 12 ø; frequency, 20 kHz; processing time, 5 min, continuously.

2.3. *Zeta potential and particle size measurement*

The zeta potential and mean particle diameter of oil droplets in each sample were measured using a zeta potential analyzer (ZEECOM ZC-3000M, Microtec Co., Ltd., Chiba, Japan). Palladium was used as the electrode material of the measurement cell, and a special quartz cell (width, 10 mm) fixed with a Teflon block with a distance between positively biased electrodes of 64 mm and a distance between reference electrodes of 36 mm was used. Data analysis was performed using personal computer measurement system software for ZEECOM ZC-3000. For the measurement of the zeta potential, oil droplet particles electrophoresed after voltage application to the positively biased electrode so that 10.0 V could be applied to the reference electrode were automatically tracked, and the zeta potential was calculated from their electrophoretic mobility using the Smoluchowski equation. For the measurement of the mean particle diameter of oil droplets, the cell containing a sample was irradiated with a laser, and the Brownian motion of particles was measured per unit of time without voltage application, and the particle diameter was calculated using the Stokes-Einstein equation..

2.4. *Rheology measurement*

For rheology measurement, a shear rete-shear stress-controlled rheometer RC20 (RheoTec Messtechnik GmbH, Ottendorf-Okrilla, Germany) as a single cylindrical rotational viscometer (Brookfield type viscometer) was used (*14,15*). Assuming room temperature, the measurement temperature was adjusted to 25 ± 0.2°C using a thermostat jacket. The external diameter of the inner cylinder used for the rheometer was 25.0 mm, the internal diameter of the cup was 26.5 mm, and their gap was 1.5 mm. For measurement, the shear velocity was increased at a rate of 2.8 (1/s) per second until reaching 500 (1/s) in the inner cylinder, maintained at 500 (1/s) for 28 seconds, and was reduced at a rate of 2.8 (1/s) per second from 500 to 0 (1/s).

3. Results

The results of changes in the thixotropy of ERI containing 3% lithium magnesium sodium silicate (GE-100) are shown in Figure 1 and Table 1. In Figure 1, the dotted and solid lines indicate thixotropy before and that after

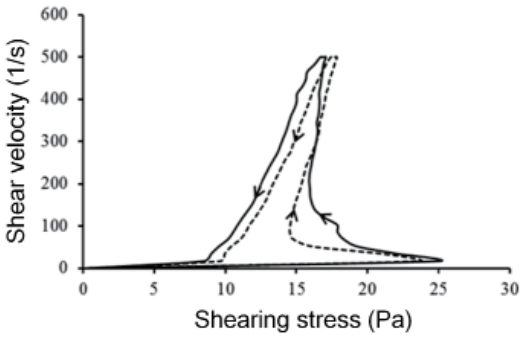

Figure 1. Thixotropic change of lithium magnesium sodium silicate gels prepared using electrolytic-reduction ion water (GE-100) sonicated by ultrasonication. Dotted line: before ultrasonication; solid line: after ultrasonication. Each linear arrow showed a direction of the hysteresis loop.

Table 1. Effect of ultrasonic treatment on hysteresis loop area of lithium magnesium sodium silicate gels prepared using GE-100

	Hysteresis loop area (Pa/s)	Area ratio (%)
GE-100 (before ultrasonication)	1,278 ± 33	100
GE-100 (after ultrasonication)	2,042 ± 67	160

GE-100: ERI containing 3% lithium magnesium sodium silicate. Mean ± SD, SD: standard deviation (n = 3).

ultrasonication, respectively. Shearing stress clearly increased after ultrasonication. In addition, as shown in Table 1, a comparison of thixotropy before and after ultrasonication showed a 60% increase in the hysteresis loop area rate after ultrasonication.

Subsequently, 0.1, 1, and 10% oil (jojoba oil, squalane, olive oil, or MCT) was added to GE-100, and the mixture was ultrasonicated. Changes in thixotropy are shown in Figure 2 and Table 2. In Figure 2, with an increase in the concentration of each type of oil, the shearing stress decreased. In Table 2 and Figure 3, the hysteresis loop area also decreased with an increase in the oil concentration. The rate (%) of the hysteresis loop area of GE-100 containing each type of oil to that of GE-100 alone (2,042 Pa/s) was the highest for MCT (143%) at a concentration of 0.1%, and the lowest also for MCT (58%) at a concentration of 10% (Figure 3). On the other hand, GE-100 containing olive oil showed high rates (134 and 103% at concentrations of 0.1 and 10 %, respectively).

Table 3 shows the zeta potential of oil droplets in each emulsion. GE-100 containing jojoba oil at concentrations of 0.1 and 1% showed low values (38.3 and –37.3 mV, respectively). However, the zeta potential did not markedly differ among the other types of emulsion (range: –23.4 - –26.8 mV).

The mean particle diameter of oil droplets in each emulsion is shown in Table 4. The mean particle diameter was small for olive oil (250-327 nm) and MCT (238-295 nm).

4. Discussion

Smectites basically have a 3-layer structure (tetrahedral layer-octahedral layer-tetrahedral layer) consisting of

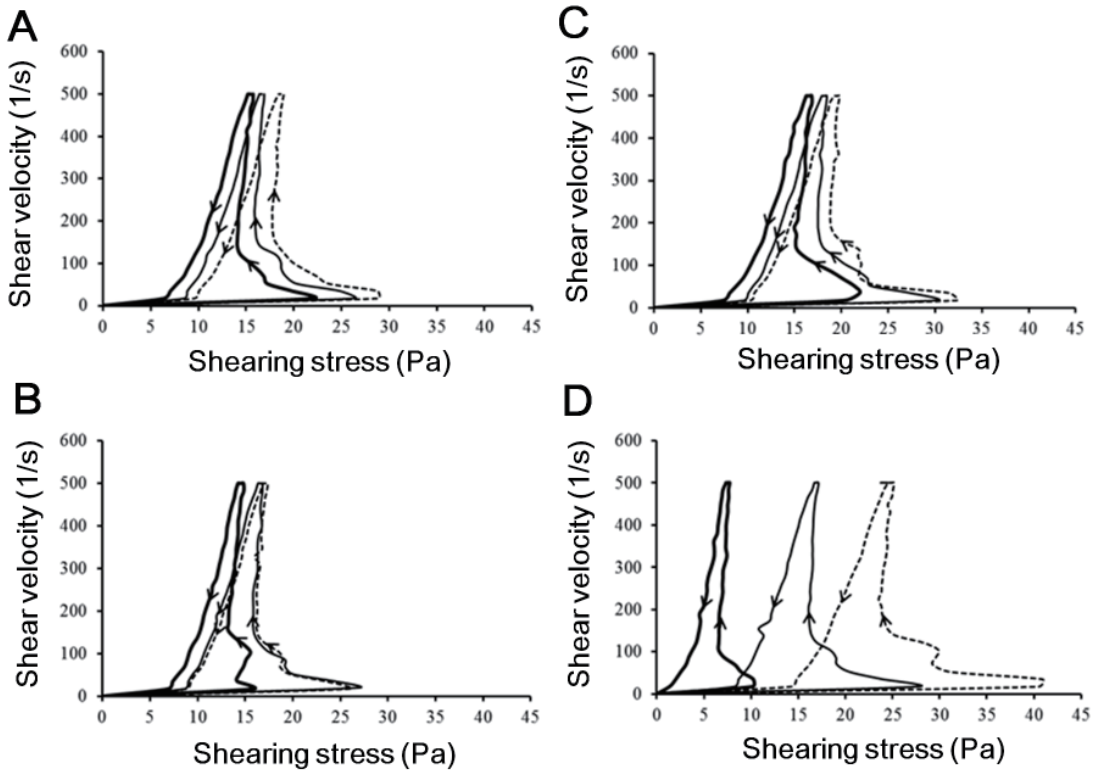

Figure 2. Thixotropic changes of different gels containing various oils. Dotted line: GE-100 + 0.1% oil; thin solid line: GE-100 + 1% oil; thick solid line: GE-100 + 10% oil. **(A)**: jojoba oil, **(B)**: squalane, **(C)**: olive oil, **(D)**: MCT. Each linear arrow showed a direction of the hysteresis loop.

Table 2. Thixotropic values of emulsions containing various oils

	Hysteresis loop area (Pa/s)		
GE-100 + Oil (%)	0.1%	1%	10%
GE-100 only	–	–	2,042 ± 67 (100)
GE-100 + jojoba oil	2,419 ± 138 (118)	2,219 ± 152 (109)	1,842 ± 62 (90)
GE-100 + squalane	2,039 ± 142 (100)	2,178 ± 129 (107)	1,485 ± 170 (73)
GE-100 + olive oil	2,741 ± 319 (134)	2,496 ± 64 (122)	2,101 ± 257 (103)
GE-100 + MCT	2,916 ± 377 (143)	2,301 ± 76 (113)	1,184 ± 124 (58)

( ): area rate (%); mean ± SD, SD: standard deviation (n = 3).

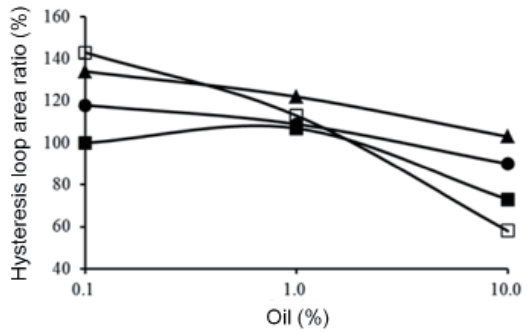

Figure 3. The relationship between the content rate (%) of various oils and hysteresis loop area. ●: GE-100 + jojoba oil, ■: GE-100 + squalane, ▲: GE-100 + olive oil, □: GE-100 + MCT.

Table 3. Zeta potential of oil droplets in various emulsions

GE-100 + Oil (%)	Zeta potential of oil droplets (mV)		
	0.1%	1%	10%
GE-100 + jojoba oil	-38.3	-37.3	-22.3
GE-100 + squalane	-26.9	-31.0	-26.4
GE-100 + olive oil	-26.1	-26.5	-25.2
GE-100 + MCT	-23.4	-30.5	-26.8

Table 4. Mean diameter of oil droplets in various emulsions

GE-100 + Oil (%)	Mean diameter of oil droplets (nm)		
	0.1%	1%	10%
GE-100 + jojoba oil	363 ± 329	573 ± 278	452 ± 244
GE-100 + squalane	355 ± 610	376 ± 832	471 ± 401
GE-100 + olive oil	250 ± 204	271 ± 267	327 ± 475
GE-100 + MCT	238 ± 182	266 ± 179	295 ± 299

Mean ± SD, SD: standard deviation (n = 220).

an octahedral sheet (composed of a magnesium atom coordinated by 6 oxygen atoms) sandwiched between 2 regular tetrahedral layers (composed of a silicate atom surrounded by 4 vertex oxygen atoms) (*1*). In lithium magnesium sodium silicate, due to the isomorphous substitution of some silicate and magnesium ions by lithium ions in the smectite structure, its surfaces are negatively charged, resulting in the formation of card-house structures. These card-house structures produce viscosity. The dispersion forms a gel with progression of card-house structures but forms a sol after shear force application, showing thixotropy. Lithium magnesium sodium silicate is widely used as a rheology modifier and texture enhancer, and particularly as a cosmetic material because it markedly increases viscosity and is highly thixotropic, well spread over the skin, and non-greasy (*13*). In this study, various types of oil were added to this GE-100, and the physiochemical characteristics of the emulsions were evaluated.

To evaluate the characteristics of GE-100 first, rheology measurement was performed before and after ultrasonication. As can be seen from Figure 1, thixotropy increased after ultrasonication. This may have been because honeycomb structures of lithium magnesium sodium silicate were more finely dispersed, resulting in denser binding.

Subsequently, various types of oil were added to GE-100 at 3 concentrations (0.1, 1, and 10%), and thixotropy was evaluated. With an increase in the concentration of each type of oil, the hysteresis loop concentration decreased (Figure 2). This was considered to be due to inhibition of the formation of card-house structures of lithium magnesium sodium silicate with an increase in the percentage of oil.

In addition, thixotropy was compared among GE-100 emulsions containing various types of oil at a concentration of 0.1 or 1% in terms of the rate (%) of the hysteresis loop area of GE-100 containing each type of oil expressed as a percentage of that of GE-100 alone (2,042 ± 67 Pa/s) (Table 2). As a result, at a low oil concentration, the hysteresis loop area rate was more than 100%, showing an increase in thixotropy. An increase in thixotropy indicates high viscosity in the static state and low viscosity after shear force application. When an emulsion is topically applied to the skin, oil added to GE-100 may allow maintenance of a highly viscous state, providing moisturizing effects, and also improve the viscosity and malleability. However, the hysteresis loop area rate was less than 100% in GE-100 containing each type of oil at a concentration of 10%. This suggested that the formation of card-house structures of lithium magnesium sodium silicate is prevented by oil with an increase in the oil concentration in GE-100. The hysteresis loop area rate was the lowest for MCT (58%). This may have been because MCT is medium chain fatty acid triglycerides of caprylic acid (8-carbon straight chain fatty acid), and its viscosity is lower than that of the other long chain fatty acids. At an oil concentration of 10%, the hysteresis loop area rate of 10% olive oil was high (103%) compared with the other types of oil. This may have been because olive oil is a long chain fatty acid mainly consisting of oleic acid (monounsaturated 18-carbon fatty acid), and, compared with MCT, the high viscosity of this oil itself affects thixotropy. At 20 °C, the dynamic viscosity of olive oil is about 85 mPa·s, on the other hand, that of MCT is about 23 mPa·s (*16*).

Comparison of the zeta potential showed low values in samples of GE-100 containing 0.1 or 1% jojoba oil (Table 3). Based on the Derjaguin, Landau, Verwey, Overbeek theory (DLVO theory) (*17,18*), these results suggest that the emulsion containing jojoba oil at a low concentration is more stable than the other emulsions.

The mean diameter of oil droplets was small in olive oil and MCT. According to the Stokes equation [I], the smaller the particle diameter, the lower the particle rise velocity. Therefore, emulsions of olive oil and MCT with a small particle diameter may be more stable.

$$v = \frac{d^2|\rho_0 - \rho|g}{18\eta} \qquad \cdots[\mathrm{I}]$$

(v: particle fall or rise velocity, d: particle diameter in the dispersed phase, ρ and ρ_0: densities of the dispersed phase and dispersion medium, respectively, g: acceleration of gravity, η: viscosity of the dispersion medium).

Based on these results concerning thixotropy and the particle diameter, when oil at a low concentration (0.1%) is added, MCT emulsions may be excellent in terms of texture and stability, and, when oil at a low (0.1%)-high (10%) concentration is added, olive oil emulsions may be excellent regarding texture and stability (Tables 2 and 4).

Concerning the reasons for the addition of oil to GE-100, when emulsions are applied to the skin, the moisturizing effects of oil can be expected (*19*). In addition, fat-soluble drugs can be added to the oil layer and used as bases for drugs for external use. In particular, as a general treatment method for atopic dermatitis, potent steroids are initially used and changed to weak ones with the alleviation of symptoms (*20*). However, as emulsifiers, synthetic surfactants such as sorbitan sesquioleate and glyceryl monostearate that markedly damage sensitive skin tissue are used. In addition, since drugs and cosmetics contain antiseptics such as paraoxybenzoic acid, which can cause allergic contact dermatitis and contact urticaria (*7*), there is a concern about the influences of their long-term use on the skin (*21*).

GE-100 used in this study is ERI containing lithium magnesium sodium silicate that is frequently used in cosmetics and improves the texture for topical application to the skin. To also add the moisturizing effects to this GE-100, oil was added, and emulsions were prepared. Various types of oil evaluated in this study are used in drugs, cosmetics, and foods. Emulsions obtained by adding each oil can prevent the influences of long-term use on the skin because of the absence of surfactants as well as the absence of the necessity of adding antiseptics due to the bactericidal effects of ERI (S-100) as the main solvent of GE-100 (*4*) or adding antioxidative agents such as dibutylhydroxytoluene, tocopherol, or ascorbic acid due to the reduction effects of ERI itself (*3*). Oil-in-GE-100 emulsions are expected to be new bases for external drugs.

References

1. Elmore A R. Final report on the safety assessment of aluminum silicate, calcium silicate, magnesium aluminum silicate, magnesium silicate, magnesium trisilicate, sodium magnesium silicate, zirconium silicate, attapulgite, bentonite, Fuller's earth, hectorite, kaolin, lithium magnesium silicate, lithium magnesium sodium silicate, montmorillonite, pyrophyllite, and zeolite. Int J Toxicol. 2003; 22:37-102.
2. Kunimine Industries Co., Ltd., Chiyoda-ku, Tokyo, Japan. Explanation of the product. *http://www.kunimine.co.jp/english/index.htm*
3. Okajima M. Abstract of Japanese society for applied research in negative ion. 2002.
4. Okajima M, Kanzaki M, Ishibashi Y, Wada Y, Hino F, Kitahara Y, Shimokawa K, Ishii F. *In vitro* bactericidal activities against periodontopathic bacteria by electrolytic-reduced ion water (ERI). Drug Discov Ther. 2011; 5:306-310.
5. Takigawa T, Shimokawa K, Ishii F. Physicochemical properties of magnesium aluminum silicates (smectites) gels prepared by electrolytic-reduction ion water (1): Rheological properties. Material Tech. 2008; 26:50-54.
6. Okajima M, Shimokawa K, Ishii F. Physicochemical properties of magnesium aluminum silicate (smectone®) gels prepared using electrolytic-reduction ion water (2): Effects of various salts on the phase diagram. Coll Surf B: Biointerf. 2009; 72:284-288.
7. Minamoto K. Skin sensitizers in cosmrtics snd skin care products. Jpn J Hyg. 2010; 65:20-29.
8. Konomatsu A, Sugibayashi K, Okajima M, Ishii F. Preparation and stability of surfactant free emulsions using electrolyzed deoxidized and ionized water. Material Tech. 2003; 21:273-285.
9. Okajima M, Shimokawa K, Ishii F. The healing effect of electrolytic-reduction ion water on burn wounds. BioSci Trends. 2010; 4:31-36.
10. Shu T, Okajima M, Shimokawa K, Ishii F. The treatment effect of the burn wound healing by electrolytic-reduction ion water lotion. BioSci Trends. 2010; 4:1-3.
11. Shu T, Okajima M, Wada Y, Shimokawa K, Ishii F. The treatment effect of the burn wound healing by electrolyticreduction ion water lotion combination therapy. Part 2: Two degree burn of forearm to the dorsum of the hand. BioSci Trends. 2010; 4:213-217.
12. Shu T, Okajima M, Wada Y, Shimokawa K, Ishii F. The treatment effect of the atopic dermatitis by electrolytic-reduction ion water lotion. Drug Discov Ther. 2010; 4:499-503.
13. Co-op Chemical Co., Ltd., Chiyoda-ku, Tokyo, Japan. Explanation of the product. *http://www.jade.dti.ne.jp/~coopchem/newsmectite.html*
14. Takeuchi M, Kageyama S, Suzuki H, Wada T, Toyoda Y, Oguma T, Ezure Y, Tsuriya Y, Kato T, Ishii F. Preparation and physicochemical properties of a reversible, thermo-setting, in situ gelling ophthalmic solution containing timolol maleate. Material Tech. 1999; 17:445-452.
15. Takeuchi M, Kageyama S, Suzuki H, Wada T, Notsu Y, Ishii F. Rheological properties of reversible thermo-setting in situ gelling solutions with the methylcellulose-polyethylene glycol-citric acid ternary system. Colloid Polym Sci. 2003; 281:1178-1183.
16. Bonnet JP, Devesvre L, Artaud J, Moulin P. Dynamic viscosity of olive oil as a function of composition and temperature: A first approach. Eur J Lipid Sci Technol. 2011; 113:1019-1025.
17. Derjaguin BV, Landau L. Theory of the stability of strongly charged lyophobic sols and of the adhesion of strongly charged particles in solution of electrolytes. Acta Physicochim. URSS 1941; 14:633-662.
18. Verwey EJW, Overbeek JTG. Theory of the stability of lyophobic colloids. Elsevier, Amsterdam, Nederland, 1952; Vols. 1 and 2.
19. Sagiv A E, Dikstein S, Ingber A. The efficiency of humectants as skin moisturizers in the presence of oil. Skin Res Technol. 2001; 7:32-35.
20. Saeki H, Furue M, Furukawa F, Hide M, Ohtsuki M, Katayama I, Sasaki R, Suto H, Takehara K. Guidelines for management of atopic dermatitis. J Dermatol. 2009; 36:563-577.
21. Cork MJ, Danby SG, Vasilopoulos Y, Hadgraft J, Lane ME, Moustafa M, RichGuy RH, MacGowan AL, Tazi-Ahnini R, Ward SJ. Epidermal barrier dysfunction in atopic dermatitis. J Invest Dermatol. 2009; 129:1892-1908.

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